SIMULTANEOUS ESTIMATION OF LUTEOLIN AND APIGENIN IN METHANOLIC LEAF EXTRACT OF *CARDIOSPERMUM HALICACABUM* BY HPLC

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Article Received on: 20/03/13 Revised on: 09/04/13 Approved for publication: 13/05/13

DOI: 10.7897/2230-8407.04724

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ABSTRACT

Cardiospermum halicacabum (Sapindaceae) widely spread in Asia, tropical and sub-tropical Africa is commonly consumed as leafy vegetable for the treatment of rheumatoid arthritis in India. The present study was aimed to develop and validate an RP-HPLC separation for the simultaneous estimation of luteolin and apigenin in the leaves of *Cardiospermum halicacabum*. HPLC separation was accomplished on an oyster ODS 3 column (5 μ m, 200 mm $\times$ 4.6 mm, i.d.) with gradient elution of potassium dihydrogen phosphate buffer (20 mM, pH 3.5 adjusted with ortho phosphoric acid, v/v) (A) and acetonitrile (B). The elution program as follows; 0-10 min 15-60% B, 10-15 min 60-75%B, 15-20 min 75-100% B, 20-25 min B hold at 100%. The method was validated as per ICH guidelines for system suitability, linearity, specificity, precision, accuracy, LOD and LOQ. The method was linear in the range of 1-70 μ g/ml. The relative standard deviation of the method was less than 2% for intra-day and inter-day precision assay and the average recoveries were between 98 and 102%. LOD, LOQ of luteolin and apigenin was found to be 0.03, 0.91 and 0.041, 0.13 μ g/ml respectively. The proposed method was found to be linear, specific, accurate, precise and robust.

Keywords: *Cardiospermum halicacabum*; apigenin; luteolin; RP-HPLC

INTRODUCTION

Cardiospermum halicacabum (C.H) commonly called as balloon vine is found in the soapwort family (Sapindaceae) and represents more than 30 recognized species throughout the world. *Cardiospermum halicacabum* is widely spread in tropical and sub-tropical Africa, Asia and in India is most commonly used for the treatment of rheumatoid arthritis¹. Besides antioxidant activity² the plant was reported for analgesic and vasodepressant activities³, antipyretic activity against yeast-induced pyrexia in rats⁴, antimalarial activity⁵, diuretic activity⁶, anti-diarrhoeal activity⁷, anti-diabetic activity⁸ and anti-ulcer activity⁹. Flavonoids viz. apigenin and luteolin was reported to be present in the leaves of *Cardiospermum halicacabum*¹⁰⁻¹². A UV and polarographic method¹³, RP-HPLC¹⁴ and a HPTLC¹⁵ method was reported for the estimation of luteolin in plant extracts. A rapid RP-HPTLC densitometry method for simultaneous determination of luteolin, apigenin and other major flavonoids in important medicinal plants was reported by Bhandari *et al*¹⁶. Two RP-HPLC methods were reported for the estimation of luteolin and apigenin in plant extract of *Cardiospermum halicacabum*^{17,18}. Li *et al*¹⁹ reported a simultaneous estimation of luteolin and apigenin in dog plasma by RP-HPLC method. There is no report available about the determination of luteolin and apigenin in *Cardiospermum halicacabum*. Hence the present study was undertaken to develop a validated RP-HPLC method for the quantification of apigenin and luteolin in *Cardiospermum halicacabum*.

MATERIALS AND METHODS

Plant material

The plant *Cardiospermum halicacabum* was collected from the hills of Palakkad district of Kerala, India and it was authenticated by Dr. G.V.S. Murthy, Scientist "E" Botanical survey of India, Coimbatore, Tamil Nadu, India. The voucher specimen for future reference was deposited in our pharmacognosy laboratory.

Chemicals, solvents and standards

Analytical grade methanol (Merck, Mumbai, India) was used for extraction, while HPLC-grade acetonitrile, methanol, orthophosphoric acid and analytical grade potassium dihydrogen orthophosphoric acid (Merck, Mumbai, India) were used in HPLC analysis. Standards of apigenin and luteolin were obtained from LGC prochem, Bengaluru, India.

Chromatographic instrumentation and conditions

The analysis was carried out with a Hewlett-packard 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) comprising a quaternary pump (HP 1311 A), a vacuum degasser (Agilent 1322 A), a variable wavelength detector (HP 1314A) and a 20 μ l sample injector (Rheodyne 7725), column thermostat (HP 1316). The separation was performed on Oyster ODS 3 column (5 μ m, 200 mm $\times$ 4.6 mm, i.d, Merck). The detection wavelength was set at 348 nm. The mobile phase consisted of solvent A (20 mM potassium dihydrogen phosphate, pH 3.5 adjusted with orthophosphoric acid v/v), solvent B acetonitrile with the elution profile as follows: 0-10 min, 15-60% B; 10-15 min, 60-75% B; 15-20 min, 75% B; 20-25 min, 75-100% B with the flow rate of 1 ml/min. The column thermostat was maintained at 30 $\pm$ 1°C.

Preparation of plant extract

Cardiospermum halicacabum leaf was finely powdered; 10 g of the powder content was transferred into a 250 ml beaker, macerated with 3 $\times$ 50 ml of methanol for 24 h, filtered through Whatman filter No 1. The extracts were concentrated in vacuum rotary evaporator (Buchi, German).

Preparation of Standard stock solutions

Ten mg each of apigenin and luteolin weighed accurately and transferred separately into a 10 ml volumetric flask, dissolved in 5 ml of methanol, sonicated for 5 min and the final volumes made up with acetonitrile to produce 1 mg/ml stock solution.

Preparation of sample solution

Ten mg of leaf extract was weighed accurately, transferred into a 10 ml volumetric flask and dissolved in 5 ml of methanol followed by sonication for 5 min and made up to volume with acetonitrile. 0.2 ml of the solutions was further diluted to 10 ml with acetonitrile, filtered through 0.45 μm nylon filter paper prior to use.

Validation of the HPLC method

The method was validated as per ICH guidelines²⁰ for system suitability, linearity, accuracy, precision, robustness, sensitivity, LOD and LOQ by the following procedures (Figure 1 to 4).

System suitability

System suitability of method was performed by calculating the chromatographic parameters namely, column efficiency, resolution, peak asymmetry factor and HETP on the repetitive of injection of standard solution.

Linearity (Calibration curve)

Linearity was performed for each apigenin and luteolin standards with five different concentrations between the ranges of 1-70 $\mu\text{g/ml}$ were analysed in triplicate for each concentration. Calibration curves were constructed by plotting peak areas against analyte concentrations. The linearity was assessed by calculating the slope, y-intercept and coefficient of determination (r^2) using Microsoft Excel 2007 program.

Precision

The precision of the method was examined by performing the intra-day and inter-day assays of six replicate injections of the mixture of standard solution at three concentration levels (1, 30 and 70 $\mu\text{g/ml}$). The intraday assay precision test was performed at intervals of 4 h in 1 day, while the interday assay precision test was performed over 3 days.

LOD and LOQ

LOD and LOQ of both the analytes were determined by k SD/ b where k is a constant (3.3 for LOD and 10 for LOQ),

SD is the standard deviation of the analytical signal, and b is the slope of the concentration/response graph.

Accuracy

The accuracy of the method was determined by calculating the recoveries of apigenin and luteolin by the method of standard addition. Known amount of the standards (80, 100 and 120%) were added to the pre-analyzed sample solutions and the amounts of these standards were estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curves.

Robustness

The robustness of the proposed method was evaluated by changing the different C18 column brand, pH and extraction time.

Specificity

Specificity is the ability of an analytical method to differentiate and quantify the analyte in presence of other components in the sample. Peak purity of the method was evaluated by calculating the difference between peak angle (θ_p) and peak threshold angle (θ_m). Peak purity was assessed using millennium 32 software for waters HPLC/PDA; system based on the degree of similarity of UV spectra across the peak in the range of 190-800 nm. The separation was performed on a Hypersil C18 column (particle size 5 μm ; 250 X 4.6 mm id).

Table 1: System suitability parameters of luteolin and apigenin

Parameters	Luteolin (1 $\mu\text{g/ml}$)	Apigenin (1 $\mu\text{g/ml}$)
Number of theoretical plates	21211	33492
Asymmetry factor	0.69	0.81
Resolution	5.42	3.11
HETP	0.94	0.59

Table 2: Peak purity profile of luteolin and apigenin

Components	Peak angle	Threshold
Luteolin (5 $\mu\text{g/ml}$)	0.669	3.773
Apigenin (5 $\mu\text{g/ml}$)	0.321	4.601

Table 3: Linear regression data for the calibration curves (n=3)

Parameter	luteolin	apigenin
Retention time (min)	7.024 $\pm$ 0.118	7.614 $\pm$ 0.107
Detection wavelength	348 nm	348 nm
LOD $\mu\text{g/ml}$	0.03	0.04
LOQ $\mu\text{g/ml}$	0.91	0.13
Linearity range $\mu\text{g/ml}$	1-70	1-70
Correlation coefficient (area)	0.9995	0.9998
Regression equation (area)	$y = 151.99x + 26.60$	$y = 124.16x + 37.51$

Table 4: Intra and interday precision of the developed method (n=6)

components	Intraday				Interday			
	Retention time		Peak area		Retention time		Peak area	
	Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
luteolin								
1 $\mu\text{g/ml}$	7.09	0.13	302	0.75	7.06	0.06	297	0.95
30 $\mu\text{g/ml}$	7.05	0.24	4492	1.18	7.01	0.26	4469	1.36
70 $\mu\text{g/ml}$	7.02	0.27	10748	0.93	6.96	0.36	10639	1.27
apigenin								
1 $\mu\text{g/ml}$	7.59	0.18	217	0.58	7.57	0.09	209	0.93
30 $\mu\text{g/ml}$	7.62	0.17	3754	1.18	7.59	0.39	3684	1.42
70 $\mu\text{g/ml}$	7.64	0.35	8649	1.32	7.62	0.27	8598	1.63

Table 5: Recovery study of luteolin and apigenin from the extract of *Cardiospermum halicacabum* (n=3)

Components	Quantity added %	Total quantity present, µg/ml	Amount quantity found, µg/ml	Recovery, %	%RSD
luteolin	0	0.130	0.128	98.46	0.84
	80	0.234	0.231	98.71	0.69
	100	0.260	0.258	99.23	1.26
	120	0.286	0.271	98.95	1.51
apigenin	0	0.436	0.431	98.85	0.93
	80	0.784	0.782	99.74	1.28
	100	0.872	0.863	98.96	1.86
	120	0.959	0.941	98.12	1.15

Table 6: Robustness of the method (n=6)

Parameters changed		Recovery, % $\pm$ SD	
		luteolin	apigenin
Extraction time	20 h	98.36 $\pm$ 1.34	98.92 $\pm$ 0.49
	24 h	99.52 $\pm$ 0.98	101.56 $\pm$ 0.72
	48 h	101.83 $\pm$ 1.19	99.82 $\pm$ 1.28
pH	3.0	98.78 $\pm$ 0.97	99.71 $\pm$ 1.32
	3.5	99.39 $\pm$ 0.70	100.39 $\pm$ 0.97
	4.0	98.38 $\pm$ 1.25	98.58 $\pm$ 1.62
Column brand	Zobrax	99.29 $\pm$ 0.73	99.37 $\pm$ 0.83
	Oyster	98.93 $\pm$ 0.83	98.49 $\pm$ 0.39
	Hypersil	101.53 $\pm$ 1.14	98.24 $\pm$ 1.85

Figure 1. Chromatogram of luteolin and apigenin standards

Fig.2. Chromatogram of *Cardiospermum halicacabum* leaf extract

Fig.3 Linearity of luteolin standard

Fig.4. Linearity of apigenin standard

RESULTS AND DISCUSSION

Optimisation of extraction

As reported by Natsume, *et al.*¹⁴ 100% methanol was selected as extraction solvent to obtain high yield of luteolin and apigenin in plant materials. The total extraction time was also evaluated as reported by Deepak, *et al.*¹⁷. However 24 h maceration of plant materials with methanol (3 × 50 ml) yielded higher amount of luteolin and apigenin compared to the previously reported methods.

Optimisation of chromatographic conditions

The HPLC-UV parameters were optimised by investigating the influence of the mobile phase composition and detection wavelength. The initial mobile phase system was tried with gradient elution of methanol and acetate buffer pH 4, but resulted in decrease resolution and tailing of peaks observed. Thus instead of methanol and acetate buffer, gradient elution of acetonitrile and potassium dihydrogen phosphate buffer (20 mM, adjusted to pH 3.5 with orthophosphoric acid, v/v) was developed. By this method separation, resolution of the peaks was good and tailing of peaks also minimal. Gradient composition was altered to get a fine separation and finally 0-10 min 15-60% B, 10-15 min 60-75% B, 15- 20 min 75% B, 20-25 min 75-100% B set as a mobile phase with the flow rate of 1 ml/min. The UV spectrum of mixer of apigenin and luteolin showed the maximum absorbance at 348 nm and it was selected as a detection wavelength. The column thermostat was maintained at $30 \pm 1^\circ\text{C}$. Before injection column was stabilised with the initial mobile phase concentration of 15% B for 45 min and after each run column was stabilised with initial gradient concentration for 10 min.

Method validation

System suitability

Six replicate injections of the system suitability solution gave %RSD values for retention time and peak area within 2%, indicating low variation of the measured values (Table 1). The symmetry of all peaks was < 2. The resolution (R) between luteolin and apigenin was 3.11, indicating a high degree of peak separation ($R > 2$). The efficiency of the column, as expressed by the number of theoretical plates, was more than 2000. These results indicate the suitability of the HPLC system and conditions of the developed method.

Specificity

Specificity evaluation was carried out by analyzing luteolin and apigenin simultaneously. It was observed that the peaks for each of the drugs were well separated and not interfered with other components. Peak threshold angle (θ_{th}) of all the peaks was found greater than that of peak angle (θ_p), values are mentioned in Table 2, indicating the purity of the peaks. Further, peaks obtained by the proposed method corresponding to each of the drugs were seen to be pure. This confirmed that the method used for estimation of the two marker compounds simultaneously was specific.

Calibration curve (Linearity)

Linear regression analysis confirms that the r^2 values for both the drugs were >0.999 confirming the linear relationship between the concentration of the drugs and area under the curve (Table 3). The calibration curves ($n=3$) constructed for the markers were linear over the concentration range of 1-70 µg/ml for each marker.

Precision

The precision results for the solution at the three concentrations are presented in Table 4. It was shown that the RSD values for retention time were <1%, while the RSD values for peak area were <2% for both intra- and interday assay precision (intraday, 4 h, six injections; interday, 3 days).

Accuracy

Recovery of the investigated components ranged from 98.12-99.74%, (Table 5) and their % RSD values were all <2%. It was known from recovery tests that the developed methods manifested reliability and accuracy for the measurement of the two components individually.

Limit of detection and Limit of quantification

The LOD and LOQ for luteolin and apigenin were found to be 0.05 and 0.15 µg/ml, 0.041 and 0.13 µg/ml, respectively.

Robustness

Selected parameters like extraction time, different C18 column and pH remained unaffected by small variations (Table 6). The recovery obtained concluded that the methods are consistent for change in extraction time, column brand and pH.

CONCLUSION

The developed validated RP-HPLC method enabled the simultaneous determination of luteolin and apigenin in the methanolic leaf extract of *Cardiospermum halicacabum*. The analytes were identified by their retention times (t_R) and by co-injection with standards. The spiked extract samples with standard compounds ensured that there was no effect of sample matrix on peak response of the analyst of interest. For quantitative determinations, the plant extracts were injected directly on to column and separated under the optimized conditions. The quantity of luteolin and apigenin in *Cardiospermum halicacabum* was found to be 1.29 and 2.16 mg/g respectively. The retention time of luteolin and apigenin was found to be 7.05 and 7.59 min respectively. Hence the newly developed method enabled the faster analysis of luteolin and apigenin in *Cardiospermum halicacabum* compared to previously reported method.

REFERENCES

- Ragupathy S, Steven G, Newmaster, Gopinadhan P, Candice BN. Exploring Ethnobiological Classifications for Novel Alternative Medicine: A case study of *Cardiospermum halicacabum* L. (Modakathon, Balloon Vine) as a traditional herb for treating rheumatoid arthritis, *Ethnobotany*; 2007.p. 19
- Kumaran A., Karunakaran RJ. Antioxidant activities of the methanol extract of *Cardiospermum halicacabum*, *Pharm. Biol* 2006; 44(2): 146-151. <http://dx.doi.org/10.1080/13880200600596302>
- Gopalakrishnan C, Dhananjayan R, Kameswaran L. Studies on the pharmacological actions of *Cardiospermum halicacabum*. *Indian J Physiol and Pharmacol* 1976; 20: 203-206. PMID:1010635
- VV Asha, Pushpangadan P. Antipyretic activity of *Cardiospermum halicacabum*. *Indian J. Exp Biol* 1999; 37: 411-414. PMID:10641181
- Waako PJ, Gumede B, Smith P, Folb PI. The *in vitro* and *in vivo* antimalarial activity of *Cardiospermum halicacabum* L. and *Momordica foetida* Schumch. *Et Thonn. J Ethnopharmacol* 2005; 99: 137-143. <http://dx.doi.org/10.1016/j.jep.2005.02.017> PMID:15848033
- Santakumari G, Pillai NR, Nair RB. Diuretic activity of *Cardiospermum halicacabum* Linn in rats. *J Sci. Res. Plant Med* 1981; 2: 32.
- Rao Venkat N, Chandraprakash K, Kumar Shanta SM. Pharmacological investigation of *Cardiospermum halicacabum* L. in different animal models of diarrhea. *Indian J Pharmacol* 2006; 38: 346-349. <http://dx.doi.org/10.4103/0253-7613.27703>
- Veeramani C, Pushpavalli G, Pugalendi KV. Anti hyperglycaemic effect of *Cardiospermum halicacabum* Linn. Leaf extract on STZ-induced diabetic rats. *J Appl. Biomed* 2008; 6: 19-26.
- MS Sheeba, VV Asha. Effect of *Cardiospermum halicacabum* on ethanol-induced gastric ulcers in rats, *J. Ethnopharmacol* 2006; 106: 105-110. <http://dx.doi.org/10.1016/j.jep.2005.12.009> PMID:16469462
- Srinivas K, Choudary KA, Rao SS, Satyanarayana T, Rao Krishna RV. Phytochemical examination of *Cardiospermum halicacabum* Linn. *Indian J Nat Prod* 1998; 14: 24-27.
- The European Agency for the Evaluation of Medicinal Products (TEAEMP) Veterinary medicines evaluation unit. EMEA/MRI/664/99-FINAL; 1999.
- Achari B, Chowdhury US, Dutta PK, Pakrashi SC. Two isomeric flavonones from *Vitex negundo*. *Phytochemistry* 1984; 23: 703-704. [http://dx.doi.org/10.1016/S0031-9422\(00\)80420-3](http://dx.doi.org/10.1016/S0031-9422(00)80420-3)
- Darina R, Anna V. UV spectrometric and DC polarographic studies on apigenin and luteolin. *Archives of Pharmacol Research* 1999; 22(2): 173-178. <http://dx.doi.org/10.1007/BF02976542>
- Natsume E, Muto Y, Fukuda K, Tokumaga T, Osakabe N. Determination of rosmarinic acid and luteolin in *Perilla frutescens* Britton (Labiatae), *J. Sci. Food Agric* 2006; 86: 891-901. <http://dx.doi.org/10.1002/jsfa.2432>
- Bazylo A, Strzelecka H. A HPTLC densitometric determination of luteolin in *Thymus vulgaris* and its extracts. *Fitoterapia* 2007; 78(6): 391-5. <http://dx.doi.org/10.1016/j.fitote.2007.01.007> PMID:17583444
- Huang MH, Huang SS, Wang BS, Wu CH, Sheu MJ, Hou WC. Antioxidant and anti-inflammatory properties of *Cardiospermum halicacabum* and its reference compounds ex-vivo and iv vivo, *J. Ethnopharmacol* 2010; 133(2): 743-750. <http://dx.doi.org/10.1016/j.jep.2010.11.005> PMID:21073940
- Deepak M, Sangli GK, Arun PC, Amit A. Quantitative Determination of the Major Saponin Mixture Bacoside A in *Bacopa monnieri* by HPLC. *Phytochem. Anal* 2005; 16: 24-29. <http://dx.doi.org/10.1002/pca.805> PMID:15688952
- Bhandari P, Kumar N, Gupta AP, Singh B, Kaul VK. A rapid RP-HPTLC densitometry method for simultaneous determination of major flavonoids in important medicinal plants. *J Sep Sci* 2007; 30(13): 2092-6. <http://dx.doi.org/10.1002/jssc.200700066> PMID:17654615
- Li L, Jiang H, Wu H, Zeng S. Simultaneous determination of luteolin and apigenin in dog plasma by RP-HPLC. *J Pharm Biomed Anal* 2005; 37(3):615-20. <http://dx.doi.org/10.1016/j.jpba.2004.11.012> PMID:15740925
- ICH. Q2B. Guidance for industry. Validation of Analytical Procedures: Methodology; 1996.

Cite this article as:

Aiyalu Rajasekaran, Vivekanandan Kalaiselvan, Nagulsamy Sarathikumar, Muthusamy Kalaivani. Simultaneous estimation of Luteolin and Apigenin in methanolic leaf extract of *Cardiospermum halicacabum* by HPLC. *Int. Res. J. Pharm.* 2013; 4(7): 109-113 <http://dx.doi.org/10.7897/2230-8407.04724>

Source of support: Nil, Conflict of interest: None Declared