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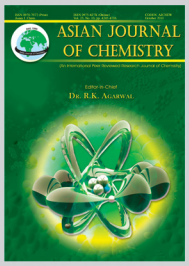

Quantitative High Performance Thin-Layer Chromatography Method for Analysis of Vitexin and Isovitexin in Extracts of Leaves of *Ficus deltoidea*

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The present study aimed to develop and validate a single step, simple, sensitive and accurate high performance thin-layer chromatography (HPTLC) method for quantification of vitexin and isovitexin in extracts of *Ficus deltoidea*. The method employed TLC plates as stationary phase with mobile phase as ethyl acetate:formic acid:acetic acid:water (100:11:11: 26). The method had shown linearity in a range of 0.1-300 µg/mL with correlation coefficient of R^2 = 0.988, 0.986 and 0.985, 0.982 with respective to peak area and height for vitexin and isovitexin, respectively. Recovery and accuracy (intra and inter day) values were found to be 92.10-101.98, 93.56-103.64 and 99.99-105.20 %, respectively with relative standard deviation (RSD) less than 5. Lowest limit of detection (LOD) and lowest limit of quantification (LOQ) were found to be 0.1 and 1.0 µg, respectively. Results of this study indicate that the proposed method is simple, sensitive, accurate and precise.

Key Words: HPTLC, Standardization, Quantification, Vitexin, Isovitexin, *Ficus deltoidea*.

INTRODUCTION

Ficus deltoidea Jack (Moraceae) is found in tropical and sub-tropical countries and has several varieties[1]. *Ficus deltoidea* was traditionally used in treating many diseases including high blood pressure, improve blood circulation, gout, pneumonia, heart problems, diarrhea, skin infection and diabetes[2]. Besides that, *F. deltoidea* is also used as aphrodisiac, specifically to increase male virility[3]. Studies have shown that *F. deltoidea* leaves possess antinociceptive, wound healing and antioxidant properties[4-7]. Shafaei *et al.*[8], reported the presence of saponin, amino acid, flavonoids and terpenoids in extracts of leaves of the plant. There is a wide variation in the amount and type of chemical constituents in samples of different species that differ in method and time of collection[9]. Thus the potency, quality and purity of drugs have to be evaluated.

The standardization of herbal products, the biggest hindrance in their wider acceptance in modern healthcare, is always challenging due their complexity and inadequacy or unavailability of standards and analytical methods[10]. In order to bring these remedies into the mainstream pharmaceutical market, solid scientific evidence is needed to support the efficacy claims of these products. The use of markers, the chemical compounds characteristic of a plant, signifies the total active constituents of an extract or correlates to pharmacological activity are used to standardize herbal products[1]. Therefore, it is important to develop analytical methods that can be used for standardization of herbal products and their batch-batch reproducibility. This can be achieved by developing analytical methods, employing new analytical techniques using marker compounds.

High performance thin layer chromatography is simple and powerful tool for high resolution chromatography and trace quantitative analysis is made possible. It is most widely used for quick and easy determination of quality, authenticity and purity of the crude drugs and market formulations[9]. However recent reviews show that the thin layer chromatography and HPTLC techniques can be used to rectify many qualitative and quantitative analytical problems in a wide range of fields including medicines, pharmaceutical, chemistry, biochemistry and toxicology[11]. So far no report has appeared on the HPTLC of the vitexin and isovitexin two C-glycosylflavones derivatives in the *Ficus deltoidea* leaves extracts. The present study is undertaken to develop a single step method for the qualitative and quantitative determination of these two pharmacologically active analytical markers (vitexin and isovitexin) to standardize the extracts of the leaves of *Ficus deltiodea*.

EXPERIMENTAL

The leaves of the plant (*Ficus deltoidea*) were purchased from Herbagus Sdn. Bhd. Penang-Malaysia and identified by Mr. Shunmugam from the school of Biological Sciences, University Sains Malaysia. A voucher sample of the plant, reference number 11204 was deposited at the herbarium of School of Biological Sciences, University Sains Malaysia.

Methanol, ethyl acetate, formic acid and acetic acid (analytical-reagent grade) solvents were purchased from Merck (Germany). Standards vitexin and isovitexin were purchased from Chromadex.

Preparation of standards and samples

Standards: The standards stock solution of vitexin and isovitexin were prepared in methanol to a concentration of 1.00 mg/mL. A series of working standard solution were prepared by diluting the stock solution with methanol to a concentration range of 0.1-300 µg/mL.

Samples: 100 g of powder dried leaves of *Ficus deltoidea* were extracted with water by refluxing and methanol using as Soxhlet extractor for 24 h. The extracts were concentrated under vacuum and dried using freeze dryer. To prepare stock solution, 0.5 g of each extract was dissolved in 10 mL methanol. Working sample solution of concentration of 5 mg/mL was prepared by diluting the stock solution with methanol.

HPTLC method and chromatographic conditions: The analysis was performed on HPTLC system of CAMAG (Berlin, Germany) comparison of densitometer (CAMAG Model-3 TLC scanner) equipped with winCATS 4 software and image recorder (CAMAG PROSTER 3). The chromatographic estimation was performed using the following conditions, stationary phase was preparative TLC plates (20 cm × 10 cm glass plates precoated with thickness 0.05 mm silica gel GF_{254}) were purchased from Merck Germany. The mobile phase used was ethyl acetate-formic acid-acetic acid-water (100:11:11:26). The plate was developed in an unsaturated glass chamber. The migration distance being 8 cm. After separation, the plate was dried in an oven at 40 ºC for 20 min.

Afterwards the plate was scanned at 366 nm and the quantification of vitexin and isovitexin were carried out by win CATS software using linear regression. Spots corresponding to peak of the samples and standards were assigned and scanned in a range of 400-200 nm for peak purity. Finally, images of the plate were taken at 366 nm for documentation (Fig. 1).

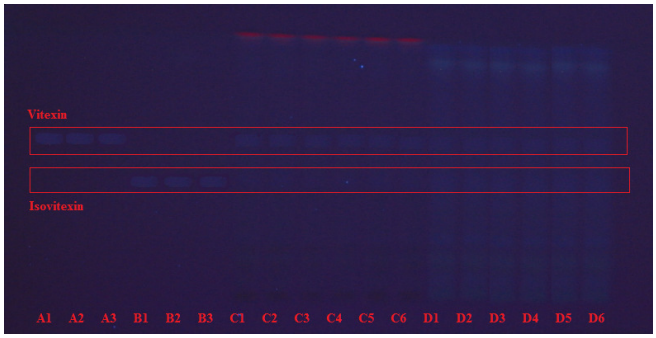

Fig. 1. Image of the plate at 366 nm; in image A1-3 (vitexin), B1-3 (isovitexin), C1-6 (methanol extract) and D1-6 (water extract)

Method validation: The developed method was validated in terms of linearity, intraday precision, interday precision and accuracy. The limit of quantification and limit of detection for vitexin and isovitexin were also determined. The lowest limit of detection (LOD) was determined at signal to noise ratio (S/N) of 3:1 by application of a series of 2-fold dilutions of the standards solutions, whereas the lowest limit of quantification (LOQ) was taken at S/N of 10:1. Linearity of the method was evaluated over the whole range of investigated standard solutions.

Accuracy of the analysis was evaluated by carrying out a recovery study. For that, a known concentration of standard in three different levels was added to preanalyzed sample. And average recovery was calculated. Three concentrations of vitexin and isovitexin 10, 40 and 100 µg/mL were used to evaluate intra-day and inter-day precision. Different concentrations of the standards were applied on TLC plate and the plate was developed and scanned at 366 nm six times in a single day for intraday precision and once daily for 6 consecutive days for inter-day precision. Accuracy was evaluated by quantifying the applied concentration from calibration curve of concentration *versus* peak area or height and precision was evaluated by relative standard deviation (RSD) among the determined values.

Application of samples and standards: Standards and samples were applied to the TLC plate by using CAMAGE Linomate-5 auto sampler equipped with 100 µL syringe: the band length was 10 mm and the application volume was 5 µL. Eighteen bands per plates were applied 8 mm from the bottom edge, 15 mm apart. The standard solutions were applied in three different concentrations (10, 40 and 100 µg/mL). All the applications were in duplicate.

RESULTS AND DISCUSSION

Linearity of the method by analyzing a series of standard solution and plotting calibration curve between concentration and peak area/peak height was found to be in the range of 0.1-300 µg/mL with correlation coefficient of R^2 = 0.988, 0.986 and 0.985, 0.982 with respective to peak area and height for vitexin and isovitexin, respectively. The limit of detection and limit of quantification was found to be 0.1 and 1 µg, respectively for vitexin and isovitexin. These values show that the method is highly selective (Table-1).

The results of recovery for both methanol and water extract presented in Table-2 indicated that these values in a range of 92.10-97.42 and 96.50-101.98 % for vitexin and isovitexin, respectively with RSD less than 4 % which indicates that the method is reproducible. Intra-day and inter-day accuracy and precision are giving in Table-3. The variations were found to be in the range of 97.66-103.64, 99.99-105.20 % for vitexin and 93.56-101.78, 100.03-102.62 % for isovitexin with RSD less than 5 %. It is clear from these results that the method is accurate and the accuracy was not compromised in intraday and inter-day analysis.

The presence of the vitexin and isovitexin in the samples was proven by the comparison of the UV-VIS spectra of the separated compounds from the samples (Figs. 2 and 3).

TABLE-1
LINEAR CORRELATION BETWEEN PEAK AREA/HEIGHT AND CONCENTRATION

Standard	Regression equation Y =		R^2 =		Linear range (µg/mL)	LOD (µg)	LOQ (µg)
	Area	Height	Area	Height			
Vitexin	35.306X + 677.34	0.8178X + 22.43	0.988	0.985	0.1-300	0.1	1
Isovitexin	51.608X + 1680.6	0.9969X + 45.041	0.986	0.982	0.1-300	0.1	1

TABLE-2
RECOVERY OF VITEXIN AND ISOVITEXIN IN METHANOL AND WATER EXTRACTS OF *Ficus deltoidea*

Standard	Concentration (µg/mL)	Methanol extract			Water extract		
		Amount (µg/mL)	Recovery (%)	RSD (%)	Amount (µg/mL)	Recovery (%)	RSD (%)
Vitexin	10	9.21	92.10	2.29	9.39	93.9	1.85
	40	38.97	97.42	2.69	38.97	97.42	2.98
	100	97.23	97.23	3.63	95.50	95.50	3.78
Isovitexin	10	9.84	98.4	2.25	9.65	96.50	2.15
	40	39.54	98.85	3.91	40.45	101.12	3.49
	100	96.96	96.96	3.75	101.98	101.98	2.91

TABLE-3
INTRA-DAY AND INTER-DAY DATA
FOR VITEXIN AND ISOVITEXIN

Standard	Concentration (µg/mL)	Intra-day (n = 6)		Inter-day (n = 6)	
		Mean (%)	RSD (%)	Mean (%)	RSD (%)
Vitexin	10	102.83	1.00	105.20	1.61
	40	97.66	1.74	99.99	1.85
	100	103.64	3.54	99.99	4.18
Isovitexin	10	93.56	1.52	102.62	1.05
	40	101.78	1.40	100.03	2.15
	100	99.09	4.68	100.36	3.98

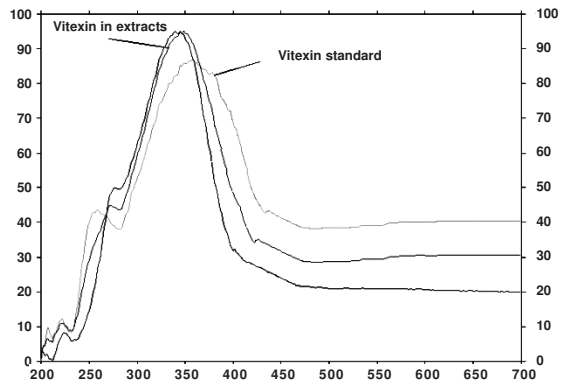

Fig. 2. UV-VIS spectra of the vitexin standard and vitexin in extracts

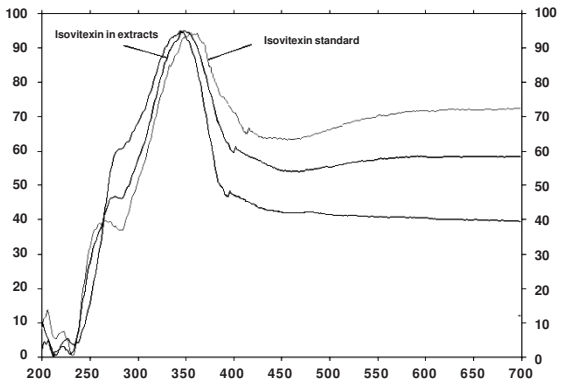

Fig. 3. UV-VIS spectra of the isovitexin standard and isovitexin in extracts

Figs. 4 and 5 shows the densitograms of the different extracts with standards. It can be observed the presence of the peak in the samples densitograms, at the same R_f values, as the peak of the standards.

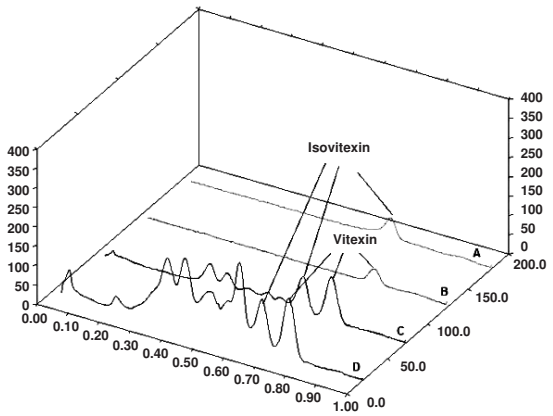

Fig. 4. 3D densitogram of the extracts and standards at 366 nm

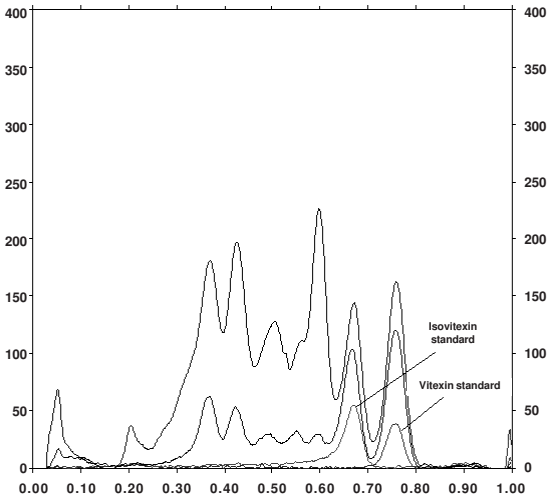

Fig. 5. 2D densitogram of the extracts and standards at 366 nm

The validated method was applied successfully to quantify vitexin and isovitexin in methanol and water extracts of *Ficus deltoidea*. For quantification of vitexin and isovitexin in the

extracts, calibration curve was constructed between concentration and peak area. For vitexin Y = 35.306X + 677.34; (R^2 = 0.988) and for isovitexin Y = 51.608X + 1680.6; (R^2 = 0.986) where Y is the peak area and X is the concentration (µg/mL). The content of vitexin and isovitexin in methanol and water extracts are giving in Table-4. The content of vitexin in both extracts was found to be higher rather than isovitexin.

Table-4
mg/g VITEXIN AND ISOVITEXIN IN THE EXTRACTS OF *Ficus deltoidea*

Standard	FDM	FDA
Vitexin	12.31 ± 0.55	4.81 ± 0.78
Isovitexin	6.20 ± 0.89	0.801 ± 0.52

The horizontal chamber was used in this experiment. The plate was positioned in the chamber with the stationary phase facing inside and chamber was allowed to saturate for 10 min before starting the development. The solvent was allowed to migrate to a distance of 8 cm from the lower edge because the development of the plate for a distance of more than 8 cm was found to be affecting linearity of the method. Therefore, optimum distance for the development of the plate was kept at 8 cm.

In the present study, we selected two C-glycosylflavones, vitexin and insovitexin, as pharmacologically active analytical markers to standardize the extracts using thin layer chromatography (TLC)-densitometry. Quantitative TLC *in situ* scanning densitometry is rapidly gaining wide acceptance in pharmacological analysis[12-14]. This is because of its simplicity, accuracy, cost effectiveness and possibility of simultaneous determination of a number of samples on a single TLC plate. The HPTLC allows the identification and the quantification of more than 20 samples in the same chromatography run. The analysis of the samples requires 15-30 min compared with more than 2 h using a typical HPLC method. Moreover, there is no need for conditioning steps, as with HPLC and each analysis by HPTLC is less expensive[15]. Simplicity, specificity and sensitivity of newly developed method makes it the appropriate choice for monitoring vitexin and isovitexin content for standardization of raw materials at the time of formulating a preparation as well as for the quality control of finished product.

Conclusion

Vitexin and isovitexin were determined qualitatively and quantitatively by densitometric method and confirmed by chromatographic and spectral method. The proposed HPTLC method was found to be rapid, simple, specific, sensitive, precise and accurate. Thus this analytical procedure permits a fast and reliable determination of these drugs in pharmaceutical dosage forms and can be used for routine analysis.

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