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IMPROVED AND RAPID HPLC-PDA METHOD FOR IDENTIFICATION AND QUANTIFICATION OF SWERTIAMARIN IN THE AERIAL PARTS OF *ENICOSTEMMA AXILLARE*

VIRENDRA SINGH RANA, TUSHAR DHANANI AND SATYANSHU KUMAR*
Directorate of Medicinal and Aromatic Plants Research
(Indian Council of Agricultural Research), Boriavi-387 310, Anand, Gujarat, India

A simple, improved, rapid and precise, reverse phase high performance liquid chromatography-photo diode array (HPLC-PDA) method has been developed and validated for the separation, identification and quantification of swertiamarin in aerial parts of Enicostemma axillare. The highest (38.12±1.74%) amount of swertiamarin was found in cold water and the lowest amount (20.13±0.84%) in hot water extracts obtained by using two different solvents. The separation of swertiamarin was achieved on a C_{18} column using the solvent system consisting of a mixture of methanol-water (1:1) as a mobile phase in a gradient flow elution mode followed by UV detection at 238 nm. The highest amount of swertiamarin was detected at 238 nm instead of detection at 227 nm and 254 nm. The developed method was validated using International Conference on Harmonisation (ICH) guidelines. Calibration curves offered good linear regression (r^2>0.998) within the test ranges. In intraday assay, the maximum relative standard deviation (RSD; %) values were found to be 0.12, 0.1 and 1.59 for low (6 µg/mL), medium (20 µg/ mL and high (60 µg/mL) concentrations of swertiamarin. In interday assay, the maximum RSD (%) values were found to be 2.68, 2.71 and 2.88 for low, medium and high concentrations, respectively. Limit of detection (LOD) and limit of quantification (LOQ) were calculated to be 4 µg/mL and 6 µg/mL whereas analytical recovery ranged from 95.80%–101.76%.

Keywords: *Gentianaceae*, *Enicostemma axillare,* Swertiamarin, HPLC-PDA

INTRODUCTION

Enicostemma Blume (Family: Gentianaceae) is a genus of four species found in the Madagascar, tropical America, tropical Africa and Asia (Kirtikar and Basu 1994). *Enicostemma axillare* Lam. Raynal (Syn. *E. littorale* Blume), a perennial glabrous herb , 10–50 cm high, is distributed throughout India up to 1500 ft from Panjub and Gangetic plain to Ceylon, found more frequently near the sea (Hooker 1997; Chopra, Nayar and Choppra 1956). The plant is bitter and used for curing of fever, snake bite, vata diseases and as tonic, antihelmintic, stomachic, laxative and hypolipidemic (Vasu *et al*. 2005; Gopal *et al*. 2004; Kirtikar and Basu 1994; Chopra, Nayar and Choppra 1956). Powdered plant is given with honey as a blood purifier and to help cure dropsy, rheumatism, abdominal ulcers, hernia, swellings, itches and insect poisoning (Chopra, Nayar and Choppra 1956). Extracts of *E. axillare* have been reported to have antidiabetic (Jaishree, Badami and Bhojraj 2008; Rafeeuddin *et al*. 2004; Srinivasan, Padmanabhan and Prince 2005; Maroo, Vasu and Gupta

*Corresponding author: Satyanshu Kumar, email: satyanshu66@gmail.com

2003; Murali, Upadhyaya and Goyal 2002; Upadhyaya and Goyal 2004), antioxidant (Vishwakarma *et al*. 2003), hepatoprotective (Baranisrinivasan *et al*. 2009) and antiinflammatory (Sadique *et al*. 1987) activities. *E. axillare* was found effective against Dalton's ascetic lymphoma (Kavimani and Manissenthilkumar 2002) and microbes (Deore *et al*. 2008; Patel and Trivedi 1995).

Swertiamarin (Fig. 1) showed antiediematogenic, antioxidant, hepatoprotective (Jaishree and Badami 2009, 2010), antiinflammatory (Jaishree, Badami and Bhojraj 2008), antinociceptive (Jaishree *et al.* 2009) and antihyperlipidimic (Vaidya *et al.* 2009) activities. Many workers have isolated swertiamarin (Jiang *et al.* 2005; Vishwakarma, Rajani and Goyal 2004; Anwar *et al.* 1996; Rai and Thakar 1966) from *E. axillare*. Gentianine, betulin (Rai and Thakar 1966), erythrocentaurin (Jiang *et al*. 2005), enicoflavine (Ghosal *et al.* 1974), apigenin, genkwanin, isovitexin, swertisin, saponarin, 5-O-glucosylswertisin and 5-O-glucosylisoswertisin (Chaudhuri, Singh and Ghosal 1975) have also been isolated. Swertiamarin content in its crude extracts and herbal formulations has been analysed by HPLC-UV method (Alam *et al.* 2009; Jun *et al.* 2008; Bhandari *et al.* 2006). Earlier report on the separation and quantification of swertiamarin by HPLC showed that the methods are time consuming varying from 12–40 min. Thus it was worthwhile to develop a rapid, method for separation, identification, quantification of swertiamarin in the aerial parts of *E. axillare*.

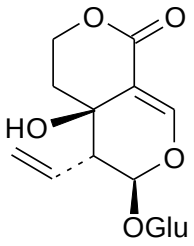


Fig. 1: Structure of swertiamarin.

METHODS

Plant Materials and Chemicals

The aerial parts of *E. axillare* were collected from the experimental field of Directorate of Medicinal and Aromatic Plants Research, Anand, Gujarat in 2010. Swertiamarin was isolated from its aerial parts and identified by nuclear magnetic resonance (NMR) and mass spectrometric (MS) data. Isolated swertiamarin (purity >95.0%), HPLC grade methanol (Merck, Mumbai) and water were used for the analysis.

Preparation of Samples

The aerial parts of *E. axillare* were washed with water, dried in shade and finely powdered using an electric grinder. Powdered sample (5 g) was extracted with methanol (50 mL X3, HPLC grade) for 24 hrs at room temperature. The hot methanol and water extracts were prepared by refluxing the material (5 g, each) with HPLC grade water and methanol

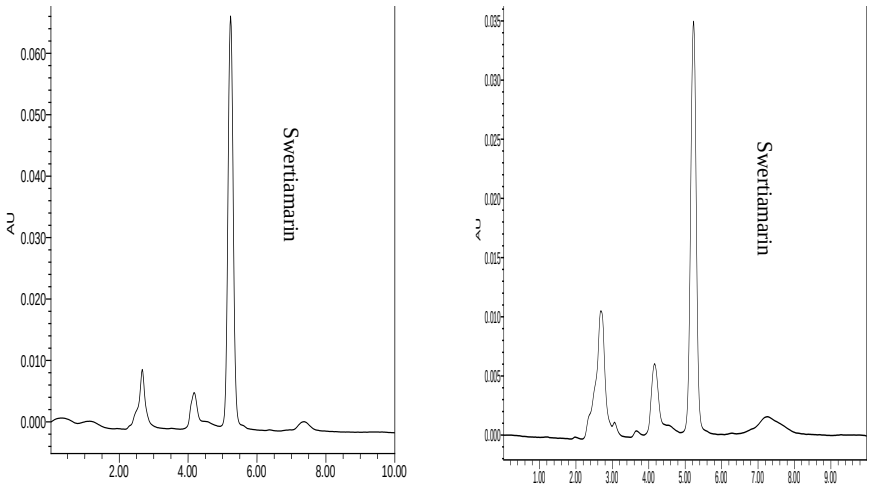
(50 mL X3) on boiling water bath for 1 hr. Solvents from the extracts were removed under reduced pressure and extracts were dried. All the extracts obtained were further dried in vacuum desiccator containing anhydrous calcium chloride for three days, prior to the analysis. The yields of the extracts prepared using cold (room temperature) percolation with methanol and water and hot (refluxing) methanol and water were found to be 45.8%, 41.5%, 44.0% and 43.6%. Working sample solutions (1.0 mg/mL) of each dried extracts were prepared by dissolving in methanol and serial dilutions from the stock solutions were prepared up to 10 µg/mL and stored at 4°C–8°C in refrigerator for analysis.

Preparation of Standard Solutions

The standard stock solutions (1.0 mg/mL) of swertiamarin were prepared in methanol and stored at 4°C in refrigerator. Working solutions of lower concentration were prepared by appropriate dilution of the stock solution in the range 10–1000 µg/mL in methanol.

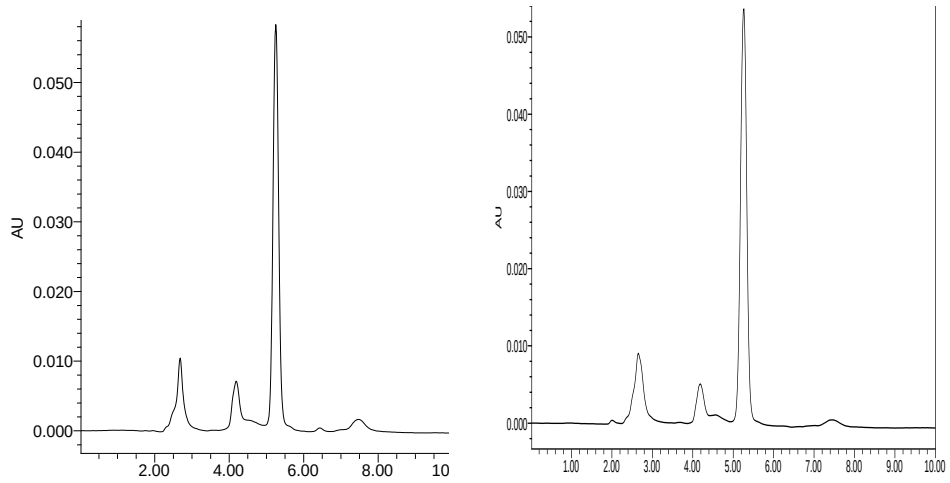
HPLC Analysis

HPLC analysis was carried out by using Waters 600 E pump (Milford, MA, USA) attached to Waters 2996 photodiode array detector and data were analysed using Empower software (Waters, USA). The mobile phase was filtered through a 0.45 µm membrane filter in solvent filtration apparatus (Millipore, USA). Samples for HPLC analysis were also filtered through a 0.45 µm membrane filter (Waters, USA). Analysis of the samples were carried out in three reverse phase LC columns, Symmetry C18 (250 × 4.6 mm, 5 µm; Waters, USA), X Terra (250 × 4.6 mm, 5 µm. Waters, USA) and Lichro CART® 250-4 C_{18} column (250 × 4.6 mm, 5 µm; Merck, Germany) for the optimal separation using acetonitrile-water (1:1) and water-methanol (1:1) as mobile phase in both isocratic as well as gradient elution using PDA detector at λ 227 nm, 254 nm and 238 nm. Column Lichro CART® 250-4 C_{18} column and methanol-water (1:1) were found to be better for separation, peak shape and resolution at UV 238 nm and thus used for further analysis. The optimised chromatographic condition is as follows: flow gradient started with solvent A (flow rate of 0.5 mL/min). At 4.9 minute, the flow rate was increased to 0.8 mL/min. The flow rate was then increased to 1.0 mL/min at 5.1 min. The flow rate was kept constant up to 5.4 min and then finally decreased to 0.5 mL/min, restoring the initial conditions at 8 min. Detector's wavelength was 238 nm and the injection volume was 10 µL. The best base line separation (peak purity >95%) for swertiamarin was achieved at λ 238 nm instead of λ 227 nm and 254 nm as reported earlier (Alam *et al.* 2009; Jun *et al.* 2008; Bhandari *et al.* 2006). Swertiamarin was found to show maximum absorption at λ_{max} 238 nm in three-dimensional ultraviolet absorption spectra using photo diode array (PDA) detector. Chromatograms of different extracts and swertiamarin analysed have been given in Figures 2 and 3. Mean retention time was of 5.6 min (Table 1).



(a) (b)

Fig. 2: HPLC chromatograms of cold (a) and hot (b) water extracts of the aerial parts of *E. axillare*.



(a) (b)

Fig. 3: HPLC chromatograms of cold (a) and hot (b) methanol extracts of the aerial parts of *E. axillare*.

Table 1: Method validation data for determination of swertiamarin by HPLC.

Parameter	Value
Mean retention time	5.26 min (RSD 0.5%)
Linear range	4–80 µg/mL
Regression equation	Y = 18000X – 1380
Regression coefficient (R^2)	0.998
Limit of detection (LOD)	4 µg/mL
Limit of quantification (LOQ)	6 µg/mL

RESULTS

Validation of HPLC Method

Selectivity

The selectivity of the method was determined by analysis of standard swertiamarin and samples. The peaks of swertiamarin in the sample solution were identified by comparing its retention time with the peak of the standard. Peak purity for swertiamarin (>95.0%) was assessed by comparing its peak at three different points, i.e. the peak start, peak apex and peak end position.

Linearity

As the linear range of analytical methods is known to be limited, linearity of the calibration curve prepared and its working range were determined. Linearity was determined based on detector's response of the different concentrations of standard used for calibration curve. Regression analysis was used to assess the linearity of the developed HPLC method using the equation, y = bx + c, where, x corresponds to the concentration of the standard solution (µg/mL), y to the peak area, b is slope of the line and c is the intercept of the straight line with y axis. In the present study, linearity was studied in the concentration range of 4–80 µg/mL of the standard solution of swertiamarin (Table 1). Calibration curve (Fig. 4) was linear in this concentration range and showed good linear regression (Y = 18000X – 1380, R^2 = 0.998).

Limits of Detection (LOD) and Limits of Quantification (LOQ)

The lowest concentration of analyte detectable and quantifiable with a stated degree of reliability is one of the many important parameters of any analytical method. LOD is the lowest amount of analyte in a sample that can be detected but not necessarily quantified. LOQ is defined as the lowest concentration, which can be reproducibly quantified above baseline level, typically an S/N = 10 or above. The LOD and LOQ for the present HPLC method developed were measured as per guidelines described by the International Conference on Harmonisation (ICH 1996). LOD and LOQ were found to be 4 and 6 µg/mL, respectively (Table 1).

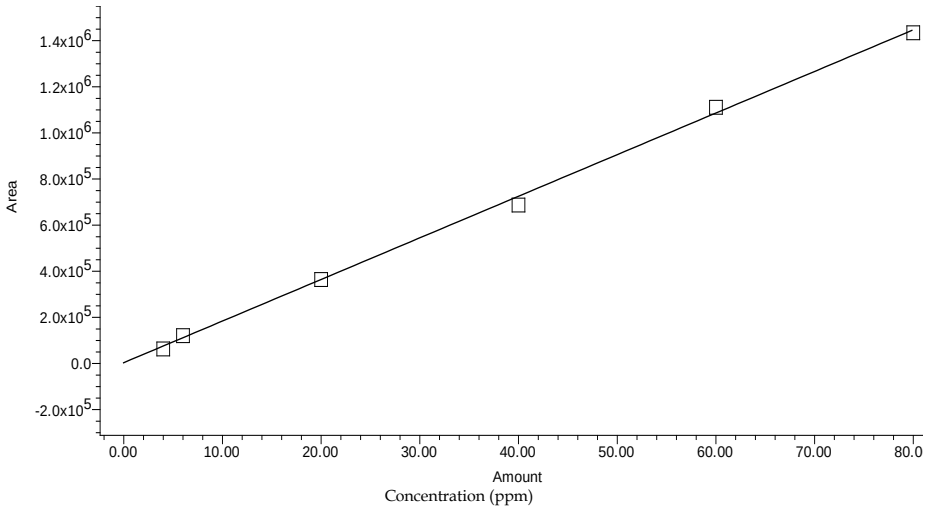


Figure 4: Calibration curve of the different concentrations (ppm) of swertiamarin (regression equation, Y = 18000X – 1380, R^2 = 0.998).

Precision

Intraday precision (repeatability) was evaluated for three concentrations of the standard with duplicate injections during the same day, under the same experimental conditions. Inter day precision was measured for the same three concentrations during three different days. Intraday precision of the analytes were in the range of 0.12%–1.59% and the inter day precision varied from 2.68%–2.88% (Table 2). The specificity of the method was examined by analysing blank sample and blank extract spiked with a known concentrations swertiamarin. No interference of the retention times of the analyte was observed.

Table 2: Precision (RSD) of the developed HPLC method at three different concentration of swertiamarin.

Concentration (µg/mL)	RSD (%)	
	Intraday	Interday
Low (6)	0.12	2.68
Medium (20)	0.10	2.71
High (60)	1.59	2.88

Accuracy and Recovery

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value and was determined based on the recovery of known amounts of analytes. Analytical and extraction recovery were performed by analysing analytes by spiking 10 µg/mL of the standard in blank extract for 6 different days. The recovery

ranged from 85.80% to 95.0%. Each determination was performed in triplicate. The quantity of swertiamarin in different extracts was calculated from the peak area of the chromatograms of each extract and is summarised (Table 3).

Table 3: Swertiamarin content (mean±SD) in different extracts of the aerial parts.

Sr. No.	Extract	Amount (%)
1	Cold methanol	34.0±1.92
2	Hot methanol	30.76±1.41
3	Cold water	38.12±1.74
4	Hot water	20.13±0.84

DISCUSSION

The aerial part of *E. axillare* is known to possess potent medicinal value and used in the treatment of many diseases in India. Swertiamarin, a seco-iridoid (Fig. 1) has also been identified as a major compound in its aerial parts and found to be a bioactive constituent. The yields of the cold (room temperature) methanol and water extraction of the aerial parts were found to be 45.8% and 41.5% and that of hot methanol and water extraction were 44.0% and 43.6%. The amount of swertiamarin was determined in the four different crude extracts by using water: methanol (1:1) as the mobile phase in a flow gradient mode using RP-18 column to establish a better extraction method with a higher yield of the compound of interest. In this method, a peak of swertiamarin in HPLC chromatogram appeared at 5.6 min (Figs. 2 and 3). The best base line separation and quantification of swertiamarin in crude samples were achieved at 238 nm instead of λ 227 nm and 254 nm as reported earlier (Alam *et al.* 2009; Bhandari *et al.* 2006). Also, swertiamarin showed maximum absorption at λ_{max} 238 nm in three-dimensional ultraviolet absorption spectra using PDA detector (Fig. 5). Cold water and methanol extracts were found to contain a higher amount (38.12±1.74% and 34.0±1.92%) of swertiamarin as compared to hot methanol and water extracts (30.76±1.41% and 20.13±0.84%). Lesser amount of swertiamarin in hot water and methanol extracts may be due to its degradation.

CONCLUSION

The development of a simple, rapid and precise HPLC method for the separation, identification and quantification of main bioactive in the presence of other compounds has always been a matter of interest. Swertiamarin has been identified as a major compound in the aerial parts of *E. axillare* and has been found to possess promising hepatoprotective, antiinflammatory, antinociceptive and antihyperlipidimic activities. A simple, rapid and precise HPLC method for the estimation of a bioactive swertiamarin in the aerial parts has been developed and validated. Quantification of swertiamarin at λ_{max} 238 nm is found to be better as compared to the wavelengths at λ_{max} 227 nm and 254 nm. This improved and rapid method can also be used for the qualitative analysis of swertiamarin in its raw extracts, drug and formulations.

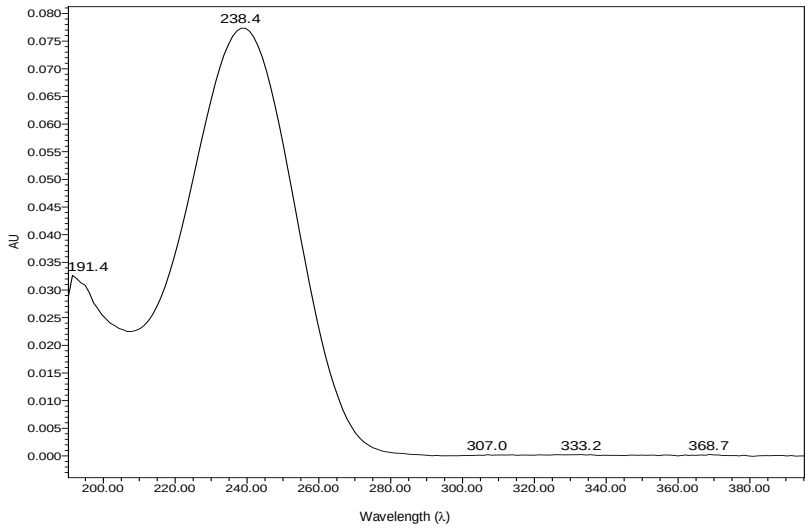


Fig. 5: Absorbance of swertiamarin in UV spectrum using PDA detector.

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