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SHORT COMMUNICATION

Analysis of scopoletin and mangiferin in botanicals and formulations of Shankhpushpi by HPLC

ALOK NAHATA[1, 2*], NEERAJ K. SETHIYA[2], NEHA JAIN[2], VINOD KUMAR DIXIT[2]

[1]Research and Development Manager
Ying Zhi Agricultural and Industries Sdn Bhd
Jitra 06000
Kedah Darulaman, Malaysia

[2]Department of Pharmaceutical Sciences
Doctor HS Gour Central University
Sagar-470003
Madhya Pradesh, India

*corresponding author: e-mail: aloknahata@gmail.com

Summary

Introduction: Shankhpushpi has been widely used in traditional Indian systems of medicine as a brain and memory boosting tonic. There are a variety of botanicals reported to be used as sources of Shankhpushpi in various parts of India. For instance, *Canscora decussata* Schult, *Clitorea ternatea* Linn., *Convolvulus pluricaulis* Choisy. and *Evolvulus alsinoides* Linn. are most commonly used as sources of Shankhpushpi by practitioners of Ayurveda in different parts of the country.

Objective: When it comes to using Shankhpushpi in herbal formulations, qualitative and quantitative analysis of the correct botanicals in the formulation decides its pharmacological effectiveness. Scopoletin and mangiferin are proven bioactive markers identified in Shankhpushpi botanicals in our previous studies. Hence the study is aimed at providing a simple analytical method for the identification of the correct variety of Shankhpushpi using proven markers.

Methods: In this study, a High Performance Liquid Chromatographic (HPLC) method has been developed for the estimation of scopoletin and mangiferin levels in four botanicals of Shankhpushpi and their marketed formulations.

Result: A simple analytical method was developed which proved to be very crucial in estimating concentrations of mangiferin and scopoletin in various test samples. This method can be used to identify the correct botanicals of Shankhpushpi present in any Ayurvedic formulation or raw material or processed powder by evaluating the content of scopoletin or mangiferin as markers.

Conclusion: The developed HPLC method is a quick and reliable method for the quantitative monitoring of mangiferin and scopoletin in herbal extracts and marketed formulations of Shankhpushpi.

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Key words: ***scopoletin, mangiferin, Canscora decussata, Clitorea ternatea, Convolvulus pluricaulis, Evolvulus alsinoides***

Słowa kluczowe: ***skopoletyna, mangiferyna, Canscora decussata, Clitorea ternatea, Convolvulus pluricaulis, Evolvulus alsinoides***

INTRODUCTION

The development and validation of proper analytical methods for formulations containing botanicals are the current healthcare professional demands in order to provide safe and effective treatment [1]. This has led to the production of phyto-pharmaceuticals from standardised botanical extracts from properly identified and controlled plant species [2]. Mostly herbal and Ayurvedic products are designed by combining the therapeutic effects of two or more drugs in one product to meet previously unmet patient needs [3]. Such products are always challenging in terms of maintaining proper quality through sophisticated validated analytical methods [4, 5].

Shankhpushpi is an official drug of Ayurveda and is regularly used as a source of raw material in the pharmaceutical industry for the development of Ayurvedic and herbal formulations (bhasma, bati, syrup and tablets) responsible for enhancing memory and intellect. Many previous studies have identified and discussed *Canscora decussata* Schult. (CD) (*Gentianaceae*), *Clitorea ternatea* Linn. (CT) (*Leguminoseae*), *Convolvulus pluricaulis* Choisy. (CP) (*Convolvulaceae*) and *Evolvulus alsinoides* Linn. (EA) (*Convolvulaceae*) as botanical sources of Shankhpushpi [6–8]. It was established in many previous experiments that mangiferin (a xanthone) and scopoletin (a coumarin) are the bioactive markers of Shankhpushpi and have a curative effect in memory dysfunction [9–11]. Phytochemical analysis of Shankhpushpi revealed the presence of scopoletin in CD, CT, CP and EA, while mangiferin is in CD [12].

Among analytical methods, some of the techniques which have been used include spectrophotometry [13], colorimetry [14], spectrofluorimetry [15, 16], liquid chromatography–mass spectrometry (LC–MS) and high-performance liquid chromatography (HPLC) methods [17-19] for the analysis of mangiferin and scopoletin. Using these analytical methods, mangiferin and scopoletin were analysed either alone or in combination with one or two other secondary plant metabolites, but attempts for simultaneous quantitative analysis of both by HPLC in a herbal drug and formulation to establish a distinct chemical profiling of Shankhpushpi are not available. In the last few decades, HPLC has emerged as an efficient tool for the quantitative analysis of different compounds found within complex natural samples [20]. Keeping in mind the utility of Shankhpushpi, and the lack of an appropriate simple HPLC method for the separation of mangiferin and scopoletin, it was proposed to develop a routine method of analysis for the qualitative and quantitative estimation of mangiferin and scopoletin (fig. 1) in botanicals and formulations of Shankhpushpi by HPLC.

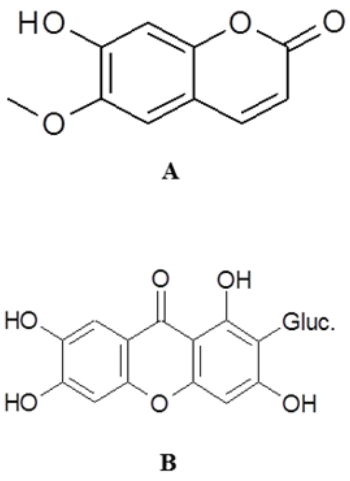

Figure 1
Chemical structures of (A) scopoletin, (B) mangiferin

MATERIAL AND METHODS

The instrument

The Shimadzu LC-10 AT VP HPLC system was used and includes a solvent reservoir, membrane degasser, binary pump, sampler, column, photodiode array detector, and PC with software.

Chemicals and solvents

Scopoletin was used as a reference standard. It was

donated by Laila Impex Research Center, Vijayawada, Andhra Pradesh, India with purity stated as 99%. Mangiferin standard (>98%) was donated by Natural Remedies, Bangalore, Karnataka, India. Methanol and water used for the study were HPLC grade solvents from Merck. Glacial acetic acid for the study was purchased from Spectrochem Chemicals, Mumbai. The petroleum ether and ethanol used for extraction purposes were of an analytical grade.

Plant materials and extraction

Aerial parts of CT, CP and EA were collected from Dharmashree, Sagar (Madhya Pradesh, India) and aerial parts of CD were collected from the outskirts of Raipur (Chhattisgarh, India). The plants were authenticated by Dr Pradeep Tiwari, Department of Botany, Dr H.S. Gour University, Sagar (Madhya Pradesh, India). All four plants are deposited in the herbarium for future reference having accession numbers viz. *Canscora decussata* (Bot/Her/2409), *Clitoria ternatea* (Bot/Her/3251), *Convolvulus pluricaulis* (Bot/Her/1260) and *Evolvulus alsinoides* (Bot/Her/1272). Aerial parts of CD, CT, CP and EA were shade-dried at room temperature. The shade-dried plant materials were coarsely powdered and subjected to extraction with petroleum ether in a Soxhlet apparatus. The defatted marc of all the drugs was subjected to ethanol (95%) extraction.

Method development

Mobile phase selection

A number of mobile phases in different ratios were tried for the separation of standard mangiferin, scopoletin and ethanol extracts of the four botanicals of Shankhpushpi. The mobile phase most suitable for analysis was found to be methanol-water-glacial acetic acid (26:55:0.5 *v/v*). Flow rate was 1.0 ml/min.

Selection of separation variable

Considering the theoretical information and after several trials, separation variables were selected which were constant during the whole experiment. All these variables are shown in table 1.

Preparation of standard solution for scopoletin and mangiferin

10 mg of mangiferin/scopoletin was weighed accurately and transferred to a 10 ml volumetric flask

Table 1.

Selection of analytical variables

Variable	Condition
Column	
Dimension	250 mm x 4.60 mm
Particle size	5 μl
Bonded phase	octadecylsilane (C18)
Mobile phase	
Glacial acetic acid (HPLC)	0.5%
Methanol (HPLC)	26%
Deionised water (HPLC)	55%
Flow rate	**1.0 ml/min**
Temperature	**40°C**
Sample size	**25** μl
Detection wavelength	
Scopoletin	345 nm
Mangiferin	254 nm
Retention time	
Scopoletin	16.35 min
Mangiferin	3.46 min

and dissolved in methanol. 1 ml was taken and further diluted to 10 ml with methanol. The concentration of the stock solution was 100 μg/ml. This stock solution was used to prepare the required dilutions containing 5–100 μg/ml of solution of mangiferin/scopoletin. To find the linearity, a series of dilutions ranging from 5 to 50 g/ml for mangiferin and scopoletin were prepared in the same manner as described above. All the solutions were filtered through a 0.45 μm syringe filter and 25 μl injected. The chromatograms were recorded. A standard curve was plotted between the areas under curve (AUC) versus respective concentrations. The chromatograms of mangiferin and scopoletin were observed at 16.35 min and 3.46 min, at 254 nm and 345 nm, respectively.

Preparation of samples from the extracts

10 mg of all four extracts viz. CD, CT, CP and EA were weighed accurately and transferred to 10 ml volumetric flasks and dissolved in methanol, 1 ml of this was taken and diluted to 10 ml with methanol. The concentration of the stock solution was 100 g/ml. 1 ml of these solutions was again diluted to 10 ml with methanol. Hence 10 μg/ml solutions of all extracts were prepared. All solutions were filtered through a 0.45 μm syringe filter and a 25 μl sample was injected. The chromatograms were recorded. A calibration curve was plotted between the area under curve (AUC) vs respective concentrations.

Marketed formulations of Shankhpushpi

Many formulations containing Shankhpushpi as a single drug or in combination with other drugs are available in the Indian market and Shankhpushpi is enthusiastically advertised for memory enhancement in print and electronic media in India. To minimise batch variations and to add scientific validity to herbal formulations, it is necessary that, like modern drugs, herbal drugs should also be analysed and proper quality control techniques developed to verify the quality and quantity of the herbs added to the formulations. Various formulations of Shankhpushpi available in the market from different manufacturers were selected for evaluation:

- Dabur Shankhpushpi
- Unjha Shankhpushpi
- Baidyanath Shankhpushpi

The above formulations contain Shankhpushpi (*Convulvulus pluricaulis)* and Brahmi (*Bacopa monnieri)* as major constituents along with other additives like sugar, citric acid, flavours and preservatives. This information is printed on their labels.

Preparation of samples from the formulations

1 ml of three marketed formulations viz. Dabur Shankhpushpi, Unjha Shankhpushpi and Baidayanath Shankhpushpi were measured accurately and transferred to 10 ml volumetric flasks and dissolved in the methanol. 1 ml of this solution was taken and diluted to 10 ml with methanol. The concentration of the stock solution was 100 μg/ml. 1 ml of these solutions was again diluted to 10 ml with methanol. 10 μg/ml solutions of all formulations were prepared. All solutions were filtered through a 0.45 μm syringe filter and 25 μl injected. The chromatograms were recorded. A calibration graph was plotted between the AUC vs respective concentrations. The regression equation was derived and the results were analysed.

Analytical method validation

Validation of linearity

Standard solutions (5 μg/ml to 50 μg/ml) were prepared in methanol and detected by HPLC. The standard curve was prepared by plotting concentration as abscissa *versus* AUC as ordinate. Linear dependence of AUC on concentration was observed throughout the concentration range tested.

Validation of precision and accuracy

The precision of the method was checked for standard solutions of the ethanol extracts of all four varieties and marketed preparations of Shankhpushpi at a concentration of 10 μg/ml prepared by appropriate dilution with methanol. The solution was analysed by HPLC and the AUC was recorded. The corresponding concentration was extrapolated from the standard curve. Then 1 μg/ml solutions of scopoletin in ethanol extract of CT, CP and EA marketed formulations and 1 μg/ml solutions of mangiferin and scopoletin, both in an ethanol extract of CD were prepared by appropriate dilution and analysed

by HPLC. The concentrations of mangiferin and scopoletin were calculated for the samples.

This solution of known concentration was added in equal volume (1 ml) to all the previous dilutions and analysed to see whether the practical concentration obtained corresponds with the theoretical or hypothetical concentration from the standard curve. Percentage recoveries were calculated on the basis of determination of analyte added to a sample containing a known amount of scopoletin and mangiferin.

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS AND DISCUSSION

The phytochemical investigations on CD, CT, CP and EA revealed the presence of scopoletin in CT, CP and EA, while both scopoletin and mangiferin were found in CD fluorescing under UV light. Mangiferin shows apricot yellow green fluorescence, while scopoletin shows intense blue fluorescence under UV light as reported earlier [10]. Thus, mangiferin was specific to CD, whereas scopoletin was present in all four varieties. These results paved the way for the development of analytical methods for the estimation of mangiferin and scopoletin in various varieties of Shankhpushpi.

Many studies discuss the advantages, precision and accuracy of the HPLC method for the analysis of herbal and Ayurvedic formulations consisting of more than one active herbal ingredient [20]. This method is economical and widely acceptable due to its rapidity, specificity, accuracy, precision and ease of automation which may be further extended for the use of routine quality control to ensure the identity, purity, potency and performance of drug products. The HPLC method also eliminates tedious extraction and isolation procedures.

The present work comprised of developing the validated HPLC method for the estimation of scopoletin in ethanol extracts of CD, CT, CP and EA and the estimation of mangiferin in CD. This was further utilised for the analysis of marketed formulations consisting of Shankhpushpi.

For the simultaneous estimation of scopoletin in CD, CT, CP and EA and mangiferin in CD by HPLC, we found the best solvent system in which both components showed better resolution is methanol-water-glacial acetic acid (26:55:0.5 *v/v*). The wavelength selected for analysis was 345 nm for scopoletin and 254 nm for mangiferin and the flow rate was adjusted at 1 ml/min in the whole experiment. Standard curves for scopoletin and mangiferin were prepared by the use of standard dilutions from 5 μg/ml to 50 μg/ml in both cases. The plots of concentration versus AUC exhibited a linear relationship. The equation for the straight line calculated for scopoletin was y=32616x (R^2=0.9934) and for mangiferin was y=4038.7x (R^2=0.9942) (fig. 2). It became feasible to estimate scopoletin in herbal extracts and marketed formulations by measuring the AUC with respect to concentration. The scopoletin content calculated from the standard curve was found to be 0.324±0.018, 0.252±0.053, 0.190±0.039, 0.062±0.023 μg/ml in CD, CT, CP and EA, respectively. The mangiferin content in CD calculated from the standard curve was found to be 0.632±0.47 μg/ml. All observations were done in triplicate (n=3). Results are shown in table 2. The order of scopoletin content in various varieties was found to be EA > CP > CT > CD.

Various marketed formulations of Shankhpushpi viz. Dabur, Unjha and Baidyanath were also standardised for this developed method of HPLC by using the same procedure. By this method we analysed that all three marketed formulations of Shankhpushpi contained only scopoletin. The amount of scopoletin in Dabur, Unjha, and Baidayanath Shankhpushpi was found to be 1.212±0.082,

Table 2.

Concentration of scopoletin and mangiferin in varieties of Shankhpushpi by HPLC

Variety of Shankhpushpi	Conc. [μg/ml]	AUC		Concentration of scopoletin from standard curve [μg/ml] Mean ± SEM; (n=3)	Concentration of mangiferin from standard curve [μg/ml] Mean ± SEM; (n=3)
		Scopoletin	Mangiferin		
Evolvulus alsinoides	10	86647	–	0.324±0.018	-
Convolvulus pluricaulis	10	84474	–	0.252±0.053	-
Clitoria ternatea	10	82589	–	0.190±0.039	-
Canscora decussata	10	78690	2553	0.062±0.023	0.632±0.47

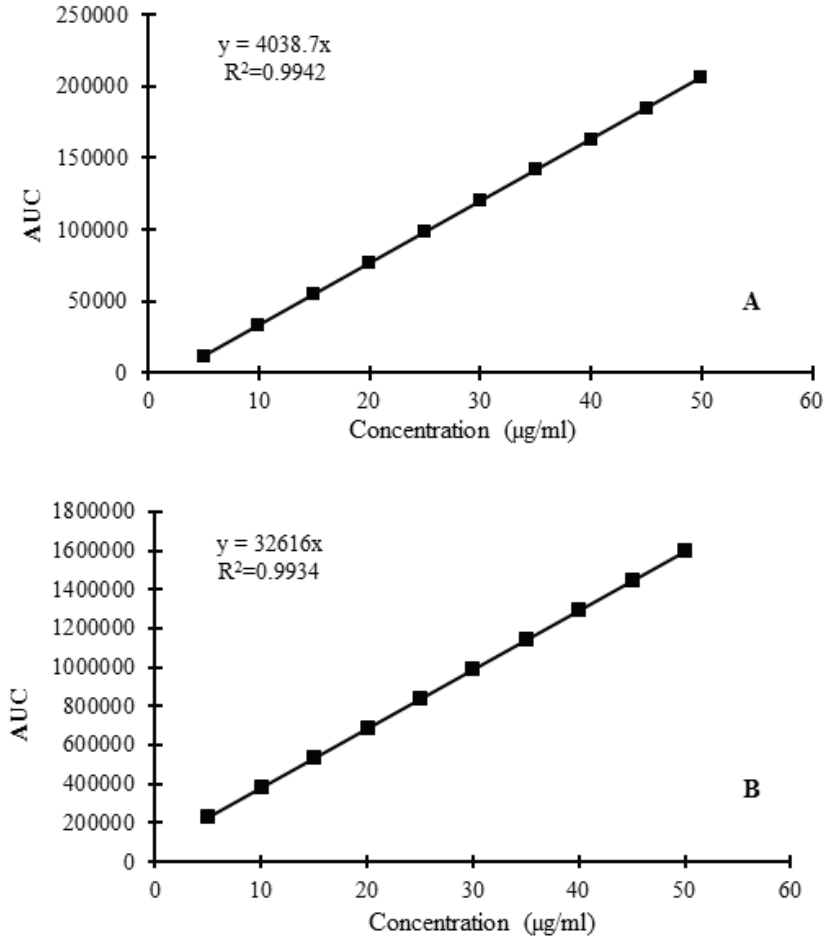

Figure 2.

Calibration plot: (A) mangiferin; (B) scopoletin

Table 3.

Concentration of scopoletin in marketed formulations by HPLC

Brand name	Conc. [μg/ml]	AUC	Concentration of scopoletin from standard curve [μg/ml] Mean ± SEM (n=3)
Dabur Shankhpushpi	10	113875	1.212±0.082
Unjha Shankhpushpi	10	132932	1.845±0.37
Baidyanath Shankhpushpi	10	103818	0.888±0.009

1.845±0.37 and 0.888±0.009 μg, respectively (tab. 3). Mangiferin was absent, suggesting the absence of CD in these formulations. The order of scopoletin content in various marketed formulations were found to be Unjha Shankhpushpi > Dabur Shankhpushpi > Baidayanath Shankhpushpi.

A simple analytical method was developed which proved to be very crucial in estimating the concentration of mangiferin and scopoletin in various test samples. The developed method was validated for linearity, reproducibility and accuracy. The linearity was found to be in the range of 5–50 μg/ml. The correlation coefficients (r^2) were 0.9934 for scopoletin in the cases of CD, CT, CP and EA and 0.9942 for mangiferin in the case of CD, indicating good linearity between concentrations versus the AUC. Scanning of the samples allowed the precision of the method to be checked. The reproducibility and accuracy of the method was checked by carrying out recovery studies. A known concentration of scopoletin was

added to known concentrations of the ethanol extracts of all four varieties, i.e. 10 μg/ml. A known concentration of mangiferin was added to known concentrations of the ethanol extract of CD. A sample of known concentration was added in equal volume to the extracts of various varieties and analysed by HPLC to see whether the observed concentration obtained corresponded to the theoretical concentration obtained from the standard curve. The percentage recovery of mangiferin and scopoletin was found to be in the range of 98–101%. The results are shown in tables 4 and 5.

Table 4.

Validation of the method and calculation of the percentage recovery of scopoletin

Extracts (10 μg/ml)	Scopoletin contained in extracts [μg/ml]	Total amount of scopoletin expected* [μg/ml]	Amount obtained [μg/ml]	Percentage recovery [%]
Clitoria ternatea	0.190±0.042	1.190±0.042	1.212±.0051	101.8±0.912
Convolvulus pluricaulis	0.252±0.027	1.252±0.027	1.261±0.030	100.7±0.301
Evolvulus alsinoides	0.324±0.022	1.324±0.022	1.321±0.026	99.8±0.419
Canscora decussata	0.062±0.085	1.062±0.085	1.049±0.076	98.7±0.916
Dabur Shankhpushpi	1.219±0.037	2.219±0.037	2.227±0.028	100.4±0.991
Baidyanath Shankhpushpi	0.888±0.042	1.888±0.042	1.893±0.041	100.2±0.128
Unjha Shankhpushpi	1.845±0.011	2.845±0.011	2.839±0.020	99.7±0.901

*Scopoletin added to the extract samples = 1 μg/ml

Table 5.

Validation of the method and calculation of the percentage recovery of mangiferin

Extract [10 μg/ml]	Mangiferin contained in extract [μg/ml]	Total amount of mangiferin expected* [μg/ml]	Amount obtained [μg/ml]	Percentage recovery [%]
Canscora decussata	3.769±0.008	4.769±0.008	4.737±0.013	99.3±0.041

*Mangiferin added to the extract samples = 1 μg/ml

CONCLUSION

The developed HPLC method is a quick and reliable method for the quantitative monitoring of mangiferin and scopoletin in herbal extracts and marketed formulations of Shankhpushpi. Our group already published similar results obtained by other methods for the estimation of mangiferin and scopoletin, such as TLC, HPTLC and spectrofluorimetry [2, 15, 16]. A quantitative evaluation of mangiferin and scopoletin in the four varieties and the presence of these markers in marketed formulations will further add value to marketed formulations of Shankhpushpi. We selected three well-known brands from the Indian market and analysed them for the presence of mangiferin and scopoletin for raw material assessment. These studies can help to control batch to batch variation and adulteration when selecting the raw material of Shankhpushpi in various formulations and help us to identify the exact variety which remains better in terms of quality and potency. Further, more precise analytical marker identification is needed in the future for differentiation among other herbs consisting similar phytochemicals.

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Conflict of interest: Authors declare no conflict of interest

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