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Influence of Seasonal Variation and particle size on Quantitative Determination of Bio-active Markers in *pluricaulis sp.*

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Abstract: *Convolvulus pluricaulis* (CP) is an indigenous perennial creeper herb in India used as a tonic in CharakSamhita that seems like morning glory. It showsvery important place in the Indian as well as Chineseherbaldrugindustrybecause, CP is one of the major ingredients in the marketed (>40)preparationsused for enhancingmemory and to treatvarious CNS disorders. The presence of coumarin component in CP i.e.scopoletin and scopolin has been foundto beresponsible for memoryenhancingactivity due to theiracetyl-cholinesteraseinhibitory action. The variation of seasonaffects the production of phyto-constituents and metabolites more thanthe otherfactors. The synthesis of chemicalmoietiesalso variesin accordance withoptimal conditions requiredatdiffernt duration of time period.In literature survey, *pluricaulis* shows maximum effect if it is collected in the month from April to August i.e. during its flowering season. Therefore, in order to find the scientific reason for collecting the plant in this season, CP was collected in two different seasons and markersquantity was determined. The modern powerful analytical technique called as planer chromatography, having separation power, performance and reproducibility superior to classic TLC method was utilized for quantitative determination of scopolin and scopoletin. Further, varient stress conditions can be investigated for both of the bio-active markers.

Keywords: Shankhpushpi, Coumarins, Hptlc, Season, Size-reduction.

1. Introduction

Convolvulus pluricaulis (CP) [syn. *Convolvulus prostrates* Forssk, *Convolvulus microphyllus* Sieb (Convolvulaceae)], commonly known as shankhpushpi, is therapeutically as whole plant reported to be helpful for improving the memory in Alzheimer's patient [1] and exhibitswide range of CNS depressant [2], hypnotic [3], antifungal [4] and antiulcerogenic activities [5-6].Worldwide, it has been utilized forthe treatment of various ailments like, liver, epileptic, microbial and viral disease, as hair tonic, brain tonic, memory enhancer, anxiolytic, anti-inflammatory, anti-oxidant,antistress, immunomodulatory, cytotoxic, hypotensive and hypolipidemic action in traditional systems [7-8].

The plant is reported to contain several constituents such as alkaloids (shankhpushpine), coumarins (scopoletin& scopolin), flavonoids (kaempferol), steroids (β-sitosterol), their glycosides [9] and fattyacids[10].

The purpose of current investigation was to determine optimum quantity of two bioactive markercontents in *pluricaulis*sp. in order to select the best season for collection of plant. The effects of twodifferent environmental seasons i.e. in the month of august and second in the December, labeled as sample 1 and 2 respectively, onthe extraction efficiency wereexamined. This led tothe way of development of a specific, cost effective and selective season for bio-active markers obtained from *pluricaulis*sp.

2. Experimental

2.1. Materials and Methods

Standard scopolin and scopoletin were purchased from Natural Remedies, Bangalore and Sigma Aldrich Chemicals Pvt. Ltd., New Delhi, India.The fresh plant material of CP collected fromRisaliaKhera, (Haryana) and was authenticated by Dr. H. B. Singh, (National Institute of Science Communication and Information Resources,vide certificate no. 2010-11/1540/138, New Delhi). Analytical grade solvents (Rankem C. Labs.,India) and distilled water,were utilized for study.

2.2. Instrumentation and chromatographic conditions

2.2.1. Sample application

The chromatographic analysis was performed with complete HPTLC system from CAMAG, Switzerland. The samples were spotted byLinomat V in the form of bands of 8 mm width, separated with 10 mm of distance from each other on the aluminium based pre-coated TLC plate of 0.2 mm thickness (20 × 10 cm) from (E. Merck, Germany). The constant automatic sample application rate (110 nLs^{-1}) was employed and the plates were developed for linear ascending development for scopolin and scopoletin, respectivelyupto a height of 90 mmin twin trough glass chamber.

2.2.2. Chromatographic condition for scopolin

The pre-saturated (10 min),twin trough glass chamber with optimized mobile phase (chloroform: methanol, 8.5:1.5 v/v) at room temperature (25 ± 2°C) with relative humidity (55 ± 5%) was used for scopolin determination.

2.2.3. Chromatographic condition for scopoletin

The pre-saturated (20 min), twin trough glass chamber with an optimized mobile phase (toluene: ether, 1:1 v/v saturated with 20% acetic acid) at room temperature (25 ± 2°C) with relative humidity (55 ± 5%) was utilized for scopoletindetermination [11].

After the development, spotted plates were dried in a current of warmair and scanned properly with the help of thin layer chromatographic scanner III. The spots of the markers (scopolin and scopoletin) were scanned in absorbance/remission mode at the maximawavelength of 340 nm.

2.3. Preparation of stock solution for bio-active markers

2.3.1.*Preparation of stock solution and calibration curve of scopolin*

A stock solution of standard scopolin (1 mg/mL) was prepared by dissolving accurately weighed 1 mg of scopolin in 1 mL methanol. The stock solution was further diluted with methanol to achieve the final concentration of 0.01 mg/mL of scopolin. The calibration curve for scopolin was investigated for its wide concentration range in order to cover the large variations in its concentration in the different extracts. Different volumes of final solution *viz*., 2, 4, 6, 8, 10, 12, 16 µL were spotted in triplicate on the TLC plate to obtain concentrations of 20, 40, 60, 80, 100, 120, 160 ng $spot^{-1}$ of scopolin.

2.3.2. *Preparation of stock solution and calibration curve of scopoletin*

A stock solution of standard scopoletin (1 mg/mL) was prepared by dissolving accurately weighed 1 mg of scopolin in 1 mL methanol. The stock solution was further diluted with methanol to achieve the final concentration of 0.02 mg/mL of scopoletin. Different volumes of final solution *viz*., 2, 4, 6, 8, 10, and 12 µL were spotted in triplicate on the TLC plate to obtain concentrations of 40, 80, 120, 160, 200 and 240 ng spot^{-1} of scopoletin.

The data of peak areas for both scopolin and scopoletin were plotted against the corresponding concentrations of the marker and treated by least-square regression analysis method.

2.4. Preparation of test solutions

2.4.1.*Preparation of test solution from crude drug*

The shade dried coarsely powdered whole plant parts, each contains accurately weighed 5 g of drug, were extracted with 50% hydro-alcoholfor sonication (0.75 h). The extracts were filtered and concentrated under vacuum (rota-evaporator), and the final volume was made up to 10 mL with solvent. The sample (10 µL) of stock solution was applied on the TLC plate, followed by development and scanning as described in the section 2.2. The analysis process was carried out in triplicate.

3. Result and discussion

3.1. Calibration curves

3.1.1. For Scopolin

The estimation of scopolin by the developed HPTLC method, showed a good correlation coefficient (r^2 = 0.9992 ± 0.0002) in the concentration range of 20–160 ng spot^{-1} with respect to the peak area. The linear regression analysis showed the mean value of slope and intercept 21.386 and 93.320, respectively (Fig. 1).

3.1.1. For Scopoletin

The estimation of scopoletin by the developed HPTLC method, showed a good correlation coefficient (r^2 = 0.9990 ± 0.0002) in the concentration range of 40–240 ng spot^{-1} with respect to the peak area. The linear regression analysis showed the mean value of slope and intercept 20.77 and 215.6, respectively (Fig. 2).

No significant difference was observed for scopolin and scopoletin in the slopes of standard curves (ANOVA, $P < 0.05$).

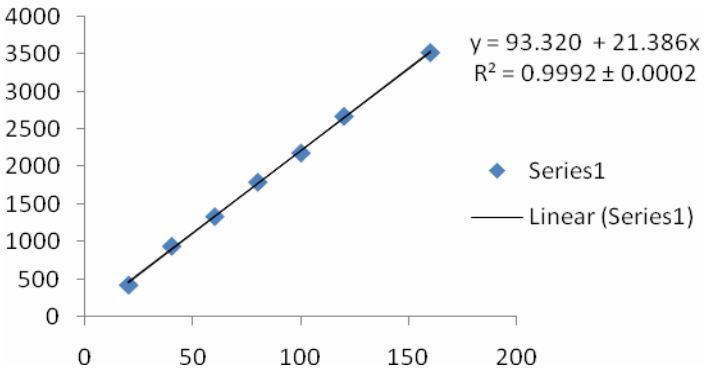

Fig.1. Calibration curve of standard scopolin at different concentrations

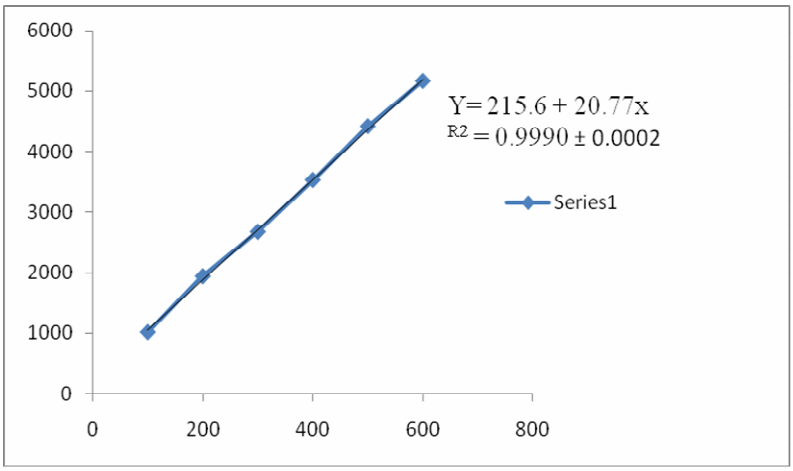

Fig.2. Calibration curve of standard scopoletin at different concentrations

4. Result & Discussion

4.1. Effect of Seasonal variation:

The whole plant of CP was collected in two separate seasons, one in that season which is recommended in Ayurveda i.e. between april- september, while another in the month of December. The drug sample was extracted with 50% hydro-alcoholic solution withthe sonication method (for 0.75 h). The results observed were contradictory to what is recommended in Ayurveda. The plant showed higher content of both the bioactive markers in December as mentioned in (Table 1), Scopolin alone attains a significant higher quantity0.0085% in the month of winter when compared to 0.0030% as per recommendedperiod.Though, the effect of seasonal variation was less on the scopoletin content but it was also slightly higher (0.023%) in winters as compared tosummer (0.022%).

4.2. Effect of Particle size:

The whole plant of CP was powdered and sieved separately for fine and very fine portions, analyzed for sonication (0.75 h). The sample shows an amount of scopolin 0.0013 and 0.0004 %, respectively (Table 2). In another sample scopoletin shows 0.0033 and 0.0017 % amount in fine and very fine plant (Table 3). This led to the estimation of scopolin and scopoletin contents more in the fine particle size as compared to very fine.

It is concluded from the above analysis that the scopolin contentin *pluricaulis* sp. was found to be more in winter season as compared to scopoletinwith minor effects on coarse as well as fine sized particles of whole plant. Therefore, seasonal variation has been of great influence during the collection of herbal drug. These results also reveals that, each phyto-constituent vary from season to season. It is better option for the drug collection as well as extraction apart from the rainy season and easy to identify its natural source. Furthermore, particle size study also demands more investigation with respect to stress conditions.

Table. 1: Quantitatve results of scopolin and scopoletin in two different samples (I, I I) of CP

Method	Particle Size mm	Time Period	Mean AUC	S.D. (%)	S.E.	% Scopolin (w/w)
Sonication	Fine (0.6)	0.75 h	1496	4.38	2.53	0.0013
	Very Fine (0.12)	0.75 h	542	4.43	2.56	0.0004

Table. 2: Analysis results of two different particle size in scopolin without any dilution.

Method	Particle Size mm	Time Period	Mean AUC	S.D. (%)	S.E.	% Scopolin (w/w)
Sonication	Fine 0.6	0.75 h	3693	1.103	0.637	0.0033
	Very Fine 0.12	0.75 h	2001	0.480	0.277	0.0017

Table 3: Analysis results of two different particle size in scopoletin without any dilution.

Marker	Time of collection	Mean AUC	S.D. (%)	S.E.	% Content (w/w)
Scopolin	Sample I	1647*	1.88	1.09	0.0030
	Sample II	1909**	1.71	0.99	0.0085
Scopoletin	Sample I	2487#	1.14	0.66	0.022
	Sample II	2601#	1.70	0.98	0.023

*Sample diluted two times; **Sample diluted five times;
Sample diluted ten times with the respective solvent

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