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Estimation of Scopoletin in Leaf and Leaf Callus of *Convolvulus microphyllus* Sieb.

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The leaf callus of *Convolvulus microphyllus* was initiated and developed in Murashige and Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid, 6-benzyl adenine, indole acetic acid and kinetin (1 ppm each). High pressure liquid chromatographic method using UV detector has been developed for the estimation of scopoletin in methanolic extracts of leaf and its calli. The method is simple, precise, accurate, specific and less time consuming for the estimation of scopoletin in crude drugs.

Convolvulus microphyllus Sieb (Convolvulaceae) is commonly known as *Shankhpushpi*, a shrub widely distributed in India (tropical and temperate regions), Sindh, Sudan and Egypt[1]. The whole plant is used in traditional system of medicine as brain tonic[2] and reported to be useful in CNS disorders[3], hypertension[4], thyrotoxicosis[5], ulcers[6], antibacterial and antifungal activity[7]. The whole plant extract was found to contain alkaloids[8], scopoletin[9], phenolics and flavonoids[10]. Since, the tissue culture work and production of secondary metabolites by callus cultures of *Convolvulus microphyllus* has not been reported, the aim of present work was to develop a methodology for the initiation and development of leaf callus of the plant in addition to propose a simple and accurate method for determination of scopoletin in the crude drug and its callus cultures.

MATERIALS AND METHODS

High pressure liquid chromatograph (Shimadzu HPLC Class VP series) with quaternary LC-10A VP pumps, variable wavelength programmable UV/VIS detector SPD-10AVP column oven (Shimadzu), CLC (M) system controller (Shimadzu) and RP C-18 column (150 mm *4.6 mm, ID; particle size 5 μm) was used.

Plant leaves were collected from herbal garden of Hamdard University New Delhi during the period of December–January. The plant was identified by the National Institute of Science and Communication (NISCOM) Pusa, New Delhi. Voucher specimens have been kept at herbarium of the university.

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Initiation and maintenance of callus:

The explants were sterilized using mercuric chloride (0.6%) for 10 min followed by washing with double distilled sterile water. The surface sterilized explants were inoculated on Murashige and Skoog (MS) medium[11] supplemented with various growth hormones. The leaf callus was initiated, developed and maintained for 3 mo on MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D)+6-benzyl adenine (6-BA)+indole acetic acid (IAA)+Kinetin (1 ppm each) at 25$\pm$2° under 16 h diffused light (1600 lux)/8 h darkness cycle.

Qualitative phytochemical analysis:

The aqueous alcoholic extracts of leaf and leaf callus were prepared and tested for the presence/absence of alkaloid, glycoside, phenolics, flavonoid, coumarin, steroid, saponin and anthraquinone by using standard qualitative chemical tests[12].

Sample preparation:

The plant leaves were dried at 60° in an oven. One gram each of dried leaves powder (fresh 60 d and 120 d old

leaf callus) was extracted with 20 ml methanol at 60° for 30 min and filtered. The filtrate was taken in 25 ml volumetric flask and made up the volume with methanol.

Standards:

Scopoletin was isolated by PTLC using toluene: ether (1:1) saturated with acetic acid in our laboratory from whole plant extract of *Convolvulus microphyllus*[13]. The compound was crystallized two times prior to use. The purity of the standard was checked by paper chromatography in two different solvents[14,15] and by UV spectroscopy[16]. Under in-house conditions the retention time of scopoletin was found to be 1.8 min.

Chromatographic conditions:

A mixture of 30% methanol in water with 5% acetic acid[17] was used as mobile phase and filtered before use through 0.45 µm membrane filter. The flow rate of mobile phase was maintained at 1.5 ml/min. The column temperature was maintained at 40°. The UV detector was set at 340 nm wavelength and injection volume was 20 µl.

Calibration curve of scopoletin:

Stock solution (1 mg/ml) of standard scopoletin isolated by preparative TLC was prepared in HPLC grade methanol. Subsequent dilutions of this solution were made to get the concentrations of 100, 200, 500, 1000 and 2000 ng/ml of the standard scopoletin. The standard solutions prepared as above were injected in triplicate into the column. The average of peak area of standard drug was calculated and the regression of the standard drug concentration over the average peak area was obtained. This regression equation was used to estimate the amount of scopoletin in leaf and its calli of *Convolvulus microphyllus*. The solutions of scopoletin containing 100 ng/ml and 200 ng/ml were subjected to proposed HPLC method of analysis for recovery studies by adding known amount of scopoletin to preanalyzed samples. The intra and inter day variations were also studied to find out precision of the proposed method.

Estimation of scopoletin in leaf and leaf callus:

One ml of each of methanolic extracts of leaf powder and leaf calli were taken separately in 100 ml volumetric flasks and made up the volume with methanol. Further 1 ml of the solutions were diluted to 10 ml with methanol and filtered through 0.45 µm membrane filter. Twenty microlitres of this solution was injected in triplicate into the column. The mean values of peak areas of leaf and leaf calli extracts of five such determinations were calculated and the content of scopoletin was quantified using the regression equation obtained from calibration curve.

RESULTS AND DISCUSSION

The leaf callus of *Convolvulus microphyllus* was successfully initiated, developed and maintained on MS medium supplemented with 2,4-D, 6-BA, IAA and kinetin (1 ppm each). Preliminary phytochemical screening of the aqueous alcoholic extract of leaf and leaf callus revealed the presence of alkaloid, amino acid, ascorbic acid, coumarin, carbohydrate, phenolics, protein, saponin and steroid. Flavonoids are present only in leaf while absent in case of leaf callus. Anthraquinones were found absent in both leaves as well as in leaf callus extracts.

The present study was carried out to develop a simple, accurate, precise, specific and rapid HPLC method for the estimation of scopoletin. The retention time for the standard scopoletin was 1.8 min as shown in fig. 1. The column pressure varied from 180 to 200 Kgf/cm^2. Each of the samples was injected 3 times and the same retention times were observed in all the cases. The peak areas of standard scopoletin for different concentrations were calculated and the average value of five such determinations was plotted against concentrations of standard scopoletin.

The peak area of the drug was found to be reproducible as indicated by low percentage RSD (2.15). A good linear relationship (r=0.9998) was observed between the

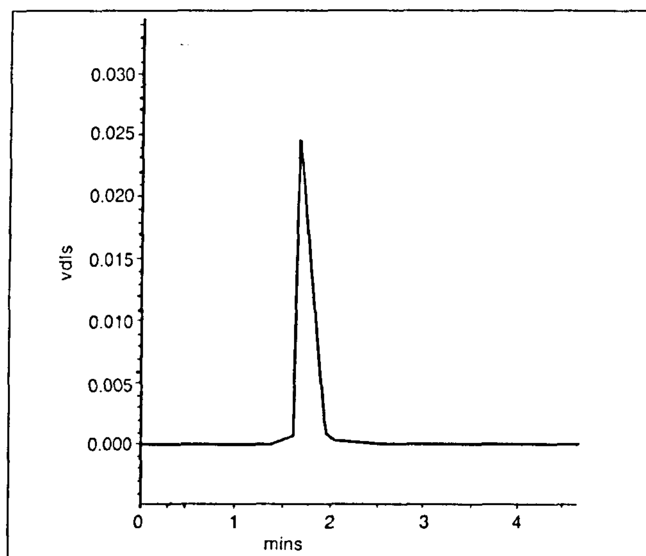

Fig. 1: A HPLC chromatogram of standard scopoletin.

TABLE 1: RECOVERY STUDIES OF PROPOSED HPLC METHOD.

Amount of scopoletin present (ng/ml)	Amount added (ng/ml)	Amount found (ng/ml)	% Recovery ±SD
	0	99.6	99.6±0.25
100	20	121.1	100.9±0.38
	50	150.2	100.1±0.68
	0	201.1	100.5±0.35
200	20	218.7	99.4±0.56
	50	249.3	99.7±0.76

concentration of scopoletin and their respective peak area. The regression equation was found to be Y=4.027X where Y is the peak area and X is the concentration of standard scopoletin in the range of 100–2000 ng/ml. From recovery studies it was found that about 99.4–100.9% of scopoletin was recovered, which indicates high accuracy of proposed HPLC method (Table 1). The low percentage RSD shown in Table 2 indicates that proposed HPLC method is highly precise.

The content of scopoletin in dried leaf powder and fresh 60 and 120 d old leaf calli was quantified using proposed analytical method and was found to be 1.7%, 1.5%, and 2.0%, respectively. The percentage RSD indicates the reproducibility of the assay of scopoletin in crude drugs, (Table 3). The results obtained in the present investigation are significant from the point of view that the biomass obtained from the leaf of the plant is capable of producing higher amount of scopoletin, which is an important bioactive constituent. Presence of scopoletin in leaf callus and its quantitative estimation in leaf and leaf callus of *Convolvulus microphyllus* is being reported for the first time. However further studies are required to be carried out for rapid growth of leaf callus in order to obtain the sufficient amount of the biomass for getting better yield of scopoletin. The work to increase the biomass of cell lines by suspension culture is currently under progress in our laboratory. The results of the study also indicated that the proposed HPLC method is simple, accurate, precise, specific, and less time consuming. Hence it is a preferred method for estimation of scopoletin in crude drugs.

TABLE 2: INTRA DAY AND INTER DAY VARIATION OF PROPOSED HPLC METHOD.

Amount of scopoletin present (ng/ml)	Amount of scopoletin found on Intra day		Amount of scopoletin found on Inter day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
100	101.7	1.9	100.4	2.1
200	201.5	1.2	199.6	1.7

TABLE 3: ESTIMATION OF SCOPOLETIN IN LEAF AND LEAF CALLUS.

Sample	Scopoletin content (% w/w)	% RSD
Leaf	1.7	1.2
60 days old leaf callus	1.5	1.3
120 days old leaf callus	2.0	1.3

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