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A Modified HPTLC Method Development and Validation for Comparative Quantification of Analgesic Salicin in Industrially Important *Bergenia* Species

Abstract

NA modified sensitive, selective, precise and robust high-performance thin layer chromatography (HPTLC) method for the determination and comparative quantification of salicin was developed and validated for the salicin in two species of Bergenia viz. Bergenia ciliata (BC) and Bergenia stracheyi (BS). Quantification of salicin was performed on TLC aluminium plates pre-coated with silica gel 60F-254 as the stationary phase. Linear ascending development was carried out in twin tough glass chamber saturated with mobile phase consisting of toluene-ethyl acetate-formic acid (4:5.5:1.5v/v) at room temperature. Camag TLC scanner III was used for densitometric scanning and analysis in visible absorbance mode at wavelength λ_{max} 500 nm. The system was found to give compact spots for salicin (R_f value of 0.37 ± 0.02). The linear regression analysis data for the calibration plots showed good linear relationship with regression equation y = 1707.5x + 1618.1, correlation coefficient (r) 0.998 in the concentration range 2-7 µg/spot with respect to peak area. According to International Conference on Harmonization (ICH) guidelines the method was validated for precision, recovery and robustness. The limit of detection and limit of quantification were determined. Statistical analysis of the data showed that the method is reproducible and selective for the estimation of analgesic salicin. From quantitative result, higher amount of salicin is found in (BS) comparative to (BC).On industrial level, Bergenia and its species can be used as an alternative source for isolation of natural analgesic compounds salicin.

Keywords: High-performance thin layer chromatography; Method validation; Quantitative analysis; *Bergenia ciliate; Bergenia stracheyi*; Salicin

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Abbreviations

BC: *Bergenia Ciliate*; BS: *Bergenia Stracheyi*; HPTLC: High-performance Thin Layer Chromatography; TLC: Thin Layer Chromatography; RSD: Relative Standard Deviation

Introduction

The genus *Bergenia* (family Saxifragraceae) and its species viz. *Bergenia ciliata* (BC) and *Bergenia stracheyi* (BS) is an evergreen perennial herb, generally distributed in Central and East Asia. It is also found in temperate Himalayas from Kashmir to Bhutan at high altitude 7000-10000 feet and in khasia hill at 400 feet [1]. Previous studies on phytochemical analysis of *B. ciliata* have been shown the isolation of bergenin (C-glycoside of 4-*O*-methyl gallic acid), gallic acid (3,4,5 trihydroxybenzoic acid), (+)-catechin, leucocyanidin, (+)-catechin-3-gallate, (+) catechin-7-*O*-beta-D-glucopyranoside, paashaanolactone, β-sitosterol, β-sitosterol-D-glucoside, and (+)-afzelechin [2]. These phytochemicals have a range of biological activities such as antioxidant [3,4] antidiarrhoeal, anti-inflammatory, menorrhagia, excessive hemorrhage [5,6] antibacterial, antitussive [7,8] and in the treatment of pulmonary infections [9].

Phytomedicines are successfully used alone or as adjuvance therapy for the treatment of degenerative rheumatism and painful arthrosis. salicin is a glycoside, which acts as a precursor compound for the synthesis of acetyl salicylic acid. Glycoside consists of a carbohydrate molecule (sugar) and a non-sugar component (aglycone). Salicin is well known for its effect on treating rheumatic fever and sub-acute bacterial endocarditis. Also salicin has been used as a traditional analgesic in Europe [10]. Salicin is a prodrug which is gradually hydrolyzed to ligenin by intestinal bacteria, and converted into salicylic acid after absorption. Thus it produces an antipyretic action without causing gastric injury [11].

The aim of our work was the development of simple and rapid methods of quantification for salicin from hydrolyzed extracts of two *Bergenia species* viz. *Bergenia ciliata* (BC) and *Bergenia stracheyi* (BS). We have chosen HPTLC for determination and quantification of salicin in extracts of *Bergenia species*. As per previous report on HPLC method quantification of the phenolic glycosides after hydrolysis to salicin in literature [12,13] we have adopted the extraction methodology reported in our previous work and further quantification is followed by development of modified HPTLC method [14,15]. Due to several advantage over other analytical methods such as the rapidity, less amount of test sample and extremely limited solvents waste, HPTLC has gained widespread interest as most acceptable technique for the determination of pharmacologically interesting compounds in the biological matrices like plants and its different parts and even in formulations [16,17].We have also validated the developed method in terms of accuracy, precision, recovery and robustness as per ICH norms [18]. From the literature survey and to our best knowledge no previous report is available on determination and comparative quantification of salicin in *Bergenia* species. However report on salicin quantification using HPTLC is available in *Salix* sp. [19].This is the first time report on determination and comparative quantification of salicin in two different *Bergenia* species.

Experimental

Chemicals and materials

Standard salicin (purity: 98% w/w) was procured from Sigma-Aldrich USA. All the solvents used were of analytical grade from Rankem laboratory, India.

Preparation of crude extracts

Bergenia ciliata (BC) and *Bergenia stracheyi* (BS) were collected from Lansdowne and Juda ka talab, Uttrakhand, India in the month of November 2012, deposited (voucher specimen no. 254013 and 262557) in repository of CSIR-National Botanical Research Institute, Lucknow (Uttar Pradesh) India(Table 1). After washing with tap water, rhizomes were chopped and dried under shade conditions at temperature 40°C. The dried rhizomes (100 g) were crushed into powdered and soaked in absolute methanol (4 × 250 mL) at room temperature (25 ± 2°C) for 5 days. The suspension was filtered and evaporated to dryness by using rotary evaporator (Buchi, USA). Methanol extract of (BC) and (BS) were further hydrolyzed in acidic medium as per reported by Srivastava et al., 2014 [14, 15].

HPTLC method

Apparatus: Camag Linomat V automated TLC applicator, Camag twin trough glass chamber, ascending. Camag TLC scanner model 3 equipped with Camag Wincats IV software were used during the study at temperature 27 ± 2°C, relative humidity.

Sample preparation: Salicin standard stock solution was prepared by dissolving pure 1 mg standard salicin into 1 ml methanol and extract of (BC) and (BS) was dissolved separately into 10 ml methanol. After dissolution both standard and samples are vortex for 10 min.

Chromatographic experiments: Sample solution and standards were applied on precoated silica gel 60F_{254} HPTLC plates with 6 mm band width using Camag 100 microlitre sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (Camag, Switzerland) under a flow of N_2 gas. The Linear ascending development was carried out with Toluene/ethyl acetate/formic acid (4:5.5:1.5 *v/v/v*) as a mobile phase in a Camag glass twin trough chamber (20 × 10 cm).The saturation time of the TLC chamber in the mobile phase was optimized to 20 min for a good resolution of the tested markers and total run time was about 25 minutes at room temperature (27 ± 2°C), 50% ± 2 % relative humidity. After run, plates were dried over hair drier and TLC image was taken on white light after derivatization (Figure 1). Scanning of TLC plate were performed by using Camag TLC Scanner 3 at λ_{max} 500 nm in Visible absorbance mode for all tracks after derivatization, TLC plate were developed at distance

Table 1: Details of collection of *Bergenia* species.

Voucher No.	Plant	State	Region explored	Collection stage	GPS Information	Material
262557	*B.stracheyi*	Uttrakhand	Juda ka talab	Pre-flowering	9400 feet, N 31⁰03.116' E 78⁰11.096'	Rhizome
254013	*B.ciliata*	Uttrakhand	Way to Taluka	Pre-flowering	7135 feet, , N 16°46'51" E 73°44'48"	Rhizome

of approximately 80 mm from the point of application and slit dimensions were 4 mm × 0.45 mm (Figure 2). Quantification evaluation of the plate was performed using peak area with linear regression of amount 2-7 μg/band (Table 2). Peak profiling was done in after derivatization in visible range region at λ_{max} 500 nm (Variable wavelength was used to get best absorbance range) check the identity of the bands, Visible absorption spectrum of standard salicin was overlaid with the corresponding band in the samples track (Figure 3). Standards six point concentration chromatogram is illustrated in (Figure 4).

Postchromatographic derivatization: Postchromatographic derivatization was necessary for the evaluation of the two dimensional stability. Derivatizing reagent anisaldehyde-sulphuric acid solution (anisaldehyde:methanol:acetic acid:sulphuric acid (0.5:85:10:5, v/v)) was used to visualize the spot of salicin in visible range. After air drying for 2 min the plates were heated at 120°C for 5 min (Figure 1).

Results and Discussion

Method validation

Specificity

The specificity of the methods was determined by analyzing the standards and samples bands. The bands for the salicin in sample solution were confirmed by comparing the R_f and Visible

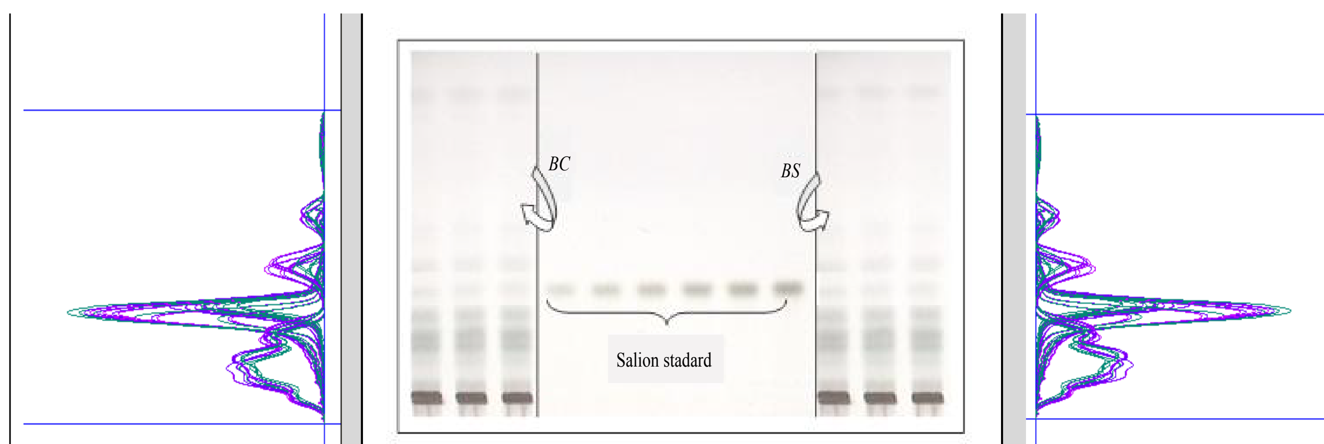

Figure 1: Image of TLC plate at white light after derivatization.

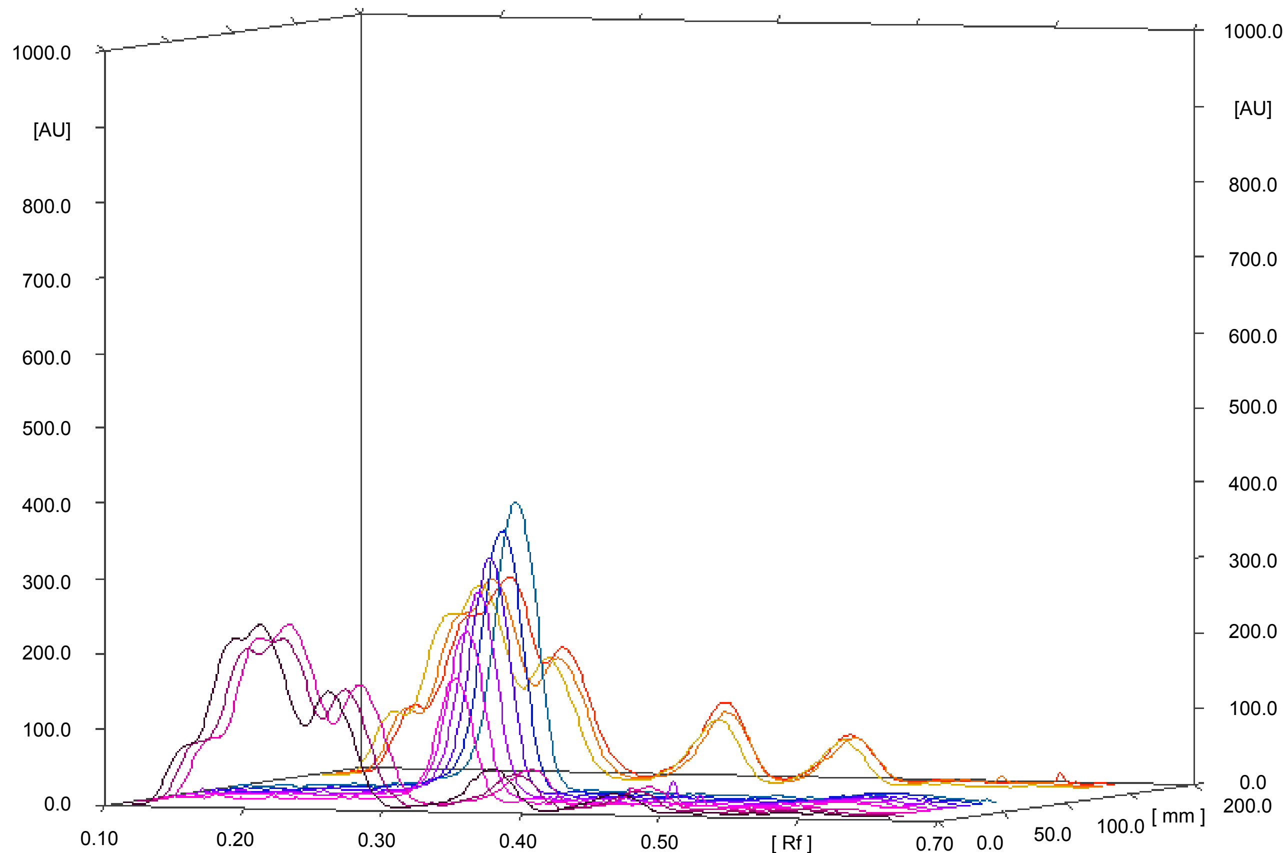

Figure 2: All track chromatogram at wavelength λmax = 500 nm. (*BC*)=*Bergenia ciliata*, (*BS*)=*Bergenia stracheyi* and Salicin.

Table 2: Quantification of Salicin in (*BC)* & (*BS*), (*BC)*= *Bergenia ciliata* and (*BS*)=*Bergenia stracheyi.*

Parameters		Salicin	
Sample	Extract (MeOH)	Applied sample volume	Content of Salicin in 10mg extract (Mean±SD)
		10mg/ml; 10µl	Salicin µg/10mg
B.ciliata	MeOH extract	10	0.88±0.02
B.stracheyi	MeOH extract	10	1.42±0.04

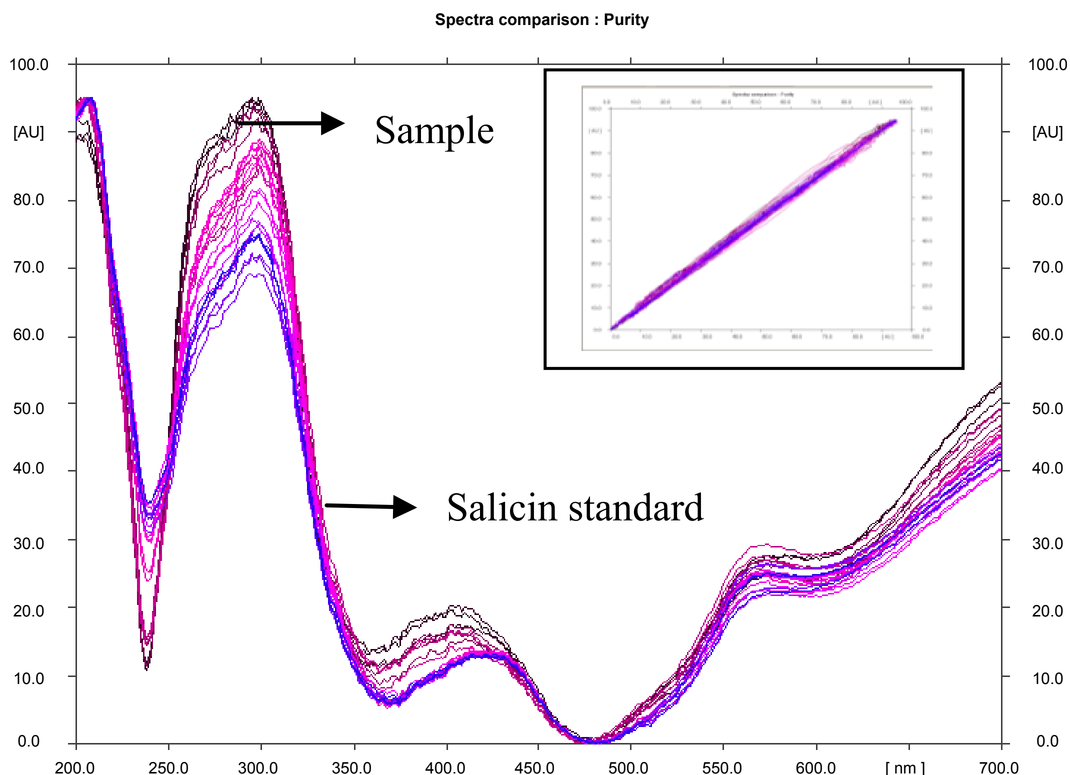

Figure 3: Overlay spectra comparison and peak purity correlation of standard Salicin with (*BC)* and (*BS*). (*BC)*=*Bergenia ciliata* and (*BS*)=*Bergenia stracheyi.*

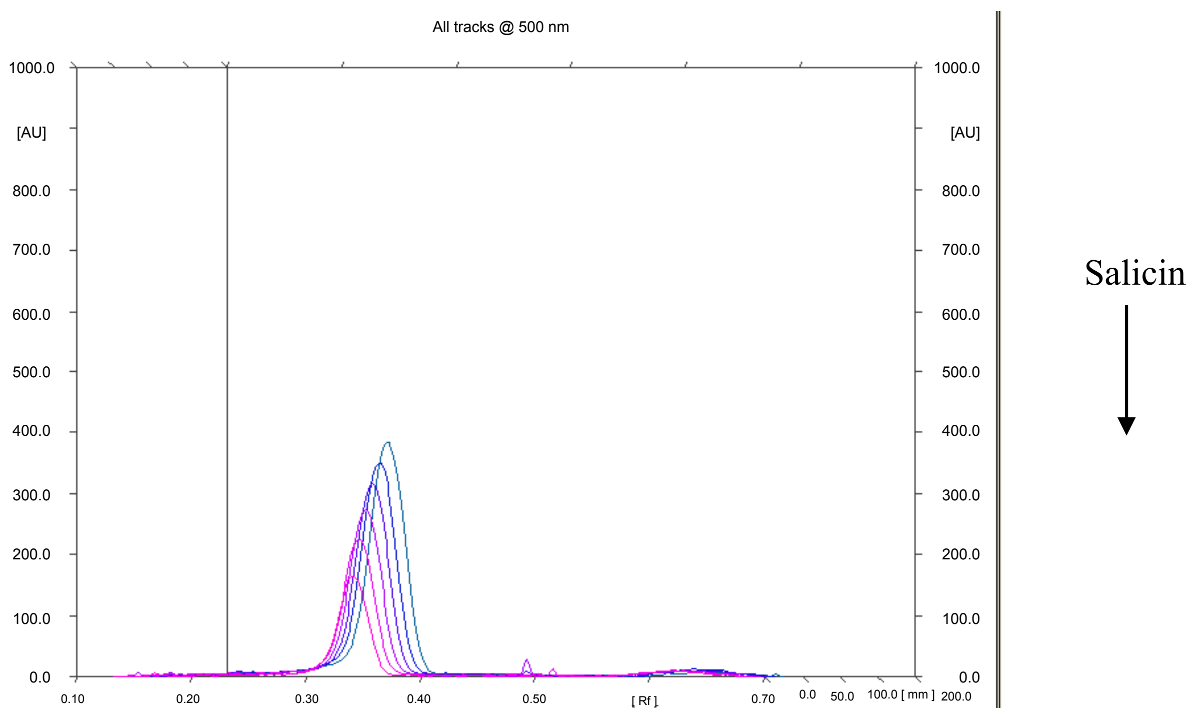

Figure 4: Calibration of standard Salicin at six concentration level.

spectra with the reference standards (Figure 4). A densitometer is used for providing whether the spot contains one compound or more by measuring its visible spectrum at the up slope (peak start), apex (peak apex) and down slope (peak end). The value of

correlation coefficient of up slope to apex ($r_{sm)}$ and apex to down slope (r_{me}) are found (≈0.99), so it can be conclude that the peak is pure (Table 3).

Calibration and quantification

The calibration curves for standard salicin was linear in the concentrations range of 2-7 µg/spot with correlation coefficient (r) 0.998 respectively. The regression data obtained showed a good linear relationship (Table 4). Plate development and spot scanning as well as quantification were performed as mentioned in chromatographic experiment section and calibration curve was constructed (Table 4).

Estimation of salicin in extracts of Bergenia species

A single spot at R_f = 0.37 Was observed in the chromatogram of the salicin in the extracts of Bergenia species. There was no interference in the analysis from the other components of extract matrix. The total salicin contents were found to be 0.88±0.02 µg/10 mg (mean ± SD) in extract of (BC) and 1.42 ± 0.04 µg/10 mg (mean ± SD) in extract of (BS) respectively (Table 2).

Accuracy

The accuracy of the methods was determined by analyzing the percentage recovery of the salicin in the both samples. To obtain it, three sets were prepared from BC. The sample was spiked with three concentrations: 50, 100 and 150 µg of salicin. The spiked sample was recovered in triplicate and then analyzed by proposed HPTLC method. The average recoveries for salicin in (BC) were found to be 99.49%, within the acceptable range. (Table 5).

Table 3: Peak purity test of Salicin, *(BC)* and *(BS)*, *(BC)=Bergenia ciliata* and *(BS)=Bergenia stracheyi.*

Standard & Sample	r (s, m)	r (m, e)
Salicin	0.999546	0.999947
B.ciliata	0.999139	0.999275
B.stracheyi	0.999314	0.999512

Table 4: Statistical analysis of calibration curves in HPTLC determination of Salicin.

Rf value	0.37±0.02
Slope	1.7075
Intercept	1618.1
Linearity equation	y = 1707.5x + 1618.1
Linearity range	2-7 µg
Regression coefficient (R^2)	0.997
Correlation Coefficient ($r=\sqrt{R^2}$)	0.998
p-value	0.002
Intercept coefficient	1618.09
Standard deviation	2.87
LOD (µg)	0.9
LOQ (µg)	2.8
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Precision

Instrumental precision was checked by repeated scanning of the spot of standards salicin five times each. The repeatability of the sample application and measurements of peak area was expressed in terms of percent relative standard deviation (%RSD). Intra-day precision study was achieved at different concentrations levels of 2-6 µg/spot of standard salicin was spotted in three times within 24 h and expressed in terms of percent relative standard deviation %RSD (Table 6). For inter-day precision study, same concentrations levels of 2-6 µg/spot of salicin used over a period of 5 days and expressed as %RSD. The results showed no significant inter and intraday variation was observed in the analysis of the salicin.

Limit of detection (LOD) and Limit of quantification (LOQ)

In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), the signal to noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1. In the present study LOD for salicin estimation in samples was found to be 0.9 µg/band, respectively whereas LOQ for estimation in samples was found to be 2.8 µg/band (Table 4).

Robustness

Robustness is a measure of the method to remain unaltered by small but deliberate variations in the method conditions, and is indicate of the reliability of the method. For robustness study different mobile phase composition, developing TLC distance and different TLC plate lots were assessed (Table 7).

Conclusion

The present study can be further utilized to understand the chemical compounds responsible for analgesic activity of extract and fractions of *Bergenia* sp. Among both species (BS) was found to contain higher amount of salicin comparative to (BC). On industrial level and in herbal medicinal system *Bergenia* and its species can be used as an alternative sources for analgesic compound salicin. The modified developed HPTLC method is precise, sensitive, accurate and robust for the determination of salicin in the extracts of *Bergenia* species. Statistical interpretation of the data proves that the method is reproducible and selective for the analysis of salicin. Since the proposed mobile phase effectively resolves salicin, the method can be used for qualitative and quantitative analysis of salicin in extract of *Bergenia* species. In future, the proposed method can be extended to study the degradation of salicin under different stress conditions, as per recommendations of I.C.H guidelines.

Table 5: Recovery study to evaluate accuracy of method.

Compounds		Intraday Interday	
	Conc.(µg/band)	%RSD	%RSD
	2	0.94	0.93
Salicin	4	0.81	1.01
	6	0.83	1.1

%RSD= Percent Relative standard deviation

Table 6: Inter- and Intra-day precision of Salicin.

Standard	Amount of Salicin present (µg/10mg)	Amount of Salicin added (µg)	Theoretical value	Mean±SD	Observed value	Recovery (%)	Average recovery (%)
	0.88	50	50.88	51.27±0.01	51.27	99.24	99.49
Salicin	0.88	100	100.88	101.5±0.14	101.5	99.39	
	0.88	150	150.88	151.1±0.05	151.1	99.85	

Table 7: Robustness testing of the HPTLC method.

Parameters	RSD% of peak area
	Salicin
Time interval difference between spotting and plate development	0.22
Mobile phase composition	0.38
Time interval between drying and scanning	0.42

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