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ORIGINAL ARTICLE

Simultaneous Quantification of Precocene I and Precocene II through High-performance Thin Layer Chromatography Validated Method in *Ageratum conyzoides* L. Germplasms from Western Himalayas

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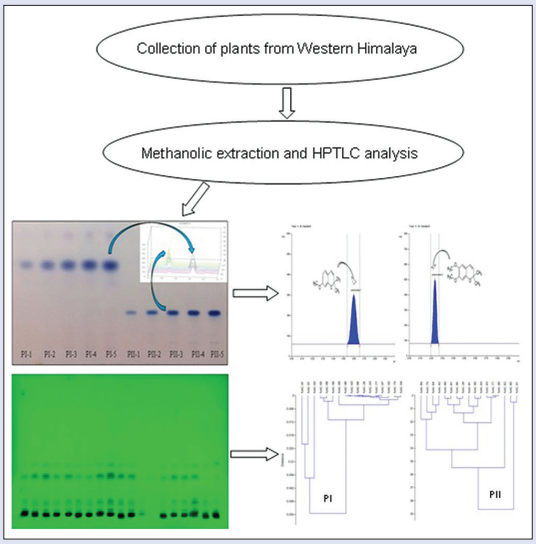
ABSTRACT

Background: *Ageratum conyzoides* L. is a traditionally used herb for various ethnoveterinary purposes. There are no earlier reports on simultaneous high-performance thin layer chromatography (HPTLC) quantification of bioactive markers. **Objective:** To develop a sensitive, robust, and replicable HPTLC method for simultaneous estimation of precocene I (PI) and precocene II (PII) in *A. conyzoides* L. collected from the Western Himalaya region of India, with an aim to understand the level of chemotypic differences arising in the intraspecific population of this ethnobotanically important plant. **Materials and Methods:** A sensitive HPTLC method was developed to resolve PI and PII using toluene: ethyl acetate (9.8:0.2 v/v) as mobile phase. The method was validated for selectivity, specificity, linearity, and precision as per International Conference on Harmonization guidelines. **Results:** Good linearity was achieved over a five-point concentration range with a correlation coefficient of 0.986 and 0.988 for PI and PII, respectively. The PI content varies in the range of 0.0016% (NAC-77) to 0.0834% (NAC-82), whereas PII was reported to be present in the range of 0.016% (NAC-85) to 0.143% (NAC-91) on a dry weight basis. A principal component analysis biplot of samples based on the content of PI and PII identified four elite chemotypes, namely, NAC-81, NAC-82, NAC-85, and NAC-91. **Conclusion:** The study identifies superior germplasms for commercial prospection and develops a validated method that can be used for the quality control of herbal drug/formulation using *A. conyzoides* as an ingredient.

Key words: *Ageratum conyzoides* L., high-performance thin layer chromatography, precocene I, precocene II, Western Himalaya

SUMMARY

- *Ageratum conyzoides* L. is a traditionally used herb for various ethnoveterinary purposes. There are no earlier reports on simultaneous high-performance thin layer chromatography (HPTLC) quantification of bioactive markers. The aim of this study was to develop a sensitive, robust, and replicable HPTLC method for simultaneous estimation of precocene I (PI) and precocene II (PII) in *Ageratum conyzoides* L. A sensitive HPTLC method was developed to resolve PI and PII using toluene: ethyl acetate (9.8:0.2 v/v) as mobile phase. The method was validated for selectivity, specificity, linearity, and precision as per International Conference on Harmonization guidelines. Good linearity was achieved over a five-point concentration range with a correlation coefficient of 0.986 and 0.988 for PI and PII, respectively. The PI content varies in the range of 0.0016% (NAC-77) to 0.0834% (NAC-82), whereas PII was reported to be present in the range of 0.016% (NAC-85) to 0.143% (NAC-91) on dry weight basis. A PCA biplot of samples based on the content of PI and PII identified four elite chemotypes, namely, NAC-81, NAC-82, NAC-85, and NAC-91. The study identifies superior germplasms for commercial prospection and develops a validated method that can be used for the quality control of herbal drug/formulation using *A. conyzoides* as an ingredient.



Abbreviations used: HPTLC: High performance thin layer chromatography, PI: Precocene I, PII: Precocene II, ICH: International conference on harmonization, SD: Standard deviation, RSD: Relative standard deviation

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INTRODUCTION

India is a huge repository of traditional knowledge and a deep-rooted system of indigenous medicine used by local people for primary health-care needs.[1] The Indian Western Himalayan region has vast and varied phytogeography which harbors several therapeutically important medicinal plants.[2] Traditionally, *Ageratum conyzoides* L. is used among the rural people and many ethnic communities in low-lying

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Bhabar to high altitude areas of Himalaya to cure cuts and wounds and as antimicrobial.[3,4] Studies on *A. conyzoides* have elucidated the presence of several therapeutically important phytochemicals such as alkaloids, flavonoids, tannins, saponins, glycosides, resins, phenols, macronutrients, and amino acids.[5,6] *A. conyzoides* has insecticidal, feeding deterrent, and repellent activities against different insects/pests of agricultural importance.[7] Reports have been published which explore the potential of *A. conyzoides* to be used in veterinary sector.[8,9]

High-performance thin layer chromatography (HPTLC) is a preferred method to undertake chemical profiling in plant extracts, mixture of compounds, etc. due to its ease of operation, sensitivity, and reproducibility. It is widely used in pharmaceutical and herbal drug research for identification and quantification of separated compounds with purity.[10]

It is a well-established fact that the synthesis of secondary metabolites in plants is affected by several biotic and abiotic factors, prevailing environmental conditions being one of the crucial factors. Since the plants exhibit a great deal of variation in their metabolite profile under the influence of existing environmental conditions, it is indispensable to map different geographical areas to identify the superior or elite chemotypes existing naturally. In an earlier study, significant variations in precocene I (PI) content in *A. conyzoides* germplasms collected from the Western Himalayan region was reported[11] which encouraged for further characterization of the identified germplasms. In another attempt to map the PI and Precocene II (PII) content along with the acaricidal potential of *A. conyzoides* from Indo-Gangetic Plains, significant variations have been observed (Kumar *et al*., unpublished data). However, the studies pertaining to the chemotypic variations in *A. conyzoides* are scanty. Hence, there is a dire need of chemical profiling studies to identify superior germplasms with a high content of therapeutically viable metabolites.

It was observed subsequent to literature review that there is no report on simultaneous quantification of PI and PII in *A. conyzoides*. Thus, the present study was aimed to establish a validated HPTLC method for simultaneous quantification of biologically active marker compounds, PI and PII in *A. conyzoides* to document the chemotypic variations existing in wild natural populations. The validated method can be used for the quality control of herbal drug/formulation using *A. conyzoides*, and the study also identifies superior quality raw material for commercial prospection.

MATERIALS AND METHODS

Chemicals and reagents

The marker compounds PI and PII were procured from Sigma-Aldrich (USA) and Santa Cruz Biotech (Mumbai, Maharashtra, India), respectively. HPTLC plates and all other chemicals for chemical and biological assay (AR grade) were procured from Merck (Germany).

Plant material

The germplasms of *A. conyzoides* were collected from wild during the flowering period from different locations of the Western Himalayan region, including Uttarakhand and Jammu states of India [Table 1]. The plant material was authenticated by Dr. AKS Rawat, Pharmacognosy and Ethnopharmacology Division, CSIR-NBRI, Lucknow, Uttar Pradesh, India. Each germplasm was assigned a voucher number, and the herbarium specimens were deposited in the institute's repository.

Preparation of plant extract

The collected germplasms (aerial part) were washed, chopped, and shade dried, followed by further drying at 40°C in hot air oven and then coarsely grinding. The coarse powder, accurately about 2 g, was subjected to cold extraction in methanol, continually shaking for 6 h and then standing at room temperature for 18 h. The sample was filtered (Whatman number 4) and the residue was soaked again in fresh solvent. Extraction procedure was repeated thrice, and the pooled filtrate was concentrated under vacuum in a rotatory evaporator (Buchi Rotavapor, Switzerland) at 40°C. The concentrated extract was finally freeze-dried and stored at 4°C for further use.

High-performance thin layer chromatography instrumentation

Chromatography was performed on Merck HPTLC precoated silica gel 60 GF_{254} (20 cm × 10 cm) plates. For sample application, a CAMAG Linomat V automated TLC sample applicator was used to dispense the aliquots of the standard stock solution and the plant samples. The plates were developed in CAMAG ascending twin trough chamber (20 cm × 20 cm). The slit dimension was 4 mm × 0.45 mm and scanning speed of 100 mm/s. Scanning of bands was performed using CAMAG TLC scanner model 3 equipped with CAMAG winCATS IV software at a wavelength of 300 nm in absorption-reflection mode.

Table 1: Brief passport data sheet of the germplasms collected

Sample code	Dist./State	Voucher no.	Altitude (Mtr.)	Latitude	Longitude	Soil type
NAC-28	Roorkee, Uttarakhand (UK.)	305928	271	29°51′15.35″N	77°53′16.80″E	Clayey loam
NAC-77	Pithoragarh, UK.	305977	2801	30°06′07.72″N	80°21′36.95″E	Sandy or gravel
NAC-79	Haldwani, UK.	305979	435	29°13′06.78″N	79°30′46.72″E	Brown or grey soil
NAC-80	Jeolicote, UK.	305980	1274	29°20′34.47″N	79°29′01.49″E	Brown or grey soil
NAC-81	Bhimtal, UK.	305981	1484	29°20′47.96″N	79°33′06.89″E	Brown or grey soil
NAC-82	Bhowali, UK.	305982	1708	29°22′56.20″N	79°31′10.54″E	Brown or grey soil
NAC-83	Sunderbani, Jammu	305983	580	33°02′58.32″N	74°29′26.38″E	Silty loam
NAC-84	Kathua, Jammu	305984	355	32°23′13.32″N	75°31′02.48″E	Loamy with little clay content
NAC-85	Mahichak, Jammu	305985	330	32°24′21.54″N	75°25′10.83″E	Loamy
NAC-86	Nowshera, Jammu	305986	818	33°05′28.57″N	74°14′07.63″E	Silty loam
NAC-87	Dedoli village, Jammu	305987	358	32°22′19.37″N	71°13′02.53″E	Gravel loam
NAC-88	Ramnagar, Jammu	305988	842	32°48′20.23″N	75°18′59.10″E	Grey brown podzolic soil
NAC-89	Billawar, Jammu	305989	730	32°36′49.59″N	75°36′14.68″E	Loamy
NAC-90	Reasi, Jammu	305990	495	33°05′03.32″N	74°50′11.30″E	clayey
NAC-91	Hiranagar, Jammu	305991	321	32°27′22.29″N	75°16′21.96″E	Loamy
NAC-92	Hatli, Jammu	305992	460	32°25′10.71″N	75°35′03.88″E	Sandy loam
NAC-93	Katra, Jammu	305993	868	32°59′30.25″N	74°55′54.38″E	Sandy loam

Chromatographic conditions

The stock solution of marker compounds and sample were freshly diluted with methanol and filtered to prepare working solution of 0.1 mg/ml and 10 mg/ml, respectively, before HPTLC profiling. Working dilutions of plant samples (15 µL) and PI and PII (3 µL) were applied on plate as 6 mm wide bands positioned 10 mm above the bottom and 15 mm from the side of the plate, using CAMAG Linomat V automated TLC applicator with the nitrogen flow providing a delivery speed of 150 nil/s from the application syringe. These conditions were kept constant throughout the study. Followed by sample application, the bands were developed in a CAMAG twin trough glass chamber, saturated with solvent system till the proper separation of bands up to a height of 8 cm. After development, the chromatogram was air dried with an air dryer.

Method validation

The developed method for simultaneous quantification of PI and PII was validated for selectivity, specificity, sensitivity, linearity, and precision studies as per the International Conference on Harmonization (ICH) guidelines.[12] Linearity of standard markers was checked at five different dilutions, and quantification of markers in sample (percentage dry weight basis) was done on the basis of regression equation of area versus concentration of marker compound dilutions.

Statistical analysis

Each sample was processed in triplicate. The data were recorded as mean ± standard deviations (SDs) and evaluated with one-way ANOVA. The correlation was analyzed using statistical software GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). The significance of the regression coefficient was evaluated by *F*-test. Differences were considered significant at $P < 0.05$. Cluster analysis was performed using PAST 2.15 software (Oslo, Norway).

RESULTS AND DISCUSSION

High-performance thin layer chromatography study

The analytical HPTLC conditions such as selection of mobile phase, absorption maxima, and slit dimensions were optimized and standardized to provide an accurate, precise, and reproducible method for simultaneous determination of PI and PII. Several combinations of solvent systems were tried on the basis of chemical nature of targeted molecules, and finally, a binary mobile phase of toluene-ethyl acetate (9.8:0.2 v/v) was selected as best-suited system for efficient separation of targeted metabolites [Figure 1]. Absorption spectrum of marker compounds was obtained at 300 nm [Figure 2]. Specificity of developed method is clearly reflected by good resolution of marker compound peaks. The PI content varies in the range of 0.0016% (NAC-77) to 0.0834% (NAC-82), whereas PII was reported to be present in the range of 0.002% (NAC-82) to 0.143% (NAC-91) on dry weight basis [Table 2]. The relationship between the concentration of marker compound and its corresponding peak area in sample band was investigated.

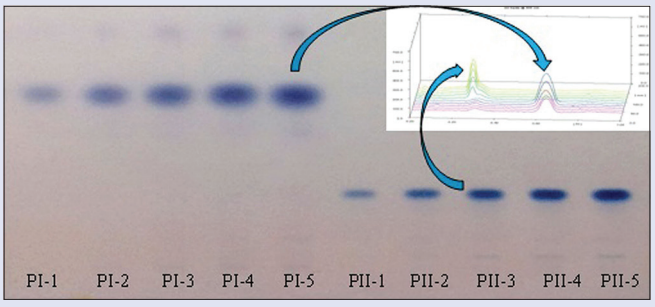

Figure 1: High-performance thin layer chromatography chromatogram and respective densitometric scan of precocene I and precocene II for chromatographic method validation. Bands from left to right: PI-1, PI-2, PI-3, PI-4, and PI-5 and PII-1, PII-2, PII-3, PII-4, and PII-5 represents 1.0, 2.0, 3.0, 4.0, and 5.0 µL of the marker compounds, respectively. Plate visualized under visible light

Calibration of Precocene I and Precocene II

A stock solution of 1000 µg/ml of PI and PII was prepared in methanol, and dilution was done to obtain a working solution of 100 µg/ml, which was used for the analysis. Different volumes (1.0, 2.0, 3.0, 4.0, and 5.0 µL) of the working solution was applied onto a HPTLC plate to furnish 100–500 ng/spot of PI and PII. Peak area versus concentration of marker compounds was treated by regression analysis. The linear relationship was also tested and found suitable for simultaneous quantification of both markers. The equation for calibration curve suggested that the method was linear with a correlation coefficient of 0.986 and 0.988 of PI and PII, respectively. Limit of detection and limit of quantification were found to be within the limit, and hence, the method was found sensitive and selective [Table 3].

Method validation

Peak area versus marker compounds' (PI and PII) concentration was subjected to regression analysis. The slope, intercept, and correlation coefficient for the

Table 2: Precocene I and Precocene II content in *A. conyzoides* L. germplasms

Sample code	Precocene I content (%)*	Precocene II content (%)*
NAC-28	0.002	0.042
NAC-77	0.0016	0.0318
NAC-79	0.0035	0.100
NAC-80	0.0019	0.049
NAC-81	0.057	0.065
NAC-82	0.0834	0.0029
NAC-83	0.0019	0.0307
NAC-84	0.004	0.0839
NAC-85	0.015	0.138
NAC-86	0.002	0.044
NAC-87	0.0017	0.051
NAC-88	0.014	0.032
NAC-89	0.0065	0.0737
NAC-90	0.012	0.058
NAC-91	0.035	0.143
NAC-92	0.0027	0.091
NAC-93	0.0018	0.056

*Values are mean (n=3)

Table 3: Calibration statistics of Precocene I and Precocene II

Parameter	Results	
	Precocene I	Precocene II
R_f	0.6	0.23
Calibration range (ng spot^{-1})	100-500	100-500
Equation	Y=2518.x+5008	Y=2863.x+5143
Slope	2518.00	2863.00
Intercept	5008.39	5143.11
Limit of Detection (µg/ml^{-1})	5.25	5.24
Limit of Quantification (µg/ml^{-1})	15.91	15.90
Linearity (Regression coefficient)	0.986	0.988
S.D.	4009.49	4552.99
Mean	12564.78	13732.35

[a]n=5

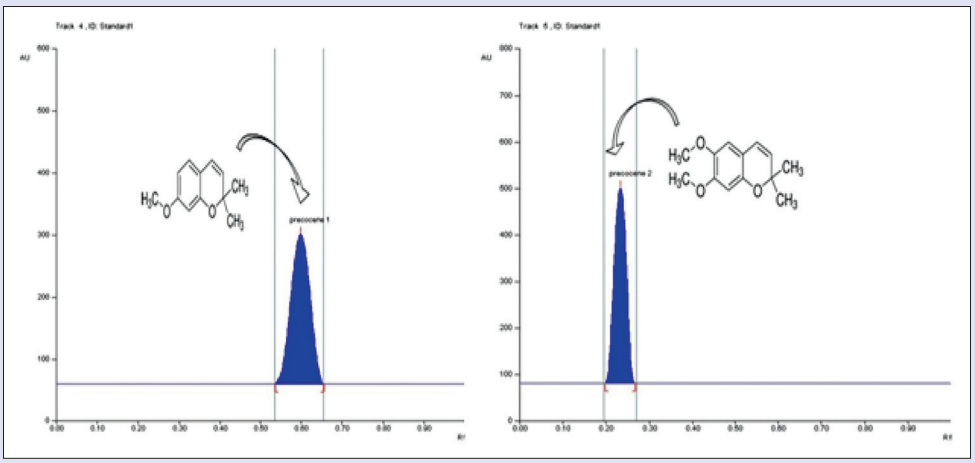

Figure 2: High-performance thin layer chromatography scan of peak area of the marker compound Precocene I and Precocene II

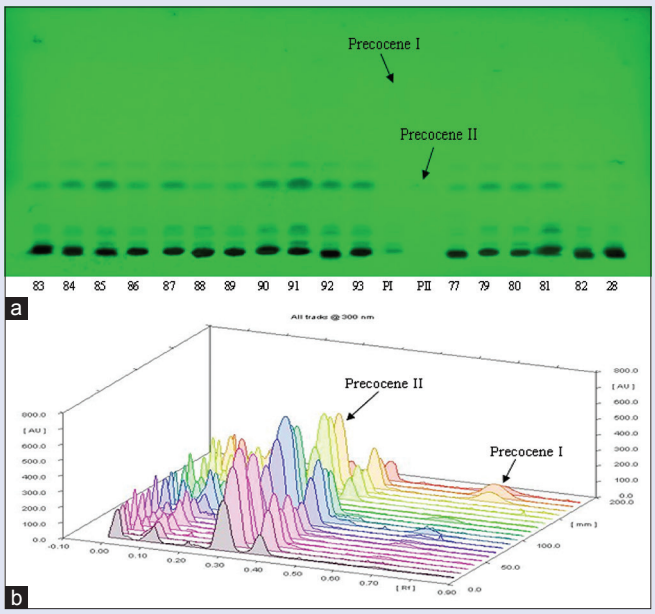

Figure 3: (a) High-performance thin layer chromatography chromatogram, visualized under 254 nm. Left to right: NAC-83, NAC-84, NAC-85, NAC-86, NAC-87, NAC-88, NAC-89, NAC- 90, NAC-91, NAC- 92, NAC-93, Precocene I, Precocene II, NAC-77, NAC-79, NAC-80, NAC-81, NAC-82, NAC-28. (PI: Precocene I, PII: Precocene II). (b) High-performance thin layer chromatography densitometric scan profile NAC-83, NAC-84, NAC-85, NAC-86, NAC-87, NAC-88, NAC-89, NAC- 90, NAC-91, NAC- 92, NAC-93, PI, PII, NAC-77, NAC-79, NAC-80, NAC-81, NAC-82, NAC-28. (PI: Precocene I, PII: Precocene II)

calibration curve were determined using five different concentrations of both the marker compounds. The results are expressed as percentage (on dry weight basis) of the total area of the identified marker compound. Method precision was studied by analyzing the marker compounds under the same analytical procedure and laboratory conditions on the same day (intraday precision) and on different days (interday precision), and the results were expressed as percentage relative SD (% RSD) [Table 4].

Precision

Intraday variability, tested at a single level of 300 ng/spot for three times in a day, reveals the mean RSD (%) values as 2.724 and 1.493 for PI and PII, respectively. The interday variability at the same level for 3 consecutive days expressed the mean RSD (%) values of 3.1758 and 2.0409 for PI and PII [Table 4]. In interday precision studies, same concentration of 300 ng/spot was scanned for 3 consecutive days. Results expressed in terms of mean RSD (%) and SD are within the limits of ICH guidelines (2005) and reflect that method is precise and reproducible for quantification of targeted metabolites underdeveloped protocols.

Table 4: Intra-day and Inter-day precision studies

Marker	Intra-day	Method precision (% RSD)[a] Inter- day		
		Day 1	Day 2	Day 3
Precocene	12637.59	11883.34	10402.67	9072.81
I (300 ng	12456.24	11291.78	10117.54	8624.52
spot^{-1})	11985.11	10786.22	09764.26	8563.82
Mean	12359.64	11320.44	10094.82	8753.71
SD	336.794	549.121	319.810	278.004
RSD (%)	2.7249	4.8507	3.1680	3.1758
Precocene	13765.77	12253.72	10654.32	9672.64
II (300 ng	13456.41	11344.56	10514.09	9624.35
spot^{-1})	13385.72	11142.98	09864.26	9313.96
Mean	13535.95	11580.42	10344.22	9536.98
SD	202.139	591.741	421.532	194.647
RSD (%)	1.4933	5.1098	4.0750	2.0409

[a]*n*=3, S.D=Standard deviation, RSD=Relative standard deviation

Specificity

The densitometeric scanning spectra of the germplasms against that of PI and PII were matching at peak start, peak apex, and peak end positions of the spot which exhibit the specificity of the method. The specificity of method was estimated by evaluating the band of marker compounds in sample solution by comparing the R_f and ultraviolet spectra. Peak purity of these compounds was assessed by comparing the each spectrum at three different levels, i.e., peak initiation, peak apex, and peak end, respectively.

Repeatability and robustness

Repeatability of method was checked by repetitive application of marker compounds (n = 5) and measurement of peak area at single concentration level of 300 ng/spot, small mean %RSD (0.0094) value represent the good repeatability of method. Method was found to be robust when tested by slight change in mobile phase composition and saturation time.

Table 5: Recovery studies of PI and PII in elite germplasm at three different levels

Marker	Amount of standard present (µg)	Amount of standard added (µg)	Theoritical value	Observed value	Recovery (%)	Mean RSD (%)*
Precocene I	0.087	25	25.0878	25.057	100.122	1.123
	0.087	50	50.0878	49.06	102.094	
	0.087	100	75.0878	74.98	100.143	
Precocene II	0.250	25	25.25	25.15	100.397	1.694
	0.250	50	50.25	48.82	102.929	
	0.250	100	75.25	75.5	99.668	

*n=3

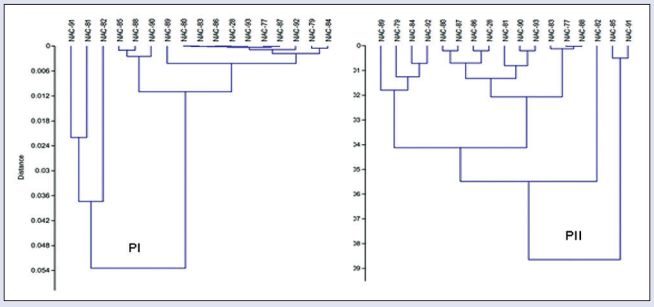

Figure 4: UPGMA dendogram of collected samples on the basis of Precocene I and Precocene II content

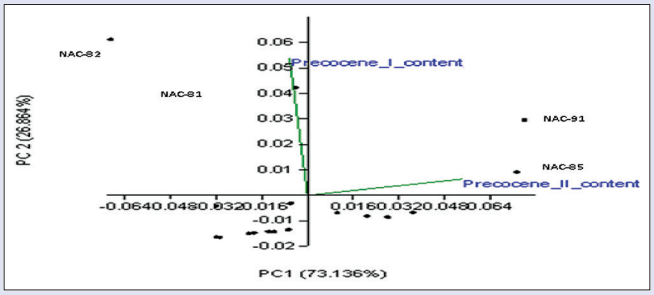

Figure 5: Biplot of collected germplasm obtained through principal component analysis based content of precocene I and precocene II

Accuracy

The accuracy of method was analyzed by recovery studies of both marker compounds at three different levels of spiking. Three sets of elite germplasm at single level for PI (NAC-82) and PII (NAC-91) were prepared and spiked with 25, 50, and 75 µg, and the data were represented in Table 5. The spiked sample was recovered and analyzed again under same chromatographic conditions of HPTLC. Recovery (%) of analytes varies from 99.66% to 102.09%. Accuracy test is effective to identify the interference of unknown metabolite(s) with the developed method for quantification of known substances.

Identification of elite chemotype

HPTLC study reveals that *A. conyzoides* germplasms contain variable amount of both the marker compounds PI and PII among the analyzed samples [Figure 3a and b]. It was noticed that, among the analyzed samples, germplasm from Uttarakhand region has higher content of PI, whereas germplasm from Jammu region are rich in PII. In addition, within the each germplasm, there is significant difference in the content of both markers at 5% level of significance ($F_{calculate} > F_{critical}$), and similarly, there is negative correlation between both the targeted markers in each sample. Hierarchical cluster analysis and construction of dendrogram on the basis of PI and PII segregate the collected population [Figure 4]. This results in identification of four elite chemotypes, namely, NAC-91, NAC-81, NAC-82, and NAC-85 respectively, from the targeted phytogeographical region.

A biplot obtained through principal component (PAST software, version 2.15, Hammer O., Oslo, Norway) based on the content of PI and PII clearly separate the four germplasms (NAC-81, NAC-82, NAC-85 and NAC-91) from rest of the population which accounts for 99.97% variation [Figure 5].

CONCLUSION

The present study aimed at developing a robust and replicable HPTLC method and its validation for quantification of two bioactive marker compounds in *A. conyzoides*. Using the validated method, the study was extended to quantify the marker compounds, PI and PII, present in germplasms collected from different locations of the same phytogeographical zone (Western Himalayas) to decipher the level of chemical variations among the germplasms. From the quantification data obtained, it is observed that there are significant differences in PI and PII content in germplasms of different locations. Therefore, this study highlights the significance of chemotypic variation studies, especially in case of medicinal plants where it becomes indispensable to identify the elite germplasm to come up with a good quality herbal drug or formulation. Such studies are also important to identify locations from where superior quality of raw material can be obtained for commercial aspects. The developed method can be used for further exploration of other phytogeographical zones with similar objectives. The marker compounds identified in germplasms can be used for quality control purposes in the herbal drugs or formulations using *A. conyzoides* as an ingredient.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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