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EXTRACTION EFFICACY, ANTIBACTERIAL POTENTIAL AND VALIDATION OF RP-HPLC COUPLED WITH DIODE ARRAY DETECTION IN *HOLARRHENA PUBESCENS*

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ABSTRACT: A rapid, simple, sensitive and reproducible RP-HPLC method was developed for the determination of conessine (bioactive steroidal alkaloid) in *Holarrhena pubescens*, a highly valued medicinal plant extensively used in pharmaceuticals. Separation was achieved on Discovery (R) RP Amide C16 Supelco column (250 µm x 4.6 µm ID) using a mobile phase of acetonitrile: water (95:5 v/v) with flow rate 0.6 ml / min detection at λ 210 nm. Assessments of six extraction techniques were also performed to evaluate the conessine content and further the efficiency of the extracts against bacterial strains was tested to address causative relationship between the both. The method was linear over a concentration range of 2.5-20 µg/ml (r^2= 0.9991). Accuracy, precision and repeatability were all within the required limits. The mean recoveries measured at the three concentrations were higher than 98.8% with RSD<2%. Extract obtained from ultra-sonication method possessed highest conessine content (1.0647%) and inhibited most of the bacteria. The study confirms ultrasonic extraction to be an ideal method to obtain conessine enriched extract possessing enhanced antibacterial potential. The proposed RP-HPLC method is simple and may be applied for the quality assays of crude drug and phytopreparations.

INTRODUCTION: *Holarrhena pubescens* Wall. ex G. Don syn. *H. antidysenterica* Wall. (Apocynaceae) is a small deciduous tree distributed in Asia, tropical areas of Africa, Madagascar, Philippines, Malayan Peninsula and India ascending up to 1200 m [1] and mainly found in the sub-Himalayan tracts.

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In India, young pods and flowers of this plant are used for edible purposes [2, 3]. Ayurveda as well as modern research highlighted the potential of *H. pubescens* as an anti-dysenteric agent. The bark and roots have been found to be an excellent remedy for both acute and chronic dysentery, especially in haemoptysis and colic pain associated with stool [4].

Besides this, the drug is carminative, anti-spasmodic, astringent, anithelmentic, lithotripter, diuretic, aphrodisiac, tonic, cardio suppressant, antihypertensive, stomachic, anti-pyretic, tonic [5], antimutagenic [6], antibacterial [7, 8], immuno-modulatory [9], antioxidant [10], antihyperglycemic [11],

anti-malarial [12], spasmolytic and spasmogenic [13] and is generally administered as an extract or decoction in amoebic dysentery and diarrhoea. Bark is given either alone or with other astringent drugs in piles, colic, dyspepsia, chest affections and diuresis; also reported to be useful in skin diseases and spleen disorders [14, 15].

Phytochemically, plant contains alkaloids, tannins and resin. Conessine as the major alkaloid is apparently responsible for much of its antibacterial activity and also acts as larval growth inhibitor, sterilant, antifeedant and antiplasmodic [16, 17]. *H. pubescens* is used in several commercial formulations viz. Mahasudarshan tablets, Dashmularishth (Dabur India Ltd., New Delhi, India), Divya mahamanjistha (Patanjali Ayurveda, Haridwar, India), Diacare, Diarex PFS, Diarex vet (Himalaya Drug Co., Bangalore, India) and many others.

The efficacy and safety of herbal drugs and formulations and other ancient formulations are always questionable. Therefore, a simple method is needed to evaluate the quality of herbal drugs, especially with respect to the amount of their active constituents to ensure their efficacy. Although, few HPTLC [18, 19] methods have been published for the determination of conessine in extract but, they suffer from some drawbacks, such as, relative lack of significance and are somewhat tedious and complex. Here, we have developed a precise extraction as well as reverse phase HPLC method for the analysis and quantification of the bioactive conessine (**Fig. 1**) in this botanical.

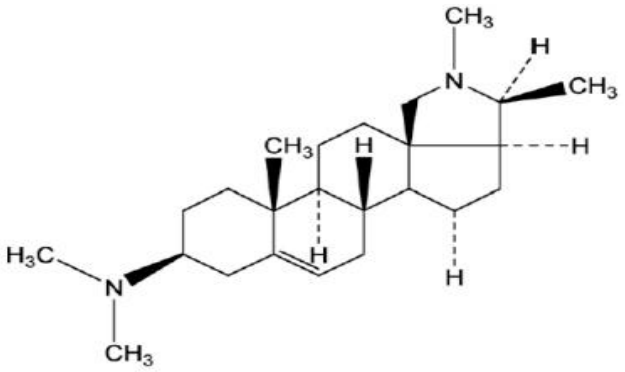


FIGURE 1: STRUCTURE OF THE STEROIDAL ALKALOID 'CONESSINE'

Extraction and product recovery are the most imperative steps in the evaluation of major phytomolecules from plants. Currently, many conventional as well as modern extraction techniques are used, to accelerate the extraction of bioactive compounds from plants [20]. Several studies have been published regarding the variation in the biological activities of extracts obtained from different extraction techniques [21, 22]. The quality of final drugs depends on the method of extraction; here we have evaluated the extraction efficacy by using different extraction techniques viz. cold percolation, shaking extraction, heat reflux extraction and ultrasonic extractions and tested the efficiency of the above extracts against some bacterial strains in order to obtain a product that can fulfil certain market requirements.

In continuation of our studied on chromatographic determination of secondary metabolites in medicinal plants [23-25], we report here simple, rapid, precise and accurate HPLC method and comparative study of different extraction methods as well as antibacterial potential of *H. pubescens.*

EXPERIMENTAL:

Plant material: Bark of *H. pubescens* was collected from arboretum of the Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow in August 2011. Authentication of the plant was established by the Botany and Pharmacognosy Department of our institute. A specimen voucher (Acc no. 13649) has been deposited in the herbarium of the institute. The collection site is located between 26.5^{o}N, 80.5^{o}E and an altitude of 120 m in north Indian plains.

Reagents: Conessine was obtained from Tocris Biosciences, USA. All HPLC/Gradient grade solvents, acetonitrile and water were purchased from Merck India. Mueller–Hinton agar and broth were from HiMedia Laboratories (Mumbai, India).

Standard stock and Sample preparation: Standard stock solution of Conessine **(Fig.1)** was prepared at concentration of 1.0 mg/ml in methanol. Shade dried Bark of *H. pubescens* were ground to powder, sieved through 20 mesh stainless steel sieve and used for the determination of proper extraction method for conessine content. To minimize the variations and to evaluate the influence of sample preparation, all analyses were performed from this homogenous sample procured from a single source.

The procedure was repeated twice with fresh solvent in each extraction method, pooled and filtered through 0.45 mm membrane (Millipore). To obtain the extractive yield (%w/w), 5 g of sample was extracted with the different methods employed for the extraction and supernatant was collected, evaporated using a vacuum evaporator (Buchi Rotavapor R-215, Switzerland) to dryness and the residue was weighed.

Extraction Methods:

1. **Cold percolation:** Five grams of powder was extracted with 250 ml of methanol (99.5%, v/v) at room temperature with occasional stirring for 24 hrs. The filtrate was concentrated and re dissolved in Methanol (5ml) and subjected for HPLC analysis.

2. **Shaking extraction**: 250 ml of methanol was added to 5 gm of grounded powder and was subjected to shaking in a temperature controlled (30^{o}C) Incubator shaker (Kuhner, Switzerland). The extract was filtered, concentrated and re dissolved in Methanol (5ml) and subjected for HPLC analysis.

3. **Heat reflux extraction:** Five gram sample was extracted with 250 ml of methanol for 6 hrs in hot water bath at 40^{o}C. The solution was filtered, concentrated under vacuum, re-dissolved in methanol (2 ml), and the final volume made up to 5ml. This solution was used for HPLC analysis.

4. **Ultrasonic Extraction:** Ultrasonic extraction was carried out using Microclean 109, Oscar Ultrasonic cleaner for 20 min. The power and frequency were set at 350 W and 40 kHz, respectively. 250 ml of methanol (99.5%, v/v) was added to 5 g of the grounded sample in a conical flask and sonicated for 20 min in the dark. The temperature was set at 45^{o}C±5^{o}C. Water in the ultrasonic bath was circulated and regulated to avoid the rise in water temperature caused by ultrasonics.

Chromatographic conditions: HPLC analysis was performed on a Shimadzu LC-10AD liquid chromatograph equipped with an SPD-M1 0A VP diode array detector, an SIL-10ADVP auto injector and a CBM-10 interface module.

Data were collected and analyzed using a class LC-10 Work Station. A pre packed Discovery (R) RP Amide C16 Supelco column (250 µm x 4.6 µm ID) column was selected for HPLC analysis. The mobile phase consisted of Acetonitrile: Water (95:5 v/v).The flow-rate was 0.6 ml/min throughout the isocratic run. Column temperature was maintained at 25±1^{o}C.

Method validation: Method validation was performed on parameters such as linearity, limit of sensitivities, selectivity, precision, accuracy, recovery and robustness as per ICH [26] guidelines.

Antibacterial assay: The antibacterial activity of all the extracts was determined against eleven strains of pathogenic bacteria by disc diffusion assay (DDA) and furthermore by micro broth dilution method (MIC) to find out the sensitivity of the bacterial strains. The activity was analyzed using strains procured as microbial type culture collections (MTCC) from the Institute of Microbial Technology, Chandigarh, India.

The bacterial strains used were *Streptococcus mutans* (MTCC497), *Raoultella planticola* (MTCC RP 530), *Bacillus subtilis* (MTCC121), *Klebsiella pneumoniae (MTCC109), Micrococcus luteus* (MTCC 2470) and *Escherichia coli* (MTCC723), Streptococcus aureus (MTCC 96), *Salmonella typhi* (MTCC 733), *Pseudomonas aeruginosa* (MTCC 741), *Enterobacter aerogenes* (MTCC 111) and *Streptococcus epidermidis* (MTCC 435).

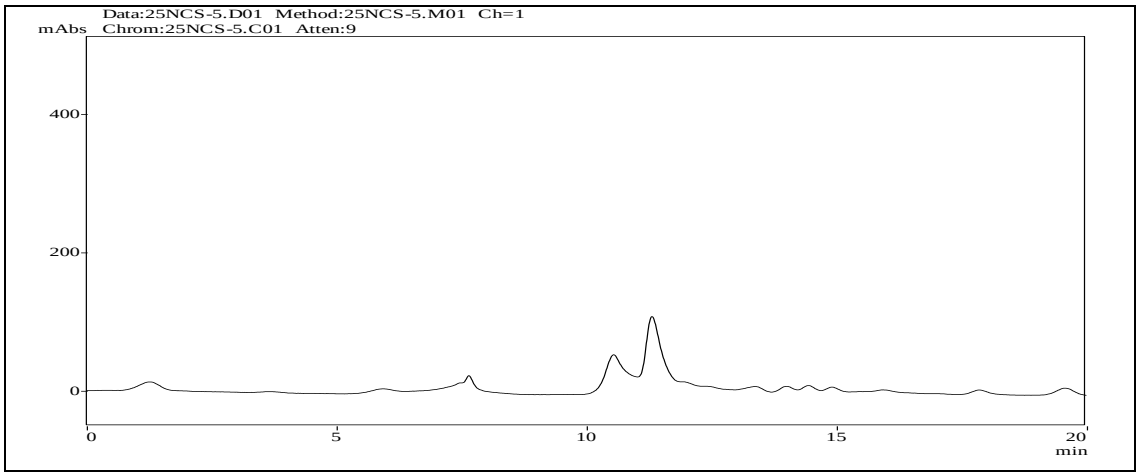
Screening of plant extracts for antibacterial activity was done by the disc diffusion as per CLSI guidelines [27]. Antibacterial activity of *H. pubescens* was determined by Micro dilution broth assay using 96 'U' bottom micro-titer plates as per CLSI guidelines [28]. The extracts were serially diluted two folds (1000-7.8125 µg/mL) in Muellar Hinton Broth (MHB). The broth was inoculated with 10.0 µL of diluted 24h grown culture of test organism with a titre equivalent to 0.5 McFarland standards. The inoculated plates were then incubated at 37ºC for 16-24h and the growth was recorded Spectro-photometrically at 600nm using spectramax 190-microplate reader (Molecular Devices, CA, and USA). The MIC value was determined from the turbidimetric data as the lowest concentration of extracts showing growth inhibition equal to or greater than 80% as compared to control.

The MFC value was detected from the turbidimetric data as the lowest concentration of extract where 99% of killing was observed. Experimental observations were performed in triplicate to rule out any error during the procedure.

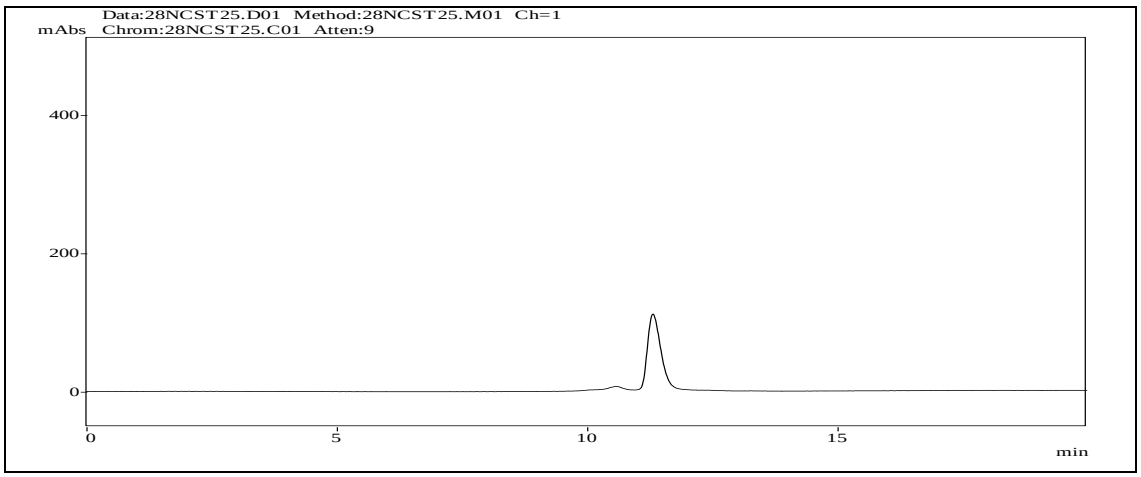
Statistical Analysis: At least 3 replicates were performed in the extraction, profile determination of extraction efficiency, 6 replicates in the method reproducibility measurement and in the spike recovery examination. Three injections were made on each sample for HPLC analysis. All mean values were subjected to analysis of variance (ANOVA) at $p < 0.05$ to determine statistical significance. The statistical analysis was done using GenStat® Release 7.21.

Optimization of Chromatographic Performance: The present study was based on HPLC coupled with photodiode array detection (PDA) Different types of columns and mobile phase compositions were carefully tested in order to determine the optimal chromatographic conditions. It was found that better separation and peak shapes were achieved with a C16 Supelco column (250 µm x 4.6 µm ID) with mixture of acetonitrile and water (95:5 v/v) provided a more efficient HPLC separation of conessine at λ 210 nm. Thus, the above mentioned chromatographic conditions were selected for the separation and quantification of marker compound in *H. pubescens*. Representative chromatograms of pure marker 'conessine' and *H. pubescens* extract are given in **Fig. 2 (a) and (b).**

RESULT AND DISCUSSION:



A



B

FIG. 2 (A) REPRESENTATIVE HPLC CHROMATOGRAM OF SAMPLE TRACK MEASURED AT λ 210 nm; (B) CHROMATOGRAM OF STANDARD TRACK MEASURED at λ 210 nm

System suitability test: System suitability was assessed by six replicate analyses of the analyte. The acceptance criterion was ± 2% for the percentage relative standard deviation (%RSD) of peak area and retention time. The resolution and tailing factors were also determined and the results are given in **Table 1.**

TABLE 1: SYSTEM SUITABILITY STUDY FOR THE DETERMINATION OF CONESSINE (STEROID ALKALOID)

Conessine	Retention time	Peak area	Tailing factor	Theoretical plates
Mean	11.316	2225428	1.46	8159
%RSD	0.184	1.32	0.028	0.002

Linearity and sensitivities: The calibration curve for conessine was plotted with five different concentrations from 2.5 to 20 µg/mL. The detector response was linear. The linearity was assessed by calculating the slope, y intercepts and correlation coefficient (r) using a least squares regression equation. The descriptions of regression curves are depicted in **Table 2.**

TABLE 2: LINEAR REGRESSION ANALYSIS PARAMETER FOR THE DETERMINATION OF CONESSINE

Linearity range	2.5-20 µg/ml
Regression coefficient (r^2)	0.991
Slope	68170
Intercept	297352
LOD	0.418 µg/ml
LOQ	1.394 µg/ml

Regression equation and coefficient of correlation (not less than 0.99) revealed a good linearity response for the method developed. A signal-to-noise ratio (S/N) of 3 and 10 was considered as limits of detection (LOD) and quantitation (LOQ) respectively. The LODs for conessine was 0.418 µg/mL and the LOQs was found to be 1.394 µg/mL. This indicates that the proposed method exhibits a good sensitivity for the quantification of SG.

Precision and accuracy: The intra- and interday precision (expressed in terms of %RSD) was observed in the range of 0.38–0.98% and 0.33–1.12%, respectively, which demonstrated good precision of the method. The low values of %RSD (< 2%) reflect the good precision of the method. The accuracy of the proposed method was expressed as the recovery of standard compounds added to the pre-analysed samples. The recovery was found to be in the range of 98.80-101.50%, indicating good accuracy of the method (**Table 3**).

TABLE 3: INTRA- AND INTERDAY PRECISION (%RSD) OF METHOD

	Amount added	Amount found	%RSD[#]	Recovery
Inter	5	4.94	0.38	98.80%
	10	10.15	0.98	101.50%
	15	14.95	0.54	99.60%
Intra	5	5.07	0.33	101.40%
	10	10.12	1.12	101.20%
	15	14.85	0.35	99.00%

[#] n=6. Results using successive injections of triplicate standard samples of conessine.

Recovery: The three different concentrations diluted from the stock solution were added to an extract with a known content of conessine and the recovery was calculated. The recovery (R) was calculated as $R = (C_{found} - C_{sample})/ C_{added}$, where C_{found} is the concentration in a spiked sample, C_{sample} is the concentration in the sample prior to spiking and C_{added} is the concentration of added standard. The results of recovery of the tests were acceptable as the average recoveries was 100.25%.

Quantification of steroidal alkaloid (Conessine): The peak of conessine was identified by comparing the retention time and UV spectra with those of standard. Stock solution containing reference compound was diluted to give series of appropriate concentration with methanol and aliquots of the diluted solutions were injected. The yield of conessine (%) was in the order of Ultrasonication extract (1.0647%) > Heat reflux method (0.7873)> Shaking extraction (0.5587)> cold percolation (0.3217). The results are summarised in **Fig 3.**

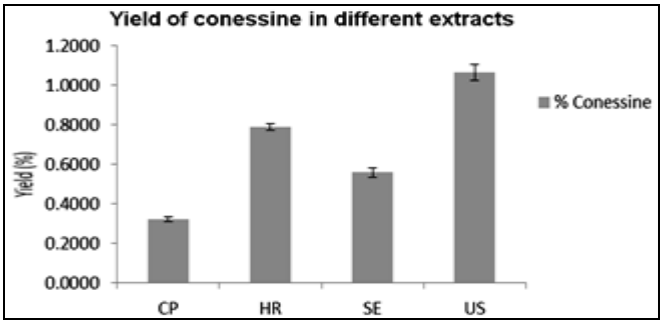


FIG. 3: YIELD OF CONESSINE FROM VARIOUS EXTRACTS OBTAINED FROM DIFFERENT EXTRACTION TECHNIQUES. Values were expressed as the mean±standard deviation (n=3). Value were significantly different (P<0.05) from each other. CP: Cold percolation; SE: Shaking extraction; HR: Heat reflux; US: Ultra sonication.

Extraction Yield: The extraction yield, obtained from different extraction techniques are presented in **Table 4.** The extraction yield depends on variety of factors such as solvents, time, temperature, as well as the chemical nature of the sample [29]. Here, we found that the effect of extraction procedure highly influenced the extractive yield of the sample. Extraction yield had no significant association with the yield of conessine in the extract. Though, it is noteworthy that the extraction yield was directly correlated with the heat applied in the extraction procedure.

The yield of ultrasonic extract (14.92%) was found to be the highest and may be due to the heating effect and its exhaustive extraction ability followed by heat refluxed extract (8.15%). The yield from cold percolation and shaking extraction were as low as 3.64% and 4.12%. It was noted that the extraction yield attained by application of heat was greater than that achieved by using cold percolation and shaking extraction methods.

TABLE 4: EXTRACTION YIELD OBTAINED FROM DIFFERENT EXTRACTION TECHNIQUES[#]

CP	SE	HR	SL
3.64 (0.11)	4.12 (0.17)	8.15(0.09)	24.71(0.21)

[#] Yield% (SD), CP: Cold percolation, SE: Shaking extraction, HR: Heat reflux, SL: Soxhletion

Extraction Efficacy and Antibacterial activity: Different extraction techniques significantly affected the yield of conessine. Ultra sonication was found to be the most efficient extraction technique for obtaining higher yield of conessine followed by heat refluxing. However, techniques like shaking and cold percolation yielded much less amount and were not found suitable for the extraction of the phytoconstituents It was interesting to note that the extracts containing higher amount of marker alkaloid, exhibited potent antibacterial activity suggesting a causative relationship between conessine and its antibacterial property.

Micrococcus luteus, Pseudomonas aeruginosa, Escherichia coli and *Bacillus subtilis* were found to be more sensitive towards the extract particularly against ultrasonic extracts (**Table 5**). Similar to the results of yield of conessine, extract from ultrasonication was found to be broad spectrum and very effective against wide range of bacteria.

It exhibited the MIC of 15.625, against *Micrococcus luteus, Escherichia coli* and *Pseudomonas aeruginosa* and was further followed by shaking, heat refluxed and cold percolated extracts. However, inhibitory action was best demonstrated by ultrasonic extract. Inhibition of *Streptococcus aureus, Streptococcus epidermidis, Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* was previously reported on the extract of *H. pubescens* [16, 30] and the result also justifies and supports it, which has also concluded that conessine is responsible for antimicrobial properties of this botanical. In view of growing interest in research concerning alternative antimicrobial active compounds including plant extracts due to their relatively less damaging effect to the mammalian health and environment our study conform with the same.

TABLE 5: GROWTH INHIBITORY ACTIVITY OF VARIOUS EXTRACTS OF *H. PUBESCENS*

	SM 497	RP 530	BS 121	KP 109	ML 2470	EC 723	SA 96	ST 733	PA 741	EA 111	SE 435
	MIC (MBC)										
CP	-	-	-	-	500 (500)	-	-	-	250 (500)	500 (>1000)	-
HR	-	-	250 (500)	-	62.5 (125)	62.5 (250)	-	-	250 (>1000)	500 (>1000)	-
SE	-	-	-	-	125 (500)	250 (500)	500 (>1000)	-	62.5 (125)	500 (>1000)	125 (250)
US	500 (>1000)	-	62.5 (125)	-	15.625 (31.25)	15.625 (62.5)	125 (250)	-	15.625 (31.25)	250 (500)	62.5 (125)

SM 497: *Streptococcus mutans*, RP 530: *Raoultella planticola*, BS 121: *Bacillus subtilis*, KP 109*: Klebsiella pneumoniea*, ML 2470: *Micrococcus luteus*, EC 723: *Escherichia coli*, SA 96: *Streptococcus aureus*, ST 733: *Salmonella typhi*, PA 741: *Pseudomonas aeruginosa*, EA111: *Enterobacter aerogenes*, SE 435: *Streptococcus epidermidis*. ZOI: Zone of inhibition, MIC: Minimum Inhibitory Concentration, MBC: Minimum bactericidal Concentration, CP: Cold percolation, HR: Heat reflux, SE: Shaking extraction, US: Ultrasonication.

CONCLUSION: In this study, different extraction techniques significantly affected the extraction yield, conessine content and antibacterial activities. The ultrasonic extraction method were simple, rapid and better methods for the extraction of antibacterial substances compared to other methods by providing a high amount of bioactive alkaloid 'conessine' with potent antibacterial activities. These results also indicate that the selection of correct extraction method is important for obtaining the extracts with the required pharmacological activity.

Moreover, a rapid, precise, accurate and specific RP-HPLC method for qualitative and quantitative analysis of conessine in *H. pubescens* was successfully developed. The proposed RP-HPLC conditions ensure sufficient resolution and the use of reference standard guarantees the precise quantification of the phytoconstituents. The method validation showed satisfactory statistical data for all the parameters tested. The precise, rapid and sensitive analytical method involving a simple and sustainable extraction procedure may be suitable for the quality control of *H. pubescens* and its products.

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