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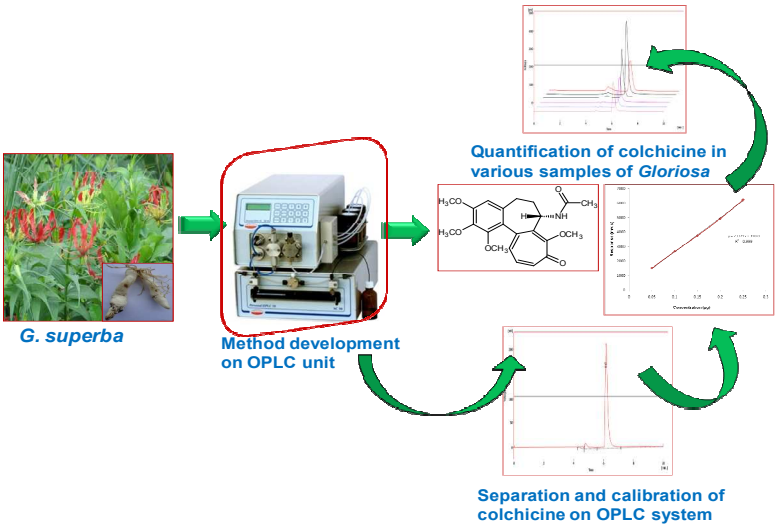
A validated (OPLC) method for separation, quantification & phytogeographical variation of Colchicine in *G. superb* tuber (s) from central India



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A validated over pressured layered chromatography (OPLC) method for separation and quantification of colchicine in *Gloriosa superba* (L.) tubers from different geographical region

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A normal phase on–line OPLC-UV technique was adopted for separation and quantification of colchicine alkaloid in *G. superba*. Analyzed and developed method was rapid, specific and selective separation was observed with high peak purity. Validation parameters prove the selectivity and reproducibility of method and thus can be applied for estimation of targeted marker in *Gloriosa*, in various pharmaceutical formulations and also to check phyto-geographical variations among the species.

OPLC is a newer, highly sensitive chromatographic technique which exploits both the benefits of simplicity of planar chromatography and the precision of liquid chromatography. This separation technique uses a solvent pump to deliver eluent into a pressurized ultra micro chamber containing an analytical or preparative planar adsorbent bed to separate the components of a mixture. The eluent is forced to flow through the adsorbent layer at an optimum, constant linear velocity. The openable planar adsorbent bed and forced flow allows OPLC to combine the benefits of TLC and HPLC. This forced flow planar chromatography is able to achieve more efficient separation than conventional methods because mobile phase in the OPLC system migrates through the entire layer with constant velocity by application of pump regulated force, in addition to capillary action and the use of optimum separation conditions (e.g. mobile phase, velocity of solvent front and external force) results in more compact spots. The uniqueness of OPLC technique lies in its ability of simultaneous multi-sample processing, fast separations, simple sample preparation and low solvent consumption. As being a newer technology there is

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little documentation available on it, especially on the characterization of plant metabolites. However, some phytochemicals like cannabinoids[1], resveratrol[2] and *Chamomile* essential oil [3] had been studied. Quantification of colchicine in plant sources as well as pharmaceutical preparations is being reported by several workers through conventional methods of chromatography, [4-10] having their own merits and demerits, but till date there is no literature available on separation and quantification of colchicine through OPLC-UV technique.

G. superba is an emerging medicinal crop of great industrial importance for its high colchicine content, although due to over-exploitation and problems faced during field cultivation; this species is now on the verge of extinction.[11] Colchicine is a traditional drug for gout, and its use in traditional medicine dates back to 1810 for treating acute conditions. Since the approval of colchicine as drug for gout in 2009 by Food and Drug Administration (FDA, USA) there has been revival of interest in colchicine research and applications.[12] Colchicine is the major chemical marker in this species (0.7% to 0.9%) whereas other chemical markers i.e. lumicolchicine, 3-demethyl-N-deformyl-N-deacetylcolchicine, 3-demethylcolchicine, N-formyldeacetylcolchcine are also present.[13] Colchicine is also used in other diseases, like cancer, scrofula and act as antipyretic, anti anthelmintic, purgative and anti abortive. Chemistry, toxicity and other pharmacological effects of the colchicine have been well established.[14] Therefore, the present study was undertaken for method development, validation and quantification of colchicine through OPLC-UV in different samples of *G. superba* (L.) tubers from Central India with an additional objective to study the role of phyto-geographical variations on the concentration of most prevalent bioactive marker.

Experimental

Chemicals

HPLC grade solvents viz. methanol, chloroform, acetone, diethyl amine and other chemicals were purchased from Merck, Mumbai, India. Analytical grade reference standard colchicine (≥95% HPLC, powder) was procured from Sigma Aldrich Co. USA. Aluminum foil backed normal phase silica gel 60 (5 µm, 0.2 mm thick), 20 x 20 cm F_{254} plates with sealed edges (JC Scientific Co. Ltd., Hong Kong) were used as stationary phase.

Plant materials and extraction protocols

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Fresh tubers of *G. superba* L. was collected in the month of September-October (2013) from six different locations of Central India of varying topographic structure, with a view to cover all possible areas having different environmental conditions. Total six samples (NBG-13 to NBG-18) were collected and after proper identification the specimens were deposited in the Herbarium of the institute with individual voucher specimen's number. Samples were washed, shade dried and powdered (40 mesh) through mechanical grinder. The coarsely powdered samples (5 g each) were macerated with methanol for 24 hr at room temperature (25 ± 2^0C). Extraction was repeated thrice, filtered and pooled filtrate was dried in rotator evaporator (Buchi, USA) under standard conditions of temperature (55 ± 2^0C) and pressure (40 mbar) and lyophilized (Labconco, USA). Before extraction, the tubers were defatted using petroleum ether to remove the fatty materials/impurity. The yield (%) of methanol extract in *Gloriosa* varies from 4.8 – 20.1 %, i.e. NBG-13: 20.1 %, NBG-14: 16.1 %, NBG-15: 16.4 %, NBG-16: 8.2 %, NBG-17: 9.3 % and NBG-18: 4.8 %.

Reference and sample preparation

The stock solutions of colchicine (1 mg/mL) were freshly prepared in methanol each day and were stored in the dark. Each day aliquots of the stock solution were diluted in 10 mL volumetric flasks with methanol to prepare a working solution of 0.1 mg/ml. Working solution of the samples were prepared (1mg/ml) in methanol from stock solution of concentration 10 mg/ml and filtered through a 0.45 µm Millipore membrane filter (Pall, USA).

Instrumentation and on-line OPLC-UV method conditions

Method development and analytical measurements were carried out on the OPLC Separation System POSU-50 (JC Scientific Co. Ltd., Hong Kong) in fully online operating mode [15] at 20 ^{0}C. The separation was carried out on 20x20 cm sealed edge TLC plate (OPLC plate) placed in a 20x20 cm, 0.2 mm development cassette. The cassette was inserted inside the hydraulic unit of POSU-50 and a pressure of 50 bar was applied over the OPLC plate. Pre conditioning was carried out with methanol at flow rate of 0.4 ml/min for 30 min. and then activated by heating at 120 ^{0}C for 10 min. Mobile phases were duly filtered through 0.22 µm Millipore filter & degassed ultrasonically for 15 min before delivering into the pressurized separation unit. Total run time

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was 10 min. Data acquisitions were performed using Data Apex Clarity software. The injection volume was 20 µl for standard and sample.

Isocratic solvent system consisting of chloroform: acetone: diethyl amine (5:2:1) was selected at a flow rate of 0.8 ml/min and eluent pressure not exceeding 40 bar. Condition for analytical on-line OPLC separation follows: injection of standard/samples in loop, separation of analyte and development of chromatogram under constant flow. The detection was achieved using online coupled flow through UV detector (Model: ECD2000; Manufacturer: ECOM spol. s r.o., Americka 3, CZ 120 00 Praha 2, Czech Republic, Deuterium lamp combined with tungsten as secondary source; wavelength detection range of 190 to 800 nm; preferably upto 600 nm) at a fixed wavelength.

Validation of method

Peak area versus reference standard concentration (50-250 µg/ml) was subjected to regression analysis. The slope, intercept and correlation coefficient for the calibration curve were determined with 5 different concentrations. The results are expressed as percentage of the total area of identified compounds. The method validation for quantification of colchicine was determined by selectivity, specificity, sensitivity, linearity, precision and accuracy. [16]

Statistical Analysis

Results were reported as means ± standard deviation (SD) of at least three replicates of the same extract. Data were subjected to one-way analysis of variance (ANOVA) and the least significant difference (LSD) between the extracts at $P < 0.01$ was calculated by *post hoc* comparison test (SPSS 11.5).

Results and discussion

As the sample under investigation is crude extract and thus contains variety of phytomolecule, it is necessary to choose sensitive mobile phase to improve reproducibility, selectivity or peak shape. Several combinations of binary and tertiary solvent systems were tried on TLC for clear separation of colchicine in *G. superba* extracts (data not shown), and finally chloroform: acetone: diethylamine (5:2:1) is selected and charged for on-line separation under optimized

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conditions. Flow rate and pressure of the system was also optimized & equilibrated based on the peak-area response. Detection was performed at 350 nm. [17] Base peak of standard colchicine (0.1 mg/ml) as shown in Figure 1 was observed at Rt 6.17 min. Repeatability (n=5) of method was tested by peak–area response of standard at one level, values of variance (0.70) and standard deviation (0.837) was within the limit of acceptance.

The proposed method was validated with selectivity, specificity, sensitivity, linearity, and precision and accuracy data. Furthermore, as an estimation of the method's analytical performance, limits of detection and quantification were also calculated. Peak area responses for calibration curve of five standards i.e. 50 - 250 µg/mL colchicine, ranges from 1514.036 - 6222.663 mV.s and the other calibration parameters for quantification of colchicine was within the specified limit (Table 1). Standard solutions of colchicine in methanol showed linear correlation between the peak area and concentration, correlation coefficient was found to be satisfactory while plotting of calibration for standards. To estimate the stability, % RSD of standard was calculated and results (not more than 3 %) shows that the analytical solutions are stable for duration of 24 h. Value of correlation coefficient for colchicine indicates good linearity. LOD and LOQ values depict that method is sensitive. The selectivity validation of method is well demonstrated by the excellent separation response of reference and peak purity was also good, it indicates that there is no merging of any unidentified peak with known identified marker (analyte) and thus confirms the selectivity of method. Recovery (%) of colchicine (0.1 mg/ml) as estimated by standard addition method (spiking at three different level of 50%, 100% and 150%) was 98.33 % (n=3), thus reflecting the accuracy. Precision of the method was accessed through intra and inter-day precision of standard (n=3) and no significant variation was observed in values (Table 2). As mentioned previously, in OPLC the vapour phase is completely eliminated from stationary bed and thus significantly improves the resolution of colchicine under controlled conditions, in multi sample analysis. In addition, with OPLC we can identify and isolate the metabolites/fractions of trace amounts and that too in no time. Hence it will be useful for studies on phyto-geographical variations of bioactive marker(s) and their concentration in high valued medicinal species.

The identification of colchicine in *G. superba* tubers (extract) was based on comparison of retention times and UV spectra of reference standards with the corresponding peaks in extract. It

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is noteworthy, that quantification of samples (NBG-13 to NBG-18) was completed within 42.06 ± 0.01 minutes and the solvent required for the same is less than 35 ml. Chromatograms shown in Figure 2 reveal the existing phyto-geographical variation in colchicine content (ranges from 0.6-2.5 %) within the samples (Table 3). Maximum content of colchicine (2.5%) was observed in sample collected from Tamiya/ Panchmarhi forest region of Madhya Pradesh i.e. NBG-14 and the minimum concentration (0.6 %) was in sample from Achanakmar, Chhattisgarh (NBG-17). The variation in content of colchicine is due to the change in topography, geography and/or environmental factors viz. soil type and elemental composition, edaphic factors etc. affecting the production of targeted metabolite/marker. However more refined study is needed to coined the specific factors responsible for the same. The results are promising for commercial and medicinal utilization of species through encouragement for cultivation in the selected phyto-geographic region as elite chemotype. Thus, with the above study we successfully represent a newer method for quantification of colchicine along with significantly validated data to propose the phyto-geographical variation in colchicine content.

Conclusions

The method was successfully developed for quantification of colchicine in the tubers of *G. superba* using on-line OPLC-UV technique. The proposed method is rapid, specific and accurate with efficient selectivity and reproducibility. Analytical performance was validated providing a powerful tool for quantification of colchicine in *Gloriosa* species, other members of family colchicaceae and pharmaceutical formulations, under the specified conditions. The novelty of work lies in method development and, validation of colchicine through OPLC-UV technique and its quantification in different samples of *G. superba* (L.) tubers from Central India, with a view to study the prevailing phyto-geographical variations among them. The high efficiency, very short time of development of chromatograms, and increasing of the number of samples analyzed on one chromatoplate for the OPLC method allowed for the proposal of the use of OPLC for quick analysis of a large number of samples. Besides this, the study will also aid in identification of existing elite chemotypes for commercial and medicinal uses of targeted chemical moiety colchicine. As the technique can detect the presence of compound in lesser concentrations, it can also be utilized further for isolation of a pure compound from herbal extract(s) or mixture of compounds, thus it becomes very useful and cost effective in herbal drug industry.

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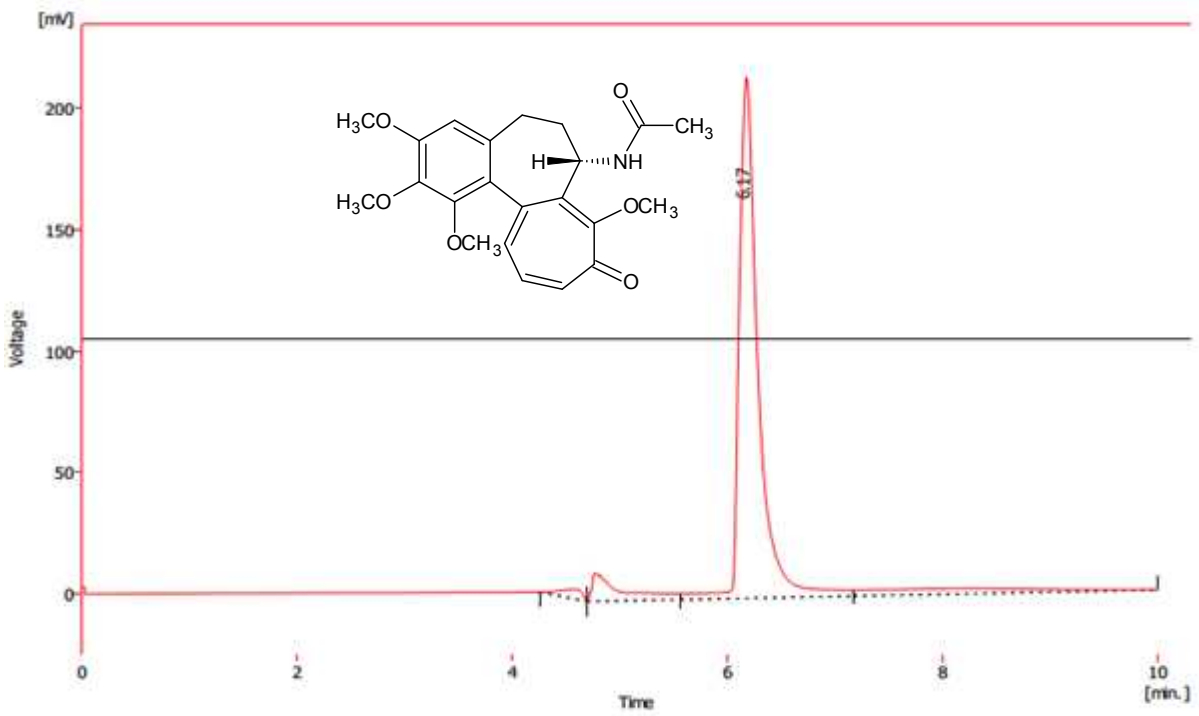

Fig. 1. On-line OPLC-UV chromatogram of standard colchicine at 0.1 mg/ml (X-axis represents the retention time in minutes and Y-axis depict peak height in mV). Area under peak is 2661.058 mV.s)

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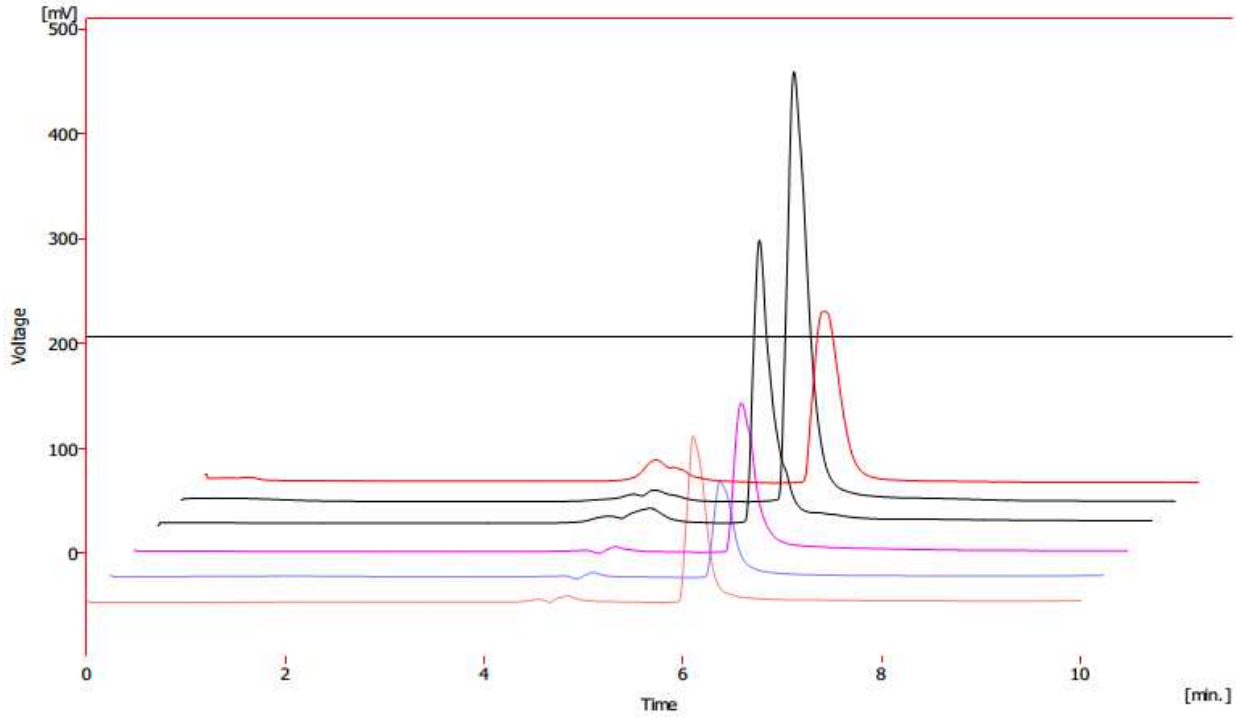

Fig. 2. On-line OPLC-UV overlay chromatogram of six samples (1 mg/ml). Sequence from top to bottom: NBG-13, NBG-14, NBG-15, NBG-16, NBG-17 and NBG-18.

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Table 1
Validation parameters for quantification of colchicine in *G. superba* samples.

Parameters	Colchicine
Slope	23370.53
Intercept	310.06
Regression coefficient (r^2)	0.9991
Linearity (µg/ml)	50 - 250
LOD (µg/ml)	0.261
LOQ (µg/ml)	0.790
Rt (min.)	6.17
RSD (%)	0.031

n=5

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Table 2
Inter day and intraday precision of colchicine standard at 0.1 mg/ml.

Colchicine standard	Response (peak area in mV. s)*			
	Inter day	**Intraday**		
		Day 1	**Day 2**	**Day 3**
	2661.058	2662.05	2665.6	2667.36
	2659.83	2663.15	2664.78	2666.55
	2659.8	2662.052	2664.68	2667.95
Mean	2660.229	2662.417	2665.02	2667.287
S.D	0.717803	0.634509	0.504777	0.702875
RSD (%)	0.026	0.023	0.018	0.026

* n=3, S.D: Standard deviation, RSD (%): Relative standard deviation.

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Table 3
Phyto-geographical variation of colchicine content in six samples of *G. superba* tubers (extracts) collected from different locations of central India.

Sample code	Location/ District/ State	Altitude (meters)	Latitude	Longitude	Soil type	Colchicine concentration (%)*
NBG-13	Bheraghat/ Jabalpur/MP	1251	$23^0$07’50.18’’N	$79^0$48’08.70’’E	Alluvial	1.3 ± 0.01
NBG-14	Tamiya/ Panchmarhi/MP	3081	$22^0$20’39.80’’N	$78^0$40’10.50’’E	Black	2.5 ± 0.00
NBG-15	Chitrakoot/ MP	475	$25^0$10’45.28’’N	$80^0$53’14.20’’E	Sandy	1.6 ± 0.01
NBG-16	Bilaspur/ Chhatisgarh	891	$22^0$04’48.59’’N	$80^0$08’21.95’’E	Loamy & clayey	1.0 ± 0.01
NBG-17	Achanakmar/ Chhatisgarh	1729	$22^0$30’18.69’’N	$81^0$46’43.40’’E	Loamy & clayey	0.6 ± 0.01
NBG-18	Amarkantak/ Chhatisgarh	3413	$22^0$40’57.53’’N	$81^0$45’11.02’’E	Loamy & clayey	0.9 ± 0.01

*Mean ± S.D, n=3, M.P: Madhya Pradesh

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