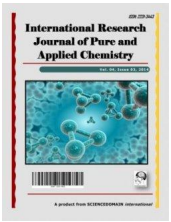

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Development and Validation of HPLC Method for the Determination and Quantification of Colchicine in *Gloriosa superba*

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: In the present study our aim is to develop and validate a reverse phase HPLC method for both determination and quantification of cochicine in different parts of *Gloriosa superba*. The developed HPLC method is validated for parameters as mentioned in ICH guidelines.
Place and Duration of Study: Study was undertaken in Department of Forest Products, University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India and in the period between June 2015 and July 2016. The plant material used for testing of developed and validated method was collected in October, 2015 and is identified in the Herbarium section of the above said Department with reference number 13900.
Methodology: The system used is of Waters binary HPLC unit with Waters HPLC pump 515, dual λ absorbance detector 2487 and Empower II software. Standard of colchicine was purchased from Sigma Aldrich, (USA) and was used for HPLC method development and validation. The developed HPLC method was validated for parameters as mentioned in ICH guidelines.
Results: The analytical column, Sunfire C_{18} (4.6×250mm, 5µm) was operated at ambient

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temperature. Isocratic elution with A acetonitrile and B 3% glacial acetic acid was at a flow rate of 1 ml/min. UV detection was done at 245nm and run time was given ten minutes for standards and fifteen minutes for samples. LOD and and LOQ of the developed method was found as 0.002 µg/ml and 0.011 µg/ml respectively.

Conclusion: Method was found to be satisfactory in terms of high accuracy, precision and robustness. The method was successfully applied to the extracts made of different plant parts of *Gloriosa superba.*

Keywords: Gloriosa superba; colchicine; HPLC; method development; validation.

1. INTRODUCTION

Gloriosa superba (Liliaceae) is mainly valued due to the presence of alkaloids which are structurally heterogeneous class of secondary biomolecules derived from basically five amino acids ornithine, lysine, phenylalanine, tyrosine and tryptophan [1]. The species produces an important alkaloid colchicine (Fig. 1), which is an amino alkaloid derived from two amino acids phenylalanine and trynosine present in it [2] and is present in seeds and tubers while the other compounds present in plant are lumicolchicine, 3-demethyl-N-deformyl-N-deacetylcolchicine, 3-demethylcolchicine, N-formyl deacetylcolchcine [3,4]. The major alkaloids colchicine and gloriosine from *Gloriosa superba* (Fig. 2) are used in treatment of gout, arthritis [5], rheumatism [6], cancer [7] and in plant breeding for inducing polyploidy [8]. Plant is also reported to be used for treating various ailments like cholera, typhus, Bright's disease, piles, skin diseases, leprosy, gonorrhea and chronic ulcers by different authors [7,9]. Tubers are thermogenic, abortifacient and antipyretic also considered useful in promoting labor and expulsion of placenta and given in rheumatic fever in powdered form [6,10]. The tuberous roots are useful against snake bite, in curing inflammation and bleeding piles [11,12,13]. Seeds are reported to be used for rheumatic pain and as a muscle relaxant [6].

HPLC method development and validation of colchicine was done by Samanidou et al. [14] which was reported applicable for colchicine estimation in pharmaceuticals & biological fluids. A number of HPLC methods were developed and validated as by several authors [15,16,17,18] but all these were reported to be applicable for pharmaceutical fluids. In the present study, objective was to develop and validate a HPLC method as per ICH guidelines for determination and quantification of colchicine in samples of *Gloriosa superba*, which is known for natural source of colchicine. This can be successfully applied in pharmaceutical industries and for further chemical evaluation studies of the plant.

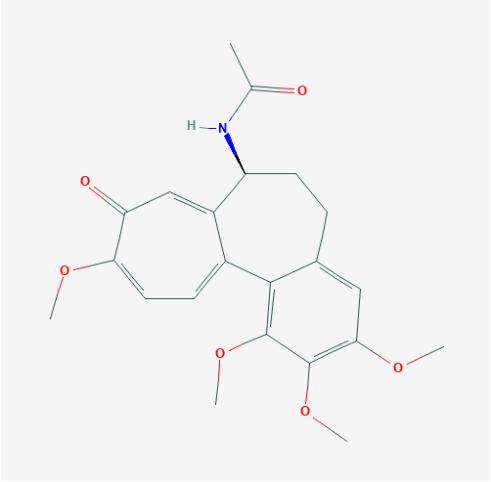

Fig. 1. Chemical structure of Colchicine (Source from PubChem.)

Fig. 2. *Gloriosa superba* plant

2. EXPERIMENTAL DETAILS

2.1 Standard, Solvents and Reagents

The colchicine standard was purchased from Sigma Aldrich (USA). All the solvents and reagents used were of HPLC grade (acetonitrile (Catalogue no. 61803025001730), glacial acetic acid (Catalogue no. 61866510001730), water (Catalogue no. 61765010001730)) of Merck brand (Darmstadt, Germany) manufactured by Merck, India. The solvent used for extraction were of AR grade.

2.2 Sample Preparation

2.2.1 Plant Material and its preparation

The plant material was collected from Nauni area of District Solan of Himachal Pradesh (India) situated at 1250m altitude, 30°51'35.85" N latitude and 77°10'22.66" E longitude. Parts of the plant separated, shade dried and was grinded mechanically and sieved by mesh size 800 microns sieve to form the uniform particle size of the plant material, which was used for quantification of colchicine in the samples under study.

2.2.2 Extraction and preparation of samples for HPLC

The samples (2 gram each) of different plant parts were extracted by soxhlet with methanol for 2 hours duration. After extraction, solvent from each sample was distilled off and the residue was completely dried to constant weight was then subjected to HPLC analysis for quantification of colchicine. The dilution of the samples was done using mobile phase (acetonitrile: water: 60: 40, v/v), centrifuged at 4000 rpm and then filtrated through 0.45 µm membrane prior to injection in the HPLC system.

2.3 Instrumentation and HPLC Method Development

The system used is of Waters binary HPLC unit with Waters HPLC pump 515, dual λ absorbance detector 2487 and program used for data analysis was Empower II software. Numerous optimization experiments on type of column, solvent system, flow rate and wavelengths *etc*. allowed the establishment of best chromatographic conditions to analytical separations of the components.

2.4 Developed Chromatographic Conditions

The optimized chromatographic conditions which clearly separate the compound of our interest *i.e.* colchicine in plant samples is as below:

Equipment	**:**	Waters HPLC unit with Waters HPLC pump 515 and dual λ absorbance detector 2487
Column	**:**	Sunfire C-18 (4.6 x 250mm, 5µm)
Mobile Phase	**:**	Acetonitrile : 3% glacial acetic acid (60 : 40)
Flow rate	**:**	1ml/min.
Mode of flow	**:**	Isocratic
Detection	**:**	245nm
Run Time	**:**	15 minutes
Retention time	**:**	3.0 ± 0.05

2.4.1 Preparation of standard stock solution

Standard stock solution containing colchicine (95.0 µg/ml) was prepared from procured reference standard of colchicine with mobile phase (acetonitrile:water :: 60:40, v/v) which was further diluted to obtain lower concentrations.

2.4.2 HPLC method validation

The developed HPLC method was validated for seven parameters as mentioned in ICH guidelines and procedure followed for testing these parameters was also as per ICH guidelines [19]. Parameters used for validation study were Linearity and range, Accuracy, Precision, Limit of detection, Limit of quantitation and Robustness as used by Sharma et al. [20].

1) Linearity and range

Six concentrations (4.750 µg/ml, 9.500 µg/ml, 19.000 µg/ml, 38.000 µg/ml, 76.000 µg/ml and 95.000 µg/ml) of the analyte solutions were used in triplicate for obtaining calibration curve of colchicine. The calibration curve was constructed by plotting the mean peak area versus the concentration of analyte. The concentration range of the method was derived from interval between upper and lower values (including these values) of linearity.

2) Accuracy

The accuracy of the method was studied by recovery studies. The accuracy of the method was determined by percentage recovery of colchicine in the spiked sample at three concentration levels. The resultant samples were then analysed (replicated three times) and the average percentage recoveries were calculated as:

$$\text{Recovery (\%)} = \frac{\text{Observed amount of compound(µg/ml)}}{\text{Actual amount of compound (µg/ml)}} \times 100$$

3) Precision

Precision of the developed method was evaluated by intra-day precision and inter-day precision study. Same concentration of standard mixture was injected for six times in a day and measuring their response for intra-day study and same concentration of standard mixture for six consecutive days and measuring their response for inter-day precision.

4) Limit of Detection (LOD)

The lowest concentration of working solution of the analyte was further diluted with mobile phase (acetonitrile : water :: 60:40, v/v) to yield a series of appropriate concentrations. Limit of detection (LOD) of the developed method was determined by injecting progressively low concentrations of the standard solutions and S/N ratio for each concentration was observed. The concentration having signal to noise ratio nearly 3 has been found as LOD.

5) Limits of Quantitation (LOQ)

The lowest concentration of working solution of the analyte were further diluted with mobile phase (acetonitrile : water :: 60:40, v/v) to yield a series of appropriate concentrations. Limit of quantitation (LOQ) of the developed method was determined by injecting progressively low concentrations of the standard solutions and observed S/N ratio of each concentration. The LOQ for investigated compound was established at signal to noise ratio approaching nearly to 10.

6) Robustness

An HPLC method is said to be robust if it can bear small but deliberate changes in the method. In the present study, change in flow rate (±0.05%) and change in mobile phase composition (±2%) was done. A fixed standard concentration was selected and injected in triplicate for each change (flow rate and mobile phase) in robustness study. The %RSD of the retention time was calculated for mean value of each factor.

2.5 Testing of Developed Method

The developed HPLC method was used for quantification of colchicine in different plant parts *viz*., leaf, stem, seeds and tubers.

3. RESULTS AND DISCUSSION

3.1 Method Validation

Developed method was validated for the said parameters:

The results obtained for linearity and range for colchicine are presented in Table 1. Linearity of colchicine was established for six concentrations ranging from 4.750 µg/ml – 95.000 µg/ml. Regression equation obtained was linear with correlation coefficient (R) value 0.999 which suggest that developed method is fit for acceptance. The regression equation derived from the linearity data was Y = 1.07e+005 X + 1.03e+005. The calibration curve was constructed by plotting the mean peak area versus the concentration of each analyte (Fig. 3).

In recovery, results showed that recovery percentage for colchicine ranged from 101.529±0.213% to 102.402±0.295% and % RSD ranged from 0.210 to 0.543. The overall recovery percentage for colchicine was found 102.101 ± 0.165. The results presented in detail in Table 2 showed that the method has good recovery as the % RSD was less than 1 (Table 2).

The intra-day precision evaluated on the basis of % RSD (coefficient of variation) which was was found 0.84% for colchicine. The %RSD for inter-day precision for colchicine, was found 1.40% (Table 3). The developed method confirms high precision as % RSD was less than 3% which has been stated satisfactory by various research workers during the precision study.

The LOD was found as 0.002µg/ml which has an average S/N ratio of 3 (Table 3). The LOQ was found as 0.011µg/ml which has an average S/N ratio of 10 (Table 3).

Table 1. Linearity data of colchicine

Phyto-constituent	Linearity (µg/ml)	Regression equation	Correlation coefficient (R)	Retention time (minutes)	
				Mean[a]	RSD%
Colchicine	4.750 – 95.000	Y = 1.07e+005 X + 1.03e+005	0.999	3.067 ± 0.008	0.270

[a] *Mean ± SD (n=18)*

Table 2. Recovery studies of Colchicine

Phyto-constituent	Initial Quantity (µg/ml)	Added Quantity (µg/ml)	Total Quantity (µg/ml)	Recovery			Overall recovery[b] (%)
				Mean recovery [a] (µg/ml)	Mean recovery [a] (%)	%RSD	
Colchicine	4.750	9.500	14.250	14.468 ± 0.030	101.529± 0.213	0.210	102.101 ± 0.165
	4.750	19.000	23.750	24.320 ± 0.070	102.402± 0.295	0.288	
	4.750	38.000	42.750	43.764 ± 0.238	102.372± 0.566	0.543	

[a]*Mean ± SD (n=3)*
[b]*Mean ± SD (n=9)*

Table 3. Precision, Limit of detection and Limit of quantitation data of Colchicine

Phyto-constituent	Precision		LOD (µg/ml)	LOQ (µg/ml)
	Intra-day (% RSD)[a]	Inter-day (% RSD)[b]		
Colchicine	0.84	1.40	0.002	0.011

[a]*Intra-day precision : data expressed as mean (n=6)*
[b]*Inter-day precision: data expressed as mean (n=6)*

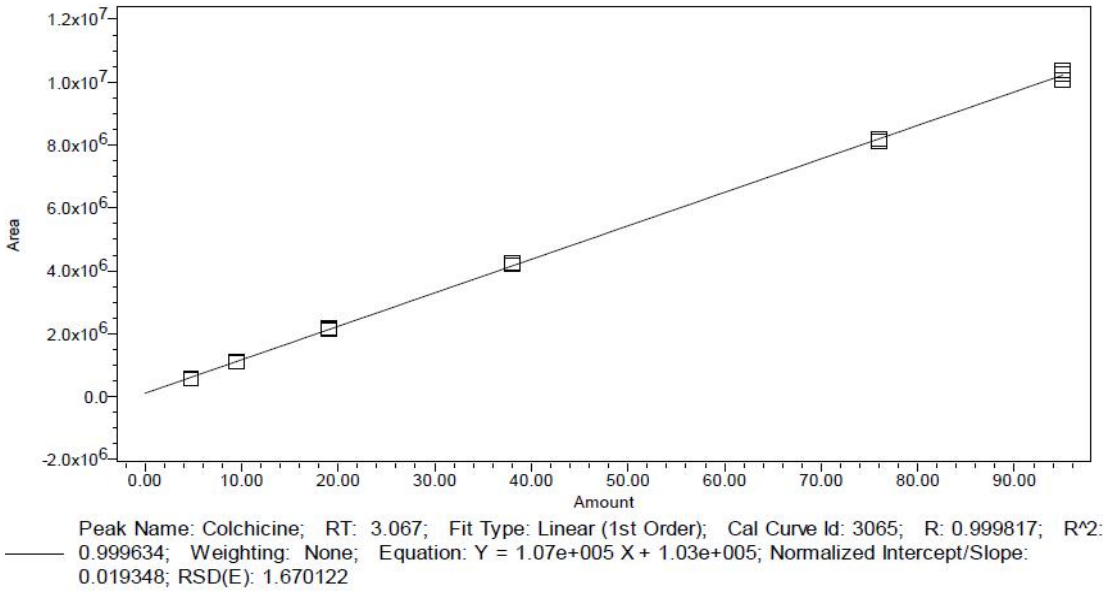

Fig. 3. Calibration curve of Colchicine (Standard compound)

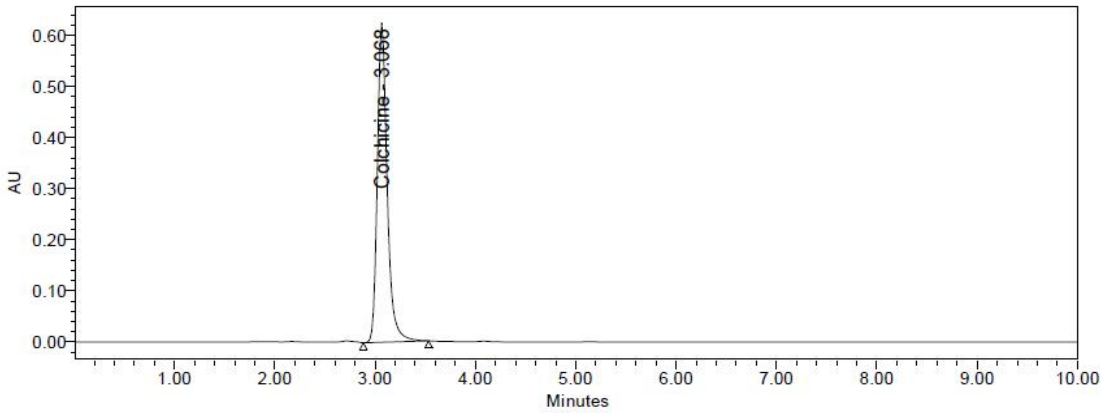

Fig. 4. Chromatogram of Colchicine (Standard compound)

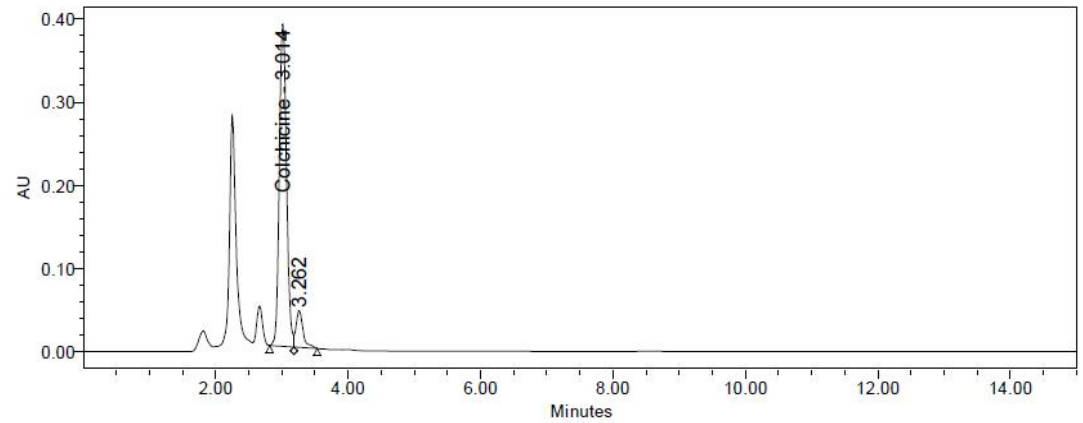

Fig. 5. Chromatogram of *Gloriosa superba* sample

Table 4. Robustness studies of Colchicine

Factor I -Flow rate (ml/min); Mobile phase (acetonitrile:3%GAA:: 60:40, v/v)	Colchicine (Retention time, minutes)[a]
0.95	3.207±0.006
1.0	3.053±0.003
1.1	2.903±0.006
Mean	3.054
S.D.[b]	0.051
%RSD	1.655
Factor II - Mobile phase (acetonitrile:3%GAA, v/v); Flow rate (1 ml/min);	
58:42	3.123±0.003
60:40	3.053±0.003
62:38	2.810±0.104
Mean	2.996
S.D. [b]	0.055
% RSD	1.830

[a]Mean ± SD (n=3)
[b]Mean ± SD (n=9)

The developed method had flow rate of 1 ml/min. and with this flow rate colchicine elutes at 3.053 minutes. When the flow rate of mobile phase was slightly decreased to 0.95 ml/min., the elution time of colchicine increased to 3.207 minutes. With the increase in flow rate to 1.1 ml/min. from 1 ml/min., the elution time of colchicine decreased to 2.903 minutes. The %RSD for retention time of colchicine was 1.655% (Table 4). The developed method had mobile phase of (acetonitrile : 3% glacial acetic acid ::60 : 40, v/v) and with this mobile phase colchicine elutes at 3.053 minutes. When the mobile phase ratio changed to acetonitrile : 3% glacial acetic acid :: 58 : 42 the elution time of colchicine increased to 3.123 minutes. With the change in mobile phase ratio as acetonitrile : 3% glacial acetic acid :: 62 :38 the elution time of colchicine decreased to 2.810 minutes. The %RSD for retention time of colchicine was 1.830% (Table 4).

In such method we can change these parameters to such extent. So the method is valid for these changes in parameter and can be stated as robust. Results showed that separation was not affected by slight but deliberate changes in chromatographic conditions.

3.2 Testing of the Developed Method

The developed method was used for quantification of colchicine in different parts *viz*., seeds, tubers, stem and leaves of *Gloriosa superba.* In the sample, compounds other than colchicine are also present so, in order to elute the sample completely from the column we increased the run time from ten minutes to fifteen minutes. The peak of colchicine in HPLC chromatograms was clearly identifiable, well resolved and without any fronting and tailing (Figs. 4 & 5). Colchicine content in samples of different plant parts ranged from 0.051% to 0.695%. The results indicate that colchicine content was highest in seeds (0.695%) followed by tubers (0.396%), leaves (0.164%) and lowest in stem part (0.051%) of cultivated plants of *Gloriosa superba* (Table 5).

Table 5. Quantification of Colchicine in different parts of *Gloriosa superba*

Sr. no.	Plant Part	Colchicine content (%)
1	Seeds	0.695±0.003
2	Tubers	0.396±0.003
3	Stems	0.051±0.007
4	Leaves	0.164±0.002

Estimation of colchicine in different parts of plant has also been done by some researchers [21,22,23]. Variation in results may be due to differences in sample source (area and time of collection) and sample preparation for HPLC quantification.

4. CONCLUSION

An HPLC method was developed and validated and shown applicable to the determination of colchicine in different parts of *Gloriosa superba*.

The method was validated as per ICH guidelines and shown the developed method to be linear, accurate, precise, and robust with low values of LOD, LOQ which shows developed method can trace cochicine in samples having very low concentration. Developed method shows good linearity over the range of 0.051% to 0.695% colchicine in samples of *Gloriosa superba* with correlation coefficient 0.999. The method has LOD and LOQ for colchicine as 0.002µg/ml and 0.011µg/ml respectively. The method has shown good precision and accuracy assessed on the basis of inter-day, intra-day and recovery studies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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