COMPARATIVE QUANTITATIVE ESTIMATION OF COLCHICINE IN CORMS OF DIFFERENT COLCHICINE BEARING PLANTS USING HPLC AND HPTLC METHOD

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ABSTRACT

Colchicine is an alkaloid which has been reported to possess anti-gout, anti-rheumatic, antiparasitic and anticancer potential. It is a toxic natural product obtained from plants of the genus *Colchicum* (*C. autumnale* and *C. luteum*), *Gloriosa superba,* and *Iphigenia indica*. In the present study, three different methods (percolation, sonication, and Soxhlation) of the extraction for colchicine from three different plants (*C. luteum*, *G.superba*, and *I. indica*) have been studied.The concentration of colchicine in different prepared extracts was quantified using high-performance liquid chromatographic (HPLC) and high-performance thin-layer chromatographic (HPTLC) methods. The developed and optimized chromatographic method was validated according to the International Conference on Harmonization (ICH) guidelines for linearity, precision, accuracy, specificity, sensitivity, recovery and robustness. The developed methods (HPLC and HPTLC) were applied for the comparative quantitative estimation of colchicine in three different plant extracts. Both the methods were found to be simple, selective and accurate with a wide range of linearity, hence useful for the quantification of colchicine content in different formulations containing colchicine-bearing plant extracts. Due to its toxic nature, the quantification of colchicine will play an important role in quality control of several herbal and Unani formulations containing colchicine-bearing plant extracts. The present studies clearly indicate that both *G. superba* and *C. luteum* contain about 0.2 %w/w of colchicine.

Keywords: Colchicine, *C. luteum*,*G. superba*, *I. indica*, estimation, HPLC, HPTLC

INTRODUCTION

Natural products, particularly secondary plant metabolites, have formed the basis of many herbal formulations. They provide an invaluable resource that has been used as leads for the development of new drug molecules[1]. The Indian Pharmacopoeia (1966) recognizes eighty five drug plants whose ingredients are used in various pharmaceutical preparations. We research confine our to a comparative study of colchicine-bearing plants by using different extraction techniques. Colchicine has been used clinically in as anti-gout agent and has been reported to decrease the production and *in vitro* release of CCF (crystal-induced chemotactic factor), the prime mediator of acute gouty attack[2]. In addition, it is also reported as an anticancer agent, which blocks mitosis by preventing tubulin polymerization to microtubules[3-4]. Microtubules are main protein filaments that make up the cytoskeleton, which is crucial in regulation of many activities including cell migration, division and polarization. It is also used to treat acute pericarditis[5], Behcet's syndrome and some forms of psoriasis[6-8]. It also been used as an antiparasitic agent in ethnoveterinary use[9].

Colchicum extract was first described by Padanius Dioscorides, a Greek surgeon in the Roman Army in the first century BC as a treatment for gout in *De Materia Medica*. It is an alkaloid first isolated in 1820 by the two French chemists, P.S. Pelletier and J. Caventon[10]. It has been found in the corms of *C. uteum* and the seeds of *Iphigenia* to the extent of about 0.25% and 0.9%, respectively[11]. In addition, *G. superba* is another plant which also contains colchicine[12]. A mixture of alkaloids consisting mainly of colchicine has been isolated from dried tubers of *G. superba*[13].

Colchicine is a toxic natural product and secondary metabolite extracted from plants of the genus *Colchicum,* commonly known as *autumn crocus*, wild saffron and

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naked lady[14]. It is present in most parts of the temperate areas of Europe, Asia and America[15]. It is an alkaloid prepared from dried corms and seeds of *C. autumnale.* The dried corms of *C. luteum* contain around 0.25% of colchicine and the seeds contain about 0.4% of colchicine. The systematic study on genus *Iphigenia,* particularly *I. indica* (L.), by A.Gray had revealed that its seeds contain as much as 0.51% colchicine. A number of *Iphigenia* species occur around Pune[16]. Colchicine levels in *G. superba* corms have been reported to the extent of around 0.9% (DM)[17].

It grows in Africa, India and South eastern Asia. Earlier studies revealed that colchicine levels are the highest during the initial growth of the plant, and these levels decline during maturation in *G. superba*[18]. Among the Indian medicinal plants, the corms of *C. luteum* and the seeds of *Iphigenia* contain colchicine to the extent of about 0.25% and 0.9%, respectively[11]. A mixture of alkaloids consisting mainly of colchicine from dried tubers of *G. superba* has been isolated[12]. *Gloriosa* is a genus of five or six species in the plant family Colchicaceae, from tropical Africa, India and South-eastern Asia. The determination of colchicine in pharmaceutical preparations, in biological fluids and in plant extracts has been described by several analytical methods[19-20].

In the present study, three different methods[21-23] of extraction of colchicine have been studied and the concentration of colchicine was quantified using high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC) in three different plant species, namely, *C. luteum*, *G. superba* and *I. indica*. Literature survey revealed that *I. indica* seeds contained maximum concentration of colchicine. However, no data on *I. indica* corms have been reported yet. So, it is essential to find out the concentration of colchicine in corms using sensitive techniques. However, there is no literature reported on comparative data of different colchicine bearing plants. So the study confined to the estimation of colchicine in corms of different colchicine-bearing plants.

MATERIALS AND METHODS

Drug and chemicals

Three plant species, namely, *C. luteum*, *G. superba*, and *I. indica* were purchased from the local market of Solan, Himachal Pradesh and used for comparative estimation. Colchicine drug was purchased from Sigma Aldrich, USA. Methanol was purchased from HiMedia and was of HPLC grade. Analytical grade water was obtained using water purification systems ELIX 03 (Millipore, USA). All the other chemicals and solvents purchased were of analytical grade and were used without any further purification.

Extraction procedure

The crushed corms of *C. luteum*, *G. superba* and *I. indica* (20 g each) were defatted with petroleum ether by percolation and the extraction of the marc was done by percolation, sonication and Soxhlation in triplicate using $CHCl_3$ as a solvent (Fig. 1).

INSTRUMENTATION

HPLC analysis was performed using WATERS (Milford, USA) system composed of 515 series pumps

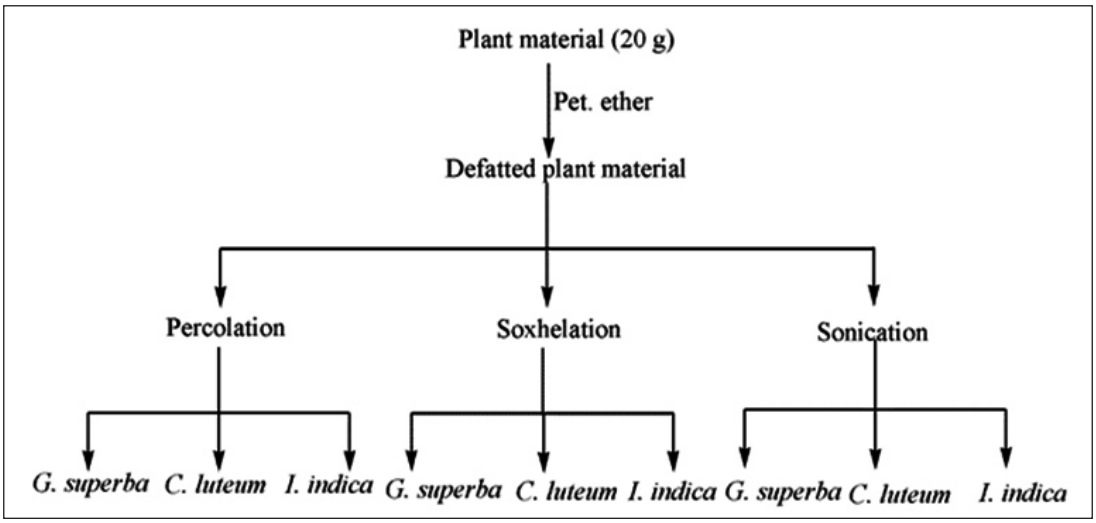

Fig. 1: Extraction method for colchicine from corms of *C. luteum*, *G. superba* and *I. indica*

combined with Waters 2707 autosampler along with Waters PDA 2998 series photodiode array detector set at wavelength range 190-800 nm with column from Waters Spherisorb® C_{18} bonded with 5 µm (4.6 x 250 mm), coupled with EMPOWER-2 software recording and processing of chromatographic data. Ultrasonic cleaner (Steryl Medi-Equip Systems Chennai) and water purification system ELIX 03 (Millipore, USA) were used.

CAMAG HPTLC system equipped with a sample applicator Linomat 5 TLC Scanner III, Reprostar and Wincats 4.02 integration software (Switzerland), twin trough glass development chamber, ultrasonic cleaner and water purification system as described earlier were used during the study.

RP-HPLC METHOD DEVELOPMENT

Sample preparation

Stock solutions of colchicine (marker) and samples, 1 mg mL^{-1}, were prepared in methanol and stored at 2-8°C until used. Aliquots from each stock solution were diluted stepwise with methanol to obtain 100 µg mL^{-1}.

Optimization of chromatographic conditions

The effects of different chromatographic conditions on the instrument response create a situation where one has to compromise between different experimental variables in order to achieve the best chromatographic separation. Chromatographic separations are significantly affected by the mobile phase conditions, such as the type and composition of the organic modifiers[24] and therefore, before selecting the conditions for the optimization, a number of preliminary trials were conducted with combinations of different organic solvents, compositions, and flow rate to check the retention time, shape, resolution, and other chromatographic parameters. Among all tried experiments, the mobile phase combination of HPLC H_2O, MeOH and formic acid in the ratio of (50: 50:0.1 V/V/V) with isocratic elution at flow rate of 1.0 mL min^{-1} was found to be most suitable. Best resolution and sensitivity of the method was obtained for colchicine at 352 nm. Typical chromatogram with optimized condition gives sharp and symmetric peak with retention time of 3.82 min (Fig. 2).

Preparation of samples for method validation

After method development and optimization, the method was validated. Optimized method was validated according to ICH guidelines for linearity, sensitivity and precision and recovery studies have been carried out[25-26].

Calibration curve (linearity)

Linearity was determined by six different concentrations of colchicine in triplicate and calibration curve was plotted in the range of 1-64 µg mL^{-1} of colchicine. The stock solution prepared was of 1mg mL^{-1} of colchicine. Further dilutions were prepared using above stock solution. Calibration curve was plotted by replicate analysis at all concentration levels and linear relationship was evaluated using the least square method with Microsoft® Excel program.

Precision

Intra-day precision and inter-day precision for the developed method was determined in terms of percent relative standard deviation (% RSD). The experiments were repeated three times a day for intra-day precision and on two different days for inter-day precision. The concentration values for both intra-day precision and inter-day precision were calculated six times separately and % RSD calculated.

Detection and quantification limits (LOD and LOQ)

Limits of detection and quantification were calculated by method based on standard deviation (σ) and slope (S) of calibration plot using formula LOD = $3.3\sigma/S$ and LOQ = $10\sigma/S$.

HPTLC densitometric method development and validation

High performance thin layer chromatography (HPTLC) method was developed and optimized for colchicine (marker). Method development involves evaluation and optimization of the various stages of sample preparation, chromatographic separation, detection and quantification. Optimization of various parameters were performed in order to develop a selective and sensitive method for analysis of colchicine on high performance thin layer chromatography (HPTLC) using photo diode array detector (PDA).

Standard stock solution

Stock solutions of colchicine (marker) and samples, 10 mg mL^{-1} were prepared in methanol and stored at 2–8 °C until used.

Sample application

Samples were spotted as a band of 8 mm width on pre-coated silica gel (10x20 mm) aluminum backed plate 60_F-254 with 200 µm thickness. Application rate of 20 µL per sample was employed and space between

bands was 10 mm. Linear ascending development was carried out in a twin trough glass chamber previously saturated with mobile phase for 20 min. Densitometric scanning was performed on CAMAG TLC scanner III in the absorbance/reflectance mode. Chromatographic separations are significantly affected by mobile phase conditions, such as type and composition of organic modifiers[24] and therefore, before selecting proper chromatographic conditions, several of preliminary trials were conducted with different combinations of different organic solvents and compositions, to obtain satisfactory retention factor, resolution, and other chromatographic parameters. From those experiments, mobile phase combination of ethyl acetate**:** ethanol: formic acid (9**:**1**:** 0.01V/V/V) was found to be most suitable.

Best resolution and sensitivity of the method for colchicine was detected at 352 nm. Typical chromatogram with optimized condition gave sharp and resolved peak with retention factor at 0.15.

Validation of optimized method

After chromatographic method development and optimization the method was validated. Optimized method was validated according to ICH guidelines for linearity, precision, accuracy, specificity, sensitivity, recovery and robustness[25-26].

Calibration curve (linearity)

Linearity was determined by six different concentrations of colchicine in triplicate and calibration curve was plotted in specified range of 200-1000 µg spot^{-1}. Calibration curve was plotted by replicate analysis at all concentration levels and linear relationship was evaluated using the least square method with Microsoft® Excel program.

Precision

Intra-day precision and inter-day precision for the developed method was determined as describe earlier as relative standard deviation (% RSD).

Detection and quantification limits (LOD and LOQ)

Limits of detection and quantification were calculated as describe earlier.

RESULTS AND DISCUSSION

RP-HPLC method development and validation

The optimization of chromatographic parameters for RP-HPLC method is depicted in Table I.

Table I: Optimization of chromatographic conditions for RP-HPLC method

Chromatographic parameter	Variation	Peak shape
Mobile phase composition varied with flow rate of 1.0 mL min^{-1} fixed	60:40:0.1	Broad peak
	40:60:0.2	Broad peak
	50:50:0.1 with isocratic flow	Sharp peak, almost symmetric
Changes in flow rate with mobile phase composition in ratio of (H_2O: MeOH: formic acid in ratio of 50**:**50:0.1 V/V/V) with isocratic elution	0.8	Broad peak
	1.0	Sharp peak, almost symmetric
	1.2	Sharp peak, not symmetric

In addition, the chromatographic conditions optimized for analysis of colchicines is depicted in Table II.

Table II: Chromatographic conditions optimized for analysis of colchicines

Stock solution	Methanol
Stationary phase	WATERS, C-18 column (4.6 X 250mm, 5µ)
Mobile phase	Methanol: water: formic acid (50:50:0.1 V/V/V)
Detector	PDA
Quantification wavelength	352 nm
Flow rate	1 mL min^{-1}
Dilution	Methanol
Retention time	3.82

The colchicine chromatogram for HPLC method is given in Fig. 2 and HPLC chromatogram of colchicine extract is given in Fig. 3.

Calibration curve (linearity)

Results of the regression analysis and the coefficient of determination (r^2) are listed in Table III. High coefficient of determination values i.e. 0.99 for colchicine in the range 1- 64 µg mL^{-1} (Fig. 4) are observed.

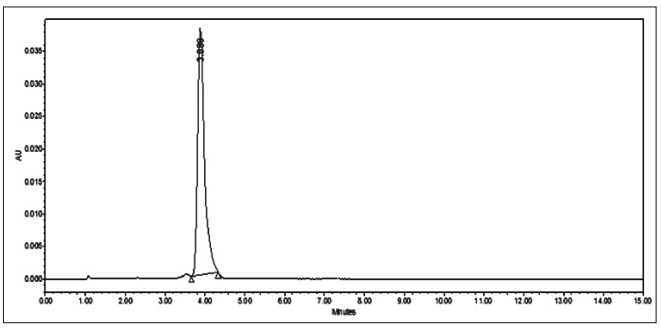

Fig. 2: Chromatogram of colchicine for optimized HPLC method

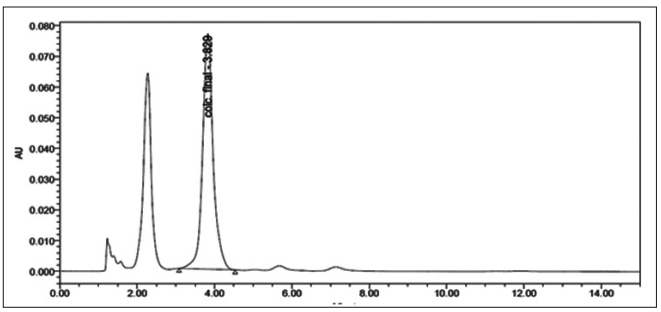

Fig. 3: HPLC chromatogram of extract showing presence of colchicine

Table III: Detailed validation parameters optimized for colchicine using HPLC and HPTLC

S. No.	Validation parameter	HPLC	HPTLC
1	UV-Detection (nm)	352	352
2	Linearity range (µg mL^{-1})	1-64 µg mL^{-1}	200-1000 µg $spot^{-1}$
3	Coefficient of determination (r^2)	0.998	0.9984
4	Regression equation	Y = 4635x	Y = 148.39x – 2839
5	Slope (b)	4635	148.39
6	Intercept (a)	00	-2839.4
7	Limit of detection LOD (ng mL^{-1})	8.53	43.35
8	Limit of quantitation LOQ (ng mL^{-1})	28.45	144.49
9	Precision (%RSD)	Intraday = 0.524 Interday = 0.695	Intraday = 1.38 Interday = 0.745

Precision

Intra-day and inter-day precision results are listed in Table IV in terms of % RSD. Intra-day and inter-day precision were found to be 0.524 and 0.695 for colchicine (% RSD< 2.0), which indicates that the proposed method is highly precise and reproducible.

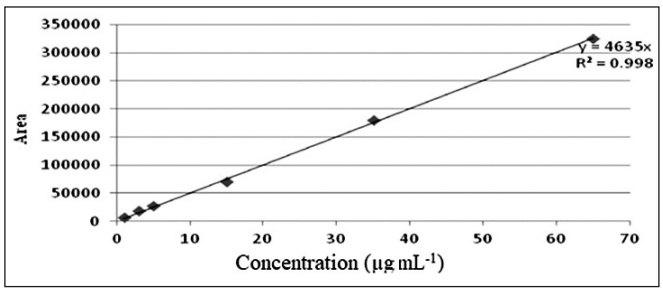

Fig. 4: Calibration curve of colchicine using HPLC

Table IV: Concentration of colchicine in different extracts of different species

Type of extract	HPLC (% w/w)	HPTLC (% w/w)
Percolation extract		
G. superba	0.16	0.12
C. luteum	0.16	0.14
I. indica	---	---
Soxhelation		
G. superba	0.18	0.14
C. luteum	0.18	0.15
I. indica	0.01	--
Sonication		
G. superba	0.17	0.13
C. luteum	**0.19**	**0.16**
I. indica	0.02	0.01

Detection and quantification limits

LOD and LOQ were found to be 8.537 and 28.459 ng mL^{-1} respectively, for colchicine. These data show that the method is highly sensitive and specific.

HPTLC densitometric method development and validation

A mobile phase consisting of ethyl acetate: ethanol: formic acid in ratio of 9:1:0.01 V/V/V was used for the analysis which gave sharp and well resolved peak of colchicine. Best resolution and sensitivity of the method was obtained at 352nm with retention factor of 0.15.

Calibration curve (linearity)

Results of the regression analysis and the coefficient of determination (r^2) are listed in Table III. High coefficient

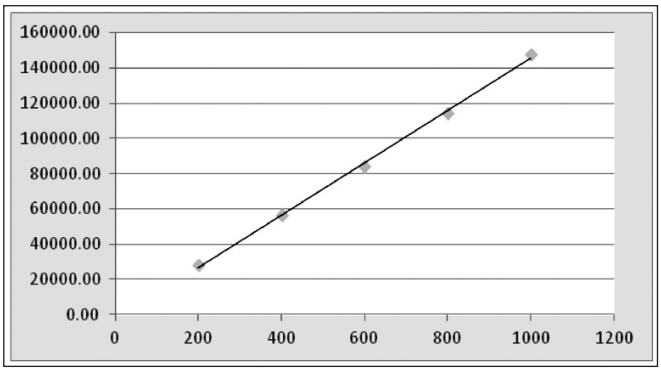

Fig. 5: Calibration curve of colchicine using HPTLC

of determination values i.e. 0.998 for colchicine in the range 200-1000 µg mL^{-1} (Fig. 5) are observed.

Precision

Intra-day and inter-day precision results are listed in Table III in terms of % RSD. Intra-day and inter-day precision were found to be 1.368 and 0.745 respectively, for colchicine, which indicated that the propose method is highly precise and reproducible.

Specificity

This was ascertained by analyzing the R_f values and spectra pattern of colchicine and extracts prepared by different methods. Peak for colchicine in different extracts was confirmed by comparing R_f values, overlay and their UV absorption spectra with that of standard (Fig. 6).

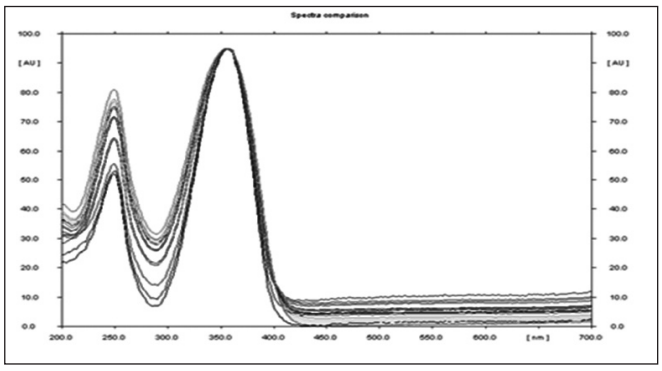

Fig. 6: UV absorption spectra of colchicine at different concentrations

CONCLUSION

Colchicine, one of the major components bearing a plethora of pharmacological activities, is widely used in the synthesis of thiocolchicoside, a muscle relaxant. Present studies have clearly indicated that colchicine content in *I. indica* is only 0.01%, contrary to the tall claims of heavy 0.9%. However, HPLC and HPTLC data revealed that the concentration of colchicine in both *G. superba* and *C. luteum* were found to be around 0.2% w/w. The data is here reported for the first time in which a comparative study related to the isolation of colchicine by different techniques is clearly described.

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