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Colchicine Determination in *Gloriosa* spp. by HPLC

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ABSTRACT

Colchicine is a phytochemical alkaloid with important pharmaceutical, biomedical, and agricultural properties. An HPLC method for colchicine analysis in *Gloriosa* spp. extract was developed in this study. The ideal compound separation and detection conditions were determined to be as follows: C18 column, with 50 mM KH_2PO_4 water: acetonitrile ratio of 40:60 in a mobile phase at a flow rate of 1 mL.min^{-1}. Colchicine was detected at 254 nm, with the linearity of the standard curve in the range of 20-100 µg.mL^{-1}. The developed method was precise with a value of 0.16 %RSD intra-day and 0.93 %RSD inter-day which was lower than 1%RSD. The limit of detection (LOD) and limit of quantification (LOQ) were 4.98 and 62.23 ng.mL^{-1}, respectively. Accuracy was evaluated using recovery (%) value, which yielded an average of 99.41 % recovery. The specificity analysis showed that interference was not observed at the retention time of colchicine (4.4 min). The method was stable without effects from mobile phase ratio, flow rate, and column temperature. This HPLC method was used to quantify colchicine from various *Gloriosa* spp. samples, the content was in the range of 117.97 - 380.66 µg.gDW^{-1}. The conditions and protocols used in this study have been rigorously modified to produce favorable outcomes for colchicine. Thus, this suggests that this approach can be used as a standardized procedure for further colchicine analysis, especially in *Gloriosa* spp. which will benefit studies in pharmaceutical science and lead to further developments.

Keywords: *Gloriosa*; Colchicine; HPLC; Validation; Extraction

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1. Introduction

Gloriosa spp., a climbing herb, is widely distributed throughout the tropical areas of Africa and Asia. Other than its use as an ornament, it is also a source of medicinal agents. The essential well-known compound in *Gloriosa* spp. is colchicine (Fig. 1), which is a phytochemical alkaloid also found in other plants such as *Colchicum autumnale.*

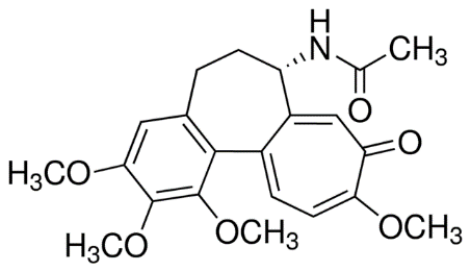

Fig. 1. Colchicine chemical structure.

Colchicine is commonly used to treat gout. Other than its involvement in several anti-inflammatory pathways, colchicine also interrupts inflammasome stimulation through blocking microtubule assembly [1]. Moreover, it is used to treat familial Mediterranean fever [2] and incident cancer in male gout patients [3].

Other than its medicinal utility, colchicine is also important in agriculture, for example, its use in the induction of polyploid plants. Polyploid induction disturbs polymerization via tubulin binding. This action alters mitosis and doubles the chromosome set. The resultant double chromosome set has been the basis for plant improvement using colchicine. For example, tetraploidy in Artemisia *annua* L., induced by colchicine, produced high-yields of artemisinin in the plant [4]. The treatment of colchicine to *Cannabis sativa* L. produced tetraploid and mixo-ploid lines and affected a change in Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) levels in which tetraploid (2n=4x) *C. sativa* L., at some stages of development, presented higher content than that of diploid plants (2n=2x) [5].

Colchicine extraction was performed by several methods. Bulbs and flowers of *C. hierosolymitanum* and *C. steveni* were extracted with petroleum ether and shaking for one hour [6]. Then, the colchicine contents in extracts were analyzed using thin layer chromatography - UV (TLC-UV) and high-performance liquid chromatography photodiode array [HPLC-UV (PAD)] methods. It was found that the colchicine content in bulbs was 1.4-1.5 times higher than that in flower. In comparing these two species, the colchicine content of *C. steveni* bulbs was 1.3-1.4 times higher than that of *C. hierosolymitanum*. Colchicine in *G. superba* and *Sandersonia aurantiaca* was extracted using maceration with methanol at 10 °C overnight [7]. Colchicine content in both species was about 0.9%. The developmental stage of the plant is one factor affecting colchicine content. Bulbs of *C. tunicatum* in the vegetating stage had higher colchicine content (0.12%w/w) than was observed in the flowering stage (0.08 %w/w) [8]. Moreover, a difference in content was observed between separate plant parts, as observed, the highest (0.16 %w/w) was in *C. tunicatum* bulbs, and the lowest was in flowers (0.4 %w/w) at the flowering stage. At the vegetating stage, the highest colchicine content was found in *C. tunicatum* roots, and was lower in bulbs, stems, leaves, and flowers, respectively [8].

As mentioned above, the content of this important secondary metabolite varies depending on various factors. In order to maximize yield, extraction and quantification protocols are crucial. In this study, we aimed to validate HPLC conditions and compare the extraction methods.

2. Materials and Methods

2.1 HPLC analysis of colchicine

Colchicine content was analyzed by HPLC and tested by modified standard of United States Pharmacopeia (USP37) [9].

2.1.1 Stock of standard colchicine

Standard colchicine was used for the validation of analytical methods before applying the procedure in the study of colchicine content in *Gloriosa* spp. Stock of colchicine was prepared by dissolving 10 mg standard colchicine into methanol in a 10 mL volumetric flask, then the volume was adjusted. The final concentration of stock standard solution was one $mg.mL^{-1}$. In order to plot the standard curve, standard colchicine at concentrations of 20, 40, 60, 80, and 100 $\mu g.mL^{-1}$ were analyzed by HPLC.

2.1.2 HPLC conditions

All extracts were dissolved in methanol (HPLC grade) and filtered through a 0.45 μm filter before HPLC analysis. The conditions of HPLC were set as follows; C18 column (4.6 x 150 mm, diameter 5 um) with guard, 50 mM KH_2PO_4 in water: acetonitrile (40:60) as a mobile phase, 1 $mL.min^{-1}$ flow rate, UV-detection at 254 nm which was the maximum absorbance of colchicine, column oven at 25 °C, and 20 μl injection volume.

2.2 Method validation

2.2.1 Linearity

Five known concentrations of colchicine standard solution in the range of 20-100 $\mu g.mL^{-1}$ (n = 3) were tested for linearity. The calibration curves were plotted with peak areas (y-axis) versus the amounts of standard (x-axis).

2.2.2 Precision

Intra- and inter-day precision values were expressed as percentage of relative standard deviation (%RSD). Colchicine standard was set at a concentration of 60 $\mu g.mL^{-1}$. The intra-day precision was analyzed within one day using three injections. The inter-day precision was analyzed over the span three consecutive days, using the same method as intra-day precision validation.

2.2.3 Limit of detection (LOD) and limit of quantitation (LOQ)

The sensitivity was estimated in terms of limit of detection (LOD) and limit of quantification (LOQ), which were defined based on the signal to noise ratios of 3:1 and 10:1, respectively.

2.2.4 Accuracy

Accuracy of HPLC conditions was evaluated using the recovery value, the percentage of which was calculated with the following equation: Recovery (%) = (Experimental value/ Theoretical value) × 100. Theoretical values were expressed in terms of three concentrations of standard colchicine (40, 60, and 80 $\mu g.mL^{-1}$) which were added to the extracts. A recovery (%) value of 100 indicates perfect accuracy.

2.2.5 Robustness

The robustness of this method was determined by slight alterations of the optimized values of chromatographic conditions and reported as colchicine content and %RSD. Those factors were the composition of mobile phase (±1 v/v), flow rate (±0.1 $ml.min^{-1}$), and oven temperature (±2 °C). Colchicine at a concentration of 60 $\mu g.mL^{-1}$ was used to evaluate the robustness.

2.2.6 Specificity

The specificity was evaluated by comparing the chromatogram of *G. superba* L. extract with those of 1) diluting solvent as a blank; 2) standard colchicine; and 3) standard colchicine mixed with *G. superba* L. extract. The purpose of the specificity test was to assess possible interference to the analyte.

2.3 Plant materials

G. superba L. was collected from Thailand and the three other *Gloriosa* spp. samples were obtained from the Netherlands. Dr. Tassanai Jaruwatanapan (Department of Horticulture, Faculty of Agriculture, Kasetsart University) provided taxonomic confirmation.

2.4 The extraction method for colchicine analysis

The bulbs of *Gloriosa* spp. were dried at 50°C and ground to powder before extraction. *G. superba* L., which was obtained from Thailand, had the highest number of bulbs. This species was found to be the most suitable plant material for extraction with the three different methods (A, B, and C). The most suitable method was thus applied to the other *Gloriosa* spp. sampels.

2.4.1 Method A

Method A using shaking followed the work of Alali et al. [6]. The powder (1 g) of *G. superba* L. was shaken for one hour in an Erlenmeyer flask containing petroleum ether (50 mL) before filtration. Then, the dried residue was re-extracted twice. Consequently, the residue of *G. superba* L. was shaken in an Erlenmeyer flask containing 20 mL dichloromethane, at room temperature for 30 min. One milliliter of 10% NH_4OH was added to the mixture, before shaking for 10 min, and then being left for 30 min. The residue was separated from the extract by filtration before removing the dichloromethane by evaporation. The dichloromethane extract was kept at -20 °C before analysis.

2.4.2 Method B

Method B was modified from Senthilkumar [10]. Ten grams of *G. superba* L. powder was extracted with methanol for three hours by soxhlet procedure. The water was added in methanol extract (1:1) before partition using petroleum ether. Then, the water layer of the extract was fractionated using chloroform. Finally, colchicine compound was in the chloroform layer and the solvent was evaporated from the extract. Then, the chloroform extract was kept at -20 °C before analysis.

2.4.3 Method C

The maceration method was modified from Finnie and Staden [7]. Five grams of *G. superba* L. was soaked overnight in 20 mL methanol at room temperature. The residue was separated from the extract by centrifugation. The methanol extract was then evaporated before re-dissolving in 10% NH_4OH. The extract was centrifuged at 6,000 g, 5 minutes, before partitioning with petroleum ether twice. The extract was extracted with dichloromethane (1:1) three times. The solvent was evaporated from the dichloromethane extract before storage at -20 °C.

2.5 Statistical treatment

Measures of central tendency and dispersion were expressed as mean ± standard deviation (SD). Data were analyzed with Analysis of Variance (ANOVA) followed by a post-hoc test.

3. Results and Discussion

3.1 Method validation

Table 1. Parameters for colchicine quantification in *Gloriosa superba* L.

Parameters	Value
Regression equation[1/]	y = 44,321.53x - 37,296.77
Correlation coefficient (r^2)	0.9993
Linear range ($\mu g.mL^{-1}$)	20.00-100.00
Precision[2/]	
Intra-day	0.16
Inter-day	0.93
LOD ($ng.mL^{-1}$)	4.98
LOQ($ng.mL^{-1}$)	62.23

[1]X is the concentration of colchicine ($\mu g.mL^{-1}$); Y is peak area at 254 nm.
[2]The intra- and inter-day precision values are expressed as percentage of relative standard deviation (%RSD).

Table 1 summarizes the validation of colchicine analysis in *Gloriosa* spp. which we examined using three parameters (linearity, precision, and accuracy). Linearity was obtained by plotting the peak areas versus the concentrations of standard colchicine and presented the outcome as correlation coefficient (r^2=0.9993). Precision outcomes from the intra-day (one day) and inter-day analyses were 0.16 and 0.93 %RSD, respectively. Both values, well below the 1.00 %RSD threshold, illustrate the remarkable precision of the HPLC system for quantifying colchicine. The LOD and

LOQ values were 4.98 and 62.23 ng.mL^{-1}, respectively.

The recovery values of 98.65% - 100.43% indicate high to perfect accuracy (Table 2). HPLC is a highly efficient method for quantitation and qualification analysis. In this study, optimized HPLC conditions were crucial for colchicine analysis.

Table 2. Recovery of colchicine.

Theoretical Value[1/] (µg.mL^{-1})	Experimental Value[2/] (µg.mL^{-1})	Recovery (%)
40.02	40.20 ± 0.03	100.43 ± 0.07
60.03	59.53 ± 0.11	99.15 ± 0.15
80.05	78.97 ± 0.03	98.65 ± 0.04

[1]Theoretical values are calculated by calculating exact amount of colchicine.
[2]Experimental values are presented as mean ± SD (n = 3).

The combined factors of linearity in the range of tested concentration, precision, and accuracy contributed to the eventual substantial and differential yields of colchicine obtained in this study.

The results of robustness testing are presented in Table 3. The tests showed that the variations in MP ratio (±1 v/v), flow rate (±1 mL.min^{-1}), and column temperature (± 2 °C) did not affect the detected colchicine.

Table 3. Robustness of colchicine analysis at a concentration 60 µg.mL^{-1}.

Parameter	Value	Colchicine content (µg.mL^{-1})	%RSD
MP ratio	59:41	60.33	0.38
(ACN:	60:40	60.39	0.02
50 mM KH_2PO_4)	61:39	60.39	0.21
Flow rate	0.9	60.41	0.01
(mL.min^{-1})	1.0	60.39	0.02
	1.1	60.40	0.01
Column	23	60.33	0.55
Temperature	25	60.39	0.02
(°C)	27	60.41	0.06

MP: mobile phase; ACN: acetonitrile; %RSD: percentage of relative standard deviation.

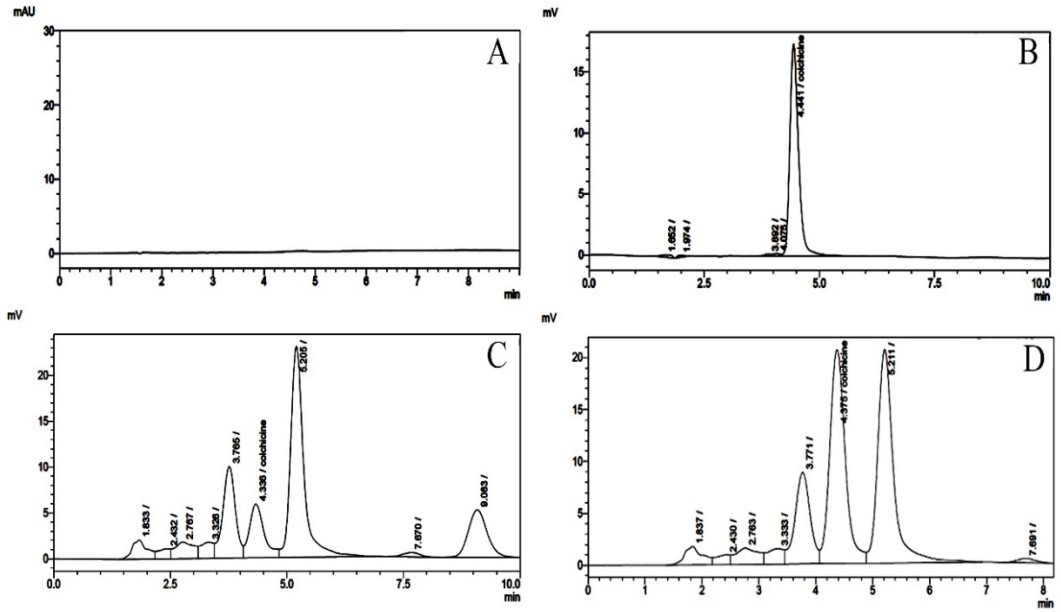

Fig. 2. Chromatograms of blank (A), standard colchicine (B), *G. superba* L. extract (C), and mixture of *G. superba* L. extract and standard colchicine.

Fig. 2 shows the specificity of the method through the chromatograms of blank, standard colchicine, *G. superba* L. extract, and the mixture of *G. superba* L. extract with standard colchicine. The peak of colchicine presents at the retention time 4.4 min (Fig 2B-2D). The blank did not interfere in the analysis which implies that this method is highly specific to colchicine.

In this study, the mobile phase approach in use of the isocratic system effectively delineated the colchicine peak in the chromatogram. A favorable separation outcome is impacted by solvent conditions in the mobile phase. In this study, we used a higher portion of acetonitrile to that of water (60:40).

Moreover, a lower polar index of acetonitrile (5.8) compared to that of water (9.0) produced better symmetric peaks in the mobile phase. Buffer was used as a mixer in the mobile phase to maintain constant pH. Since variations in pH lead to inconsistent results, judicious use of a buffer would disallow this unfavorable outcome. Thus, good use of buffer allows the consistent separation of the peaks of interest from the interferences. In this study, the use of 50 mM KH_2PO_4 yielded the favored outcome of consistent separation of colchicine. Our study revealed that the rapid isocratic system was optimized and could minimize running time and costs for routine analyses. This method has been validated, confirming that it is suitable for its intended use.

3.2 Appropriate method of colchicine extraction from *Gloriosa* spp.

Of the three methods used, petroleum ether followed by dichloromethane for extraction (method A) proved the most efficient, with a value of 0.18 mg.gDW^{-1} yield (Table 4). The colchicine contents from the three extraction methods were compared, with method A (348.93 µg.gDW^{-1} or equal to 0.03%) being significantly higher (1.01-1.28 times) than methods B or C ($p < 0.01$).

Table 4. Yield and colchicine content of *G. superba* L. extracts derived from different extraction methods.

The extraction	Yield[1/] (mg.gDW^{-1})	Colchicine content[2/] (µg.gDW^{-1})	% RSD[3/]
A	0.18	348.93± 0.27 a[4/]	0.06
B	0.10	346.17± 0.02 b	0.00
C	0.04	273.46± 0.27 c	0.08

[1]DW: dry weight
[2]Colchicine content are presented as mean ± SD
[3]RSD: Relative Standard Deviation
[4]The letter represents significant statistical differences after analysis by ANOVA, Duncan's test with $p \leq 0.01$.

From previous studies, colchicine in *G. superba* L. derived by an extraction method similar to method A yielded content in the range of 0.01-0.70 % [11-13]. The existence of a previous study addressed a similar theme as in ours which we compared in terms of methodology and results. Methodology-wise, both studies used the same extraction methods and recommended petroleum ether followed by dichloromethane as the choice solvent of extraction [11]. Petroleum ether was used in method A to defatuate the fatty acid compounds in *Gloriosa* spp. which could interfere in the extraction of colchicine.

In terms of results, the colchicine content in this study (0.27-0.35 mg.gDW^{-1}) (*G. superba* L.) was higher than in a previous investigation (0.14-0.21 mg.gDW^{-1}) [11]. The higher content may be attributed to the variation in plant materials which could be influenced by environmental factors during cultivation such as: temperature, light period, geographical location, and genetic background. For example, differences in the contents of diterpene lactones in *Andrographis paniculata* L. germinated from seeds collected from various locations [14] or colchicine content (0.6-2.5 %) in *G. superba* L. derived from tubers cultivated in different geographical regions [15] were observed. Random Amplified Polymorphic DNA (RAPD) revealed polymorphisms among *G. superba* L. collected from five different locations with variations of colchicine content (0.06-0.37%) [Ghosh, 2008].

Ranked by yield of colchicine extraction, methods C and A produced the least and the most, respectively, with method B in between. In terms of time, method C took the longest (one night) and method A the shortest (~ 2 h) with method B in between (3h). However, method B required a higher temperature than method A. Given these outcomes, method A is recommended for extracting colchicine from the other *Gloriosa* spp.

3.3 Colchicine content analysis in other *Gloriosa* spp.

For the sake of consistency, *G. superba* L. was re-analyzed with the other *Gloriosa* spp. in triplicate. Table 4 shows the range of colchicine content values (117.97 - 380.66 μg.gDW^{-1}). ANOVA comparisons among the *Gloriosa* spp. yielded a significant omnibus p value (< 0.01). Based on our significant finding, we are confident that *G. superba* L. is a suitable plant material for colchicine extraction and for use as a base species for tissue culture establishment or other applications. Notably, the other *Gloriosa* spp. might produce differential yields of colchicine based on their genetics.

Table 5. Colchicine content in *Gloriosa* spp. collected from various sources.

Plant sp.	Colchicine content[1/] (μg.gDW^{-1})
G. superba L.	380.66 ± 0.00 a[2/]
G. carsonii Baker	253.31 ± 0.28 b
G. lutea auct.	244.09 ± 0.46 c
G. rothschildiana	177.97 ± 0.04 d

[1]Colchicine content is presented as mean ± SD
[2]The letter represents significant statistical differences after ANOVA, Duncan's test with $p \leq 0.01$.

Table 5 details variations in yields of extracted colchicine from the different *Gloriosa* spp. Use of these plant sources would warrant rigorous quantitation and quality control before application.

From a previous study, colchicine contents in various *Gloriosa* spp. derived from petroleum ether followed by dichloromethane extraction were compared. Both the previous and present studies used three *Gloriosa* spp. in common (*G. superba* L., *G. lutea* auct., *G. rothschildiana*) [11]. In two of the species (*G. superba* L. and *G. rothschildiana*), the colchicine yields were 1.1-1.8 times higher than theirs, which could be the reason for the variation in *Gloriosa* spp. materials as discussed.

4. Conclusion

With our use of various methodologies and parameters, we have determined the optimal approach to identifying, quantifying, and extracting colchicine from *Gloriosa* spp. Our finding in *G. superba* L. establishes this plant as the most optimal source of colchicine. The results of this study point to the use of method A under our validated HPLC conditions as a possible protocol for standardization of colchicine content, especially in *Gloriosa* spp.

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