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Determination of (–)-demecolcine and (–)-colchicine content in selected Jordanian *Colchicum* species

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In our continuing attempt to investigate Jordanian *Colchicum* species as a potential and viable source of colchicinoids; the distributions of the most interesting colchicinoid alkaloids (−)-demecolcine and (−)-colchicine in different plant parts of wildly growing *C. crocifolium* Boiss., *C. ritchii* R. Br., and *C. triphyllum* Kunze, were analyzed. The method is based on the use of external reference standards and a reversed-phase gradient HPLC. While (−)-colchicine was found in all plant parts of the three investigated *Colchicum* species, (−)-demecolcine was not detected in the leaves and daughter corms of *C. triphyllum*, the corms of *C. crocifolium*, and the flowers, stems, and mother and daughter corms of *C. ritchii*. *C. triphyllum* was found to be the highest in total (−)-colchicine content of 0.10% (wt/wt), while *C. crocifolium* was the highest in total (−)-demecolcine content of 0.09% (wt/wt). During flowering, leaves and corms in *C. crocifolium*, and leaves and stems in *C. ritchii* are the main storages of (−)-colchicine. (−)-Colchicine showed a homogenous distribution in all plant parts (stems, leaves, and mother and daughter corms) of *C. triphyllum* of 0.10% (wt/wt). Regarding (−)-demecolcine, stems and leaves of *C. crocifolium*, stems of *C. triphyllum*, and leaves of *C. ritchii* are the main storages. This work reports for the first time the presence of (−)-colchicine and (−)-demecolcine in *C. crocifolium*.

1. Introduction

Out of the 225 species of the genus *Colchicum* (Nordenstam 1998), nine are found in Jordan, namely: *C. brachyphyllum* Boiss. & Haussk. ex Boiss., *C. crocifolium* Boiss, *C. heirosolymitanum* Feinbr, *C. tauri* Siehe ex Stefanov, *C. ritchii* R. Br., *C. shemperi* Janka & Stefanov, *C. stevenii* Kunth, *C. triphyllum* G. Kunze, and *C. tunicatum* Feinbr (Feinbrun-Dothan 1986; Al-Eisawi 1998; Oran and Al-Eisawi 1998).

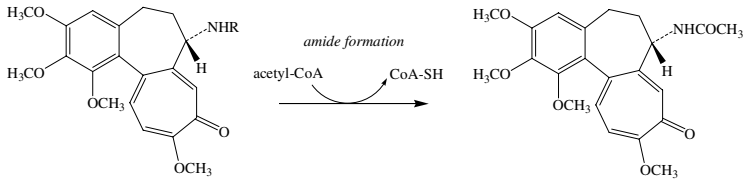
Plants which belong to the family Colchicaceae are of great interest. They are source of colchicinoid alkaloids which possess different and valuable therapeutic activities. Among these colchicinoids and due to their profound potency, (−)-colchicine and (−)-demecolcine are of primary interest (Samuelsson 1992). (−)-Colchicine possesses anti-inflammatory properties as it is the drug of choice in the treatment of gout (Terkeltaub 2003), familial Mediterranean fever (Drenth and Van Der Meer 2001), and Behcet's disease (Sakane and Takeno 2000). In addition, clinical studies have proved (−)-colchicine to possess a potent anti-tumor activity. Due to the lack of tumor selectivity and high toxicity, (−)-colchicine use as an anti-neoplastic drug is limited (Eigsti and Dustin 1955). As an anti-neoplastic agent, (−)-demecolcine has a wider margin of safety than (−)-colchicine and is used for the treatment of myelocytic leukemia and malignant lymphoma (Samuelsson 1992).

(−)-Colchicine occurs as yellowish-white amorphous scales, freely soluble in water, alcohol and chloroform (Trease and Evans 1989; Dollery et al. 1999). It has a molecular weight of 399.4, a melting point of 155–157 °C, and $[\alpha]_D^{20} - 121°$ ($CHCl_3$) (Cordell et al. 1989; Dollery et al. 1999). (−)-Demecolcine occurs as pale yellow prisms from ethyl acetate and ether, soluble in acidified water, alcohol, ether, chloroform, and benzene. It has a molecular weight of 371.43, a melting point of 186 °C, and $[\alpha]_D^{20} - 129.0°$ ($CHCl_3$) (Budavari 1996).

Biosynthetically, (−)-colchicine (**4**) is biosynthesized from (−)-demecolcine (**1**) by oxidation through *N*-formyl-deacetylcolchicine (**2**). Loss of formyl group produces deacetyl-(−)-colchicine (**3**), which is acetylated to (−)-colchicine (**4**) (Scheme) (Samuelsson 1992; Dewick 1998).

Several worldwide research groups have assayed different *Colchicum* species for their colchicinoid content (Baytop et al. 1980; Sütlüpinar et al. 1988; Vicar et al. 1993; Ondra et al.1995). *Colchicum ritchii* has been the subject of a phytochemical study by Freyer et al. (1987). That study has revealed the presence of (−)-colchicine and (−)-demecolcine along with a number of other colchicinoids (Freyer et al. 1987). Ondra *et al*. (1995) reported the presence of (−)-colchicine and (−)-demecolcine in the corms of *C. triphyllum*. In our lab, a reversed-phase gradient HPLC method was developed for the quantitative determination of (−)-colchicine and structurally related colchici-

Scheme



[**1**] R = CH_3 (−)-Demecolcine
[**2**] R = CHO *N*-formyl-deacetyl-(−)-colchicine
[**3**] R = H Deacetyl-(−)-colchicine

[**4**] (−)-Colchicine

noids in wildly growing Jordanian *Colchicum* species. So far, four Jordanian *Colchicum* species, *C. brachyphyllum*, *C. heirosolymitanum*, *C. stevenii*, and *C. tunicatum* have been assayed for their (−)-colchicine content (Al-Fayyad et al. 2002; Alali et al. 2004, 2006). This paper describes the results of quantitative determination of (−)-colchicine and (−)-demecolcine in flowers, leaves, stems, corms, mother corms, and daughter corms of *C. crocifolium*, *C. ritchii*, and *C. triphyllum*. To the best of our knowledge, no such study was reported for any of the investigated species.

C. crocifolium, a rare species, is found flowering from March to April in North Eastern desert of Jordan, usually in clayey sandy desert, and characterized as perennial herb, with corms covered by thick, dark brown to reddish scales. While *C. ritchii* is characterized as a sandy perennial herb, with underground, huge corms, covered by many dark brown-blackish, thick scales, and found flowering between January and February in sandy mountains and sandy soil in Southern Jordan. *C. triphyllum* is a sandy, perennial herb, with very deep, underground corms covered by light brown scales, and found flowering from January to March in Southern Jordan, usually in sandy soil and sand dunes (Feinbrun-Dothan 1986; Al-Eisawi 1998).

2. Investigations, results and discussion

While (−)-colchicine was observed in all plant parts of the three investigated *Colchicum* species, (−)-demecolcine was not detected in the leaves and daughter corms of *C. triphyllum*, the corms of *C. crocifolium*, and the flowers, stems, and mother and daughter corms of *C. ritchii*. Total (−)-colchicine content in *C. crocifolium*, *C. triphyllum*, and *C. ritchii* was found to be 0.02 (± 0.01), 0.10 (± 0.02), and 0.06% (± 0.03) (wt/wt), respectively, while that of (−)-demecolcine was found to be 0.09 (± 0.00), 0.04 (± 0.02), and 0.02% (± 0.01) (wt/wt), respectively. *C. triphyllum* was found to be the highest in total (−)-colchicine content of 0.10% (wt/wt), while *C. crocifolium* was the highest in total (−)-demecolcine content of 0.09% (wt/wt). Leaves and corms in *C. crocifolium*, and leaves and stems in *C. ritchii* showed the highest (−)-colchicine content of 0.02 and 0.10% (wt/wt), respectively. (−)-Colchicine showed a homogenous distribution in all plant parts of *C. triphyllum* (stems, leaves, and mother and daughter corms) of 0.10% (wt/wt). Regarding (−)-demecolcine, stems and leaves in *C. crocifolium*, stems in *C. triphyllum*, and leaves in *C. ritchii* showed the highest (−)-demecolcine content of 0.09, 0.06, and 0.02% (wt/wt), respectively (Table 1).

Judging from the linearity of the calibration curve, low % *RSD* and peak width, the HPLC method used showed acceptable reproducibility, accuracy and resolution (Table 2, Fig.). Based on this study, significant statistical differences were observed in (−)-colchicine and (−)-demecolcine content among different plants parts of *C. crocifolium, C. triphyllum,* and *C. ritchii.* In this study, stems and leaves of *C. crocifolium* showed the highest (−)-demecolcine content of 0.09% (wt/wt), while stems, leaves, and mother and daughter corms of *C. triphyllum* showed the highest

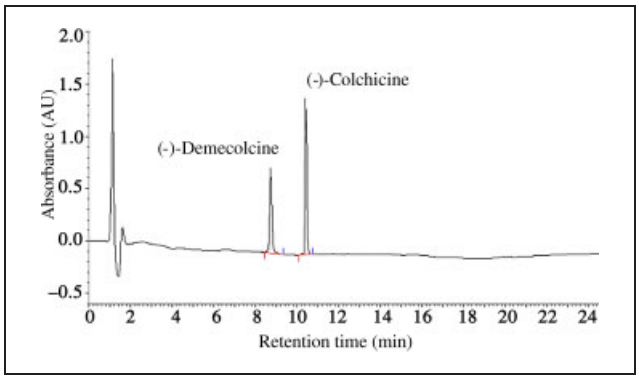


Fig.: HPLC chromatogram for quality control samples of (−)-colchicine (75 ppm) and (−)-demecolcine (75 ppm)

Table 1: (−)-Colchicine and (−)-demecolcine content of *C. crocifolium*, *C. ritchii*, and *C. triphyllum*

Plant part	% w/w (−)-Demecolcine content[a]			% w/w (−)-Colchicine content[a]		
	C. triphyllum	*C. crocifolium*	*C. ritchii*	*C. triphyllum*	*C. crocifolium*	*C. ritchii*
Flowers	NA	NA	ND	NA	NA	0.02 ± 0.01
Stems	0.06 ± 0.01	0.09 ± 0.01	ND	0.12 ± 0.01	0.01 ± 0.00	0.10 ± 0.01
Leaves	ND	0.09 ± 0.01	0.02 ± 0.01	0.11 ± 0.01	0.02 ± 0.01	0.10 ± 0.01
Corms	–	ND	NA	–	0.02 ± 0.01	–
Mother corms	0.02 ± 0.01	NA	ND	0.10 ± 0.01	NA	0.04 ± 0.01
Daughter corms	ND	NA	ND	0.08 ± 0.01	NA	0.05 ± 0.01

[a] (−)-Colchicine and (−)-demecolcine content is expressed as w/w g% derived from the average of two extraction replicates, each run in duplicate. NA: Not analyzed. ND: Not detected

Table 2: Accuracy validation using quality control (QC) points

Actual concentration[a]	Measured concentration[a]		Average measured concentration[a]	%RSD
	1st trial	2nd trial		
(−)-Colchicine				
40	41.79	41.25	41.52	3.80
75	82.68	82.97	82.83	10.44
(−)-Demecolcine				
40	36.74	36.65	36.70	8.25
75	77.59	78.57	78.08	4.12

[a] Measured in μg/mL

(−)-colchicine content of 0.10% (wt/wt). This study reports for the first time the presence of (−)-colchicine and (−)-demecolcine in *C. crocifolium*.

Taking into consideration the adaptability of local *Colchicum* species to grow under drought conditions, further agricultural and agro-economic studies are needed to explore the feasibility of developing these species as a source of colchicinoids.

3. Experimental

3.1. Equipment

HPLC analysis was performed on a SHIMADZU® (Kyoto, Japan), SPD-10A VP, UV-VIS detector, SIL-10 AD VP, auto injector, LC-10AD VP, liquid chromatograph, DGU-12A, degasser, model SPD-10A VP, CAT. No. 228-40000-38, serial No. C20994009812 LP. The analytical HPLC column used was LiChroCART® 125-4, Purospher® STAR RP-18 endcapped (5 μm).

3.2. Reagents

Petroleum ether (analytical grade), dichloromethane HPLC grade, ammonia solution 25% (synthesis grade), ethanol absolute (analytical grade), methanol HPLC grade, acetonitrile HPLC grade, and acetic acid (glacial, analytical grade) were all from Scharlau Chemie S.A. (Barcelona, Spain). (−)-Colchicine standard was purchased from Fluka Chemie (Buchs, Switzerland). From *Colchicum brachyphyllum*, (−)-demecolcine was isolated, purified, and spectroscopically fully characterized in our institution (Alali et al. 2005).

3.3. Plant material

Flowers, leaves, stems, mother corms and daughter corms of *C. ritchii,* and leaves, stems, mother corms and daughter corms of *C. triphyllum* were collected during their flowering and vegetating stages, respectively. *C. ritchii* was collected from Al-Dair in Petra, Southern Jordan and *C. triphyllum* from Wadi Rum, Southern Jordan in February 2004. Leaves, stems, and corms of *C. crocifolium* were collected during its vegetating stage from Ar-Ruaished, Eastern part of Jordan in April 2005. The collected materials of the three species were identified by Dr. Khaled Tawaha, a pharmacognosit and field taxonomist, Faculty of Pharmacy, University of Jordan. Voucher specimens of *C. ritchii* (PHC # 108), *C. triphyllum* (PHC # 109), and *C. crocifolium* (PHC # 110) were registered and deposited at the Herbarium Museum of the Faculty of Pharmacy, Jordan University of Science and Technology, Irbid, Jordan. The plant raw material of *C. ritchii*, *C. triphyllum*, and *C. crocifolium* were divided into six parts: flowers, leaves, stems, corms, mother corms, and daughter corms; air-dried at room temperature with the corms sliced into small pieces to speed up their dryness. After drying, plant parts were grounded to powder using a laboratory mill and passed through a sieve to provide homogeneous powder for the analysis. Powdered material was maintained at room temperature (22–23 °C), protected from light until required for analysis.

3.4 Sample preparation and analysis

0.5 g (± 0.1 mg) of each finely ground plant part of *C. crocifolium*, *C. ritchii*, and *C. triphyllum* were accurately weighed and placed into 100-mL Erlenmeyer flasks. Petroleum ether (2 × 10 mL) was added with frequent shaking for 1 h, followed each time by filtration. Solid residues were air dried at room temperature and then extracted with 10 mL HPLC-grade dichloromethane with frequent shaking for 45 min. Ammonium hydroxide (12.5%), 0.5 mL, was added, followed by vigorous shaking for 15 min, and then the mixture was left for 30 min. Afterwards, plant residues were filtered and the filtrates were saved. Plant residues were washed twice with 10 mL HPLC-grade dichloromethane and then filtered. The collected filtrates and washes were combined. The organic phase was evaporated to dryness under vacuum and then dissolved in 1 mL of 70% ethanol. The solution was filtered through a 0.45 μL Teflon filter, 0.5 mL was transferred into 2 mL amber HPLC vials, and then 1 mL of 70% ethanol was added. A portion, 25 μL, was injected into the HPLC. Two extraction replicates were prepared for each plant part.

A stock solution of combined (−)-colchicine and (−)-demecolcine standards 1000 ppm was prepared by accurately weighing 25 mg of (−)-colchicine and 25 mg (−)-demecolcine reference standards into 25 mL volumetric flask, 10 mL of HPLC grade methanol was added, shaken until all solids were dissolved then diluted to volume with HPLC grade methanol and mixed to ensure complete solubility. The stock solution was then diluted using HPLC grade methanol to construct two calibration curves of five-, and six-points, for (−)-colchicine and (−)-demecolcine, respectively, namely (25, 50, 100, 150, and 200 ppm), (25, 50, 100, 150, 200 and 250 ppm) and two quality control (QC) points (40, 75 ppm) for the two calibration curves, respectively (Table 2). HPLC was used to assay (−)-demecolcine and (−)-colchicine. Mobile phase was a gradient blend of acetonitrile (solution A) and 3% acetic acid in water (solution B) as follows: 0–11 min, 10% A; 11–15 min, 60% A; and 15–20 min 10% A. Flow rate used was 1 mL/min, detector set at 245 nm and injection volume was 25 μL. (−)-Colchicine eluted at 10.5 min; while (−)-demecolcine eluted at 8.8 min; the total run time was 25 min. All plant samples, calibration points and QC samples were injected twice. Measuring peaks areas, two linear calibration curves were constructed with r^2 values of 0.990, and 0.998 for (−)-colchicine and (−)-demecolcine, respectively. Two quality control (QC) samples at 40 and 75 ppm were accurate within 3.8, 10.44% and 8.25, and 4.12% (ppm, *RSD* %) based on (−)-colchicine and (−)-demecolcine's calibration curves, respectively, from actual concentration (Table 2). All plant samples were injected twice; results are given in Table 1. The percent of (−)-colchicine and (−)-demecolcine in the samples were calculated against external standards of (−)-colchicine and (−)-demecolcine based on the following equation:

$$\%\,W/W = C \times FV \times D \times 100\,\%/W, \quad (1)$$

where C is the sample's colchicine/demecolcine concentration (mg/mL), extrapolated from the calibration curves' linear regression, FV is the final volume of the sample in mL, D is the dilution factor, and W is the sample weight in mg.

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