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Isolation and Structural Characterization of Tropolonic Alkaloids *Colchicum speciosum* Steven bulbs

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ABSTRACT

The *Colchicum* genus, is well-known for its significant biological activities. Among its bioactive constituents, tropolonic alkaloids, particularly colchicine, are widely used in the treatment of gout and have shown potential in treating proinflammatory conditions such as familial Mediterranean fever and Behcet's disease. Furthermore, colchicine has emerging applications in anti-tumor therapies. *Colchicum speciosum* Steven, a perennial herb native to Iran, is a valuable source of these bioactive compounds. This study investigates the phytochemical profile of *C. speciosum* bulbs collected from Savadkouh, Iran. Methanol (MeOH) extracts were analyzed using high-performance liquid chromatography with photodiode array detection and mass spectrometry (HPLC-PDA-MS), along with nuclear magnetic resonance (NMR) spectroscopy. The MeOH extract was sequentially partitioned with dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and water. Electronic circular dichroism (ECD) spectral calculations were used to determine the absolute configurations of compounds 3, 4, and 5. Chromatographic separation of the CH_2Cl_2 fraction led to the isolation and identification of five tropolonic alkaloids, including four known compoundscolchicine (**1**), demecolcine (**2**), speciosamine (**4**), and N-formyldemecolcine (**5**) and one novel compound 3. The structures were confirmed by 1D (^{1}H NMR) and 2D-NMR (COSY, HSQC, and HMBC). ECD analysis confirmed that compounds 3, 4, and 5 exhibited the S configuration. This study expands the chemical profile of *C. speciosum*, identifying one new tropolonic alkaloid and four known compounds, thereby advancing our understanding of the phytochemistry of this plant.

Keywords: *Colchicum speciosum* Steven, tropolonic alkaloids, 1D and 2D NMR, Absolute Configuration.

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Introduction

The genus *Colchicum*, a genus of the Colchicaceae (Liliales) family, consists of 19 genera and 225 species, each characterized by unique distributions across diverse climates and regions. These plants have garnered significant attention for over two millennia due to their exceptional biological properties (1). Across various cultures, *Colchicum* species, also known as "Sorenjân" in some regions, have been traditionally utilized for the treatment of a broad range of ailments, including osteoarthritis, gout, cancer, inflammations, jaundice, and sexual impotence, underscoring their significance as a reservoir of medicinal resources. Additionally, *Colchicum* species, along with other members of the Colchicaceae family, are a rich source of diverse bioactive compounds, including alkaloids (especially tropolone alkaloids and isoquinoline alkaloids), phenolic compounds, tannins, flavonoids, and carbohydrates. The composition and concentration of these compounds can fluctuate in response to the plant's developmental stage and seasonal variations (2-4).

Among the constituents, tropolonic alkaloids, including colchicine and its derivatives, hold particular significance. These compounds are localized in various plant parts, including the bulbs, leaves, seeds, and flowers of Colchicum species, as well as in other genera within the Colchicaceae family. Colchicine, a well-established remedy for gout, has a history of therapeutic application dating back to 1810 for the management of acute gout episodes (5). Its clinical utility was further solidified with its approval as a gout medication by the Food and Drug Administration (FDA) in the United States in 2009 (6, 7). In addition to its use for gout, colchicine has demonstrated efficacy in treating fever and is a cornerstone treatment for Familial Mediterranean Fever (8-10). Its therapeutic also extend to inflammatory and fibrotic conditions, as well as diseases such as Behcet's disease, pericarditis, and coronary artery disease (11, 12).

One notable species within the *Colchicum* genus is *Colchicum speciosum*, a flowering plant indigenous to mountainous regions of northern Turkey and northern Iran (13). Recent pharmacological investigations have highlighted the therapeutic potential of *C. speciosum* anti-diabetic effects by significantly enhancing glucose uptake while reducing insulin secretion (14). Additionally, this species exhibits cholinergic activity (15) and has been shown to possess an antileukemia effect (16). Notably, *C. speciosum* represents a valuable source of isoquinoline and tropolone alkaloids (17).

Phytochemical analyses have identified *C. speciosum* as s a rich source of isoquinoline and tropolone alkaloids. In particular, colchicoside and colchicine have been detected at concentrations of 51.9 mg/100 g and 75.9 mg/100 g of dry weight, respectively (18).

Despite these findings, comprehensive data on the full chemical profile of *C. speciosum* remains scarce. Accordingly, the present study aims to investigate the phytochemical composition of the MeOH extract obtained from the bulbs of *C. speciosum*, with a specific emphasis on tropolonic alkaloids. Furthermore, the study reports the isolation and structural elucidation of five compounds, including the identification of a novel constituent, thereby contributing to a deeper understanding of the chemical constituents of this species.

Material and methods

General experimental procedures

All extractions and chromatographic separations were performed using technical-grade solvents, which were distilled prior to use to ensure analytical purity (Emertat, Iran). Open-column chromatography was conducted using silica gel (70–230 mesh, Merck), and

the progress of separations was monitored by thin-layer chromatography (TLC) on pre-coated silica gel F_{254} plates (20 × 20 cm; Merck, Darmstadt, Germany). TLC spots were visualized under UV light (254 and 366 nm) and, when necessary, by spraying with anisaldehyde or Dragendorff's reagent followed by gentle heating. For structural elucidation, purified samples were dissolved in deuterated methanol (MeOD-d_4; Armar Chemicals, Döttingen, Switzerland) prior to nuclear magnetic resonance (NMR) spectroscopy. NMR spectra, including ^{1}H, ^{13}C, COSY, HSQC, and HMBC, were recorded on a Bruker Avance III 500 MHz spectrometer. All chemical shifts (δ) are reported in parts per million (ppm) with reference to the residual solvent peak.

Concentration of extracts and chromatographic fractions was carried out under reduced pressure using a Heidolph Laborota 4000 rotary evaporator (Germany). Electronic circular dichroism (ECD) spectra were acquired using a Chirascan ECD spectropolarimeter (Applied Photophysics, Leatherhead, UK), and spectra were analyzed for stereochemical assignments.

Semi-preparative high-performance liquid chromatography (HPLC) was conducted on a Knauer WellChrom system (Berlin, Germany), comprising a K-1001 pump, D-14163v degasser, and K-2800 photodiode array (PDA) detector. Chromatographic separations were achieved using reversed-phase SunFire™ C18 columns (5 µm, 19 × 50 mm and 3.5 µm, 3 × 150 mm; Waters), with mobile phases consisting of water and acetonitrile, each containing 0.1% formic acid, under gradient elution conditions. The PDA detector was coupled to a triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source, allowing for high-sensitivity detection of alkaloid constituents. Data acquisition and analysis were performed using LabSolutions software.

NMR Spectroscopy Analysis

For NMR analysis, purified and fully dried samples were dissolved in deuterated methanol (MeOD-d_4). All spectra were recorded on a Bruker Avance III 500 MHz spectrometer operating at 500.13 MHz for ^{1}H NMR and 125.7 MHz for ^{13}C NMR. The spectroscopic experiments included one-dimensional (^{1}H, ^{13}C) and two-dimensional (^{1}H–^{1}H COSY, HSQC, and HMBC) techniques to facilitate complete structural elucidation.

Extraction and Isolation

Plant Material

Bulbs of *Colchicum speciosum* (500 g) were collected in May 2016 from the Savadkouh district, Mazandaran Province, Iran. The plant material was taxonomically identified by Dr. Ali Sonboli (Shahid Beheshti University), and a voucher specimen (MPH-2257) has been deposited in the Herbarium of the Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran, for future reference.

Extraction Procedure

The collected bulbs were air-dried at room temperature and finely ground into a powder. A total of 500 g of the powdered dried material underwent maceration with methanol (7 × 2 L) at room temperature. The extracts obtained were combined, and the solvent was evaporated under vacuum using a rotary evaporator.

The resulting dried methanolic extract (65 g) was dissolved in 400 mL of distilled water and partitioned sequentially with n-hexane (5 × 400 mL). The organic layers were combined, dried, and concentrated under reduced pressure. To selectively enrich the colchicine-containing fraction, hydrochloric acid (HCl) was added to the aqueous phase, adjusting the pH to 3. This acidic aqueous phase was then extracted with dichloromethane (CH_2Cl_2; 5 × 400 mL). The pooled organic extracts were concentrated under reduced pressure, yielding 2.75 g of material.

Column Chromatography

The CH_2Cl_2 fraction (2.75 g) obtained in the previous step was subjected to silica gel column chromatography (70–230 mesh, 3.5 × 100 cm, 140 g). Elution was performed using a gradient of n-hexane/ EtOAc (100:0 → 0:100), followed by increasing concentrations of MeOH up to 100%. Fractions were collected in volumes of 250 mL and 500 mL, resulting in a total of 78 fractions. These fractions were subsequently combined based on similarities in their chemical profiles as determined by TLC, yielding 19 combined fractions.

Colchicine (**1**) (850 mg), a dark orange sediment, was obtained from fraction 13 (eluted with EtOAc/n-hexane, 12:88). Demecolcine (**2**) (172 mg), also a dark orange sediment, was isolated from fraction number 16 (eluted with MeOH/EtOAc, 25:75) by elution with acetone. Moreover, fraction 12 (273 mg, eluted with MeOH/EtOAc, 10:90) was subjected to further purification via silica gel column chromatography (70–230 mesh, 1.5 × 15 cm, 20 g). Elution was performed using a gradient of CH_2Cl_2/MeOH (90:10 to 60:40), resulting in the isolation of N-formyldemecolcine (**5**).

Fraction 14 (45 mg, eluted with EtOAc/n-hexane, 12:88) was subjected to preparative thin-layer chromatography (PTLC) utilizing a mobile phase system of $CHCl_3$/MeOH/Formic acid (90:9.9:0.1), leading to the formation of six subfractions. Subfraction 14.2 (12 mg) was further purified using semi-preparative HPLC.

HPLC analysis

Further purification of subfraction 14.2 (12 mg) was achieved through applying semi-preparative HPLC. The HPLC system (Knauer WellChrom, Berlin, Germany) consisted of a K-1001 pump, D-14163v degasser, and a k-2800 photodiode array detector (PDA) detector. Reversed-phase SunFire™ C18 columns (5 µm, 19 × 50 mm and 3.5 µm, 3 × 150 mm) from Waters were employed. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The elution gradient was as follows: 0-12 min, 10-21% B; 12-15 min, 21-100% B; 15-20 min, 100% B; 20-21 min, 100-10% B; 21-25 min, 10% B. The flow rate was set at 3 mL/min, and the column temperature was maintained at 20 °C. This procedure led to the isolation of two demecolcine (**2**) and compounds **3**, which were further purified using HPLC-PDA-MS.

HPLC-PDA-MS analysis was performed using a Waters system (Milford, MA, USA) comprising of a binary solvent manager, a PDA detector, and a G2 Q/TOF mass spectrometer equipped with an ESI source. Mass spectra were acquired in both negative and positive ion modes, cover a mass range from m/z 160 to 1500. Separation was carried out at a flow rate of 0.4 mL/min using a gradient of water (solvent A) and acetonitrile (solvent B), both containing 0.1% (v/v) formic acid. The gradient profile was as follows: 0-12 min, 10-21% B; 12-15 min, 21-100% B; 15-20 min, 100% B; 20-21 min, 100-10% B; 21-25 min, 10% B. The ESI source parameters were set as follows: spray voltage of 4.5 kV, capillary temperature of 500 °C, and nitrogen gas (>99.98% purity) was employed as the sheath, auxiliary, and sweep gas. Data acquisition was performed at a scan rate of 6000 u/s with a polarity switching time of 0.150 s in both positive and negative ion modes.

Computational Methods

Conformational analyses for compounds **3**-**5** were conducted using MacroModel 9.1 software (Schrödinger LLC) with the OPLS 2005 force field in water (H2O). Geometrical optimization and energy calculations were performed on selected conformers within a 2 kcal/mol energy window from the global minimum to obtain the most stable conformations. Density Functional Theory (DFT) calculations were carried out using Becke's nonlocal three-parameter

exchange-correlation functional, combined with the Lee-Yang-Parr (LYP) correlation functional (cam-B3LYP), and the B3LYP/6-31G** basis set, as implemented in the Gaussian 09 software package. Vibrational analysis was also performed at the same level of theory to confirm the stability of the conformers by ensuring that no imaginary frequencies were present.

Excitation energies (reported as wavelengths in nm), rotatory strengths (Rvel), and dipole velocity (Rlen) in methanol (MeOH) were calculated using Time-Dependent DFT (TD-DFT) with the cam-B3LYP/6-31G** functional and the SCRF method, using the CPCM model to account for solvent effects. Electronic Circular Dichroism (ECD) spectra were generated from the rotatory strengths with a half-bandwidth of 0.3-0.4 eV. For UV shift analysis, SpecDis v1.64 software was used to further analyze and interpret the spectral data.

Spectroscopic data of isolated compounds

Colchicine (**1**): Dark orange sediment (850 mg); UV (MeOH) λ_max (log ε) 242, 318 nm; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.51 (1H, d, J = 7.5 Hz, NH), 7.16 (1H, s, H-8), 7.11 (1H, d, J = 11 Hz, H-12), 7.01 (1H, d, J = 11 Hz, H-11), 6.75 (1H, s, H-4), 4.35 (1H, dt, J = 12.0, 7.0, 5.8 Hz, H-7), 3.87 (3H, s, H-17), 3.83 (3H, s, H-15), 3.79 (3H, s, H-14), 3.54 (3H, s, H-13), 2.58 (1H, m, H-5), 2.23 (1H, m, H-5), 2.02 (1H, m, H-6), 1.85 (1H, m, H-6, overlapped with H-16); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 178.5 (C-9), 169.5 (C-18), 164.2 (C-10), 153.4 (C-3), 151.7 (C-7a), 151.2 (C-1), 141.0 (C-2), 135.9 (C-12a), 134.8 (C-12), 134.6 (C-4a), 130.5 (C-8), 126.0 (C-1a), 112.5 (C-11), 107.8 (C-4), 61.3 (C-13), 61.2 (C-14), 56.5 (C-15), 56.4 (C-17), 51.7 (C-7), 36.4 (C-6), 29.9 (C-5), 23.2 (C-16); ESI-MS m/z 400 $[M+H]^+$.

Demecolcine (2): Dark orange sediment (170 mg); UV (MeOH) λ_max (log ε) 238, 350 nm; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 7.48 (1H, s, H-8), 7.13 (1H, d, *J* = 10.6 Hz, H-12), 7.03 (1H, d, *J* = 10.9 Hz, H-11), 6.74 (1H, s, H-4), 3.88 (1H, s, H-16), 3.83 (3H, s, H-14), 3.78 (3H, s, H-15), 3.53 (3H, s, H-13), 3.20 (1H, dd, *J* = 10.9, 5.4 Hz, H-7), 2.54 (1H, m, H-5), 2.22 (1H, m, H-6), 2.21 (1H, m, H-5), 2.15 (3H, s, H-17), 1.62 (1H, m, H-6); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 178.8 (C-9), 163.5 (C-10), 153.4 (C-3), 150.5 (C-1), 149.2 (C-7a), 141.1 (C-2), 136.3 (C-12a), 135.0 (C-4a), 134.9 (C-12), 131.5 (C-8), 125.6 (C-1a), 112.4 (C-11), 107.9 (C-4), 62.0 (C-7), 61.8 (C-13), 61.5 (C-14), 56.5 (C-15), 56.4 (C-16), 37.0 (C-6), 33.6 (C-17), 29.7 (C-5); ESI-MS (positive mode) m/z 372 $[M+H]^+$.

Compound 3: Yellow sediment (170 mg); UV (MeOH) λ_max (log ε) 243, 350 nm; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 7.88 (1H, s, H-8), 7.52 (1H, d, *J* = 10.8 Hz, H-12), 7.33 (1H, d, *J* = 11 Hz, H-11), 7.16 (1H, dt, *J* = 6.12, 1.78 Hz, H-4′), 7.02 (1H, m, H-6′), 6.83 (1H, s, H-4), 6.82 (1H, m, H-3′), 6.78 (1H, dt, *J* = 6.47, 1.28 Hz, H-5′), 4.71 (1H, s, OH), 4.19 (3H, s, H-16), 4.07 (1H, s, H-17), 4.02 (3H, s, H-15), 3.98 (3H, s, H-14), 3.80 (1H, m, H-17), 3.61 (1H, m, H-7), 3.55 (3H, s, H-13), 2.71 (1H, m, H-5), 2.48 (1H, m, H-5/H-6), 1.93 (1H, m, H-6); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 179.0 (C-9), 163.3 (C-10), 155.9 (C-2′), 153.0 (C-3), 151.2 (C-7a), 149.8 (C-1), 140.1 (C-2), 137.5 (C-12a), 135.4 (C-12), 134.0 (C-4a), 130.7 (C-8), 128.5 (C-6′), 127.5 (C-4′), 124.8 (C-1a), 122.8 (C-1′), 118.5 (C-5′), 114.6 (C-3′), 112.8 (C-11), 106.7 (C-4), 59.5 (C-13/14), 57.9 (C-7), 54.9 (C-16), 54.6 (C-15), 37.4 (C-6), 33.6 (C-17), 29.0 (C-5); ESI-MS (positive mode) m/z 464.5 $[M+H]^+$.

Speciosamine (4): Yellow sediment (4 mg); UV (MeOH) λ_max (log ε) 242, 350 nm; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.08 (1H, s, H-8), 7.46 (1H, d, *J* = 10.6 Hz, H-12), 7.31 (1H, d, *J* = 10.6 Hz, H-4′), 7.23 (1H, d, *J* = 10.7 Hz, H-11), 7.18 (1H, m, *J* = 7.2 Hz, H-6′), 7.13 (1H, m, H-4′), 6.82 (1H, t, *J* = 7.4 Hz, H-5′), 6.81 (1H, d, *J* = 3.8 Hz, H-3′), 6.80 (1H, s, H-4), 4.77 (1H, s, OH), 4.09 (3H, s, H-16), 4.00 (3H, s, H-15), 3.98 (3H, s, H-14), 3.77 (1H, d, *J* = 7.2 Hz, H-17), 3.67 (3H, s, H-13), 3.56 (1H, d, *J* = 12.5 Hz, H-17), 3.27 (1H, dd, *J* = 11.1,

5.9 Hz, H-7), 2.67 (1H, dd, *J* = 12.6, 5.3 Hz, H-5), 2.48 (1H, m, H-6), 2.35 (1H, m, H-5), 2.29 (1H, s, H-18), 1.95 (1H, m, H-6); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 180.3 (C-9), 163.1 (C-10), 155.2 (C-2′), 153.0 (C-3), 152.7 (C-7a), 149.4 (C-1), 140.7 (C-2), 138.5 (C-12a), 135.1 (C-12), 134.1 (C-4a), 131.7 (C-8), 128.5 (C-6′), 127.2 (C-4′), 124.8 (C-1a), 123.2 (C-1′), 118.5 (C-5′), 114.3 (C-3′), 112.8 (C-11), 106.4 (C-4), 66.8 (C-7), 59.5 (C-13/14), 55.2 (C-17), 54.9 (C-16), 54.6 (C-15), 38.7 (C-18), 34.6 (C-6), 29.2 (C-5); ESI-MS (positive mode) m/z 478.1 $[M+H]^+$.

N-Formyldemecolcine (5): Yellow sediment (30 mg); UV (MeOH) λ_max (log ε) 242, 350 nm; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.10 (1H, br s), 7.90 (1H, s, H-17), 6.89 (1H, s, H-8), 6.65 (1H, s, H-11), 6.38 (1H, s, H-4), 4.57 (1H, s, H-7), 3.78 (3H, s, H-16), 3.73 (3H, s, H-14), 3.70 (3H, s, H-15), 3.46 (3H, s, H-13), 3.06 (3H, s, H-18), 2.45 (1H, m, H-5), 2.25 (1H, m, H-5), 2.12 (1H, m, H-6), 1.98 (1H, m, H-6); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 179.0 (C-9), 164.2 (C-10), 162.9 (C-17), 153.7 (C-2), 151.1 (C-1), 149.8 (C-3), 149.7 (C-7a), 135.9 (C-12a), 135.4 (C-12), 133.3 (C-4a), 130.2 (C-8), 125.5 (C-1a), 112.1 (C-11), 107.3 (C-4), 55.4 (C-7), 60.0 (C-13/14), 56.5 (C-15), 56.1 (C-16), 33.8 (C-18), 32.7 (C-6), 29.4 (C-5); ESI-MS (positive mode) m/z 400 $[M+H]^+$.

Results and Discussion

In the present study, a combination of open-column chromatography on silica gel and semi-preparative reverse-phase HPLC was employed to isolate and purify secondary metabolites from the methanolic (MeOH) extract of *Colchicum speciosum* bulbs. Following the extraction, the MeOH extract was partitioned with dichloromethane (CH_2Cl_2), affording a bioactive fraction that was further subjected to chromatographic separation. This approach led to the successful isolation of five tropolonic alkaloids. Among these, compound 3 represents a novel structure and is reported herein for the first time (Fig. 1). The chemical structures of all isolated compounds were elucidated through comprehensive spectroscopic analyses, including 1D and 2D NMR techniques (^{1}H NMR, ^{13}C NMR, COSY, HSQC, and HMBC). Among the isolated metabolites, colchicine (**1**) a prominent tropolonic alkaloid was first identified in 1820 from *C. autumnale* and is well-documented for its pharmacological relevance (19). Colchicine (**1**) has been extensively investigated for its therapeutic potential in a range of medical conditions, including the management of acute gout (20), cirrhosis of the bile and liver, and amyloidosis (21, 22)

Colchicine (1) exhibited a ^{1}H NMR spectrum with four well-resolved aromatic proton signals. The doublets at δ_H 7.01 (1H, *d*, *J* = 11 Hz, H-11) and δ_H 7.11 (1H, *d*, *J* = 11 Hz, H-12) indicated two ortho-coupled aromatic protons. Additional singlets at δ_H 7.16 (1H, s) and 6.75 (1H, s) were consistent with two isolated aromatic protons. The presence of four methoxy groups was confirmed by singlets in the range of δ_H 3.54–3.87. A downfield doublet at δ_H 8.51 (1H, *d*, *J* = 7.3 Hz), with no observable HSQC-DEPT correlation, was attributed to an NH proton, supporting the presence of a phenylamine moiety adjacent to a carbonyl group. HMBC correlations from the NH proton (δ_H 8.51) and H-16 (δ_H 1.85) to the carbonyl carbon at δ_C 169.5 (C-18) confirmed the substitution pattern. Electrospray ionization mass spectrometry (ESI-MS) revealed a molecular ion peak at *m/z* 400 $[M + H]^+$, consistent with the molecular formula $C_{22}H_{25}NO_6$. These spectroscopic features are in agreement with previously reported data for colchicine, thereby confirming the identity of compound **1** (23, 24).

Table 1. ^{1}H and ^{13}CNMR spectroscopic data of compounds 1-5 (δ in ppm, J in Hz) [a].

Position	colchicine (**1**)		demecolcine (**2**)		compound (**3**)		speciosamine (**4**)		N-formyldemecolcine (**5**)	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	-	151.2, C	-	150.5, C	-	149.8, C	-	149.4, C	-	151.1, C
1a	-	126.0, C	-	125.6, C	-	124.8, C	-	124.8, C	-	125.5, C
2	-	141.0, C	-	141.1, C	-	140.1, C	-	140.7, C	-	153.7, C
3	-	153.4, C	-	153.4, C	-	153.0, C	-	153.0, C	-	149.8, C
4	6.75, s	107.8, CH	6.74, s	107.9, CH	6.83, s	106.7, CH	6.8	106.4, CH	6.38	107.3, CH
4a	-	134.6, C	-	135.0, C	-	134.0, C	-	134.1, C	-	133.3, C
5	2.23 2.58	29.9, CH_2	2.21 2.54	29.7, CH_2	2.71 2.48	29.0, CH_2	2.67 2.35	29.2, CH_2	2.45 2.25	29.4, CH_2
6	2.02 1.85	36.4, CH_2	2.22 1.62	37.0, CH_2	2.48 1.93	37.4, CH_2	2.48 1.95	34.6, CH_2	2.12 1.98	32.7, CH_2
7	4.35	51.7, CH	3.2	62.0, CH	3.61	57.9, CH	3.27	66.8, CH	4.57	55.4, CH
7a	-	151.7, C	-	149.2, C	-	151.2, C	-	152.7, C	-	149.7, C
8	7.16, s	130.5, CH	7.48	131.5, CH	7.88	130.7, CH	8.08	131.7, CH	6.89	130.2, CH
9	-	178.5, C	-	178.8, C	-	179.0, C	-	180.3, C	-	179.0, C
10	-	164.2, C	-	163.5, C	-	163.3, C	-	163.1, C	-	164.2, C
11	7.01, (d, 11 Hz)	112.5, CH	7.03, (d, 11 Hz)	112.4, CH	7.33. (d, 11 Hz)	112.8, CH	7.23, (d, 10.7 Hz)	112.8, CH	6.65	112.1, CH
12	7.11, (d, 11 Hz)	134.8, CH	7.13, (d, 11 Hz)	134.9, CH	7.52	135.4, CH	7.46	135.1, CH	7.04	135.4, CH
12a	-	135.9, C	-	136.3, C	-	137.5, C	-	138.5, C	-	135.9, C
13	3.54	61.3, CH_3	3.53	61.8, CH_3	3.55	59.5, CH_3	3.67	59.5, CH_3	3.46	60.0, CH_3
14	3.79	61.2, CH_3	3.83	61.5, CH_3	3.98	59.5, CH_3	3.98	59.5, CH_3	3.73	60.0, CH_3
15	3.83	56.5, CH_3	3.78	56.5, CH_3	4.02	54.6, CH_3	4.00	54.6, CH_3	3.7	56.5, CH_3
16	1.85	23.2, CH_3	3.88	56.4, CH_3	4.19	54.9, CH_3	4.09	54.9, CH_3	3.78	56.1, CH_3
17	-	169.5, C	2.15	33.6, CH_3	3.8 4.07	33.6, CH_3	3.77, 3.56 3.56	55.2, CH_2	7.9	162.9, CH
18	3.87	56.4	-	-	-	-	2.29	38.7, CH_3	3.06	33.8, CH_3
1`	-	-	-	-	-	122.8, C	-	123.2, C		
2`	-	-	-	-	-	155.9, C	-	155.2, C	-	-
3`	-	-	-	-	6.82	114.6, CH	6.81	114.3, CH	-	-
4`	-	-	-	-	7.16	127.5, CH	7.31	127.2, CH	-	-
5`	-	-	-	-	6.78	118.5, CH	6.82	118.5, CH	-	-
6`	-	-	-	-	7.02	128.5, CH	7.18	128.5, CH	-	-
NH	8.51, d	-	-	-		-	-	-	-	-
OH	-	-	-	-	4.71	-	4.77	-	-	-

Demecolcine (2) was identified based on a combination of mass spectrometric and NMR spectroscopic data. ESI-MS analysis revealed a protonated molecular ion at *m/z* 372 $[M + H]^+$, consistent with a molecular formula of $C_{21}H_{25}NO_5$. The overall NMR profile of compound 2 shared strong similarities with that of colchicine (1), reflecting the conserved tropolone core structure. However, the ^{13}C NMR spectrum of compound 2 notably lacked the carbonyl carbon signal observed in colchicine, indicating the reduction or substitution of the corresponding functional group. Instead, the presence of a methyl group attached to the nitrogen was confirmed by a singlet at δ_H 2.15 (3H, s), which correlated in the HSQC spectrum to a carbon resonance at δ_C 33.6. This key structural modification distinguishes demecolcine from colchicine and supports its identity as the N-desacetyl analog. Together, the spectral features align well with previously reported data for demecolcine, confirming its structure (25).

Compound 3 displayed molecular ions at m/z 464 [M + H]+, m/z 927 [2M + H]+, and m/z 462 [M + H]- in the ESI-MS spectrum, with a calculated degree of unsaturation of 11, indicating the molecular formula C27H29NO6. The 1H NMR spectrum showed eight aromatic proton signals between δH 6.77–7.88 ppm, along with signals assigned to the methoxy groups. A signal in the aliphatic region at δH 4.71 (1H, s), which did not correlate with any carbon in the HSQC-DEPT spectrum, suggested the presence of a hydroxyl group in the structure of compound 3. Multiple aliphatic signals between δH 3.55–4.19 ppm and δH 1.93–2.71 ppm provided further information about the substitution pattern. Based on these spectral data, the structure of compound 3 was determined to be 3-hydroxy-2-methoxycolchicine (Fig. 1).

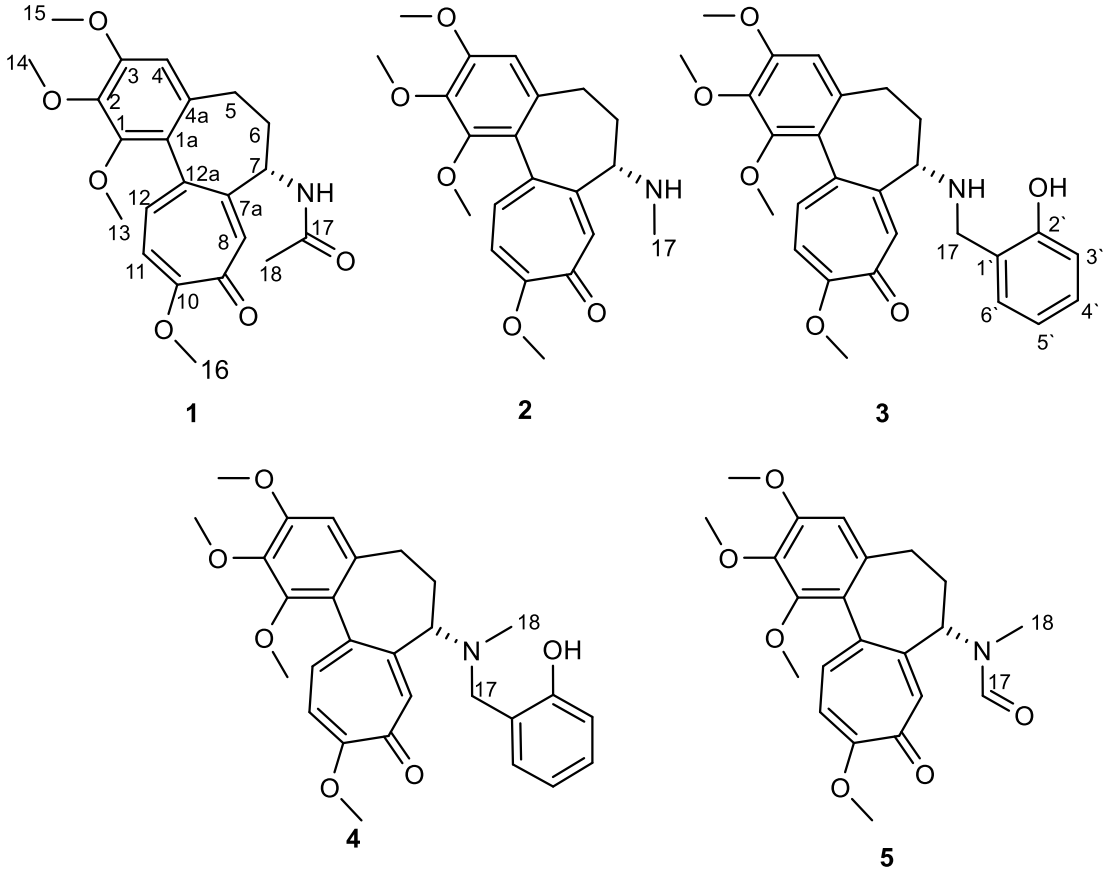

Figure 1. Chemical structures of compounds 1-5 isolated from *Colchicum speciosum*

The ESI-FMS analysis of speciosamine (4) revealed a molecular weight of 477 Da, with molecular ions observed at m/z 478 [M + H]+, m/z 955 [2M + H]+, and m/z 476 [M + H]-. Its degree of unsaturation was calculated to be 14. Consequently, the molecular formula of speciosamine (4) was determined as C28H31NO6. The NMR data of speciosamine (4) (Table 1) were similar to those of compound 3, with the addition of a methyl group at δH 2.29 (3H, s, H-18), which correlated with C-18 (δC 38.7) in the HSQC spectrum, indicating its attachment to the nitrogen atom. COSY correlations, such as those between H-7 (δH 3.27) and H-6 (δH 1.95), and between H-6 and H-5 (δH 2.35, 2.67), further supported the structure elucidation. The final structure of speciosamine (4) is illustrated in Figure 1.

The NMR data for N-formyldemecolcine (5) showed a structural similarity to colchicine (1), with some minor spectral differences. Notably, the shift of C-17 in colchicine (1) (δC 169.5) to δC 162.9 in compound 5 indicated the presence of an aldehyde carbonyl group. The remaining 1H and 13C NMR signals were largely consistent with colchicine, suggesting a conserved core structure. COSY experiments confirmed correlations among key protons (H-7, H-6, H-5), supporting this assignment.

ESI-MS confirmed a molecular mass of 477 Da, with corresponding peaks at m/z 478 [M + H]+, 955 [2M + H]+, and 476 [M + H]–. With one degree of unsaturation, the molecular formula of N-formyldemecolcine (5) was determined to be C22H25NO6, completing its structural identification (Fig. 1).

Absolute Configuration Determination

To elucidate the absolute configuration of compounds 3, 4, and 5, time-dependent density functional theory (TDDFT) calculations were carried out based on the ECD data of their possible stereoisomers. The calculated ECD spectra showed positive and negative Cotton effects (CEs), primarily associated with π → π* transitions within the aromatic moieties. Figure 2 displays the computationally derived ECD spectra for the two enantiomeric forms of compounds 3, 4, and 5.

Compound 3 exhibited experimental ECD bands with positive CEs at approximately 230 nm and a shoulder at 250 nm, alongside negative CEs at 220 nm, 280 nm, and 345 nm. These transitions are attributed to π → π* excitations within the aromatic rings and the extended conjugated system, indicative of its structural features. The theoretical ECD spectrum of the (7S)-stereoisomer of compound 3 displayed comparable features, including positive CEs at 230 nm and 260 nm, and negative CEs at 220 nm, 310 nm, and 350 nm. The congruence between the experimental data and the calculated spectrum for the (S)-enantiomer strongly suggests that compound 3 possesses the S absolute configuration (Fig 2).

Compounds 4 and 5 displayed analogous patterns in their experimental ECD spectra. Compound 4 exhibited a positive CE at 236 nm and negative CEs at 216 nm and 352 nm, while compound 5 showed a positive CE at 230 nm and negative CEs at 216 nm, 281 nm, and 352 nm. The computationally generated ECD spectra for the (7S)-stereoisomers of both compounds demonstrated a high degree of similarity to their respective experimental counterparts. Consequently, compounds 4 and 5 were also assigned the S absolute configuration at the corresponding chiral center. These findings are consistent with prior literature that established the S-configuration for colchicine based on ECD spectroscopic analysis (26).

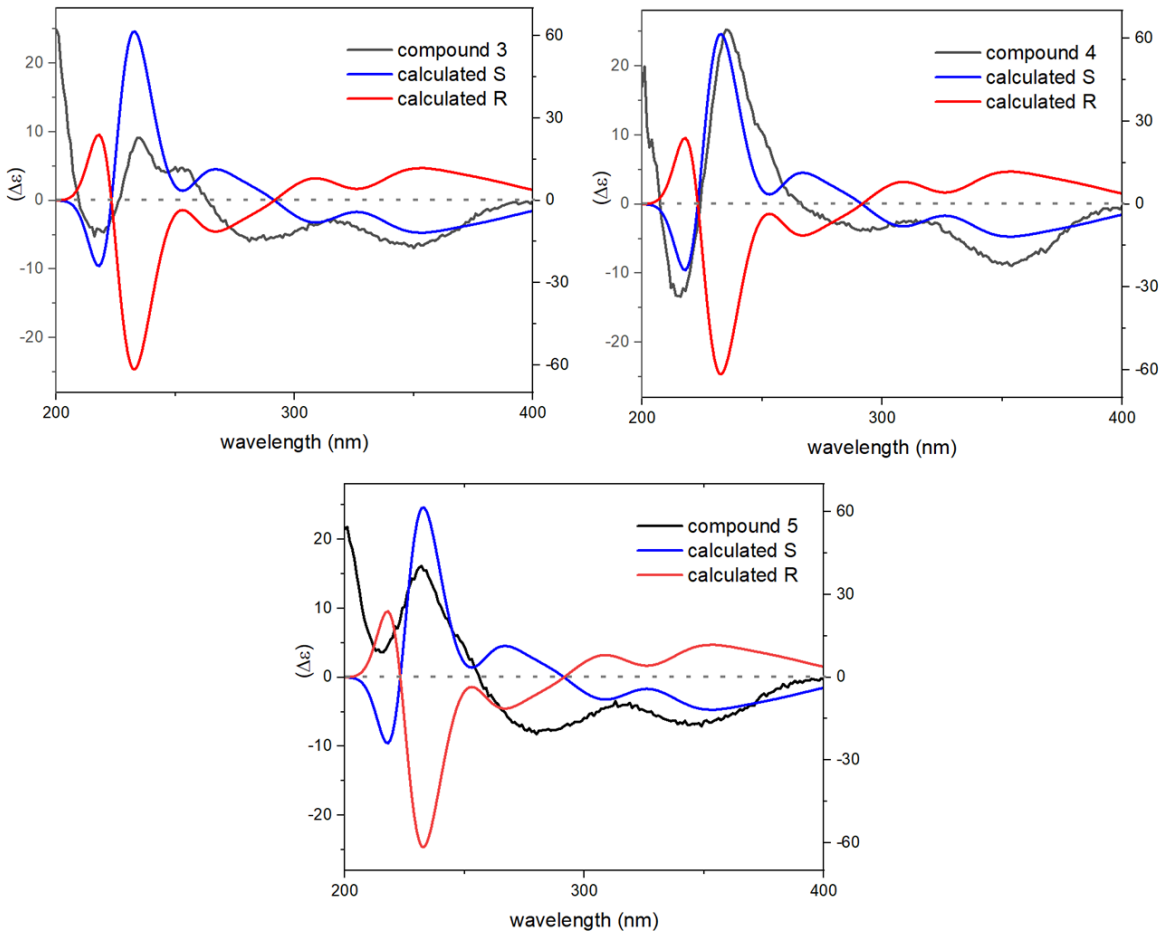

Figure 2. Calculated ECD spectra of compounds 3, 4, and 5. The spectra were obtained using time-dependent density functional theory (TDDFT) at the CAM-B3LYP/6-31G level of theory in methanol.**

Conclusion

This study provides a comprehensive phytochemical analysis of the methanol (MeOH) extract of *Colchicum speciosum* bulbs. Using a combination of open column chromatography and semi-preparative RP-HPLC, five tropolonic alkaloids were successfully isolated, including colchicine (1), demecolcine (2), speciosamine (4), N-formyldemecolcine (5), and compound (3). The structures of these compounds were thoroughly elucidated using 1D and 2D NMR spectroscopic techniques. The absolute configurations of compounds 3, 4, and 5 were determined through a comparative approach involving experimental electronic circular dichroism (ECD) spectroscopy and time-dependent density functional theory (TDDFT)-based computational simulations. The calculated ECD spectra of the potential stereoisomers exhibited excellent agreement with the experimentally obtained spectra, confirming the S absolute configuration for all three compounds. These results align with previously reported configurational assignments for similar compounds such as colchicine. Moreover, the close match between the experimental and calculated UV spectra of compounds 3, 4, and 5 further supports the validity of the computational predictions and overall conclusions. In summary, this study successfully isolated and characterized five tropolonic alkaloids from *C. speciosum*, contributing both known and novel structural information. The determination of the absolute configurations of compounds 3- 5 offers critical stereochemical insights. These findings enhance our chemical understanding of *C. speciosum*

and provide a foundation for future investigations into the biological activities and potential applications of these isolated compounds.

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Authors' Contributions

Marzeieh Omrani: Conceptualization, Investigation, Formal analysis, Writing-original draft. Saheb Taieb: Methodology, Visualization, Mohamad Hossein Mirjalili: Visualization, Writing-review and editing. Samad N. Ebrahimi: Conceptualization, Investigation, Formal analysis, Methodology, Visualization, Validation, Writing—review and editing, Supervision, Resources, Funding acquisition, Project administration.

Declaration of Interest

The authors of this article declared no conflict of interest.

Ethical Considerations

All ethical principles were adheried in conducting and writing this article.

Transparency of Data

In accordance with the principles of transparency and open research, we declare that all data and materials used in this study are available upon request.

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