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Chromatographic determination of constituents of the genus *Colchicum* (Liliaceae)☆

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Abstract

A high-performance liquid chromatographic method for the determination of colchicinoid alkaloids in plant material is described. The determination is performed separately in neutral and basic alkaloid fractions using a C_{18}-bonded silica column. Nine alkaloids, i.e., 3-demethylcolchicine, 2-demethylcolchicine, colchifoline, N-deacetyl-N-formylcolchicine, colchicine, cornigerine, 2-demethyldemecolcine, 3-demethyldemecolcine and demecolcine, in seven *Colchicum* plants were assayed. For identification of phenolic compounds, a method using gas chromatography–mass spectrometry was elaborated and twenty phenolic compounds were identified in extracts from five *Colchicum* species. As the presence of luteolin appeared to be of value for chemotaxonomic purposes, its simple densitometric determination was developed.

1. Introduction

The plants of the genus *Colchicum* have been known for more than 2000 years for their marked biological effects. Colchicine, the main alkaloid, was isolated from all species of genera *Colchicum*, *Merendera* and *Gloriosa* (subfamily Wurmbaeoideae, Liliaceae). Colchicine and its congeners are chemotaxonomic markers for the subfamily Wurmbaeoideae. The genus *Colchicum* includes 42 species, most of which are endemic for the Middle East [1]. In most of the earlier studies, column and thin-layer chromatography were used for the isolation and identification of these alkaloids; other constituents, with the exception of flavones, were not studied [2].

For systematic phytochemical studies of *Colchicum* growing wild in Turkey, we developed methods of analysis for colchicinoid and phenolic compounds in plant extracts. So far, only three Turkish *Colchicum* species, *C. bivonae* [3], *C. micranthum* [4] and *C. sovitsii* [5] have been qualitatively investigated for their alkaloid content. This paper describes the results of the HPLC determination of colchicinoids in seven plants, GC–MS identification of phenolics in five

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plants and densitometric determination of luteolin in seven *Colchicum* plants.

2. Experimental

Alkaloids and luteolin used as reference compounds originated from the collection at our Institute and their structures were confirmed by their melting points and ultraviolet, infrared and nuclear magnetic resonance spectra. Phenolic reference compounds were obtained from Aldrich (Milwaukee, WI, USA) and ICN Biomedicals (Bucks, UK).

2.1. Extraction of plant material

Dried powdered plant material was extracted with methanol in a Soxhlet apparatus. The dried methanolic extracts were taken up in 0.01 *M* H_2SO_4 and extracted with diethyl ether. The solvent was evaporated after drying and the phenolic compounds were identified by GC–MS in the crude evaporation residue; luteolin was determined by densitometry. The aqueous solution was then extracted with chloroform and the extract was dried and evaporated, yielding a crude mixture of neutral alkaloids. The acidic aqueous residue was made alkaline (pH 9–10) with ammonia and extracted again with chloroform. After drying and evaporation of the extract, a crude mixture of basic alkaloids was obtained. The alkaloids in both fractions were identified and determined by HPLC. Details of extraction procedure are given in Ref. [6].

2.1. HPLC of alkaloids

Extracts were analysed using an SP 8700 apparatus and SP 4290 detector (Spectra Physics,

Table 1
Alkaloid composition in corms of the studied Turkish *Colchicum* species

Plant	Alkaloid[a] (μg/g dried drug) (R.S.D., %)[b]								
	2MCO	3MCO	CFNE	NFOCO	COL	COR	2MDE	3MDE	DEM
C. macrophyllum [7]	[c]	2276 (0.05)	[c]	168 (0.05)	2223 (0.05)	[c]	[c]	1 (0.03)	67 (0.01)
C. turcicum [8]		56 (0.03)	19 (0.03)	[c]	323 (0.04)	7 (0.23)	4 (0.14)	2 (0.15)	225 (0.02)
C. cilicicum [9]	n.a.[d]	n.a.	n.a.	n.a.	300 (0.02)	n.a.	n.a.	n.a.	1100 (0.01)
C. kotschyi [10]	109 (0.01)	289 (0.01)	16 (0.01)	[c]	1058 (0.04)	[c]	0.9 (0.01)	5 (0.03)	6 (0.01)
C. bornmuelleri	[c]	844 (0.03)	91 (0.01)	[c]	3063 (0.01)	5 (0.03)	23 (0.04)	18 (0.02)	720 (0.02)
C. speciosum	13 (0.05)	10 (0.06)	220 (0.01)	61 (0.03)	4245 (0.01)	187 (0.01)	92 (0.03)	106 (0.03)	3159 (0.03)
C. triphyllum	54 (0.02)	88 (0.03)	63 (0.03)	[c]	958 (0.01)	75 (0.02)	65 (0.01)	187 (0.03)	105 (0.02)

Quantification of alkaloids in other plant parts is given in refs. [7–10].

[a] 3MCO 3-demethylcolchicine, 2MCO 2-demethylcolchicine, CFNE colchifoline, NFOCO N-deacetyl-N-formylcolchicine, COL colchicine, COR cornigerine, 2MDE 2-demethyldemecolcine, 3MDE 3-demethyldemecolcine, DEM demecolcine. Retention times (min) in elution system for neutral alkaloids: 3MCO 5.9, 2MCO 6.7, CFNE 9.7, NFOCO 10.4, COL 11.0, COR 12.4; in elution system for basic alkaloids: 2MDE 3.9, 3MDE 4.7, DEM 6.3.

[b] In parentheses.

[c] Below detection limit.

[d] n.a. = Not analysed.

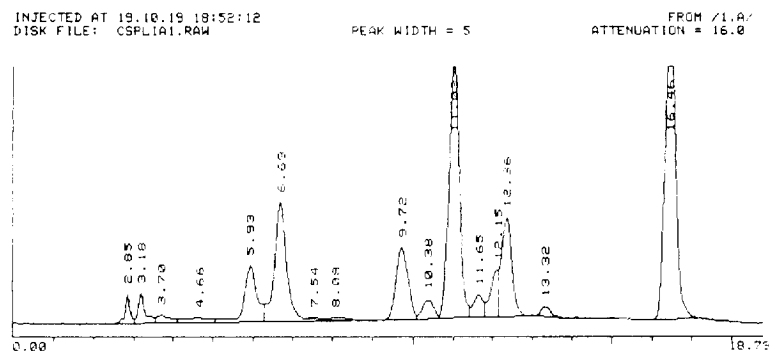

Fig. 1. HPLC separation of neutral alkaloid fraction from *Colchicum speciosum*. Separation was performed on a Separon SGX C_{18} octadecylsilyl column (250 × 4.6 mm I.D.) with a gradient of MeCN–MeOH in 0.02 *M* phosphate buffer (pH 7.5) (0–6 min, 13:20; 16–21 min, 18:25; 23 min, 13:20, v/v) at ambient temperature and a flow-rate of 1.5 ml/min, with UV detection at 353 nm. Peaks: 5.93 min = 3-demethylcolchicine; 6.69 min = 2-demethylcolchicine; 9.72 min = colchifoline; 10.38 min = N-deacetyl-N-formylcolchicine; 11.03 min = colchicine; 12.36 min = cornigerine; 16.46 min = demecolcine.

Santa Clara, CA, USA), equipped with a 250 × 4.6 mm I.D. column filled with octadecyl-modified Separon SGX C_{18} (Tessek, Prague, Czech Republic), particle size 7 μm. The solvent system for neutral alkaloids was a gradient of MeCN–MeOH in 0.02 *M* phosphate buffer (pH 7.5) as follows: 0–6 min, 13:20; 16–21 min, 18:25, 23 min, 13:20 (v/v); for basic alkaloids 13% tetrahydrofuran in 0.02 *M* acetate buffer (pH 5.5) was used; in both instances the flow-rate was 1.5 ml min. UV absorbance detection at 353 nm was applied.

The methanolic solutions of the chloroform extracts (2 mg) were percolated through modified silica gel cartridges (Separcol SI C_{18}) and the solvent was evaporated under nitrogen. The dry residues were dissolved in 1 ml of mobile phase and filtered through a 0.45-μm filter. Samples were injected in amounts of 10 μl and the alkaloids were identified by their retention

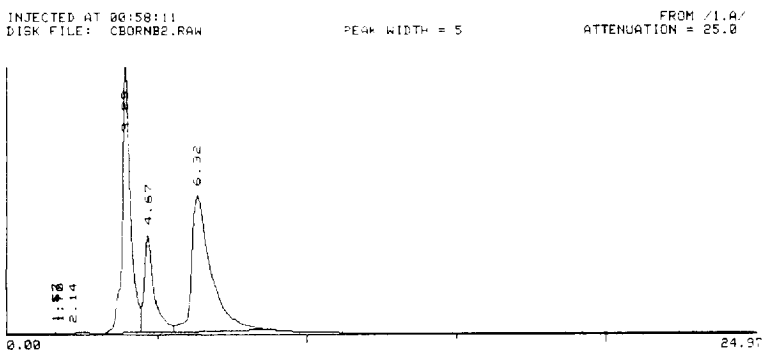

Fig. 2. HPLC separation of basic alkaloid fraction from *Colchicum bornmuelleri*. Separation was performed on a Separon SGX C_{18} octadecylsilyl column (250 × 4.6 mm I.D.) with 13% tetrahydrofuran in 0.02 *M* acetate buffer (pH 5.5) at ambient temperature and a flow-rate of 1.5 ml/min, with UV detection at 353 nm. Peaks: 3.89 min = 2-demethyldemecolcine; 4.67 min = 3-demethyldemecolcine; 6.32 min = demecolcine.

Table 2
Phenolic compounds found in the studied Turkish *Colchicum* species

Species	Compound	Retention time (min)	Plant part			
			Corms	Leaves	Seeds	Flowers
C. bornmuelleri	4-Methoxybenzoic acid	16.6	–	+	–	–
	4-Hydroxy-3-methoxybenzaldehyde (vanillin)	16.7	+	–	+	–
	2,4-Dihydroxybenzoic acid	17.4	–	+	–	–
	2,5-Dihydroxybenzoic acid	19.2	+	–	+	+
	4-Hydroxybenzoic acid	17.6	+	+	+	+
	3,4-Dihydroxybenzaldehyde	18.8	–	+	+	–
	4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	19.3	–	+	+	–
	3,4-Dihydroxybenzoic acid	19.9	+	+	+	+
	3-(4-Hydroxyphenyl)-2-propenoic acid (coumaric acid)	21.7	+	+	+	+
	3-(3,4-Dimethoxyphenyl)-2-propenoic acid	22.2	–	+	–	–
	3-(4-Hydroxy-3-methoxyphenyl)-2-propenoic acid (ferulic acid)	24.0	+	+	+	+
	3-(3,4-Dihydroxyphenyl)-2-propenoic acid (caffeic acid)	24.8	+	+	+	+
	3′,4′,5,7-Tetrahydroxyflavone (luteolin)	38.6	+	+	–	+
C. speciosum	4-Hydroxyphenylmethanol	15.3	+	–	–	+
	4-Hydroxy-3-methoxybenzaldehyde (vanillin)	16.7	+	–	–	–
	3-Phenyl-2-propenoic acid (cinnamic acid)	16.8	+	–	–	–
	2,5-Dihydroxybenzoic acid	19.2	–	+	–	+
	2,6-Dihydroxybenzoic acid	19.1	–	–	–	+
	4-Hydroxybenzoic acid	17.6	+	+	+	+
	4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	19.3	+	+	+	+
	3,4-Dihydroxybenzoic acid	19.9	+	+	+	+
	3-(4-Hydroxyphenyl)-2-propenoic acid (coumaric acid)	21.7	+	+	+	+
	3-(3,4-Dihydroxyphenyl)-2-propenoic acid (caffeic acid)	24.8	–	+	–	+
	3′,4′,5,7-Tetrahydroxyflavone (luteolin)	38.6	–	+	–	+
C. kotschyi	4-Hydroxy-3-methoxybenzaldehyde (vanillin)	16.7	+	–	–	n.a.[a]
	2,5-Dihydroxybenzoic acid	19.2	–	+	–	n.a.
	4-Hydroxybenzoic acid	17.6	–	+	+	n.a.
	3,4-Dihydroxybenzaldehyde	18.8	–	–	+	n.a.
	3-(4-Hydroxyphenyl)propanoic acid	19.2	–	–	+	n.a.
	4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	19.3	+	+	+	n.a.
	3-(4-Hydroxy-3-methoxyphenyl)propenol	19.7	+	–	–	n.a.
	3,4-Dihydroxybenzoic acid	19.9	–	+	+	n.a.
	3-(4-Hydroxy-3-methoxyphenyl)propanoic acid	20.7	+	–	+	n.a.
	3-(4-Hydroxyphenyl)-2-propenoic acid (coumaric acid)	21.7	+	+	+	n.a.
	3-(4-Hydroxy-3-methoxyphenyl)-2-propenoic acid (ferulic acid)	24.0	+	–	–	n.a.
	3-(3,4-Dihydroxyphenyl)-2-propenoic acid (caffeic acid)	24.8	+	+	+	n.a.
	3′,4′,5,7-Tetrahydroxyflavone (luteolin)	38.6	–	–	–	n.a.
C. macrophyllum	2,5-Dihydroxybenzoic acid	19.2	+	+	–	+
	2,6-Dihydroxybenzoic acid	19.1	+	+	–	+
	4-Hydroxybenzoic acid	17.6	+	–	–	–
	3-(4-Methoxyphenyl)propanoic acid	18.7	–	+	+	+
	4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	19.3	–	+	–	+
	3,4-Dihydroxybenzoic acid	19.9	–	–	+	+
	3-(4-Hydroxyphenyl)-2-propenoic acid (coumaric acid)	21.7	+	+	–	+
	3-(4-Hydroxy-3-methoxyphenyl)-2-propenoic acid (ferulic acid)	24.0	–	+	+	+
	3-(3,4-Dihydroxyphenyl)-2-propenoic acid (caffeic acid)	24.8	+	+	–	+
	3′,4′,5,7-Tetrahydroxyflavone (luteolin)	38.6	+	+	+	+
C. triphyllum	4-Hydroxy-3-methoxybenzaldehyde (vanillin)	16.7	+	+	+	–
	2,5-Dihydroxybenzoic acid	19.2	+	–	–	–
	2,6-Dihydroxybenzoic acid	19.1	+	–	–	–
	4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	19.3	+	+	–	+
	3,4-Dihydrobenzoic acid	19.9	+	+	–	–
	3-(4-Hydroxyphenyl)-2-propenoic acid (coumaric acid)	21.7	+	–	–	–
	3-(4-Hydroxy-3-methoxyphenyl)-2-propenoic acid (ferulic acid)	24.0	–	–	+	+
	3-(3,4-Dihydroxyphenyl)-2-propenoic acid (caffeic acid)	24.8	+	+	–	+

[a] n.a. = Not analysed.

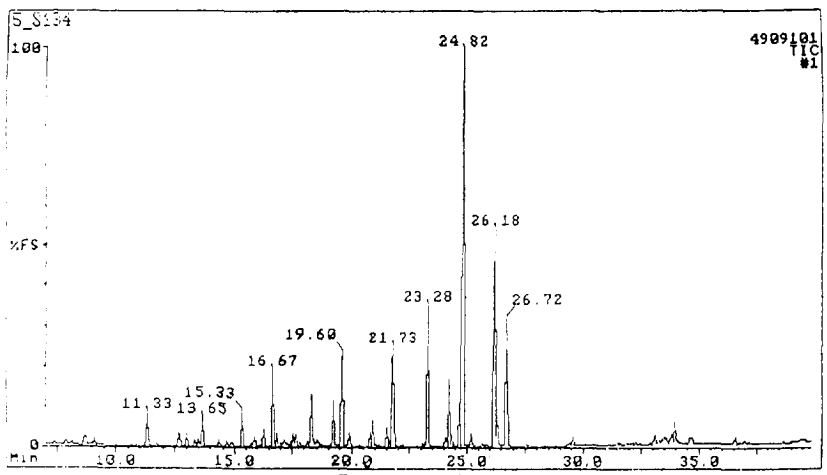

Fig. 3. GC profile of diethyl ether fraction from corms of *Colchicum speciosum*. Chromatographic conditions: column, Fisons 60 m × 0.32 mm I.D., coated with 0.5-μm DB-5 bonded phase; carrier gas, helium; flow-rate, 1.5 ml/min; column temperature gradient, 100°C for 7 min, then increased at 20°C/min to 220°C, at 4°C/min to 300°C and at 7°C/min to 320°C; injector temperature, 300°C. Peaks: 15.33 min = 4-hydroxyphenylmethanol; 16.67 min = vanillin; 16.81 min = cinnamic acid; 17.58 min = 4-hydroxybenzoic acid; 19.30 min = vanillic acid; 19.89 min = 3,4-dihydroxybenzoic acid; 21.73 min = coumaric acid; 24.82 min = caffeic acid.

times in comparison with reference compounds. Determination was carried out by the external calibration method. The results of three parallel determinations were processed using Student's *t*-test at the 95% confidence level for evaluation of the confidence interval. The limit of determination was ca. 1 μg of each substance per gram of dried drug.

Table 3
Content of luteolin in the studied Turkish *Colchicum* species

Plant	Concentration (μg/mg dry material)[a]			
	Corms	Leaves	Seeds	Flowers
C. macrophyllum	110.0	115.0	[b]	161.6
C. speciosum	56.1	153.8	[b]	278.9
C. kotschyi	145.7	42.1	[b]	n.a.
C. bornmuelleri	241.1	466.7	[b]	484.5
C. burtii	111.6	n.a.[c]	n.a.	85.0
C. triphyllum	219.1	202.2	[b]	150.9
C. umbrosum	89.4	118.7	1.8	334.8

[a] R.S.D. < 0.09%.
[b] Below detection limit.
[c] n.a. = Not analysed.

2.3. Capillary gas chromatography–mass spectrometry (GC–MS) of phenolic compounds

A diethyl ether extract (1 mg) after addition of 50 μl of pyridine and 100 μl of bis(trimethylsilyl) trifluoroacetamide containing 1% of trimethylchlorosilane was sealed and heated for 30 min at 100°C to produce trimethylsilyl derivatives for gas chromatography.

The derivatized samples were separated and analysed in an HRGC 5160 apparatus (Fisons, Middlewich, UK), equipped with a Fisons 60 m × 0.32 mm I.D. silica column, coated with 0.5-μm DB-5 bonded phase, and a splitless injector with a 7-min flush after sample injection to remove residual gases. The end of the column was introduced directly into the analyser chamber of a mass spectrometer. The system was operated under the following conditions: carrier gas, helium; flow-rate, 1.5 ml/min; injector temperature, 300°C; GC column temperature gradient, 100°C for 7 min, then increased at 20°C/min to 220°C, at 4°C/min to 300°C and at 7°C/min to 320°C. The mass spectrometer was set to scan 40–650 u per nominal second with an ionizing voltage of 70 eV. Samples of 0.3–0.5 μl

were injected. The identities of phenolic compounds in the ether extracts were established after GC separation by comparison of their retention times and mass spectra with those of reference compounds.

2.4. Determination of luteolin

Quantitative densitometric measurements were carried out on a Chromoscan MK II instrument (Joyce Loebl, Gateshead, UK) equipped with a device for evaluation of thin-layer chromatograms. The chromatograms (Silufol UV ready-to-use plates; Kavalier, Votice, Czech Republic) were used in duplicate, 0.1 mg of crude ether extract being applied to the plate. After elution with benzene–chloroform–ethanol (3:2:1), the quenching of fluorescence was measured at 260 nm. The spot area showed a linear dependence on the concentration of luteolin obtained in standard samples. The limit of determination was ca. 10 μg/mg dried drug.

3. Results and discussion

The alkaloid composition found in corms of the studied *Colchicum* species is presented in Table 1; representative chromatograms of neutral and basic alkaloid fractions are displayed in Figs. 1 and 2. Using the method described here, we determined nine of the 31 alkaloids found in *Colchicum* species [11]. Owing to the limit of determination (1 μg/g dried drug), we do not discuss the presence of other colchicine congeners in the plants studied. For colchiceines (colchicine derivatives with a hydroxyl instead of a methoxy group at C-10), the HPLC conditions used do not allow their determination. Compounds of this type can be determined in the form of Cu(II) complexes [12]. Lumi-colchicines, formed as artifacts during the extraction and which are without chemotaxonomic importance, cannot be determined by the method described.

Using GC–MS, we identified twenty phenolic compounds in five *Colchicum* species (see Table 2 and Fig. 3). Caffeic acid and leuteolin being the major phenolic compounds in studied plants, we consider luteolin to be the most suitable candidate for chemotaxonomic purposes. Therefore, a densitometric method for its determination was developed. The results for luteolin content in seven *Colchicum* species are given in Table 3.

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