Liquid Chromatography-Mass Spectroscopy and Liquid Chromatography-Ultraviolet/Visible Photodiode Array Analysis of Selected *Colchicum* Species

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Z. Naturforsch. **67c**, 451–460 (2012); received September 21, 2011/July 22, 2012

An in-house strategy to dereplicate colchicinoid alkaloids was recently developed by our team. It aimed at quickly identifying *Colchicum* constituents using LC-MS (liquid chromatography-mass spectroscopy) and LC-UV/Vis PDA (liquid chromatography-ultraviolet/visible photodiode array) techniques. In this project, our goal was to validate the developed method through analysing the alkaloid-rich fractions of three *Colchicum* species that had been previously studied phytochemically using the traditional bioactivity-guided fractionation methodology. The analysed species were *Colchicum tauri* Siehe ex Stefanoff, *Colchicum stevenii* Kunth, and *Colchicum tunicatum* Feinbr., all belonging to the family Colchicaceae. In addition to identifying the compounds previously isolated and characterized by the traditional methodology, the new strategy succeeded in tentatively identifying a set of known compounds, but new to the species.

Key words: Dereplication, Colchicinoids, LC-APCI-MS

Introduction

Most natural products drug discovery programs rely on a traditional bioactivity-guided fractionation approach. Although this approach succeeds in isolating most of the well-known drugs of natural origin, it is tedious, costly, time-consuming, and may end up in isolating previously known compounds (Ghisalberti, 1993; Kingston, 1996). In the field of natural products, interest has been growing in the last twenty years in developing fast and efficient strategies to enable the discrimination between previously isolated known compounds and new compounds at the level of the crude extract. This strategy which is termed dereplication is very important for the efficient use of human and financial resources. By adopting a suitable dereplication method, tedious and time-consuming isolation of previously known compounds can be avoided, and hence efforts are focused on the targeted isolation of compounds showing novel or uncommon spectroscopic features (Hostettmann *et al.*, 2001; Wolfender *et al.*, 2003). In a pioneering work, Corley and Durley (1994) developed strategies to search through scientific and technical network files using molecular weight, carbon count, structure fragments, and taxonomy for rapid identification of known compounds.

Hyphenated techniques, which can be defined as the coupling of high performance liquid chromatography (HPLC) separation technologies with on-line spectroscopic/spectrometric detection technologies, are the most commonly used techniques in dereplication strategies. Of these, liquid chromatography-mass spectroscopy (LC-MS) has a wide range of applications in the development of dereplication tools for the tentative identification of plant and microbial secondary metabolites (Ackermann *et al.*, 1996; Alali and El-Alali, 2005;

Carmona *et al.*, 2006; Jin *et al.*, 2006; Liau *et al.*, 2007; Liu *et al.*, 2007; Petsalo *et al.*, 2006; Sarker and Nahar, 2012).

A dereplication strategy was recently introduced by our research group for the analysis of colchicinoid alkaloids using LC-MS and liquid chromatography-ultraviolet/visible photodiode array (LC-UV/Vis PDA) techniques (Alali *et al.*, 2008a). Briefly, this strategy used a three-step approach. First, LC-MS was used to acquire the molecular ion and, hence, the molecular weight of the compound. These data were searched across natural product databases, particularly the Dictionary of Natural Products (Chapman & Hall, London, UK; Version 15:2) and against a self-compiled library of colchicinoid alkaloids. Next, LC-UV/Vis PDA analysis was used to acquire the UV/Vis spectrum of a compound that serves as a fingerprint grouping each compound into one of the different structural classes that are typical for colchicine analogues. Finally, the mass fragmentation data and the chromatographic retention times (and hence, relative polarity) were used to identify the structures of the compounds. The developed method was used to analyse *Colchicum brachyphyllum* Boiss. & Haussk. ex Boiss. (Colchicaceae), a *Colchicum* species that has been previously studied phytochemically in our laboratory (Alali *et al.*, 2005, 2008b). The interesting results obtained by the dereplication method encouraged us to investigate three *Colchicum* species, previously studied with the bioactivity-guided fractionation approach, applying the above described method to further validate our method and to detect the presence of any interesting compounds that were not separable at the preparative level.

The alkaloid-rich fractions of *Colchicum tauri* Siehe ex Stefanoff, *Colchicum stevenii* Knuth, and *Colchicum tunicatum* Feinbr. were analysed using our dereplication strategy (Alali *et al.*, 2008a). Using the bioactivity-guided fractionation approach, the following compounds had previously been isolated: from *C. tauri*: (-)-colchicine, 2-demethyl-(-)-colchicine, 3-demethyl-(-)-colchicine, (-)-demecolcine, *N*-methyl-(-)-demecolcine, 3-demethyl-*N*-methyl-(-)-demecolcine, *β*-lumicolchicine, 3-demethyl-*β*-lumicolchicine, and apigenin (Alali *et al.*, 2006a); from *C. stevenii*: (-)-colchicine, 2-demethyl-(-)-colchicine, *β*-lumicolchicine, cornigerine, isoandrocymbine, and *O*-methyl androcymbine (Al-Mahmoud *et al.*, 2006); from *C. tunicatum*: (-)-colchicine, 3-demethyl-(-)-colchicine, cornigerine, *β*-lumicolchicine, and (-)-androbiphenyline (Alali *et al.*, 2006b). In the present study, we report compounds tentatively identified using the dereplication method and compare them with those isolated by the traditional bioactivity-guided fractionation methodology.

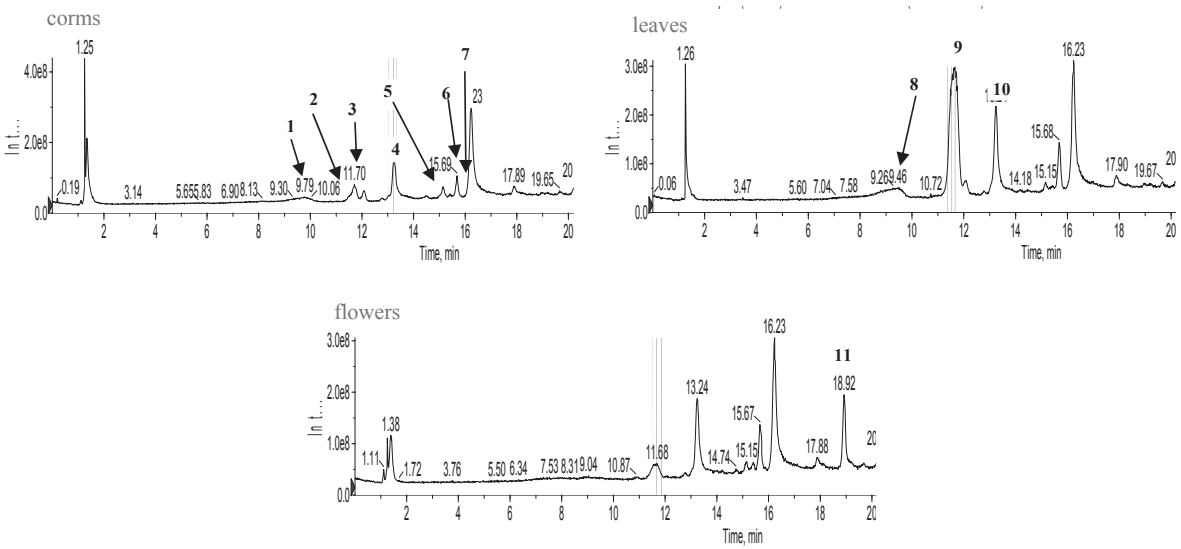
Results and Discussion

The alkaloid-rich fractions of corms, leaves, and flowers of *C. tauri*, and corms and leaves of *C. stevenii* and *C. tunicatum*, respectively, were analysed analytically as outlined in the Experimental Section using LC-MS and LC-UV/Vis PDA techniques. Fig. 1 shows typical LC-MS total ion chromatograms (TICs) of the analysed parts of *C. tauri*, *C. stevenii*, and *C. tunicatum*. The molecular ion for each peak of each part is shown in Fig. 2. Table I summarizes the UV_{max} and mass spectral data of each peak as well. The identified compounds were given consecutive numbers corresponding to the order of their elution during reversed phase (RP)-HPLC as well as the order of the investigated species and their parts as arranged in Fig. 1.

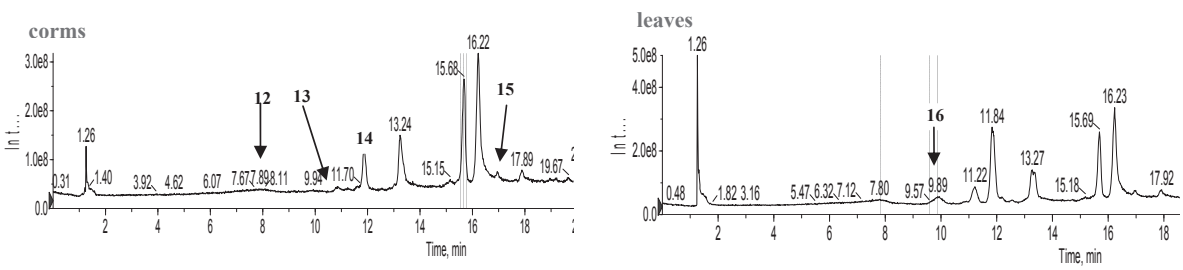
The (+)-APCI mass spectrum of peak 1 at t_R = 9.8 min of the corms of *C. tauri* showed a parent molecular ion at *m/z* 372 for $[M+H]^+$, 28 Da less than the analogous peak in (-)-colchicine (Figs. 1 and 2), and the compound was eluted earlier (*i.e.* it is more polar) than (-)-colchicine. The UV/Vis PDA spectrum had two absorption maxima at 245 and 354 nm, implying structural similarities to (-)-colchicine. These data suggested that this compound was (-)-demecolcine (Alali *et al.*, 2006a; Freyer *et al.*, 1987). The (+)-APCI mass fragmentation spectrum of the molecular ion peak showed a peak at *m/z* 341 which was attributed to the ion $[MH–NH_2CH_3]^+$, which was followed by loss of CO tropolonic to *m/z* 313.

In a way analogous to the identification of peak 1, peaks 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, and 18 were identified as *N*-methyl-(-)-demecolcine, 3-demethyl-*N*-methyl-(-)-demecolcine, 2-demethyl-(-)-colchicine, (-)-colchiciline or (-)-colchifoline, (-)-colchicine, *β*-lumi-(-)-colchicine, 2-demethyl-(-)-demecolcine or 3-demethyl-(-)-demecolcine, 3-demethyl-(-)-colchicine, apigenin, (-)-isoandrocymbine, crociflorinone, *O*-methyl-(-)-androcymbine, (-)-cornigerine, and (-)-colchiceine, respectively (Figs. 1 and 2 and Table I) (Alali *et al.*, 2005, 2006a; Alali and El-Alali, 2005;

A) *Colchicum tauri:*



B) *Colchicum stevenii*



C) *Colchicum tunicatum:*

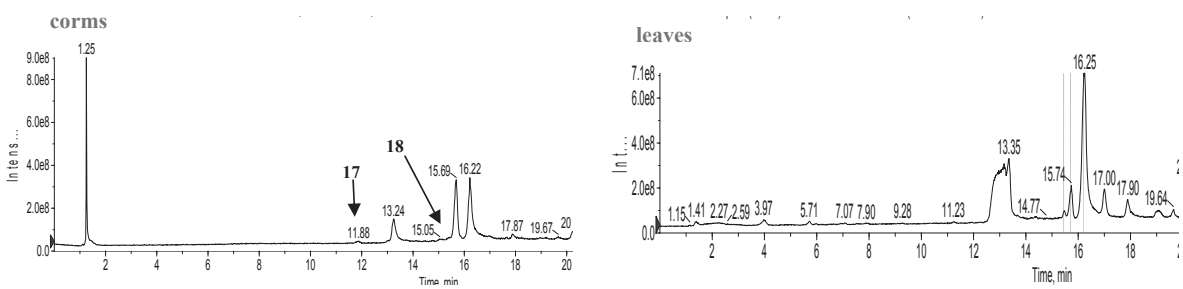
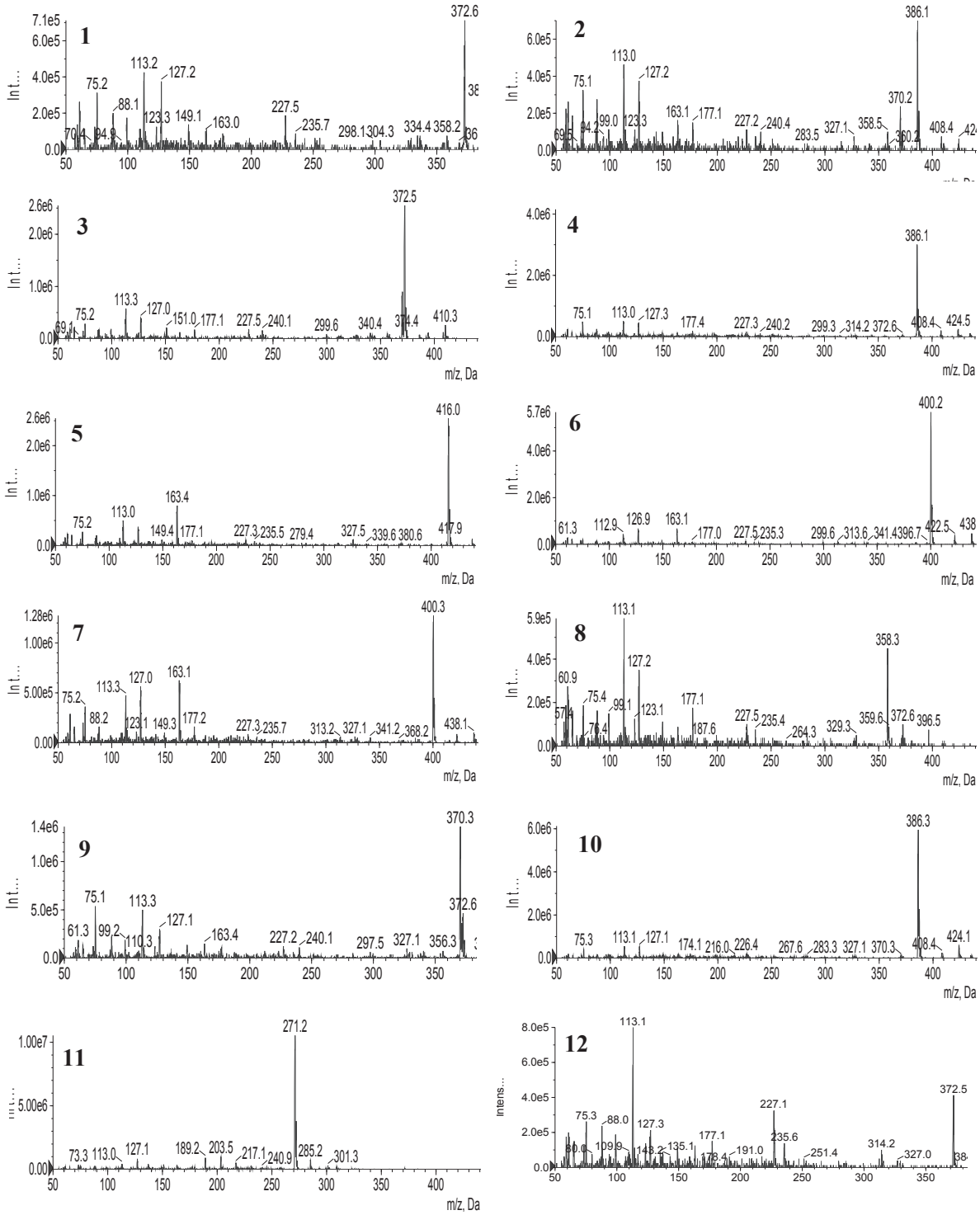


Fig. 1. (+)-APCI total ion chromatograms of the alkaloid-rich fractions of (A) *C. tauri*, (B) *C. stevenii*, and (C) *C. tunicatum*.

Al-Mahmoud *et al.*, 2006; Chommadov *et al.*, 1990; Freyer *et al.*, 1987; O'Neil *et al.*, 2001; Potesilova *et al.*, 1977; Rosso and Zuccaro, 1998; Roth and Rupp, 1995; Turdikulov *et al.*, 1974).

For *C. tauri*, we were unable to suggest a chemical structure for peak 9 at t_R = 11.7 min in the flowers and leaves that showed a parent molecular ion at *m/z* 370 for $[M+H]^+$. The retention


1
2
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4
5
6
7
8
9
10
11
12
m/z, Da
Int...
intens...

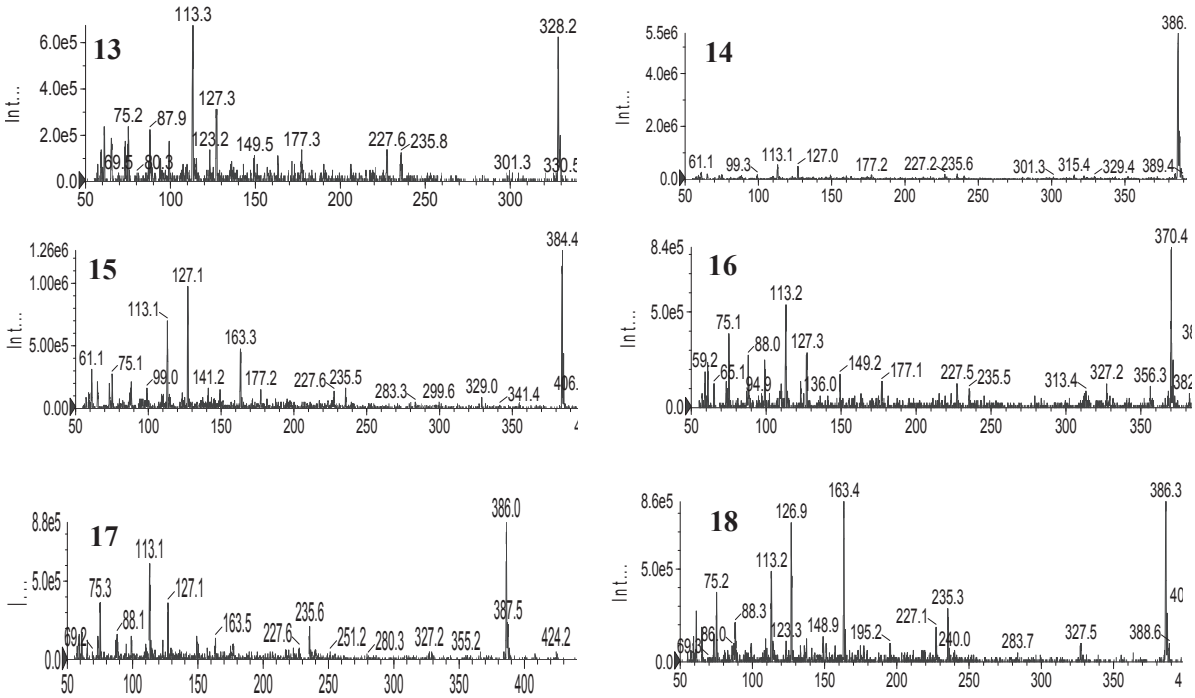


Fig. 2. (+)-APCI mass spectra of the TIC chromatographic peaks of *C. tauri*, *C. stevenii*, and *C. tunicatum* arranged based on the appearance of the corresponding chromatographic peaks in Fig. 1.

Table I. Retention times, UV_{max}, and mass spectral data of the (+)-APCI TIC chromatographic peaks.

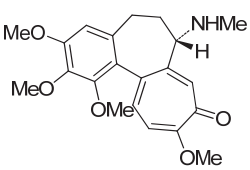
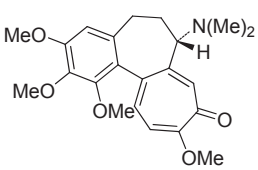
Peak number	*Colchicum* species (plant part)	t_R [min]	UV_{max} [nm]	LC-MS and LC-MS/MS-(+)-APCI mass fragments (*m*/*z*)	Compound
1	*C. tauri* (corms)	9.8	245, 354	372 $[M+H]^+$ (base peak), 358, 341, 334, 313, 304, 298, 235, 227, 163	 (-)-Demecolcine (**1**)
2	*C. tauri* (corms, flowers)	11.4	243, 350	386 $[M+H]^+$ (base peak), 370, 358, 355, 343, 327, 315, 283, 240, 227, 177	 *N*-Methyl-(-)-demecolcine (**2**)

Table I continued.

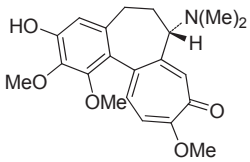
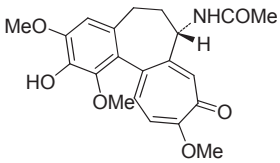
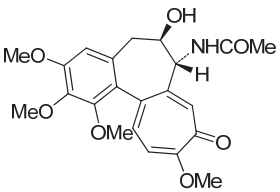
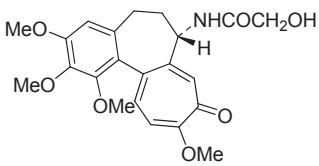
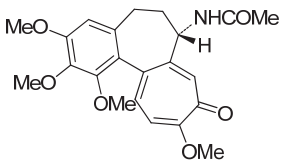
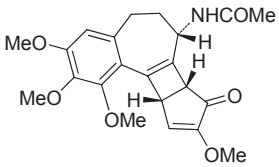
Peak number	*Colchicum* species (plant part)	t_R [min]	UV_{max} [nm]	LC-MS and LC-MS/MS-(+)-APCI mass fragments (*m*/*z*)	Compound
3	*C. tauri* (corms)	11.7	244, 356	372 $[M+H]^+$ (base peak), 340, 329, 302, 299, 240, 227, 177	 3-Demethyl-*N*-methyl-(-)-demecolcine (**3**)
4	*C. tauri* (corms, leaves) *C. stevenii* (corms, leaves)	13.2 13.4	246, 354	386 $[M+H]^+$ (base peak), 372, 327, 314, 299, 240, 227, 177	 2-Demethyl-(-)-colchicine (**4**)
5	*C. tauri* (corms, leaves, flowers) *C. stevenii* (corms, leaves)	15.2	245, 353	416 $[M+H]^+$ (base peak), 327, 235, 227, 163	 (-)-Colchiciline (**5**) or  (-)-Colchifoline (**5**)
6	*C. tauri* (corms, leaves, flowers) *C. stevenii* (corms, leaves) *C. tunicatum* (corms, leaves)	15.7	247, 354	400 $[M+H]^+$ (base peak), 341, 313, 299, 282, 235, 227, 177, 163, 126	 (-)-Colchicine (**6**)
7	*C. tauri* (corms, leaves, flowers) *C. stevenii* (corms, leaves) *C. tunicatum* (corms)	15.8	225, 273	400 $[M+H]^+$ (base peak), 368, 341, 327, 313, 235, 227, 177, 163, 127, 113	 *β*-Lumi-(-)-colchicine (**7**)

Table I continued.

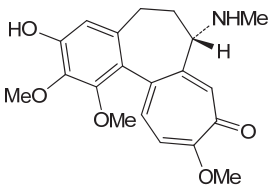
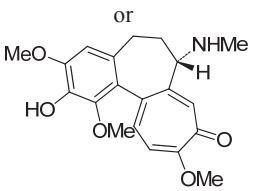
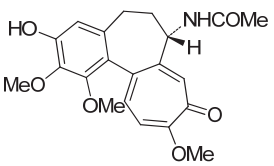
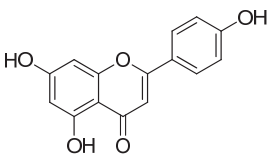
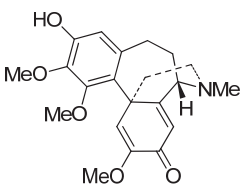
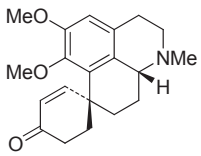
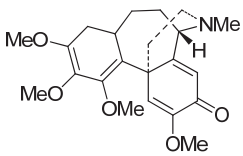
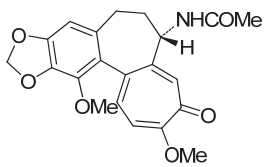
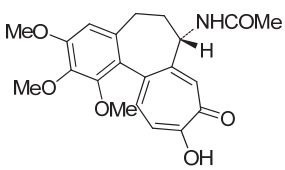
Peak number	*Colchicum* species (plant part)	t_R [min]	UV_{max} [nm]	LC-MS and LC-MS/MS-(+)-APCI mass fragments (*m*/*z*)	Compound
8	*C. tauri* (leaves)	9.7	242, 352	358 $[M+H]^+$, 329, 327, 264, 235, 227, 187, 177	 3-Demethyl-(-)-demecolcine (**8**) or  2-Demethyl-(-)-demecolcine (**8**)
9	*C. tauri* (leaves, flowers)	11.7	ND	370 $[M+H]^+$ (base peak), 356, 327, 297, 240, 227, 163	Unknown
10	*C. tunicatum* (corms, leaves) *C. tauri* (leaves, flowers)	13.2 13.3	243, 355	386 $[M+H]^+$ (base peak), 327, 253, 235, 227, 163	 3-Demethyl-(-)-colchicine (**10**)
11	*C. tauri* (flowers)	18.9	239, 259, 342	271 $[M+H]^+$ (base peak), 240, 217, 203, 189	 Apigenin (**11**)
12	*C. stevenii* (corms, leaves)	7.8	225, 254, 288	372 $[M+H]^+$, 327, 314, 251, 235, 227, 191, 177	 (-)-Isoandrocymbine (**12**)

Table I continued.

Peak number	*Colchicum* species (plant part)	t_R [min]	UV_{max} [nm]	LC-MS and LC-MS/MS-(+)-APCI mass fragments (*m/z*)	Compound
13	*C. stevenii* (corms, leaves)	10.9	ND	328 $[M+H]^+$, 301, 235, 227, 177	 Crociflorinone (**13**)
14	*C. stevenii* (corms, leaves)	11.8	227, 256, 292	386 $[M+H]^+$ (base peak), 329, 315, 301, 235, 227, 177	 *O*-Methyl-(-)-androcymbine (**14**)
15	*C. stevenii* (corms, leaves) *C. tunicatum* (leaves)	17.0	244, 353	384 $[M+H]^+$ (base peak), 341, 329, 325, 299, 297, 283, 235, 227, 177, 163	 (-)-Cornigerine (**15**)
16	*C. stevenii* (leaves)	9.9	ND	370 $[M+H]^+$ (base peak), 356, 327, 313, 235, 227, 177	Unknown
17	*C. tunicatum* (corms)	11.9	ND	386 $[M+H]^+$ (base peak), 355, 327, 280, 251, 235, 227, 163	Unknown
18	*C. tunicatum* (corms, leaves)	15.4	244, 356	386 $[M+H]^+$ (base peak), 370, 327, 299, 283, 267, 226, 216, 174	 (-)-Colchiceine (**18**)

ND, not detected.

time and UV/Vis PDA spectra were not conclusive. For the same reasons, we were unable to suggest chemical structures for peak 16 (t_R = 9.9 min) in the leaves of *C. stevenii* and peak 17 (t_R = 11.9 min) in the corms of *C. tunicatum* that showed molecular ion peaks at *m/z* 370 and 386, respectively.

Of the nine compounds previously isolated from *C. tauri* by bioactivity-guided fractionation (see Introduction) (Alali *et al.*, 2006a), we were able to identify all except 3-demethyl-*β*-lumicolchicine. Moreover, we were able to tentatively identify two compounds new for the species, namely: 3-demethyl-(-)-demecolcine [or 2-demethyl-(-)-demecolcine],

(-)-colchiciline [or (-)-colchifoline], in addition to one compound, which was not identified and thus considered unknown.

All six compounds previously isolated from *C. stevenii* (Al-Mahmoud *et al.*, 2006) were identified here, and we were able to tentatively identify two new compounds, namely: (-)-colchiciline [or (-)-colchifoline] and crociflorinone, in addition to one compound which was not identified and thus considered unknown.

Of the five colchicinoids previously isolated from *C. tunicatum* (Alali *et al.* 2006b), four were identified here except for (-)-androbiphenyline. On the other hand, one compound new to the species was tentatively identified namely (-)-colchiceine, in addition to one compound which was not identified and thus considered unknown.

These findings, from the three studied species, clearly demonstrate the advantage of using LC-MS and LC-PDA dereplication strategies as powerful and economical tools for dereplication of natural products in crude plant extracts. Although it took around a year of hard and costly work to isolate and identify the active components from *Colchicum* species phytochemically, we were able to tentatively identify the majority of the constituents from the crude alkaloid fraction in a single linear analytical run. Moreover, the strategy was able to discriminate between previously isolated, known compounds and new compounds hitherto not described. Hence, the application of this strategy before setting out a large-scale isolation project is of great importance to avoid the tedious isolation of known constituents, and thus focus on the targeted isolation of constituents presenting novel or unusual spectroscopic features. In comparison with our already published paper (Alali *et al.*, 2008a), in the current work, we used a different LC-MS instrument (triple quadrupole mass analyser rather than ion trap) in a different laboratory and operated by a different technician, and the results thus demonstrate the robustness of the developed method.

Experimental

General

LC-MS data were collected using an Applied Biosystems (Foster City, CA, USA) triple quadrupole LC-MS instrument (API 3200) operated in the APCI positive ionization mode and an Agilent 1200 series HPLC instrument (Santa Clara, CA, USA). The separation was achieved using a Hypersil BDS (125 mm × 4 mm; 5 μm) column (Thermo Electron, Auchtermuchty, UK). The mobile phase used was: (A) H_2O acidified with 0.1% formic acid; (B) MeOH. The flow rate was 1 mL/min in the following gradient combinations: 0–2 min, 90% (v/v) A/10% B; 2–27 min, 10% A/90% B; 27–30 min 90% A/10% B. The injection volume was 20 μL, and the total run time was 30 min. The mass detector conditions were as follows: APCI positive ionization mode; full scan mode from *m/z* 50 to 800; corona discharge voltage, NC, 5 kV; APCI temperature, 500 ºC; gas flow rate, 5 L/min.

UV/Vis PDA spectra were obtained on an Agilent 1200 series HPLC instrument, equipped with an Agilent 1200 quaternary pump, a 1200 photodiode array detector in the range between 200 and 400 nm, and a 1200 series auto-sampler. Mobile phase, flow rate, analytical column, injection volume, and run times were the same as those used for LC-MS.

Formic acid (extra pure) and methanol (HPLC grade) were obtained from Scharlau Chemie S.A. (Barcelona, Spain). (-)-Colchicine standard was purchased from Fluka Chemie (Buchs, Switzerland).

Sample preparation and analysis

The alkaloid-rich fractions of the different parts of *C. tauri*, *C. stevenii*, and *C. tunicatum* came from our earlier work (Al-Mahmoud *et al.*, 2006; Alali *et al.*, 2006a, b) and were stored in glass vials wrapped with aluminum foil, sealed with Parafilm® and maintained at –2 °C. For the dereplication studies, an aliquot of the alkaloid-rich fraction of each plant part (2 mg) was dissolved in the mobile phase, filtered through a 0.45-μm Teflon filter, and then transferred to 2-mL amber HPLC vials. A 20-μL aliquot was injected into the LC-UV/Vis PDA and LC-MS systems. (-)-Colchicine standard was used for retention time matching.

Acknowledgements

The authors acknowledge the financial support from the Deanship of Scientific Research, JUST, Irbid, Jordan, and the technical help of Mr. Ashraf Al-Matalq, Pharmaceutical Research Center, JUST, Irbid, Jordan.

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