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LC-MS and LC-PDA vs. phytochemical analysis of *Colchicum brachyphyllum*

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In this paper, reliability and validity of a recently in-house developed dereplication strategy based on LC-MS and LC–UV/Vis PDA techniques were verified. The dereplication strategy was applied to investigate the alkaloid rich fraction of *Colchicum brachyphyllum* Boiss. & Haussk. ex Boiss. (Colchicaceae) which has been previously analyzed phytochemically. Both studies results, LC-MS and LC-PDA and phytochemical analysis, were matched in seven compounds namely: (−)-colchicine (**5**), 3-demethyl-(−)-colchicine (**4**), (−)-cornigerine (**7**), β-lumi-(−)-colchicine (**6**), (−)-demecolcine (**3**), 3-demethyl-(−)-demecolcine (**2**), and 2,3-didemethyl-(−)-demecolcine (**1**). LC-MS and LC-PDA based dereplication strategy was not able to detect two of the compounds that were reported in the phytochemical study namely: (+)-demecolcinone and (−)-androbiphenyline. This finding could raise a question about the "naturality" of (+)-demecolcinone. On the other hand apigenin (**8**) was identified only using this dereplication strategy. Four compounds which had molecular ions at m/z 372 and 400 in the stems, and at m/z at 372 and 358 in flowers, could not be dereplicated, and were thus considered unknown. Their spectral data were not matched with neither the compounds that have been isolated previously from this species nor with any other colchicinoid; hence they should be pursued as potential new compounds.

1. Introduction

Traditional natural drugs discovery programs utilize bioactivity-directed fractionation approaches to isolate, purify and characterize bioactive lead compounds from crude extracts, wherein the bioassay results drive the purification processes. This process is often tedious, costly, and time consuming; moreover, it may end up with isolation of a previously known bioactive compound (Ghisalberti 1993; Kingston 1996). To accelerate drug discovery timelines of new bioactive natural products, it is of crucial importance to discriminate between previously isolated, known compounds and new compounds, at the level of the crude extract. Thus, a crucial step in the overall process is to establish whether or not an interesting bioactivity can be ascribed to known or new compounds. This process, which is termed dereplication, is a key component of natural products studies. It is used to insure that resources are expended on only the most promising samples, which are those most likely to yield bioactive and structurally unique compounds (Cordell and Shin 1999; Hostettmann et al. 2001; Wolfender et al. 2006). Thus, quick and easy methods for dereplication of natural product extracts are essential (Ackermann et al. 1996; Constant and Beecher 1995).

UV spectroscopy and mass spectrometry are of prime importance in the field of dereplication procedures. When combined with HPLC, they create hyphenated techniques, of which LC-UV/PDA and LC-MS are the most popular. The UV profile of compounds in an extract, obtained using LC-UV/PDA, serves as a fingerprint that is characteristic of certain structural classes (Alvi et al. 1995; Sedlock et al. 1992; Su et al. 2002; Sun et al. 2002; Tsao and Yang 2003). Also, LC-MS has become invaluable for the dereplication of plant and microbial natural products studies (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; Nielsen and Smedsgaard 2003; Petsalo et al. 2006; Smelcerovic et al. 2006). It is considered a selective, sensitive, and powerful tool (Niessen 1999; Sarker and Nahar 2005), as the molecular weight provides an entry point into the molecular formula of compounds.

In the research laboratory of Alali's group, a dereplication strategy was recently introduced for investigating colchicinoid alkaloids using LC-MS and LC–UV/Vis PDA techniques (Alali et al. 2008). Briefly, the developed dereplication strategy used a three-step approach. First, LC-MS is used to acquire the molecular ion and hence, the molecular weight of the compound. These data are searched across natural product databases, particularly the Dictionary of Natural Products (Chapman & Hall; Version 15 : 2) and against a self compiled library of colchicinoid alkaloids. Next, LC-UV/PDA is used to acquire the UV/Vis spectra of a compound which serves as a fingerprint grouping each compound into one of the different structur-

al classes that are typical for colchicine analogs. Finally, the mass fragmentation data and the chromatographic retention times (and hence, relative polarity) are used to identify the structures of the compounds.

In this paper reliability and validity of the above described dereplication strategy were verified. The alkaloid rich fraction of *Colchicum brachyphyllum* Boiss. & Haussk. ex Boiss. (Colchicaceae), previously investigated using bioactivity-directed fractionation (Alali et al. 2005), was analyzed using this newly developed dereplication strategy (Alali et al. 2008). In the phytochemical approach, nine colchicinoids were isolated and characterized. One of these has a novel ring system, (+)-demecolcinone, and represent the first naturally-occurring dextrorotatory colchicinoid. Another isolated compound was a new colchicinoid analog: 2,3-didemethyl-(−)-demecolcine (**1**); in addition to seven known colchicinoids. Here we report a comparison between the results obtained by the two different approaches; the phytochemical and the dereplication strategy.

2. Investigations, results and discussion

The alkaloid rich fraction of leaves, flowers, stems, and corms of *C. brachyphyllum* were analyzed analytically as outlined in the Experimental section using LC-MS and LC-UV/PDA techniques. Figure 1 shows typical LC-MS total ion chromatograms (TICs) of leaves, flowers, stems and corms of *C. brachyphyllum*. The molecular ion for each peak of each part is shown in Fig. 2. The Table summarizes the UV max (nm), and mass spectral data of each peak of each plant part as well. By application of the dereplication strategy as described by Alali et al. (2008); seven known compounds and one known but new to the species were tentatively identified in the (+)-APCI TIC chromatograms. The known compounds were (−)-colchicine (**5**), 3-demethyl-(−)-colchicine (**4**), 2,3-didemethyl-(−)-demecolcine (**1**), 3-demethyl-(−)-demecolcine (**2**), (−)-demecolcine (**3**), and (−)-cornigerine (**7**); all belongs to the colchicine-type alkaloids, a lumiderivative: β-lumi-(−)-colchicine (**6**), in addition to the flavone flavonoid: apigenin (**8**) which is known but new to the species.

The (+)-APCI mass spectrum of (−)-colchicine (**5**) (peaks 3, 7, 4, and 4, $t_R = 14.1$, 14.6, 14.3, and 14.4 min for the corms, stems, leaves and flowers, respectively) showed a parent molecular ion at m/z 400 $[M + H]^+$. The (+)-APCI-MS/MS fragmentation spectrum of the molecular ion peak showed a peak at m/z 341 which was attributed to the ion $[MH-NH_2COCH_3]^+$, followed by loss of $[CO]^+$ to give a peak at m/z 313, which then either followed by loss of $[OCH_3]^+$ to give m/z 282, or loss of $[CH_3]^+$ to give m/z 298. The UV/PDA spectrum of (−)-colchicine showed two absorption maxima at 244 and 352 nm, typical values for (−)-colchicine, supporting the identity of this compound (O'Neil et al. 2001; Rosso and Zuccaro 1998). This was further verified by comparison of the APCI mass spectrum, UV/PDA spectrum, and HPLC retention time of an authentic standard of (−)-colchicine, where complete matching was observed (Alali et al. 2005).

The (+)-APCI mass spectrum of peaks 2, 5, 3 and 3, $t_R = 12.2$, 12.4, 12.5, and 12.3 min in the corms, stems, leaves and flowers, respectively, showed a parent molecular ion at m/z 386 for $[M + H]^+$, 14 daltons less than the analogous peak in (−)-colchicine. This compound was also eluted at an earlier retention time (i.e. more polar) than (−)-colchicine. The UV/PDA spectrum of the compound showed two absorption maxima at 246 and 354 nm, similar to colchicine-type alkaloids. These data suggested that this compound was either 2-demethyl-(−)-colchicine or 3-demethyl-(−)-colchicine (Chommadov et al. 1990). 3-Demethyl-(−)-colchicine was reported previously from this species (Alali et al. 2005), hence the compound was identified as 3-demethyl-(−)-colchicine (**4**). The (+)-APCI-MS/MS fragmentation spectrum of the

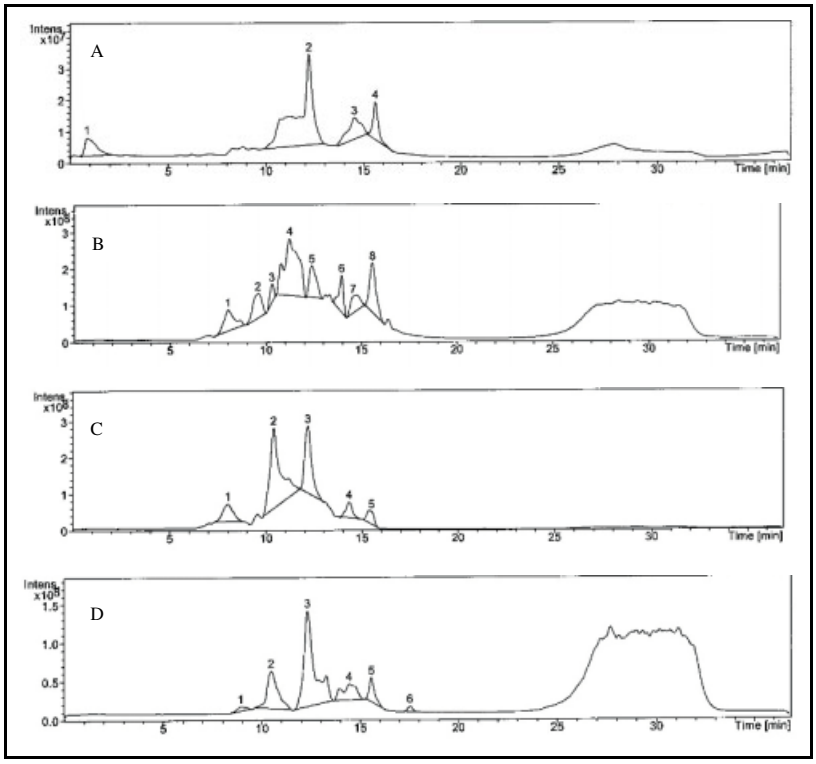


Fig. 1:
(+)-APCI TIC chromatograms of fraction C of the corms (A), stems (B), leaves (C), and flowers (D) of *C. brachyphyllum*

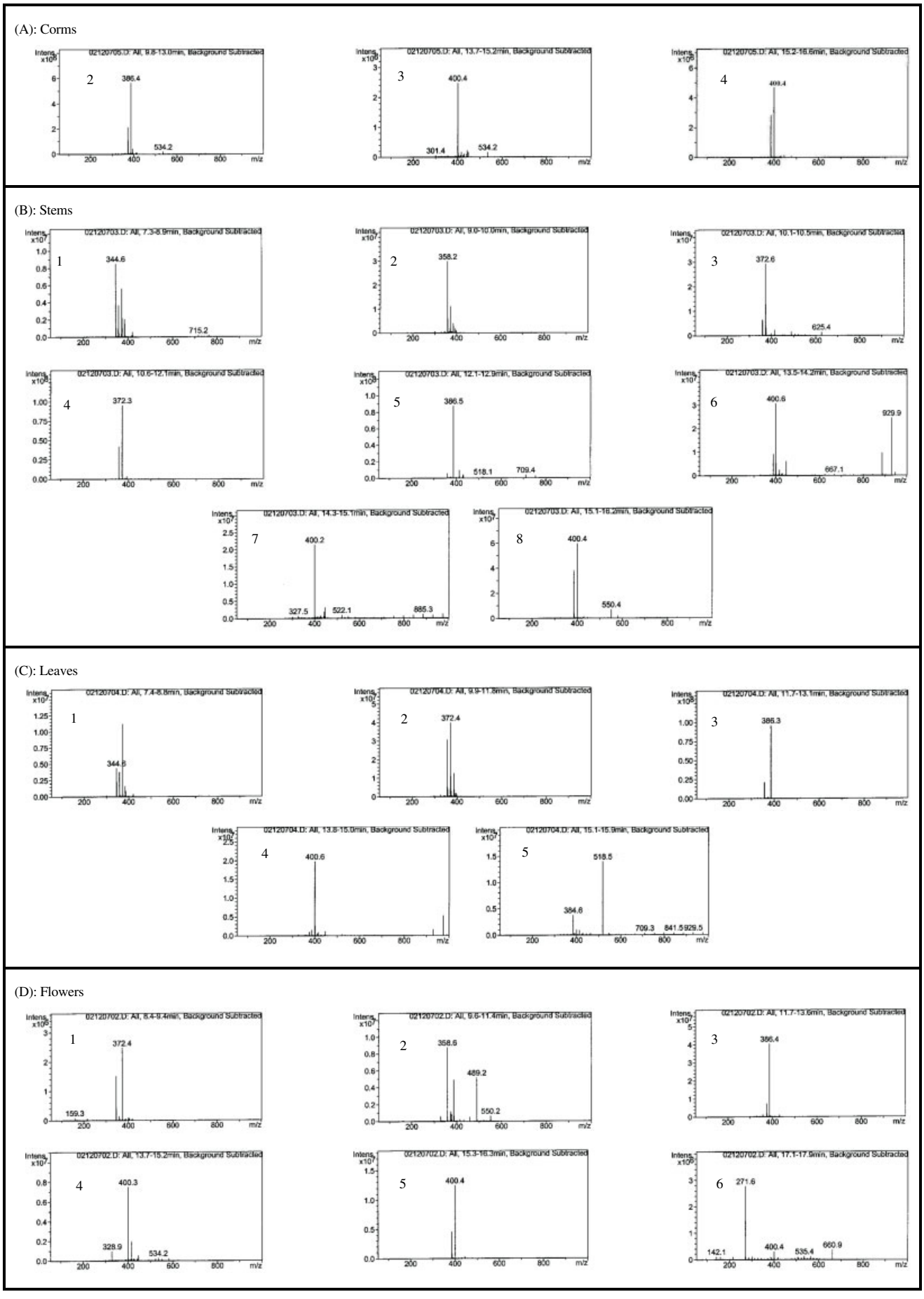


Fig. 2: (+)-APCI Mass spectra of the TIC chromatographic peaks of the (A) corms, (B) stems, (C) leaves, and (D) flowers of *C. brachyphyllum*

molecular ion peak showed a peak at m/z 327 which was attributed to the ion $[MH-NH_2COCH_3]^+$, followed by loss of $[CO]^+$ to give a peak at m/z 299, loss of $[COCH_2]^+$ from the molecular ion peak gives m/z 344.

The (+)-APCI mass spectrum of peaks 4, 8, and 5, $t_R = 15.6$ min in the corms, stems, and flowers, respectively, showed a parent molecular ion at m/z 400 for $[M + H]^+$, which had the same molecular weight as (−)-colchicine. This compound was also eluted at a later retention time (i.e. less polar) than (−)-colchicine. The few and small intensity fragments of this compound were not very informative. However, the UV/PDA spectrum of the compound showed two absorption maxima at 234 and 268 nm, indicating that this compound was a photoisomer of (−)-colchicine. These data suggested that this compound was β-lumi-(−)-colchicine (**6**) (Alali et al. 2005; Chommadov et al. 1990).

The (+)-APCI mass spectrum of peak 1, $t_R = 8.0$ min in stems and leaves, showed a parent molecular ion at m/z 344 for $[M + H]^+$, 28 and 14 daltons less than the analogous peaks in (−)-demecolcine and 3-demethyl-(−)-demecolcine, respectively. This compound was also eluted at an earlier retention time (i.e. more polar) than (−)-demecolcine and 3-demethyl-(−)-demecolcine. The UV/PDA spectrum of the compound showed two absorption maxima at 242 and 357 nm, similar to colchicine-type alkaloids. These data suggested that this compound was 2,3-didemethyl-(−)-demecolcine (**1**), a compound that was isolated previously for the first time from this species in 2005 (Alali et al. 2005). The (+)-APCI-MS/MS fragmentation spectrum of the molecular ion peak showed a peak at m/z 313 which was attributed to the ion $[MH-NH_2CH_3]^+$.

The (+)-APCI mass spectrum of peak 2, $t_R = 9.6$ min in stems showed a parent molecular ion at m/z 358 for $[M + H]^+$, 14 daltons less than the analogous peaks in (−)-demecolcine. This compound was also eluted at an earlier retention time (i.e. more polar) than (−)-demecolcine. These data suggested that this compound was either 2-demethyl-(−)-demecolcine or 3-demethyl-(−)-demecolcine, but since only 3-demethyl-(−)-demecolcine was reported previously from this species, the compound was presumed to be 3-demethyl-(−)-demecolcine (**2**) (Alali et al. 2005). The (+)-APCI-MS/MS fragmentation spectrum of the molecular ion peak showed a peak at *m/z* 327 which was attributed to the ion $[MH-NH_2CH_3]^+$.

The (+)-APCI mass spectrum of peak 3 and 2, $t_R = 10.3$ and 10.4 min in the stems and leaves, respectively, showed a parent molecular ion at m/z 372 for $[M + H]^+$, 28 daltons less than the analogous peak in (−)-colchicine. This compound was also eluted at an earlier retention time (i.e. more polar) than (−)-colchicine. The UV/PDA spectrum of the compound showed two absorption maxima at 242 and 348 nm, implying structural similarities to (−)-colchicine. These data suggested that this compound was (−)-demecolcine (**3**) (Freyer et al. 1987). The (+)-APCI-MS/MS 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.

The (+)-APCI mass spectrum of peak 5, $t_R = 15.5$ min in the leaves showed a parent molecular ion at m/z 384 for $[M + H]^+$, 16 daltons less than the analogous peak in (−)-colchicine. This compound was also eluted at a later retention time (i.e. less polar) than (−)-colchicine. The few and low intensity mass fragments of this compound where not diagnostic. The UV/PDA spectrum of the compound showed two absorption maxima at 244 and 353 nm, implying structural similarities to (−)-colchicine. These data suggested that this compound was (−)-cornigerine (**7**) (Freyer et al. 1987).

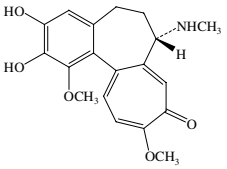
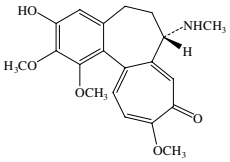
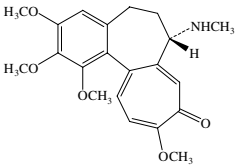
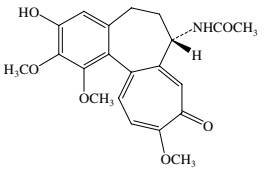
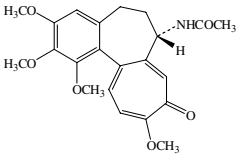
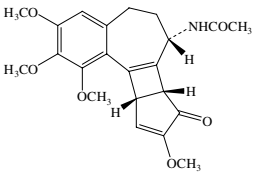
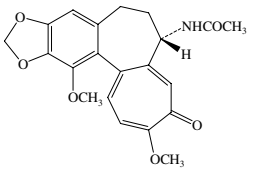
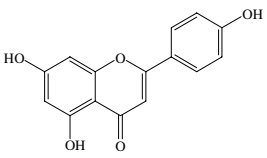
The (+)-APCI mass spectrum of peak 6, $t_R = 17.5$ min in the flowers showed a parent molecular ion at m/z 271 for $[M + H]^+$. The few and low intensity mass fragments of this compound where not diagnostic. The UV/PDA spectrum of the compound showed three absorption maxima at 240, 258 and 344 nm, typical of flavone type flavonoid. These data suggested that this compound was apigenin (**8**) (Schultze and Roth 1993), a compound that was previously isolated from other related *Colchicum* species (Alali et al. 2006), but reported here for the first time from *C. brachyphyllum*.

We were unable to suggest chemical structures for peaks 4 and 6 in the stems which had molecular ions at m/z 372 and 400, respectively for $[M + H]^+$, and for peaks 1 and 2 in the flowers which had molecular ions at m/z at 372 and 358, respectively for $[M + H]^+$. Their retention time and UV/PDA spectra were not conclusive and their spectral data were not matched with neither the compounds that have been isolated previously from this species nor with any other colchicinoid; hence they would be a potential new compounds to pursue.

In the phytochemical study of *C. brachyphyllum* which was carried out by Alali et al. (2005), nine colchicinoids were isolated and characterized, namely (+)-demecolcinone which has a novel ring system, 2,3-didemethyl-(−)-demecolcine (**1**) a new colchicinoid analog; in addition to (−)-colchicine (**5**), 3-demethyl-(−)-colchicine (**4**), (−)-cornigerine (**7**), β-lumi-(−)-colchicine (**6**), (−)-androbiphenyline, (−)-demecolcine (**3**), and 3-demethyl-(−)-demecolcine (**2**) (Alali et al. 2005). Comparing the compounds that were isolated and characterized by the phytochemical study of Alali et al. (2005) with this current LC-MS and LC-PDA dereplication strategy of *C. brachyphyllum*, both study results matched in seven compounds namely: 2,3-didemethyl-(−)-demecolcine (**1**), (−)-colchicine (**5**), 3-demethyl-(−)-colchicine (**4**), (−)-cornigerine (**7**), β-lumi-(−)-colchicine (**6**), (−)-demecolcine (**3**), and 3-demethyl-(−)-demecolcine (**2**). LC-MS and LC-PDA dereplication strategy was not able to detect (−)-androbiphenyline and the compound with the novel ring system (+)-demecolcinone. This finding could raise the question whether (+)-demecolcinone is naturally occurring as it could be an artifact that formed in the column via oxidation from 2,3-didemethyl-(−)-demecolcine (**1**). On the other hand apigenin (**8**) was identified only by this dereplication strategy, in addition to four compounds which were unidentified and thus considered unknown.

These findings clearly demonstrate the promise 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 *C. brachyphyllum* using the bioactivity-guided fractionation approach (Alali et al. 2005), the LC-MS and LC-PDA strategy was able to identify most 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. 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.

Table: Retention times, UVmax (nm), and mass spectral data of the (+)-APCI TIC chromatographic peaks

Compound	Peak number	t_R (min)	UV max (nm)	LC-MS and LC-MS/MS-(+)-APCI mass fragments (m/z)
 2,3-Didemethyl-(−)-demecolcine (**1**)	1[b] 1[c]	8.0[b] 8.0[c]	242, 357	344 $(M + H)^+$ (base peak), 343, 342, 314, 313, 312, 311, 286, 280
 3-Demethyl-(−)-demecolcine (**2**)	2[b]	9.6[b]	242, 352	358 $(M + H)^+$ (base peak), 357, 356, 328, 327, 326, 325, 324, 295
 (−)-Demecolcine (**3**)	3[b] 2[c]	10.3[b] 10.4[c]	242, 348	372 $(M + H)^+$ (base peak), 345, 344, 341, 340, 326, 313, 311, 310, 309, 206, 167
 3-Demethyl-(−)-colchicine (**4**)	2[a] 5[b] 3[c] 3[d]	12.2[a] 12.4[b] 12.5[c] 12.3[d]	246, 354	386 $(M + H)^+$ (base peak), 368, 366, 354, 344, 342, 327, 326, 325, 312, 299, 267, 234, 235, 207
 (−)-Colchicine (**5**)	3[a] 7[b] 4[c] 4[d]	14.1[a] 14.6[b] 14.3[c] 14.4[d]	244, 352	400 $(M + H)^+$ (base peak), 382, 368, 367, 358, 341, 340, 326, 313, 310, 309, 298, 282, 268, 235
 β-Lumi-(−)-colchicine (**6**)	4[a] 8[b] 5[d]	15.6[a] 15.6[b] 15.6[d]	234, 268	400 $(M + H)^+$ (base peak), 388, 387, 386, 385, 376, 375, 374, 372
 (−)-Cornigerine (**7**)	5[c]	15.5[c]	244, 353	384 $(M + H)^+$
 Apigenin (**8**)	6[d]	17.5[d]	240, 258, 344	271 $(M + H)^+$ (base peak)

[a] corms, [b] stems, [c] leaves, [d] flowers

3. Experimental

3.1. Equipment

LC-MS data were determined using an Agilent® (Palo Alto, CA, USA) ion-trap mass spectrometer equipped with APCI positive ionization mode and an Agilent® 100 series HPLC. 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% A; 2–27 min, 10% A; 27–30 min 90% A. The injection volume was 100 μL and the total run time was 30 min. The mass detector conditions were set as follows: APCI positive ionization mode, full scan mode from 50 to 800 m/z, capillary voltage set at – 4500 v, APCI temperature 400 °C, gas flow rate 4 L/min.
LC-UV/Vis PDA spectra were obtained on a Lachrom® MERCK-HITACHI (Tokyo, Japan) HPLC, equipped with quaternary gradient L-7150 pump, L-7455 Diode-Array Detector, L-7200 auto-sampler, and D-7000 Interface. The diode array detector was used to monitor UV signals in the range between 200 and 400 nm. Mobile phase, flow rate, analytical column, injection volume, and run times were the same as those used for LC-MS.

3.2. Reagents

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).

3.3. Plant material

Corms, flowers, leaves, and stems of *C. brachyphyllum* were collected during the flowering stage in February 2003 in the northern part of Jordan from al-Mazzah, al-Mafraq. A voucher specimen (PHC-106) was deposited in the herbarium of the Faculty of Pharmacy, Jordan University of Science and Technology, Irbid, Jordan.

3.4. Plant samples preparation and analysis

The alkaloid rich fraction of the leaves, flowers, stems and corms of *C. brachyphyllum* came from an earlier work (Alali et al. 2005). The fractions 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 mobile phase, filtered through a 0.45 μm Teflon filter, and then transferred to 2 mL amber HPLC vials. A 100 μL aliquot was injected onto the LC-UV/PDA and LC-MS systems. (−)-Colchicine standard was used for retention time matching.

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