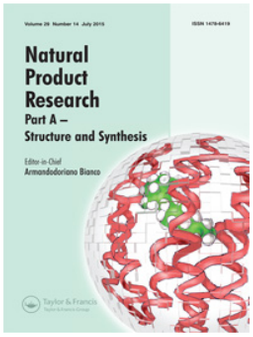

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Metabolites profiling of *Chrysanthemum pacificum* Nakai parts using UPLC-PDA-MS coupled to chemometrics

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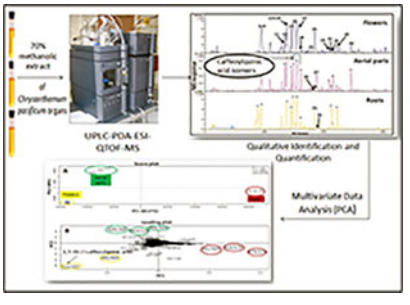

Methanol-soluble constituents from the flowers, non-flowering aerial parts and roots of *Chrysanthemum pacificum* Nakai were analysed *via* high resolution UPLC-PDA-qTOF-MS followed by chemometrics. Forty-seven chromatographic peaks belonging to various metabolite classes were detected. Most metabolite classes showed qualitative and quantitative differences across parts, with luteolin conjugates being mostly enriched in flowers whereas non-flowering aerial parts contained mostly quercetin and methoxylated flavone conjugates. Root sample ranked the lowest for all flavones and dicaffeoylquinic acids. In contrast, 1,5-di-caffeoylquinic acid levels were found at high levels in flowers and aerial parts reaching 3145 and 1390 μg/g, respectively, suggesting that *C. pacificum* could serve as a natural resource of this well-recognised anti-hepatotoxic phenolic. Principal component analysis was further used for organs classification in an untargeted manner. This study provides the first map of secondary metabolites distribution in *C. pacificum* Nakai organs.

Keywords: *Chrysanthemum pacificum*; metabolomics; UPLC/MS; hydroxycinnamic acids; chemometrics

1. Introduction

Chrysanthemum L. (Asteraceae) is a genus comprising 40 species, most of which are distributed in East Asia (Liu et al. 2012). *Chrysanthemum pacificum Nakai* is a herbaceous perennial flowering plant native to Japan (Honshū). The plant is cultivated in Egypt as an ornamental. To date, there has been no systematic detailed characterisation of secondary metabolites profile of *C. pacificum* organs, despite the fact that many *Chrysanthemum* species could present a valuable resource of phenolic compounds, well recognised for their potential hepatoprotective,

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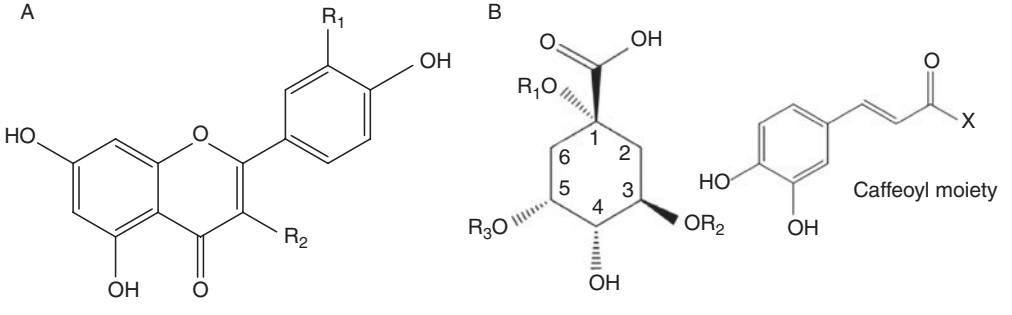
antioxidant, anticancer, phytotoxic and antihypertensive effects (Ukiya et al. 2002; Beninger et al. 2004; Kim & Lee 2005; Clifford et al. 2007; Lai et al. 2007; Wang et al. 2008; Nikolova & Dzhurmanski 2009; Sugawara & Igarashi 2009; Wu et al. 2010). Herein we report on an ultra-high performance liquid chromatography method (UPLC) coupled to photodiode array detector and quadrupole high resolution time of flight mass spectrometry (qTOF-MS) for the metabolites profiling of *C. pacificum* organs; flowers (capitulum inflorescence), aerial parts (leaf, stem) and roots. This study provides the first detailed map for polyphenols and fatty acids composition in *C. pacificum* parts and revealing for the distinct chemical profiles among them.

2. Results and discussion

2.1. *Identification of metabolites in C. pacificum Nakai parts*

To assess for differences in *C. pacificum* metabolite composition, a non-targeted metabolite profiling of extracts prepared from the flowers, aerial parts and roots samples was conducted. Methanol-soluble constituents from various parts were analysed *via* a high resolution UPLC-PDA-qTOF-MS followed by chemometrics. Following the identification strategy, 47 chromatographic peaks were detected (Table 1) belonging to various metabolite classes. Metabolite assignments were made by comparing retention time, UV spectra and MS data (accurate deprotonated mass, isotopic distribution and fragmentation pattern in negative ionisation mode) of the compounds detected with *Chrysanthemum* compounds reported in the literature and searching in the existing phytochemical dictionary of natural products database. Metabolites belonged to various classes including hydroxycinnamic acid conjugates, flavonoid glycosides/methyl ether derivatives and fatty acids (Figure 1).

UPLC-PDA-MS traces of *C. pacificum* flowers (Figure S1) are in general characterised by two main regions: (150–500 s) with peaks principally due to hydroxycinnamic acids and flavonoids and region (500–650 s) for fatty acids. It showed similar MS and PDA chromatograms in the first elution region (100–400 s), whereas for the later eluents (400–800 s), the peaks for fatty acids had a much higher response in MS than in UV. UPLC-MS traces of *C. pacificum* organs are presented (Figure S2) for comparison between them.



Kaempferol	R_1=H, R_2=OH 1,5-di-*O*-Caffeoylquinic acid R_1, R_3=Caffeoyl moiety, R_2=H
Quercetin	R_1=OH, R_2=OH 3,5-di-*O*-Caffeoylquinic acid R_2, R_3=Caffeoyl moiety, R_1=H
Isorhamnetin	R_1=OCH_3, R_2=OH3-*O*-Caffeoyl quinic acid R_2=Caffeoyl moiety, R_1, R_3=H
Luteolin	R_1=OH, R_2=H5-*O*-Caffeoyl quinic acid R_3=Caffeoyl moiety, R_1, R_2=H
Apigenin	R_1,R_2=H
Chrysoeriol	R_1=OCH_3, R_2=H

Figure 1. Major classes of natural products; flavonoids (a) and hydroxycinnamic acids (b) detected in *C. pacificum* and discussed throughout the manuscript.

Table 1. Metabolites detected in *Chrysanthemum pacificum* organs: flowers (F), aerial parts (AP), roots (R) methanol extracts using UPLC-PDA-MS in negative ionisation mode.

Peak no.	Rt (s)	UV	Name	Mol. Ion $[M-H]^-$ /m/z (−)	Error (ppm)	El. Comp.	MSn ions m/z (−)	F.	AP.	R.
1	170	264, 358	Unknown	479.0900	0.3	$C_{21}H_{19}O_{13}^-$	317	+	+	+
2	179	264. 358	Unknown	479.0897	0	$C_{21}H_{19}O_{13}^-$	317	+	+	+
3	188	326	3-*O*-Caffeoyl quinic acid[a]	353.0975	0.8	$C_{16}H_{17}O_9^-$	191	+	+	+
4	193	326	5-*O*-Caffeoyl quinic acid (chlorogenic)[a]	353.0898	0.8	$C_{16}H_{17}H_9^-$	191	+	+	+
5	207	290	Benzyl alcohol – pentosyl hexoside	401.1525	0.3	$C_{18}H_{25}O_{10}^-$	353, 248, 174	+	+	+
6	218	285	Unknown	387.1738	3.1	$C_{18}H_{27}O_9^-$	305	+	+	–
7	220	ND	Sinapyl alcohol hexoside	371.1409	2.8	$C_{17}H_{23}O_9^-$	305, 225	+	–	–
8	224	ND	*p*-Coumaroyl quinic acid	337.1566	1.3	$C_{16}H_{17}H_8^-$	337, 191	+	+	+
9	246	324	Phenyl butanol hexoside	327.1429	6.3	$C_{16}H_{23}O_7^-$	197	+	–	–
10	252	283, 332	Eriodictyol glucuronide	463.0893	2.3	$C_{21}H_{19}O_{12}^-$	287	+	+	–
11	268	253, 345	Rutin	609.1504	3.9	$C_{27}H_{29}O_{16}^-$	301	+	+	–
12	272	255, 347	Luteolin-7-*O*-rutinoside (scolymoside)[a]	593.1558	7.7	$C_{27}H_{29}H_{15}^-$	285, 265	+	–	–
13	275	284, 332	Eriodictyol glucuronide isomer	463.1006	5.4	$C_{21}H_{19}O_{12}^-$	287	+	+	+
14	279	255, 348	Luteolin glucuronide	461.0742	3.6	$C_{21}H_{17}O_{12}^-$	285	+	+	–
15	287	326	3,5-di-*O*-Caffeoylquinic acid[a]	515.1236	3.4	$C_{25}H_{23}O_{12}^-$	369, 353, 191	+	+	+
16	293	328	1,5-di-*O*-Caffeoylquinic acid[a]	515.1195	6.1	$C_{25}H_{23}H_{12}^-$	353, 191, 161	+	+	+
17	309	329	Dicaffeoylquinic acid isomer	515.1223	5.4	$C_{25}H_{23}O_{12}^-$	353, 191	+	+	+
18	323	287, 328	Quercetin dihexosyl glucuronide	801.167	7.6	$C_{33}H_{37}O_{23}^-$	639, 463, 301	+	+	+
19	336	269, 332	Jaceosidin glucuronide	505.1086	4.7	$C_{23}H_{21}O_{13}^-$	329	+	+	+
20	339	327	di-*O*-Caffeoylquinic acid isomer	515.1280	4.3	$C_{25}H_{23}H_1^{2-}$	353, 161	+	+	+
21	343	326	Apigenin hexoside	431.1418	0.5	$C_{21}H_{19}O_{10}^-$	269	+	–	–
22	349	291	Tetrahydroxyflavanone-pentoside	419.1057	0.4	$C_{20}H_{19}O_{10}^-$	353, 295	+	+	+
23	352	ND	Quercetin caffeoyl hexoside	625.1214	0.3	$C_{30}H_{25}O_{15}^-$	463, 301	–	+	+
24	358	289	Tetrahydroxyflavanone	287.0560	0.3	$C_{15}H_{11}O_6^-$	151	+	+	+
25	368	255, 365	Quercetin	301.0424	1	$C_{15}H_9O_7^-$		+	+	+
26	369	266, 347	Luteolin[a]	285.0409	1.7	$C_{15}H_9O_6^-$	175	+	+	+
27	375	273, 334	Methoxyluteolin	315.0503	2.4	$C_{16}H_{11}O_7^-$	300, 285	–	+	–
28	386	296, 353	Methoxyluteolin isomer	315.0578	5.4	$C_{16}H_{11}O_7^-$	300, 285	+	+	+
29	393	342	Tetrahydroxy-dimethoxyflavone	345.0672	3.9	$C_{17}H_{13}O_8^-$	327	+	+	–
30	400	296	Unknown	585.2032	0.8	$C_{23}H_{37}O_{17}^-$	487	+	+	+
31	409	ND	Apigenin[a]	269.0508	0.3	$C_{15}H_9O_5^-$		+	+	+
32	416	ND	Kaempferol	285.0460	2.3	$C_{15}H_9O_6^-$	174	+	+	+
33	417	ND	Trihydroxy-octadecadienoic acid	327.2238	3.5	$C_{18}H_{31}O_5^-$	299	+	+	+

34	418.9	273, 334	Chrysoeriol	299.0621	3.8	$C_{16}H_{11}O_6^-$		+	+	−
35	426	254, 371	Isorhamnetin	315.0577	2.2	$C_{16}H_{11}O_7^-$	301, 174	+	+	+
36	431.2	273, 345	Jaceosidin	329.0730	1	$C_{17}H_{13}O_7^-$	314	+	+	−
37	432	ND	Unknown	785.3660	2.5	$C_{45}H_{53}O_{12}^-$	523, 329	+	−	−
38	444	ND	Trihydroxy-octadecenoic acid	329.2395	0	$C_{18}H_{33}O_5^-$		+	+	+
39	445	345	Jaceosidin	329.0728	1.5	$C_{17}H_{13}O_7^-$	314	+	+	+
40	454	345	Trihydroxy-trimethoxyflavone	359.0761	2.8	$C_{18}H_{15}O_8^-$	344	+	+	−
41	469	ND	Unknown	567.2328	0.2	$C_{42}H_{31}O_2^-$	429, 248, 174	+	−	−
42	491	273, 342	Dihydroxy-trimethoxyflavone	343.0815	0.6	$C_{18}H_{15}O_7^-$	328	+	+	−
43	504	ND	Dihydroxy-trimethoxyflavone	343.0878	2.5	$C_{18}H_{15}O_7^-$	328	+	+	−
44	569	ND	Unkown	505.237	0.6	$C_{23}H_{37}O_{12}^-$	279, 180	+	+	−
45	591	ND	Unkown	667.2858	1.5	$C_{36}H_{43}O_{12}^-$	316, 248, 174	+	−	−
46	606	ND	Unkown	519.2457	2	$C_{24}H_{39}O_{12}^-$	311, 180	+	+	−
47	653	ND	Hydroxy-octadecadienoic acid	295.2262	4.7	$C_{18}H_{31}O_3^-$	248, 174	+	−	−

Note: (+) and (−) indicate presence and absence of a metabolite, respectively; ND, not detected; Rt, retention time; El. Comp., Elemental composition.
[a]Denotes metabolites confirmed by comparison with reference standards.

2.1.1. *Identification of flavonoids*

UV spectra interpretation (200–600 nm) allowed for identification of different flavonoid subclasses including flavonols (peaks 11, 18, 23, 25, 32 and 35), flavones (peaks 12, 14, 19, 21, 26–29, 31, 34, 36, 39, 40, 42 and 43) and flavanones (peaks 10, 13, 22 and 24). Eriodictyol glucuronide (flavanone derivative) being abundant among the three organs and quercetin conjugates most abundant in the aerial parts. A flavone conjugate of luteolin was detected as the major glycoside in flowers and this is in agreement with *C. morifolium* (Sugawara & Igarashi 2009; Sun et al. 2010). In MS/MS analysis, identification of sugar type in glycosides was established from the mass difference between the glycoside and the aglycone i.e., 162 amu for hexose or 132 amu for pentose. In detail, MS interpretation allowed for the detection of luteolin (*m/z* 285.0409, $C_{15}H_9O_6^-$), as aglycone in peaks 12, 14 and 26–28, apigenin signals (*m/z* 269.0508, 16 amu lower than luteolin, $C_{15}H_9O_5^-$) in peaks 21 and 31, jaceosidin signals (*m/z* 329.073, $C_{17}H_{13}O_7^-$) in peaks 19, 36 and 39 with a main fragment ion at *m/z* 314 derived from the loss of a methyl from its methoxy group (− 15 Da). Chrysoeriol presented another methylated derivative of luteolin (*m/z* 299.0621, $C_{16}H_{11}O_6^-$) found in peak 34. Similarly, in flavonols, MS interpretation allowed for the detection of quercetin signals (*m/z* 301.0424, $C_{15}H_9O_7^-$) in peaks 11, 18, 23 and 25, kaempferol signals (*m/z* 285.046, 16 amu lower than quercetin, $C_{15}H_9O_6^-$) in peak 32, and isorhamnetin signals (*m/z* 315.0577, $C_{16}H_{11}O_7^-$) in peak 35. Two flavanone peaks 10 and 13 $[M - H]^-$ ion at *m/z* 463.1006 $[M - H - 176]^-$ were identified showing a fragment at (*m/z* 287) derived from the loss of a glucuronide moiety, and tentatively identified as eriodictyol glucuronide and its isomer, respectively. Other glucuronide-containing flavonoid peaks showing similar mass loss of 176 amu include peak 14, $[M - H]^-$ *m/z* 461.0742 and peak 18, $[M - H]^-$ *m/z* 801.167, identified as luteolin glucuronide and quercetin dihexosyl glucuronide, respectively. Peak 23 showed $[M - H]^-$ ion at *m/z* 625.1214 and produced fragment ions at 301 due to a loss of a caffeoyl hexose moiety tentatively identified as quercetin caffeoyl hexoside. Several methoxylated flavone peaks 27–29, 40, 42 and 43, showing a λ_{max} at 345 nm typical for flavones and exhibiting the loss of 15 amu from the methoxy group in its tandem MS spectrum, were found in the aerial parts and flowers, which is in agreement with other *Chrysanthemum* species (Lin & Harnly 2010; Wang et al. 2010; Uehara et al. 2012).

2.1.2. *Identification of phenolic acids (hydroxycinnamates)*

Conjugates formed from the reaction of hydroxycinnamic acids with quinic acid are of common occurrence in different *Chrysanthemum* species (Clifford et al. 2007). Several of the hydroxycinnamoyl quinic acid conjugates, such as caffeoyl- and *p*-coumaroyl quinic acids were identified in this study, in addition to di-*O*-caffeoylquinic acids. The predominant fragment of 191 amu for quinic acid in the MS and characteristic UV_{max} at (325–330) nm are diagnostic for hydroxycinnamic acid conjugates. Peaks 3, 4, 15 and 16 exhibited UV spectra comparable to caffeoylquinic acids with high resolution masses of 353.0898 for 3 and 4 and *m/z* 515.1236 for 15 and 16 identified as positional isomers of 3-*O*-caffeoylquinic acid (3); 5-*O*-caffeoylquinic acid (chlorogenic acid, 4); 3,5-di-*O*-caffeoylquinic acid (15) and 1,5-di-*O*-caffeoylquinic acid (cynarin, 16).

Chlorogenic acid and cynarin are well recognised for their hepato-protective, choleretic and antimicrobial activities in artichoke (Zhu et al. 2004; Matuschowski et al. 2005), whether such an effect is present in *C. pacificum* has yet to be examined.

2.1.3. *Identification of fatty acids*

The negative ion MS revealed for several hydroxy fatty acids in peaks 33, 38 and 47 displaying a molecular ion of 327.2238, 329.2395 and 295.2262 amu, with predicted molecular formulas of $C_{18}H_{31}O_5^-$, $C_{18}H_{33}O_5^-$ and $C_{18}H_{31}O_3^-$, respectively. A mass difference of 2 amu between peaks

33 and 38 is indicative of an extra double bond and was tentatively identified as trihydroxyoctadecadienoic, trihydroxyoctadecenoic and hydroxyoctadecadienoic acids. In this MS study, it was not possible to provide an unambiguous structural assignment of the hydroxylated fatty acid isomers with respect to the location of the functional groups or the *E/Z* geometry of double bonds. The detailed analysis and full structural elucidation to determine position of hydroxylation in oxygenated fatty acids is still under way.

2.2. *Quantification of metabolites*

The data collected from UPLC-PDA analysis were also used to determine the absolute amounts of major metabolites in different parts (Table S1 & Figure S3)

Metabolites showing differential accumulation among parts were subjected to absolute quantification using standards. A total of 13 phenolics were quantified including flavone conjugates (luteolin), flavonols (quercetin/isorhamnetin) and hydroxycinnamic acids. Flavones were detected mostly in flowers, whereas flavonols were major peaks in roots. 1,5-Dicaffeoylquinic acid levels were found at high levels reaching 3145 and 1390 μg/g in flowers and aerial parts, respectively, suggesting that *C. pacificum* could serve as another natural resource of this well-recognised antihepatotoxic phenolic.

2.3. *Multivariate principal component analysis of UPLC/MS data*

Principal component analysis (PCA) was used to explore the relative variability within the different samples of UPLC-MS derived data-set in a more holistic way. The PC1/PC2 scores plot (Figure S4A) shows that three major distinct clusters are formed, corresponding to the three organs studied. On the right side of the plot, root samples are positioned (positive PC1 values), whereas on the far left side, flower samples are located (negative PC1 values), while the aerial parts are spread in between. Tight intragroup clustering for each organ samples indicates good overall method reproducibility. The separation observed in PCA can be explained in terms of the identified compounds, using the loading plots for PC1, exposing the most discriminatory signals. MS signals for luteolin-7-*O*-β-rutinoside (12), luteolin-7-*O*-glucuronide (14), 1,5-di-*O*-caffeoylquinic acid conjugates (16) and luteolin (26) contributed negatively to PC1 and were found more enriched in flowers (Figure S4B). In contrast, MS signals for two flavonols aglycones namely isorhamnetin (35), quercetin (25) and methoxylated luteolin (jaceosidin, 39) showed a positive effect on PC1 and were found more enriched in root samples (Figure S4B). Further examination of PC2 loadings plot revealed that mass variables assigned for quercetin glycosidic conjugates (18 and 23), methylated and methoxylated flavones (27, 34, 36 and 42) contributed the most to the discrimination of aerial parts from flowers and roots samples.

3. Experimental

3.1. *Plant material*

Samples of the flowers, aerial parts and roots were collected during 2012–2013 from the plants cultivated in the Experimental Station of Medicinal plants, Pharmacognosy Department, Faculty of Pharmacy, Cairo University. The plant was kindly identified by Mrs Therease Labib, taxonomy specialist. A voucher specimen (27.2.2014) was deposited at the herbarium of Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

3.2. *Chemicals and reagents*

Acetonitrile and formic acid (LCMS grade) were obtained from J.T. Baker (Deventer, The Netherlands), milli Q water was used for LC analysis. Luteolin-7-*O*-rutinoside, luteolin (≥98%)

and apigenin (≥99%) were all purchased from Chromadex (Wesel, Germany). 1, 3O and 1,5-*O*-dicaffeoylquinic acids were purchased from Phytolab (Vestenbergsgreuth, Germany). Umbelliferone (≥98%), chlorogenic acid (≥95%) and linolenic acid (≥97%) were obtained from Sigma Aldrich (St. Louis, MO, USA).

3.3. *Plant extraction procedures*

Dried plant organs were ground with a pestle in a mortar using liquid nitrogen. The powder (30 mg) was then homogenised with 2.5 mL 70% MeOH containing 5 μg/mL umbelliferone (an internal standard for relative quantification using a Turrax mixer (11,000 RPM) for five 20 s periods. To prevent heating, a period of 1 min separated each mixing period. Extracts were then vortexed vigorously and centrifuged at 3000 *g* for 30 min to remove plant debris. For UPLC-MS analyses, 500 μL was aliquoted and placed on a (500 mg) C18 cartridge preconditioned with methanol and water. Samples were then eluted using 3 mL of 70% MeOH and 3 mL of 100% MeOH, the eluent was evaporated under a nitrogen stream and the obtained dry residue was re-suspended in 500 μL methanol. Three microlitres were used for analysis.

3.4. *High resolution UPLC-MS analysis*

Chromatographic separation was performed on an Acquity UPLC system (Waters) equipped with a HSS T3 column (100 × 1.0 mm, particle size 1.8 μm; Waters). The analysis was carried out by applying the following binary gradient at a flow rate of 150 μL min^{-1}: 0 to 1 min, isocratic 95% A (water/formic acid, 99.9/0.1 [v/v]), 5% B (acetonitrile/formic acid, 99.9/0.1 [v/v]); 1 to 16 min, linear from 5 to 95% B; 16 to 18 min, isocratic 95% B; 18 to 20 min, isocratic 5% B. The injection volume was 3.1 μL (full loop injection). Eluted compounds were detected from *m*/*z* 100 to 1000 in negative ion mode using the following instrument settings: nebuliser gas, nitrogen, 1.6 bar; dry gas, nitrogen, 6 L min^{-1}, 190°C; capillary, −5500 V; in-source CID energy, 0 V; hexapole RF, 100 Vpp; quadrupole ion energy, 5 eV; collision gas, argon; collision energy, 10 eV; collision RF 200/400 Vpp (timing 50/50); transfer time, 70 μs; prepulse storage, 5 μs; pulser frequency, 10 kHz; spectra rate, 3 Hz. Internal mass calibration of each analysis was performed by infusion of 20 μL 10 mM lithium formate in isopropanol: water, 1:1 (v/v), at a gradient time of 18 min using a diverter valve.

3.5. *UPLC-MS data processing and multivariate analysis*

Relative quantification of metabolite profiles after UPLC-MS was performed using XCMS data analysis software, which can be downloaded freely as an R package from the Metlin Metabolite (Smith et al. 2006; Farag et al. 2013).

4. Conclusion

This study is the first report that shed light on metabolites profiling in *C. pacificum* Nakai on an organ basis using UPLC-MS. Hydroxycinnamic acid derivatives present the major class found in flowers, whereas flavonoids are the most abundant class in root and aerial parts. The metabolic profiling results showed that there is certain analogy of *C. pacificum* constituents and artichoke, later is considered an important medicinal herb for treatment of liver disorders. Further studies are required to evaluate *C. pacificum* antihepatotoxic effect.

Supplemental material

Supplementary material is available online, alongside Figures S1–S4 and Table S1.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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