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Research Article

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Characterization of Triterpenoid Saponins, Phenylethanoids and Flavonoids in *Bacopa Caroliniana* (Walt.) B.L. Robins Using LC-ESI-QTOF-MS/MS Technique

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Abstract

This research reports upon the chemical constituent of the aerial part of *Bacopa caroliniana* identified using the liquid chromatography with electrospray ionization/mass spectrometry (LC-ESI-QTOF-MS/MS) technique. The LC separation along with high resolution mass spectrometry data revealed significant differences in phytochemicals of *B. caroliniana*. The mass spectral fragmentation patterns in both positive and negative modes indicated the numbers, positions, and linkages of sugar moieties in the saponin structures. The distinct *m/z* 455 $[Aglycone + H\text{-}H_2O]^+$ from in source fragmentation suggested that the triterpenoid saponins in this plant are jujubogenin type whereas *m/z* 473 $[Aglycone + H]^+$ are pseudojujubogenin type. This fragmentation pattern led to identification of bacoside A_3 (1), bacopaside X (2) and their derivatives (3-6). The four derivative compounds (3-6) are differed in the glycosidic part with the malonyl moiety conjugated to the hexose or/and pentose moieties. Additionally, four phenylethanoid glycosides e.g.acteoside (I), acetylacteoside (III) and two of their isomeric forms (II, IV) were identified. Nine flavonoids (a-i), two flavone and seven *O*-methoxy flavonoids were also reported. All the identified compounds were reported for the first time in *B. caroliniana*. This chemical diversity can be very important as supporting data for further uses in pharmaceutical and food industries.

Keywords

Bacopa caroliniana, LC-ESI-QTOF-MS/MS, Triterpenoid saponins, Phenylethanoid glycosides, Flavonoids, Structure elucidation

Introduction

Bacopa caroliniana (Walt.) B.L. Robins or Lemon bacopa (Plantaginaceae) is a perennial aquarium plant. The leaves of this plant are succulent and release smell like the lemon-mint when disturbed and the flowers are bright blue [1]. In Thailand, there are three species of *Bacopa* genus, i.e. *B. monnieri*, *B. caroliniana* and *B. floribunda* [2]. *B. monnieri* (Brahmi) has traditionally been reported as a herbal medicine in Ayurvedic medicine for memory improvement [3]. The compounds responsible for memory enhancing effects of Brahmi are triterpenoid saponins i.e. bacoside A_3, bacopaside I, bacopaside II, bacopasaponin C and bacopaside X [4,5]. The investigation of triterpenoid saponins has drastically increased due to their diverse and potentially attractive biological activities. *B. caroliniana*, one of bacopa species, might be a source of saponin glycosides in the same fashion as *B. monnieri*. In addition, *B. caroliniana* possessed significant activity against microorganisms, especially *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* [6]. The eighteen volatile compounds from *B. caroliniana* have been identified and tested on the inhibition of acetylcholinesterase and insecticidal activities. It was found that α-terpinolene exhibited the highest activities [7]. In the recent report, methanolic extractfrom aerial part of *B. caroliniana* showed moderated anti-lipid peroxidation activity [8].

Previously, we have reported a great number of saponins found in *B. monnieri* [8,9]. In this study, we aim to search for

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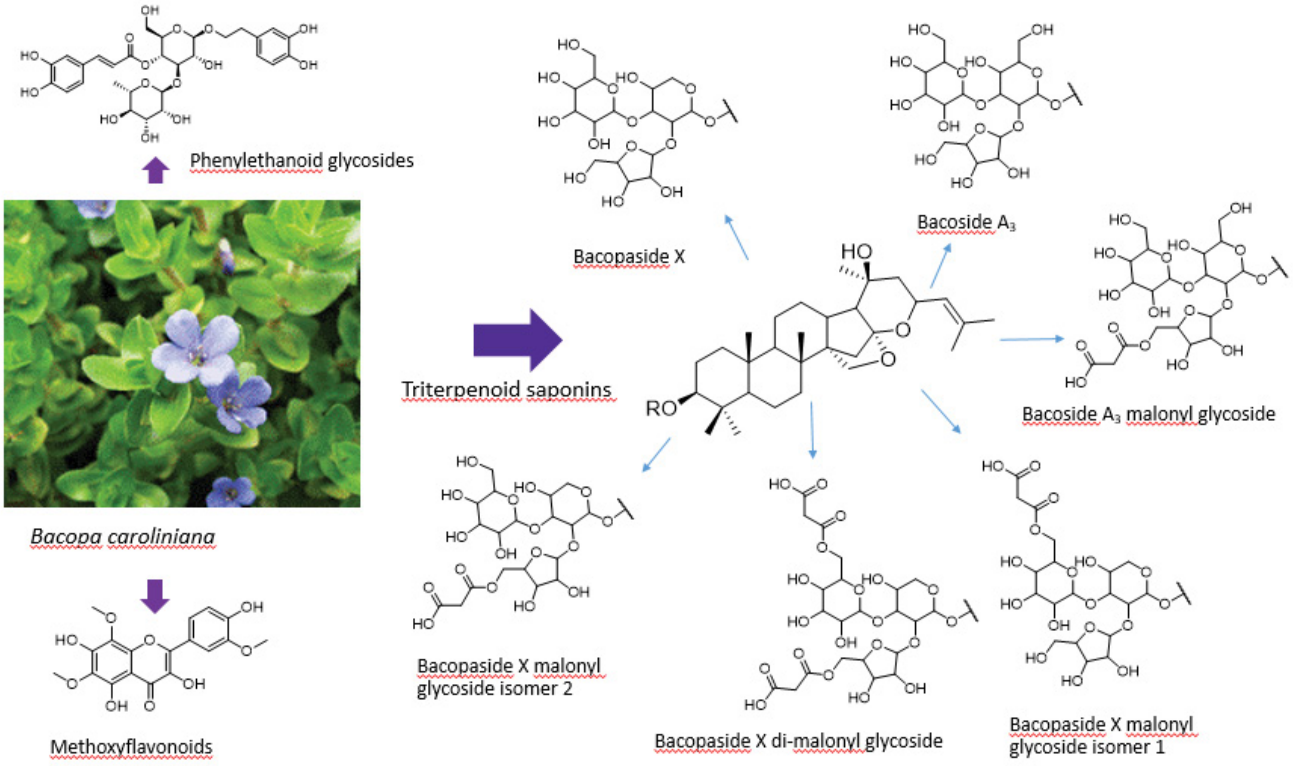


Graph 1: Chemical constituents in *Bacopa caroliniana* elucidated using LC-ESI-QTOF/MS.

new saponins and other compounds in *B. carolianiana* using LC-ESI-QTOF-MS/MS method. This method is a powerful tool for the analysis of complex mixtures, especially those for crude extracts of natural products. A method is using simultaneous acquisition of exact masses at high and low collision energies, to obtain fragmentation information allowing the elucidation of elemental composition and structural identification of compounds. The results of this study for finding new saponin and other chemical constituents could provide new opportunities to promote this plant which possesses high amounts of bioactive compounds in the pharmaceutical and food industries.

Experimental Section

Chemicals and plant materials

Acetonitrile (ACN) and methanol (MeOH) with LC-MS grade were purchased from Lab scan (RCI Labscan, Thailand). Formic acid was bought from Merck (Darmstadt, Germany). Water type I was purified by a Milli-Q purification system from Millipore (Bedford, MA, USA).

The authentic compounds: Bacoside A_3 and bacopaside X were purchased from Natural Remedies Pvt. Ltd. (Bangalore, India). Luteolin and apigenin were obtained from Chromadex (CA, USA).

B. caroliniana was planted in January - July 2017 at Faculty of Pharmaceutical Sciences, Naresuan University. This plant was identified by Assist. Prof. Dr. Pranee Nangngam and its voucher specimen (Saesong013) have been deposited at Department of Biology, Faculty of Science, Naresuan University. The aerial part (10 cm from the top) of the sample was collected based on a previous method [10]. The plant was cleaned, and oven dried at 50 °C for 24 h. After that, the plants were ground and passed through a 60 mesh sieve and stored in airtight plastic containers under refrigeration at -20 °C for later use.

Sample preparation

A 20 mg pulverized sample was added to a plastic tube and was then extracted with 2.0 mL of 70% (v/v) methanol/water for 20 sec on a Vortex Genie 2 mixer (Scientific Industries, New York, USA) and followed by 15 min extraction in an ultrasonic bath (Grant MXB14, Keison Products, UK). Prior to LC-MS analysis, the extract was filtered through a Nylon 0.22 µm, 13 mm syringe filter (ANPEL Laboratory Technologies, Shanghai Inc.).

LC-ESI-QTOF-MS/MS analysis

Chromatographic experiments were performed with an Agilent 6540 Q-TOF-MS spectrometer (Agilent Technologies, Singapore) coupled with an Agilent 1260 Infinity Series High performance liquid chromatography (HPLC) system (Agilent, Waldbronn, Germany). The separation was performed with the ZORBAX Eclipse plus C18, 4.6 mm × 100 mm, 3.5 µm column (Agilent Technologies, USA) at a flow rate of 600 µL/min and the column temperature was set to 35 °C. The mobile phase A was water type I and B was acetonitrile. Both phases contained 0.1 % (v/v) formic acid. The gradient mode started with 35% solvent B for 0-8 min, 35-38% B for 8-10 min, 38-50% B for 10-14 min, 50-80% B for 14-25 min and post-run for 5 min. The injection volume was 20 µL. The operating pa-

rameters for MS detection were as follows: drying gas (N_2) flow rate 10.0 L/min; temperature 350 °C; nebulizer pressure 30 psig; capillary 3500 V; skimmer 65 V; octapole RFV 750 V; and fragmentor voltage 250 V in negative mode and 100 V in positive mode. The mass range was set at *m/z* 100-1200 with a 250 ms/spectrum. The non-target MS/MS mode was set up at three collision energies of 10, 20, and 40 V using high purity nitrogen gas. All acquisition and analysis of the data were controlled by MassHunter Data Acquisition Software version B.05.01 and MassHunter Qualitative Analysis Software B 06.0

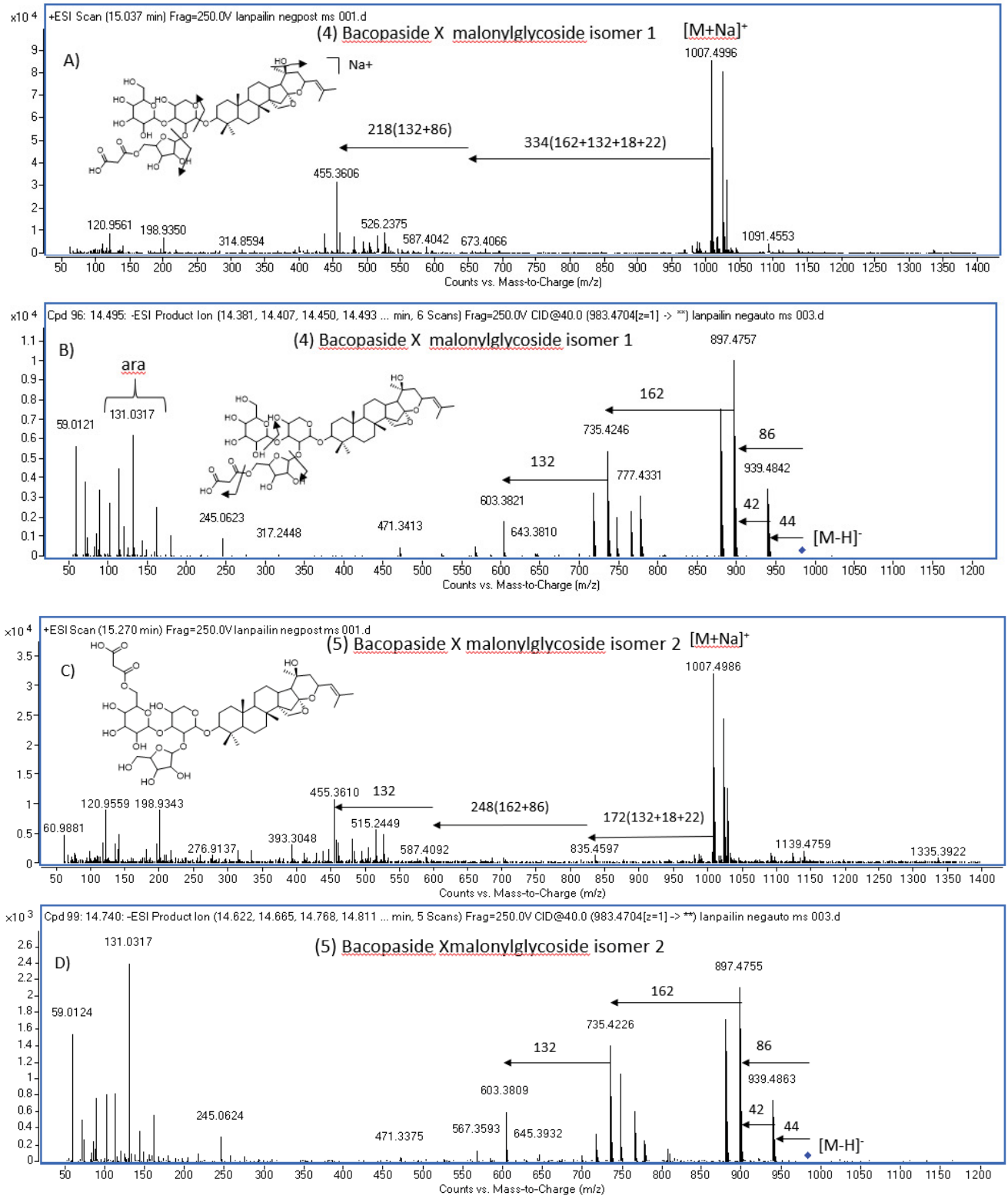


Figure 1: Representative fragmentations of bacopaside X malonyl glycosides,i.e. compound **4** in positive mode (A) and negative mode (B) and compound **5** in positive mode (C) and negative mode (D).

respectively (Agilent Technologies, USA). Analysis of each sample was performed both in positive and negative ionization modes including non-targeted MS/MS mode to provide abundant information for structural identification.

Results and Discussion

Structure elucidation of triterpenoid saponins

HPLC coupled to ESI-QTOF-MS/MS (tandem mass spectrometry) was applied to separate, characterize and identify triterpenoid saponins (**1**-**8**) in *B. caroliniana* methanolic extract. The previous work indicated that jujubogenin glycosides and pseudojujubogenin glycosides commonly found in *Bacopa* species can be discriminated by the characteristic of in source fragmentation at *m/z* 455 [aglycone + H-H_2O]$^+$ of jujubogenin glycosides and *m/z* 473 [aglycone + H]$^+$ of pseudojujubogenin glycosides [9]. The ESI-QTOF-MS/MS data of *B. caroliniana* shown at *m/z* 455 [aglycone + H-H_2O]$^+$ in compounds **1**- **6** proposed the presence of jujubogenin type in this plant.

Furthermore, in negative ion mode of MS/MS fragmentation, saponins under went glycosidic cleavages using high energy (CE 40 V) resulting in sequential loss of sugar moieties leaving one sugar unit attached to aglycone with the lowest *m/z* peak. The sugar attached at C3 position would be glucose if the typical of *m/z* 633; 471 + 162; [aglycone + glucose]$^-$ was observed or arabinose if *m/z* 603; 471+132; [aglycone + arabinose]$^-$ was present. The neutral loss of sugar; glucose (m/z162) and arabinose/xylose (m/z 132) can be seen in the hydration form, loss of 18 Da (-H_2O) due to the conjugation process. The aglycone-sugar linkage of glucose could be confirmed by *m/z* 161,113,101, while that of arabinose could be observed at *m/z* 131, which were highly abundant in the left side of the mass spectrum (Figure 1B and Figure 1D). So clearly define that the sugar moiety link to aglycone is arabinose. Consequently, identification of saponins in *Bacopa* species based on both positive and negative characteristic fragmentation patterns were successfully conducted (Table 1).

Compounds **1** and **2** were elucidated using the above-mentioned characteristic fragmentations and confirmed with the reference standards as bacoside A_3 and bacopaside X, respectively. These two compounds have been found in *B. monnieri* [9].

Compound **3** showed *m/z* 1037.5103[M + Na]$^+$, which gained 86 Da higher than that of bacoside A_3. Its fragmentation in positive mode showed loss of 248Da (162 + 86: Glucosyl + malonyl). It is likely that this malonyl group is attached at the 6-position of the glucosyl moiety. In negative mode, the loss of 44Da (CO_2), 42Da (CH_2=CO) and 60Da (CH_3COOH) represented the typical loss of malonyl saponin [11]. This compound was, therefore, proposed to be bacoside A_3 malonylglycoside.

Compounds **4** and **5** with the same *m/z* 983.4861 [M-H]$^-$ and 1007.4996 [M + Na]$^+$ in negative mode showed the loss of 44 and 42 Da implying the presence of a malonyl moiety in their structures. In positive mode, the sequential loss of 86 Da in compound **4** occurred together with the loss of xylosyl (218Da = 132 + 86), where as compound **5** malonyl group was lost together with glucosyl moiety (248Da = 162 + 86). These two compounds are tentatively identified as bacopaside X malonyl glycoside isomer 1 (jujubogenin 3-*O*-(5-*O*-mal-

Table 1: LC (+/-) ESI-QTOF-MS/MS data for structural elucidation of triterpenoid saponins in *B. caroliniana.*

Peak	t_R (min)	Measured mass	MS/MS	Molecular formula	Error (ppm)	Identified compound
1	12.11	951.5094 [M + Na]$^+$	617.41,455.36	$C_{47}H_{76}O_{18}$	-17.90	Bacoside A_3
2	12.58	943.4914 [M + HCOO]$^-$	897.47,735.42,603.37	$C_{46}H_{74}O_{17}$	-0.63	Bacopaside X
		921.5091 [M + Na]$^+$	749.46,587.41,455.36			
3	13.01	1013.496 [M - H]$^-$	969.49,927.48,765.42, 633.38	$C_{50}H_{78}O_{21}$	0.28	Bacoside A_3 -malonylglycoside
		1037.5103 [M + Na]$^+$	921.49,789.45,541.24, 455.35		-16.90	
4	14.54	983.4861 [M - H]$^-$	939.48,897.47,735.42, 603.37	$C_{49}H_{76}O_{20}$	-0.39	Bacopaside X malonylglycoside isomer 1
		1007.4996 [M + Na]$^+$	673.40,455.36		-17.20	
5	14.82	983.4866 [M - H]$^-$	939.48,897.47,735.42,603.38	$C_{49}H_{76}O_{20}$	-0.90	Bacopaside X malonylglycoside isomer 2
		1007.4990 [M + Na]$^+$	835.46,587.40,455.36		-16.70	
6	15.85	1069.4865 [M - H]$^-$	981.49,939.48,879.45,735.42, 603.37	$C_{52}H_{78}O_{23}$	-0.36	Bacopaside X dimalonyl glycoside
		1093.5012 [M + Na]$^+$	455.36		-17.00	
7	19.44	487.344 [M - H]$^-$	469.33.443.35	$C_{30}H_{48}O_5$	-2.26	Centellasapogenol A
		511.3487 [M + Na]$^+$	467.35		-18.20	
8	20.51	487.3438 [M - H]$^-$	469.32,425.33	$C_{30}H_{48}O_5$	-1.85	Centellasapogenol A
		511.3468 [M + Na]$^+$	467.35		-14.50	

onyl)-α-L-arabinofuranosyl (1-2)-[*β*-D-glucopyranosyl(1-3)]-α-L-arabinopyranoside) (**4**) and bacopaside X malonyl glycoside isomer 2 (jujubogenin 3-*O*-α-L-arabinofuranosyl (1-2)-[*β*-D-(6-*O*-malonyl)-glucopyranosyl (1-3)]-α-L-arabinopyranoside) (**5**). The difference of mass fragmentation can be seen in (Figure 1). Compound **4** has been previously isolated from ethanol extraction of the stems of *Colubrina retusa* [12].

Compound **6** with *m/z* 1069.4865 $[M-H]^-$and 1093.5012 $[M + Na]^+$ showed 86 Da higher than compounds **4**,**5** and the neutral loss of 88Da (2-CO_2) and 42 Da (CH_2 = CO). The 42 Da might be 2 groups of malonyl that conjugated with glucosyl and xylosyl moieties. Thus, this compound was proposed as bacopaside X di-malonylglycoside.

The other two triterpenoid saponins eluted at the retention time 19.4 and 20.5 min (**7** and **8**) with the same *m/z* 487.34 $[M-H]^-$ and 511.34 $[M + Na]^+$ are tentatively identified as centellasapogenol A and its isomer by comparison with the literature [13]. Without the standard or NMR data, it is still inconclusive whether 7 or 8 is centellasapogenol A.

The MS data of triterpenoid saponins are illustrated in Table 1. The total ion chromatogram (TIC) of *B. caroliniana* is presented in Figure 2.

Structure elucidation of phenylethanoid glycosides

Compounds **I** and **II** eluted at the retention time of 1.69 and 2.11 min with the *m/z* 623 $[M-H]^-$, and 647 $[M + Na]^+$ showed the similar fragmentation patterns, which indicated that they might be isomers (Table 2). In the same fashion, compounds **III** and **IV** at 2.25 and 2.42 min were isomers with the *m/z* of 665 $[M-H]^-$ and 689 $[M + Na]^+$. By comparison of MS data with the previous reports, compounds **I**-**IV** can be proposed as phenylethanoid glycoside, in which **I** was acteoside and **II**-**IV** were its derivatives. Acteoside (**I**) (*m/z* 623.1971) produced the fragment at *m/z* 461 by the loss of the caffeic acid (162Da) moiety, and then generated an ion at *m/z* 315 by a further loss of rhamnose (146Da). The dominant fragment at *m/z* 161 originated from caffeic acid with the loss of water molecule. Isoacteoside (**II**) showed the same *m/z* and fragmentation pattern as acteoside but eluted with a longer retention time. The other two compounds (**III** and **IV**) with the *m/z* 665.2100 $[M-H]^-$, 689.2176 $[M + Na]^+$ which were 42Da greater than that of acteoside, were tentatively identified as acetylacteoside and acetylisoacteoside [14]. These compounds were previously found in Cistanches Herba, a famous traditional Chinese medicine from the dried succulent stems of *Cistanche deserticola* Y. C. Ma and *Cistanche tubulosa* (Schrenk) Wight. Both herb have been used for the treatment of kidney deficiency, impotence, female infertility, morbid leucorrhea, profuse metrorrhagia, and senile constipation [14,15]. Phenylethanoid glycosides found in *B. coroliniana* contain one more rhamnose moiety than *B. monnieri* [8].

Structure elucidation of flavonoids

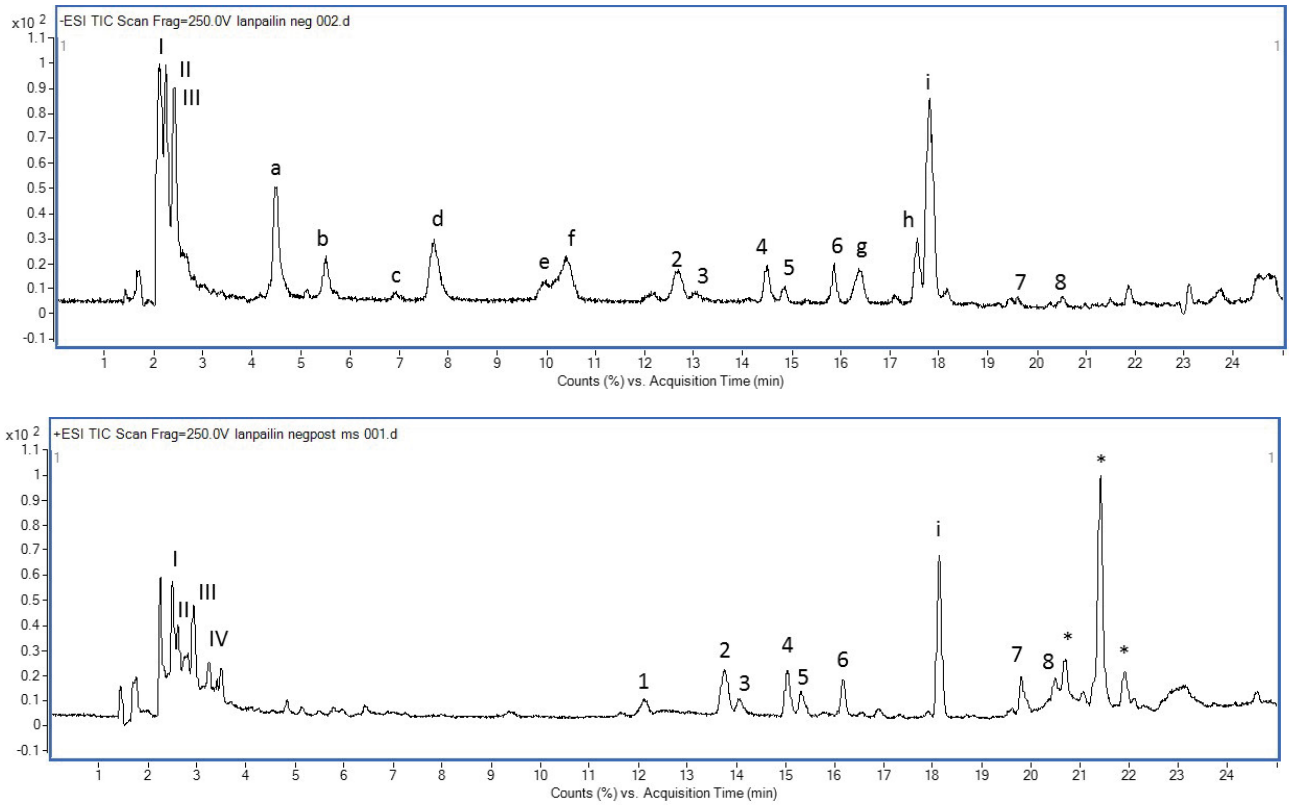


Figure 2: The total ion chromatograms (TIC) from ESI-QTOF-MS at 10 mg/mL of *B. caroliniana* methanolic extract. The upper trace was obtained from negative mode and the lower trace was obtained from positive mode. The peak numbers and compound identifications are presented in Table 1, Table 2 and Table 3. The number 1-8 represent triterpenoid saponins, I-IV was phenylethanoid glycosides and a-i was flavonoids. The compounds are unidentified mark as*.

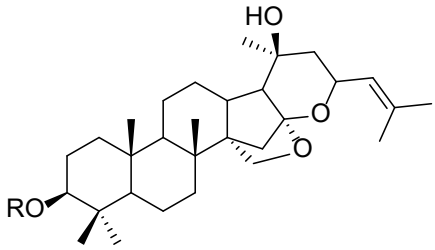
Table 2: LC (+/-) ESI-QTOF-MS/MS data for structural elucidation of phenylethanoid glycosides in *B. caroliniana.*

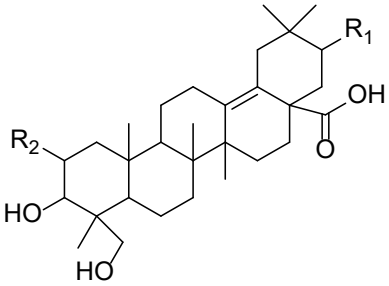
Peak	t_R (min)	Measured mass	MS/MS	Molecular formula	Error (ppm)	Identified compound
I	1.69	623.1987 $[M-H]^-$	461.16,315.11,161.02	$C_{29}H_{36}O_{15}$	-0.89	Acteoside
		647.2050 $[M + Na]^+$	501.14,163.04,85.03		-16.00	
II	2.11	623.1996 $[M-H]^-$	461.15,315.10,161.02	$C_{29}H_{36}O_{15}$	-2.34	Isoacteoside
		647.206 $[M + Na]^+$	501.14		-17.55	
III	2.25	665.2100 $[M-H]^-$	461.15,315.10,161.02	$C_{31}H_{38}O_{16}$	-1.94	2´Acetylacteoside
		689.2176 $[M + Na]^+$	543.15		-18.00	
IV	2.42	665.2101 $[M-H]^-$	461.16,315.10,161.02	$C_{31}H_{38}O_{16}$	-2.09	2´Acetyl isoacteoside
		689.2176 $[M + Na]^+$	543.15		-18.00	

Table 3: LC (+/-) ESI-QTOF-MS/MS data for structural elucidation of flavonoids in *B. caroliniana.*

Peak	t_R (min)	Measured mass	MS/MS	Molecular formula	Error (ppm)	Identified compound
a	4.57	285.0415 $[M-H]^-$	133.02	$C_{15}H_{10}O_6$	6.88	Luteolin
		287.0607 $[M + H]^+$	153.02			
b	5.57	375.073 $[M-H]^-$	360.04,345.02,246.01,218.02	$C_{18}H_{16}O_9$	2.00	Limocitrol
c	7.08	269.0463 $[M-H]^-$	117.03	$C_{15}H_{10}O_5$	7.24	Apigenin
d	7.85	299.0577 $[M-H]^-$	284.03,256.03, 227.03	$C_{16}H_{12}O_6$	-5.31	Diosmetin
e	10.06	389.0885 $[M-H]^-$	374.05,359.03	$C_{19}H_{18}O_9$	-1.78	Trihydroxy tetramethoxy-flavonoid
f	12.01	359.0779 $[M-H]^-$	344.04,329.02	$C_{18}H_{16}O_8$	-1.84	Trihydroxy trimethoxy-flavonoid
g	16.45	373.0941 $[M-H]^-$	358.06,343.03	$C_{19}H_{18}O_8$	-3.24	Dihydroxy-tetramethoxy flavonoid
h	17.58	373.094 $[M-H]^-$	358.06,343.04	$C_{19}H_{18}O_8$	-2.97	Dihydroxy tetramethoxy flavonoid
i	17.83	403.1052 $[M-H]^-$	388.07,373.04	$C_{20}H_{20}O_9$	-4.33	Dihydroxy pentamethoxy flavonoid
		405.1261 $[M+ H]^+$	390.09,375.07		-20.00	
*	20.69	799.2355 $[2M + Na]^+$	411.11			Unidentified
*	21.40	859.2577 $[2M + Na]^+$	441.12			Unidentified
*	21.90	1083.731 $[2M + Na]^+$	533.36			Unidentified

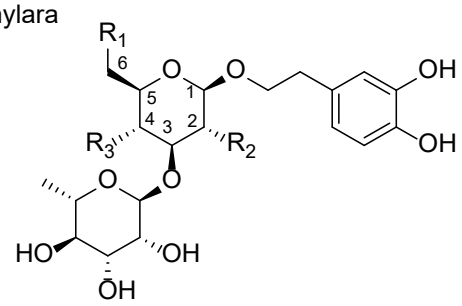
Besides saponins and phenylethanoid glycosides, flavonoids were also tentatively identified (Table 3). Compounds **a** and **c** (Figure 2) with the *m/z* 285 $[M-H]^-$ and 269 $[M-H]^-$ were unambiguously assigned as luteolin and apigenin, respectively and confirmed with authentic compounds. These two compounds were also found in *B. monnieri.* Compound **d** with the *m/z* 299 $[M-H]^-$ was assigned as diosmetin due to containing of 30Da ($-OCH_3$) higher than apigenin. Compound **b** with the *m/z* 375 $[M-H]^-$ was proposed as a tetrahydroxy trimethoxy flavonoid, which was tentatively identified as limocitrol. Compounds **e**-**I** also provided similar fragmentation patterns of flavonoids with the 15Da distinct loss. Therefore, they were proposed as flavonoid derivatives that contain hydroxyl and/or methoxy groups attached to the C atom of the flavonoid backbone (flavone and flavonol). Compound **e** with *m/z* 389 $[M-H]^-$ was identified as trihydroxy tetramethoxy flavonoid. Compound **f** with *m/z* 359 $[M-H]^-$; 30Da less than compound **e** may be trihydroxy trimethoxy flavonoid. Compounds **g** and **h** showed the same *m/z* 373 $[M-H]^-$, were proposed as dihydroxy tetramethoxy flavonoid. Compound **i** with *m/z* of 403 $[M-H]^-$ was identified as dihydroxy pentamethoxy flavonoid. However, the positions of hydroxyl and methoxy group on these compounds cannot be pointed out by this LC-MS/MS information. The compound identities still need proof by other techniques such as NMR. Three compounds eluted at 20.69, 21.40 and 21.9 min are unidentified in this time. Luteolin, apigenin, diosmetin and limocitrol are commonly found in citrus fruit [16]. The bioactive properties of flavonoids have

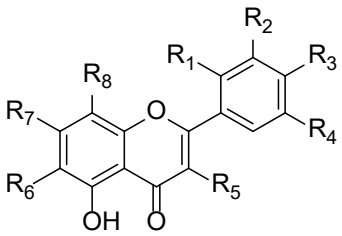




Bacoside A_3 (**1**); R=Glc-Glc-Ara
Bacopaside X(**2**); R=Ara-Glc-Ara
Bacoside A_3 malonylglycoside(**3**); R=Glc-Malonylglc-Ara
Bacopaside X malonylglycoside isomer 1(**4**); R=Ara-Glc-Malonylara
Bacopaside X malonylglycoside isomer 2(**5**); R=Ara-Malonylglc-Ara
Bacopaside X dimalonyl glycoside(**6**); R=Ara-Malonylglc-Malonylara

Centellasapogenol A(**7**); R_1=H, R_2=OH
Centellasapogenol A isomer(**8**); R_1=OH, R_2=H





Acetoside(**I**); R_1=OH, R_2=OH, R_3=Caffeic acid
Isoacetoside(**II**); R_1=Caffeic acid, R_2=OH, R_3=OH
2'acetylacetoside(**III**); R_1=OH, R_2=OCH_3CO, R_3=Caffeic acid
2'acetylisoacetoside(**IV**); R_1=Caffeic acid, R_2=OCH_3CO, R_3=OH

Luteolin(**a**); R_1=R_4=R_5=R_6=R_8=H, R_2=R_3=R_7=OH
Limocitrol(**b**); R_1=R_4=H, R_3=R_5=R_7=OH, R_2=R_6=R_8=OCH_3
Apigenin(**c**); R_1=R_2=R_4=R_6=R_8=H, R_3=R_7=OH
Diosmetin(**d**); R_1=R_4=R_5=R_6=R_8=H, R_2=R_7=OH, R_3=OCH_3
Trihydroxy tetramethoxy flavonoid(**e**); R_1=R_4=OH, R_3=R_5=R_6=R_7=OCH_3, R_2=H
Trihydroxy trimethoxy flavonoid(**f**); R_1=R_2=R_4=H, R_3=R_5=R_6=OCH_3, R_7=R_8=OH
Dihydroxy tetramethoxyflavonoid(**g**); R_1=R_4=R_6=H, R_2=R_3=R_5=R_7=OCH_3, R_8=OH
Dihydroxy tetramethoxyflavonoid(**h**); R_1=R_5=R_6=H, R_2=R_4=R_7=R_8=OCH_3, R_3=OH
Dihydroxy pentamethoxy flavonoid(**i**); R_1=R_4=R_6=H, R_2=R_3=R_5=R_7=OCH_3, R_8=OH

Figure 3: Structures of phytochemical compounds found in *B. caroliniana* were tentatively identified using LC-ESI-QTOF-MS/MS. Glc: Glucose; Ara: Arabinose; Malonylglc: Malonylglucose; Malonylara: Malonylarabinose

been primarily associated with decreased oxidative stress and free radical damage activity [17] (Figure 3).

Conclusions

High-resolution LC-ESI-QTOF-MS/MS has led to the tentative identification of 21 compounds in *B. caroliniana*. Eight compounds found belong to triterpenoid saponin group; six of them were bacopa saponin i.e., bacoside A3 and bacopaside X with their derivative that differ in glycosidic part with the malonyl one or two groups conjugated to the hexose or/and pentose moieties. The other two saponin compounds were proposed as centellasapogenol A and its isomer. Beside this, four phenylethanoid glycosides; acteoside and isoacteoside with their acetyl group were elucidated. Additionally, two flavones; luteolin and apigenin were found. Seven *O*-methoxy flavonoids were also reported in this plant. All the identified compounds were reported for the first time in *B. caroliniana*.

Declaration of Competing Interest

The authors declare no conflict of interest.

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