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Characterization of Triterpenoids, Flavonoids and Phenolic Acids in *Eclipta prostrata* by High-performance Liquid Chromatography/diode-array Detector/electrospray Ionization with Multi-stage Tandem Mass Spectroscopy

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Abstract – High-performance liquid chromatographic method with diode-array detector and electrospray ionization with multi-stage tandem mass spectroscopy (HPLC/DAD/ESI-MS^n) was used to identify the major constituents in a methanolic extract of *Eclipta prostrata*. The chromatographic separation was performed on a C18 column. Acetonitrile-water was used as a mobile phase. HPLC/DAD/ESI-MS^n allowed the characterization of constituents of *E. prostrata*, mainly triterpenoids (eclalbasaponin I, II, III, IV, VI), flavonoids (luteolin 7-*O*-glucoside, demethylwedelolactone, wedelolactone, luteolin, demetylwedelolactone sulfate, luteolin sulfate, apigenin sulfate) and phenolic acids (5-*O*-caffeoylquinic acid, 3, 4-*O*-dicaffeoylquinic acid, 3, 5-*O*-dicaffeoylquinic acid, 4, 5-*O*-dicaffeoylquinic acid).

Keywords – *Eclipta prostrata*, HPLC/DAD/ESI-MS^n, triterpenoids, flavonoids, phenolic acids

Introduction

Eclipta prostrata (synonyms: *Eclipta alba,* Compositae) is a small, branched annual herb with white flower heads inhabiting tropical and subtropical regions of the world. *E. prostrata* has been used in the treatment of hepatic diseases, hyperlipidemia and snake venom poisoning in folk medicine (Bae, 2000; Ma-Ma *et al*., 1978). Thiophenes, triterpenoids, coumestanes and flavonoids have been reported as constituents of *Eclipta* species (Singh and Bhargava, 1992; Yahara *et al*., 1997; Wagner *et al*., 1986). In addition, *Eclipta* species have been reported to exert diverse biological activity including hepatoprotective, anti-inflammatory, antihemorrhagic, antihyperlipidemic and antihyperglycemic activities (Wagner *et al*., 1986; Saxena *et al*., 1993; Melo *et al*., 1994; Kumari *et al*., 2006).

The coupling of LC with spectroscopic techniques such as UV, MS or NMR provides a useful tool for rapid data collection and structure elucidation (Wolfender *et al*., 2003). LC/DAD is an effective technique for a rapid screening of mixtures, however, the light absorbance data obtained are insufficient for structure elucidation (Snyder *et al*., 1997). The use of hyphenated LC/UV/MS instrumentation has been reported in numerous applications (Wolfender *et al*., 1998). This technique is fast and the short time of exposure of the analysts to light and air limits their degradation.

Earlier we published the preliminary outcomes of a study of triterpenoids of *E. prostrata* (Lee *et al*., 2008). Here we present the results of a more comprehensive investigation of chemical composition of *E. prostrata* using reversed phase liquid chromatography with a tandem UV-photodiode array and mass selective detection (HPLC/DAD/MS^2).

Experimental

Chemicals and herb materials – The aerial parts of *E. prostrata* were purchased from Kyung-dong Market, Seoul, Korea in June 2004, and identified by Dr. Jong Hee Park, a professor of the College of Pharmacy, Pusan National University. A voucher specimen (SNUPH-EP2004-06) has been deposited in the Herbarium of the Medicinal Plant Garden, College of Pharmacy, Seoul National University.

Three standard compounds, elcalbasaponin I (**8**), II (**13**), and III (**7**) were isolated and purified from aerial parts of *E. prostrata* at our laboratory. Detailed experimental procedures of isolation and identification of triterpenoid glycosides from methanolic extract were shown in our previous work (Lee *et al*., 2008). A standard solution of each compound was prepared by dissolving it

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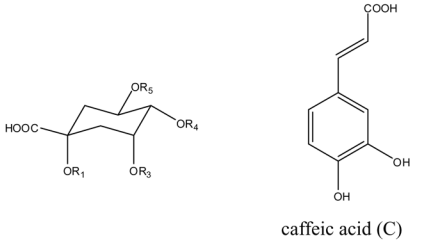
in methanol to the proper concentration.

HPLC grade solvents (acetonitrile, water and methanol) and reagents were obtained from BDH chemicals (Poole, UK). Phosphoric acid (analytical grade) was purchased from Merck (Darmstadt, Germany). Triple deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

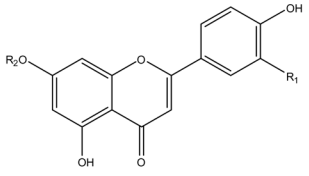
Instrumentation – The Finnigan Surveyor MS Pump Plus (Thermo Electron Corporation, San Jose, CA, USA) was a dual-piston, quaternary, low-pressure mixing pump with a built-in vacuum degasser and pulse dampener. The sample injector was Surveyor AS (Thermo Electron Corporation, San Jose, CA, USA). The Finnigan Surveyor PDA Plus Detector (Thermo Electron Corporation, San Jose, CA, USA) was a full-featured, time-programmable, photodiode array detector. It was controlled remotely via an ethernet communication link from a computer using the Xcalibur 2.0 data system. The Xcalibur 2.0 data system was also used for data acquisition and processing.

The Finnigan LCQ Advantage MAX (Thermo Electron Corporation, San Jose, CA, USA) was an advanced analytical instrument that included a syringe pump, an optional divert/inject valve, an atmospheric pressure ionization (API) source, a mass spectrometer detector, and the Xcalibur 2.0 data system. The Finnigan LCQ Advantage MAX Ion Trap mass spectrometer with ESI source was used for the mass spectrometric determination.

Separation was achieved on a Waters XTerra^{TM} RP18 (5 μm, 4.6 mm I.D. × 150 mm). A linear gradient elution of A (0.03% formic acid) and B (100% acetonitrile) was used (0 min, 20% B; 50 min, 80% B; 51 min 20% B; 60 min, 20% B; v/v). The flow rate was 0.3 mLmin^{-1}, and the injection volume 10 μL. The conditions were as follows: capillary temperature 270 °C, i spray voltage 5 kV, sheath gas flow rate 22 arbitrary units, aux/sweep gas flow rate 0 arbitrary units. The precursor ions were

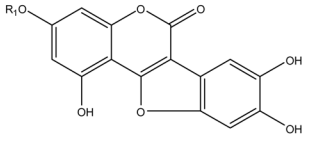


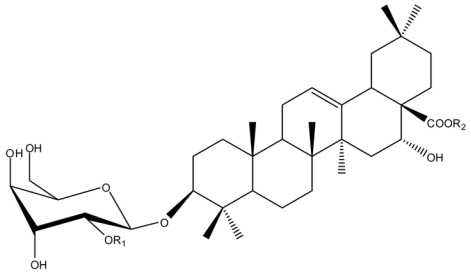
caffeic acid (C)



	R_1	R_3	R_4	R_5
5-*O*-caffeoylquinic acid (**1**)	H	H	H	C
3,4-*O*-dicaffeoylquinic acid (**3**)	H	C	C	H
3,5-*O*-dicaffeoylquinic acid (**4**)	H	C	H	C
4,5-*O*-dicaffeoylquinic acid (**5**)	H	H	C	C

	R_1	R_2
luteolin 7-*O*-glucoside (**2**)	OH	glc
luteolin (**10**)	OH	H
luteolin sulfate (**14**)	OH	SO_3H
apigenin sulfate (**16**)	H	SO_3H





	R_1
demethylwedelolactone (**6**)	H
wedelolactone (**9**)	CH_3
demethylwedelolactone sulfate (**11**)	SO_3H

	R_1	R_2
eclalbasaponin III (**7**)	glc	glc
eclalbasaponin I (**8**)	H	glc
eclalbasaponin IV (**12**)	glc	H
eclalbasaponin II (**13**)	H	H
eclalbasaponin VI (**15**)	SO_3H	glc

Fig. 1. Structures of compounds **1** - **16**.

isolated with an isolation width of 2 *m/z* units and fragmented using collision energy of 40% for MS experiments.

Sample preparation for HPLC – For the preparation for HPLC experiment, 10.0 g of *E. prostrata* was weighed accurately. The herb extracted with 80% MeOH (100 mL) 3 times in an ultrasonic apparatus. The extract was filtered and evaporated *in vacuo*, and then suspended to 100 mL of 50% methanol. This sample solution was filtered through 0.45 μm membrane filter (Millipore, Nylon, 170 μm) and analyzed with HPLC.

Results and discussion

Various mixtures of water and acetonitrile in combination with formic acid were tested as a mobile phase. In our chromatographic condition, addition of formic acid in water increased the volatility. The gradient profile used in this work allowed the separation of all the compounds. The LC-MS^2 examination of *E. prostrata* resulted in detection and identification of 16 diverse and distinctive components. UV and total ion current chromatograms of the extract of *E. prostrata* are shown in Fig. 2. Table 1 presents their MS and MS^2 fragment ions.

Caffeoyl and dicaffeoyl quinic isomers – Peaks **1**, **3**, **4** and **5** had the same UV spectra, with a maximum at 324 - 330 nm and a shoulder at 296 nm, typical of caffeic acid derivatives (Fig. 3). The mass spectrum of peak **1** showed a $[M-H]^-$ ion at *m/z* 353 in the ESI-MS with negative ion mode, and ions at *m/z* 191 and 707. The LC retention time and UV and MS spectra of peak **1** were identical to standard chlorogenic acid, which correspond to caffeic acid esterified to quinic aicd (5-*O*-caffeoylquinic acid, Fig. 1). Ions at *m/z* 191 would correspond to deprotonated quinic acid fragment, while ion *m/z* 707 was dimeric adduct of the caffeoyl quinic acid molecule. Peaks **3**, **4** and **5** all shared the same UV spectra, similar to that of chlorogenic acid, although the MS spectra showed a $[M-H]^-$ ion at *m/z* 515 and a fragment with *m/z* 353 (Table 1). The molecular ion at *m/z* 515 is indicative of dicaffeoyl quinic acid isomers. The ion at *m/z* 353 would correspond to the loss of one dehydrated molecule of caffeic acid [M-caffeic acid-H_2O]$^-$. The three isomers can be differentiated by their secondary peaks; identification of *m/z* 191 and 179 in MS^2 spectra of peak **4** besides the base peak at *m/z* 353 allowed the peak to be identified as 3,5-*O*-caffeoylquinic acid (Schutz *et al*., 2005). The presence of *m/z* 335, besides *m/z* 353, *m/z* 173 and 179 in MS^2 spectra of peak **3** was characteristic of 3,4-*O*-caffeoylquinic acid (Clifford *et al*., 2003). Considering the retention times and elution profile in C18 columns reported for dicaffeoyl quinic acid isomers (Clifford and Ramirez-Martinez, 1990; Yoshimoto *et al*., 2002), peaks **3**, **4** and **5** corresponded to 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid (isochlorogenic acid A), and 4,5-*O*-dicaffeoylquinic acid, respectively (Fig. 1).

Flavonoids – Peaks **2**, **10**, and **14** had the same UV spectra, with maxima at 347, 252 nm, typical of flavonoid

Table 1. UV spectra and characteristic ions of triterpenoids, flavonoids and phenolic acids from *Eclipta prostrata*.

Compd.	Rt (min)	λ_{max}	$[M-H]^-$	MS^2	Proposed structure
1	9.67	326, 296sh	353	191, 179	5-*O*-Caffeoylquinic acid
2	14.29	347, 253	447	327, 285	Luteolin 7-*O*-glucoside
3	17.17	324, 296sh	515	353, 335, 179	3, 4-*O*-Dicaffeoylquinic acid
4	18.26	330, 296sh	515	353, 191, 179	3, 5-*O*-Dicaffeoylquinic acid
5	19.21	327, 296sh	515	353, 299, 203	4, 5-*O*-Dicaffeoylquinic acid
6	20.58	350, 249, 215	299	255	Demethylwedelolactone
7	21.60	–	1003*	957, 633	Eclalbasaponin III
8	23.51	–	841*	795, 633	Eclalbasaponin I
9	26.78	350, 249, 215	313	298	Wedelolactone
10	27.32	347, 252	285	–	Luteolin
11	28.28	349, 248, 214	379	299	Demethylwedelolactone sulfate
12	31.59	–	841*	795, 633	Eclalbasaponin IV
13	36.21	–	679*	633, 471	Eclalbasaponin II
14	37.58	347, 252	365	285	Luteolin sulfate
15	40.91	–	875	713	Eclalbasaponin VI
16	49.99	357, 266	349	269	Apigenin sulfate

* $[M+HCOOH-H]^-$

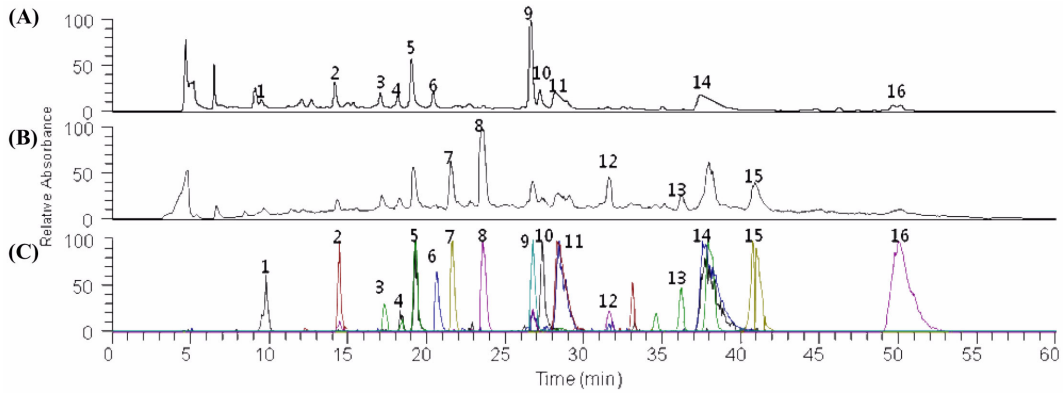


Fig. 2. HPLC-UV (254 nm) (A), HPLC-MS total ion current (negative mode) (B) and extracted ions (C) chromatograms of the extract of *E. prostrata*.

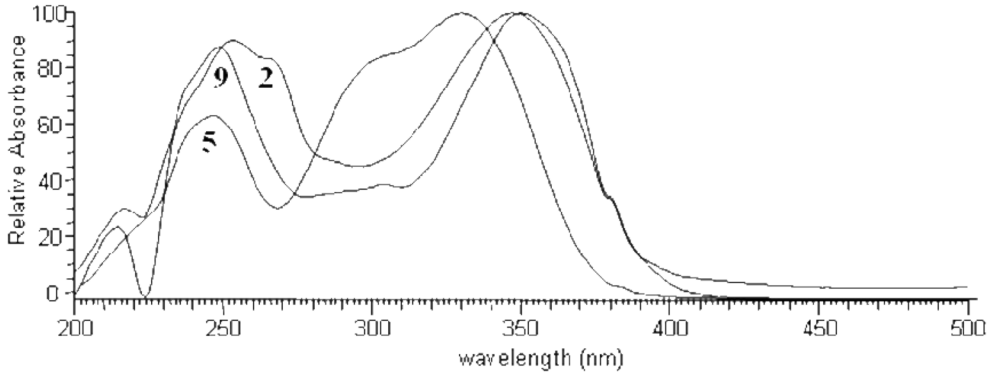


Fig. 3. UV spectra of (**2**) luteolin 7-*O*-glucoside, (**5**) 4,5-*O*-dicaffeoylquinic acid, and (**9**) wedelolactone.

derivatives. Peak **2** investigated in the full-scan chromatogram was *m/z* 447, where a peak with a retention time of 14.29 min was present. The product ion spectrum of this peak at a CID gave a product ion at *m/z* 285, which suggested a kaempferol or a luteolin glycoside. Because kampferol and luteolin are isomers, kaempferol 3-*O*-glycoside and luteolin 7-*O*-glycoside have the same mass and UV λ_{max} for band I around 346-348 nm. However, they have significant differences in the λ_{max} for UV band II. The different oxygenation of their B ring (3'- and 4'-hydroxyls for luteolin as opposed to the 4'-hydroxyl for kaempferol) gives luteolin and its 7-*O*-glycosides two peaks in the UV band II position while kaempferol and its 3-*O*-glycosides have only one peak (Lin and Harnly, 2008). Peak **2** showed two peaks in UV band II and identified as luteolin 7-*O*-glucoside (Fig. 3). Peak **10** (*m/z* 285) showed two peaks in UV band II and identified as luteolin. Peak **14** (*m/z* 365) showed two peaks in UV band II. In addition, a fragment ion at *m/z* 285 ([M-H-SO_3]$^-$) from CID of the molecular ion of *m/z* 365 was detected, indicating that was a sulfate group substituted in the molecule. Peak **14** was identified as luteolin sulfate.

Peaks **6**, **9**, and **11** had the same UV spectra, with three maxima at 215, 249, and 350 nm, typical of coumestan derivatives (Chen *et al*., 2008). Wagner *et al*. (1986) reported that wedelolactone was the main constituent of the ethyl acetate fraction of *E. prostrata*, followed by demethylwedelolactone. Peak **6** (*m/z* 299) was identified as demethylwedelolactone. Peak **9** showed molecular specie [M-H]$^-$ ion at *m/z* 313 in the negative ion mode. In addition, a fragment ion at *m/z* 298 ([M-H-CH_3]$^-$) from CID of the molecular ion of *m/z* 313 was detected, indicating that was a metyl group substituted in the molecule. Peak **9** was identified as wedelolactone. Peak **11** showed molecular specie [M-H]$^-$ ion at *m/z* 379 in the negative ion mode. In addition, a fragment ion at *m/z* 299 ([M-H-SO_3]$^-$) from CID of the molecular ion of *m/z* 379 was detected, indicating that was a sulfate group substituted in the molecule. Peak **11** was identified as demethylwedelolactone sulfate.

Peak **16** had the UV spectrum, with maxima at 357, 266 nm, typical of flavonoid derivative. It showed molecular

specie $[M-H]^-$ ion at *m/z* 349 in the negative ion mode. In addition, a fragment ion at *m/z* 269 ($[M-H-SO_3]^-$) from CID of the molecular ion of *m/z* 349 was detected, indicating that was a sulfate group substituted in the molecule. Peak **16** was identified as apigenin sulfate.

Triterpenoid glycosides – Triterpenoid glycosides (peaks **7**, **8**, **12**, **13**, and **15)** were not be detected with conventional UV absoption detection. The LC/MS analysis of triterpenoid glycosides showed that its $[M+HCOOH-H]^-$ ion was much more intense than $[M-H]^-$ ion except peak **15**. When a pure standard was available, the compound was identified by comparing its HPLC retention time, and mass spectra with those of the standard. When no standard was available, the structures were proposed mainly based on the mass spectra. Comparing the retention times and the MS^2 spectra with those of the authentic standards, peaks **7**, **8**, and **13** were identified as eclabasaponin III, I, and II, respectively. Peak **12** was detected with its $[M+HCOOH-H]^-$ ion at *m/z* 841 as peak **8**. In addition, two fragment ions at *m/z* 633 (base peak) and *m/z* 795 from CID of the molecular ion of *m/z* 841 were detected, indicating that was a glc unit substituted in the molecule. Peak **12** was identified as eclalbasaponin IV (Yahara *et al*., 1994). Peak **15** was detected with its $[M-H]^-$ ion at *m/z* 875 as peak **8**. Because compound **15** have sulfate group in the structure, it seems that its $[M-H]^-$ ion was much more intense than $[M+HCOOH-H]^-$ ion different from other triterpenoid saponins. In addition, a fragment ion at *m/z* 713 from CID of the molecular ion of *m/z* 875 was detected, indicating that was a glc unit substituted in the molecule. Peak **15** was identified as eclalbasaponin VI (Yahara *et al*., 1997).

Conclusion

HPLC/DAD/ESI-MS^n allowed the characterization of constituents of *E. prostrata*, mainly triterpenoids (eclalbasaponin I, II, III, IV, VI), flavonoids (luteolin 7-*O*-glucoside, demethylwedelolactone, wedelolactone, luteolin, demetylwedelolactone sulfate, luteolin sulfate, apigenin sulfate) and phenolic acids (5-*O*-caffeoylquinic acid, 3, 4-*O*-dicaffeoylquinic acid, 3, 5-*O*-dicaffeoylquinic acid, 4, 5-*O*-dicaffeoylquinic acid).

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