ISSN 0974 – 5211

Research Paper

Journal of Natural Products
Volume 4 (2011)
www.JournalofNaturalProducts.com

Identification of the Phenolic Components of *Vernonia amygdalina* and *Russelia equisetiformis*

C.E. Johnson[1]*, Long-Ze Lin[2], J. M. Harnly[2], F. O. Oladeinde [1, 3,4], A.M. Kinyua[1, 6], R. Michelin[5], Y. Bronner[1]

[1] School of Community Health and Policy, Morgan State University, Baltimore, MD, USA.
[2]Food Composition and Methods Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service, USDA, Beltsville, MD, USA.
[3]Dept. of Chemistry, Morgan State University, Baltimore, MD, USA
[4]Dept. of Pharmaceutical Chemistry, University of Ibadan, Ibadan, Nigeria.
[5] Dept. of Biology, Morgan State University, Baltimore, MD, USA.
[6]Dept. of Physics, Morgan State University, Baltimore, MD, USA.
* Corresponding Author

(Received 18 October 2010; Revised 21 October -1o December; Accepted 11 December 2010)

ABSTRACT

The leaves of *Vernonia amygdalina* (Family-Compositae) and *Russelia equisetiformis* (Family-Scrophulariacae), were analyzed using LC-DAD-MS to identify 26 phenolics components. Among them, 9 caffeoylquinic acids, verbascoside, isoverbascoside and 7 glycosylated flavonoids were positively identified. This is the first report of the existence of caffeoylquinic acids in *Vernonia amygdalina* and most of the flavonoids in *Russelia equisetiformis*.

Keywords: Caffeoylquinic acids; Glycosylated flavonols; LC-DAD-ESI/MS analysis.

INTRODUCTION

Vernonia amygdalina Del. is a vegetable found in some African countries. The leaves are used in traditional African medicine and are reputed to have hepatoprotective, antioxidant, antibacterial, cytoprotective, antimalarial, and antidiabetic activities (Masaba, et al., 2000; Izevbigie, 2003; Iwalokun, et al., 2006; Ibrahim, et al., 2009; Ong, et al., 2010). Chemical studies on the bioactive components of this plant have led to the identification of some sesquiterpene lactones and steroids (Jisaka, et al., 1992; Erasto, et al., 2006). *Russelia equisetiformis* is a medicinal plant considered to have anti-inflammatory, analgesic and membrane stabilizing properties (Awe, et al., 2004; 2009). Two phenylethanoid glycosides of this plant, russectinol and russeliaoside, were identified as its active constituents (Awe, et al., 2004; 2007).

The total phenolic content of both plants used in this study was previously quantified in a previous study (Johnson, et al., 2008). The aim of this study was to identify their phenolic components using a standardized profiling method based on

liquid chromatography with diode array and electro-spray ionization mass spectrometric detection (LC-DAD-ESI/MS) (Lin and Harnly, 2007; 2008).

MATERIALS AND METHODS

***Chemicals*:** Chlorogenic acid from Sigma Chemical Co. (Saint Louis, MO, USA), apigenin 7-O-glucoside, vitexin and orientin from Extrasynthese (Genay Cedex, France), verbascoside, isoverbascoside, 1,4-, 1,5-dicaffeoyquinic acids from ChromaDex Inc. (Irvine, CA), and the isolated 3-, 4-caffeoylquinic acids, 3,5-, 3,4-, 4,5, 1,4-dicaffeoylquinic acids in the USDA laboratory (Lin & Harnly, 2007) were used as the standards. Formic acid and HPLC solvents (acetonitrile, methanol) from VWR Scientific (Seattle, WA), and prepared HPLC grade water were used for HPLC-MS analysis.

Plant materials: The leaves of *Vernonia amygdalina* and the whole plants of *Russelia equisetiformis* were collected from southwestern Nigeria during the rainy season and identified using descriptions in literature (Hostettmann, et al., 2001). Authentication was done in the Department of Pharmacognosy, University of Ibadan, Ibadan, Nigeria and a voucher specimen is deposited. The plant materials were carefully air-dried indoors at room temperature for three days, dried in an oven between 40-50˚C; comminuted into small powders and passed through 20 mesh sieves prior to the extraction experiments.

Plant extracts: Dried powdered *Vernonia amygdalina* leaves (200 mg) and *Russelia equisetiformis* whole plants (1000 mg) were extracted with methanol-water (5.00 ml, 60:40, v/v) by sonication at room temperature for 60 minutes. Extracts were filtered and a 50 µl sample of each was injected onto the analytical column for analysis.

LC-DAD and ESI-MS conditions: The LC-DAD-ESI/MS instrument and operating parameters have been previously described (Lin and Harnly, 2007). Briefly, the LC-DAD-ESI/MS consisted of an Agilent 1100 HPLC coupled to a diode array detector and mass spectrometer (MSD, SL mode) (Agilent, Palo Alto, CA). A 250 x 4.6 mm i.d., 5 µm Symmetry (or SymmetryShield) C18 column (C18, 5 µm,) with a 20 x3.9 mm i.d., 5 µm sentry guard column (Symmetry, 3.9 x 20 mm) (Waters Corp., Milford, MA) was used at flow rate of 1.0 ml/min. The column oven temperature was set at 25°C. The mobile phase consisted of a combination of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The gradient was varied linearly from 10% to 26% B (v/v) in 40 minutes, to 65% B at 70 minutes. The DAD was set at 350, 310, and 270 nm to provide real-time chromatographic traces and the UV spectra were recorded from 190-450 nm for plant component identification. Mass spectra were simultaneously acquired using electro-spray ionization in the positive and negative ionization (PI and NI) modes at low and high fragmentation voltages (70 V and 250 V) over the range of *m/z* 100-2000.

RESULTS

***Identification of the extract of Vernonia amygdalina*:** HPLC chromatograms (at 350 nm) of the aqueous methanol extract of *Vernonia amygdalina* leaves were recorded on both a Waters Symmetry column and a SymmetryShield column (Fig. 2A and B, respectively). The structures of the caffeoylquinic acids are shown in Figure 1. The retention times (t_R), wavelength of maximum absorbance (λ_{max}), deprotonated molecules ($[M-H]^-$), and major fragment ions are listed in Table 1. In Table 1, the asterisk "*" denotes compounds identified by comparison to standards or references all other compounds were reported previously in the plant. The two columns provided

separation of the 14 peaks (Fig. 2A and 2B). Neither column, by itself, provided satisfactory separation. The UV λ_{max} at 240, 300sh, 326 nm spectra is typical for caffeic acid, suggesting that these compounds are caffeic acid conjugates. The masses suggest that they are quinic acid conjugates.

Positive identification of the common isomeric caffeoylquinic acids was achieved by comparison to purchased or isolated dicaffeoylquinic acids and monocaffeoylquinic acids (Lin and Harnly, 2008). Positively identified 1-mono-, 1, 4,5-, and 1,3,5-tri-caffeoylquinic acids in arnica flower and burdock roots, and 3,4,5-tricaffeoylquinic acid in sweet potato leaves were previously profiled using the standardized method, and 13 naturally occurring isomeric caffeoylquinic acids in plants have been recorded and stored in the collected food phenolic component database in the USDA laboratory (Lin and Harnly, 2008).

The compounds in Table 1 were identified by direct comparison of their retention time and UV and mass spectra with those of the standard. Thus, it was possible to identify peaks 1, 2, 3 and 4 as 1-, 3-caffeoylquinic acid, chlorogenic acid (5-caffeoylquinic acid), and 4-caffeoylquinic acid ($[M-H]^-$ at *m/z* 353). Peaks 5-10 were identified as 1,3-, 1,4-, 3,4-, 3,5-, 1,5- and 4,5-dicaffeoylquinic acids ($[M-H]^-$ at *m/z* 515). Finally, peaks 11, 12 and 13 were identified as 1,4,5-, 1,3,5- and 3,4,5-tricaffeoylquinic acids ($[M-H]^-$ at *m/z* 677) and peak 14 was identified as feruloyldicaffeoylquinic acid ($[M-H]^-$ at *m/z* 691).

The detection limit for these compounds is depended upon their concentration in the extract and the injection volume. Thus, as mentioned in the Materials and Methods section, the detected phenolics in these leaves have concentrations equal to or larger than 0.0005 % by dry weight, i.e., 0.005 mg/g of dried leaves. The 3,4-, 1,5-, 3,5-, and 4,5-dicaffeoylquinic acids (peak 7,8,9 and 10) and 3,4,5-tricaffeoylquinic acid (peak 14) are the main phenolics of the leaves and are present at concentrations much greater than the detection limit.

***Identification of the phenylethanoid glycosides and flavonoids in whole plants of Russelia equisetiformis*:** HPLC chromatograms (at 350 nm) of the aqueous methanol extract of the whole plant *Russelia equisetiformis* were recorded on Waters Symmetry column (Fig. 2C). The retention times, wavelengths of maximum absorbance (λ_{max}), protonated and deprotonated molecules ($[M+H]^+$ and $[M-H]^-$), and major fragment ions are listed in Table 2. The structures of these compounds are shown in Fig. 1.

Eighteen peaks were observed in the chromatograms of this plant. Based on a direct comparison of the data in Table 2 with our collected data for standards and reference compounds (Lin and Harnly, 2008), 9 of the peaks were identified as phenylethanoids, and 9 as flavonoids. Peaks 11 and 13 were identified as verbascoside and isoverbascoside and peaks 2, 5, 6, 9, 12, 15 and 18 were identified as 6, 8-diglucosylapigenin, 6-arabinosyl-8-glucosylapigenin, orientin (8-glucosylluteolin), vitexin (8-glucosylapigenin), luteolin 7-*O*-glucuronide, apigenin *O*-glucuronide and apigenin, respectively. The remaining peaks were minor components and provisionally assigned as listed in Table 2. Based on an extraction of 1g of plant material with 5.0 ml of solvent and an injection volume of 50 µl, the detection limit for each compound was equal to 0.0001 % (i.e., 1 mg/ kg of dry plant material) by dry weight. The major component verbascoside, however, was present at much higher concentrations.

DISCUSSION

A literature search revealed that caffeoylquinic acids as identified in *Vernonia*

amygdalina, have many biological activities. In addition to the common antioxidant, radical-scavenging, and anti-inflammatory activities of plant phenolic compounds, the caffeoylquinic acids, especially 3,4,5-tricaffeoylquinic acid, have been reported to show antimutagenicity, anti-human cancer cell, and anti-human immunodeficiency virus activities (Yoshimoto, et al., 2002; Tamura, et al., 2006; Kurata, et al., 2007). Verbascoside was previously isolated as one of the active antinociceptive components from the leaves of *Russelia equisetiformis* and named russetinol (Awe, et al., 2007). Verbascoside was also reported to exist in many herbs with biological activities similar to many of the common plant phenolics (Bilia, et al., 2008). Further research is necessary to determine biological activity of the newly identified flavanoids in *Russelia equisetiformis.*

CONCLUSION

With the use of this method, 10 caffeoylquinic acids were detected in the aqueous methanol extract of *Vernonia amygdalina* leaves and 7 phenylethanoids and 9 glycosylated flavonoids were detected in *Russelia equisetiformis*. This is the first report of the existence of caffeoylquinic acids and most of the flavonoids in these plants.

Acknowledgements: This work was supported by the Agricultural Research Service of the U.S. Department of Agriculture and an Interagency Agreement with the Office of Dietary Supplements of the National Institutes of Health, and a grant from National Center for Complementary and Alternative Medicine (NIH-NCCAM # 1-T32-TA01058-01). A special thanks to Dr. Janet Makinde for her contributions to this study.

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Table -1: Peak assignment for aqueous methanol extracts of *Vernonia amygdalina* leaves.

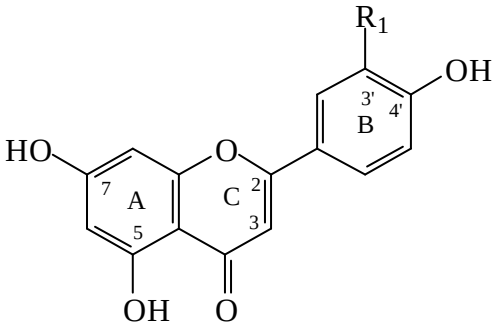
Peak No	t_R-s(min)	t_R-ss (min)	[M-H]$^-$ (*m/z*)	NI, aglycone, and other ions (*m/z*)	UV λ_{max}(nm)*	Identification
1	6.42	10.07	353	191, 179, 135	ud	1-caffeoylquinic acid*
2	7.32	10.07	353	191, 179, 135	ud	3-caffeoylquinic acid*
3	10.62	16.07	353	191, 179, 135	240, 300sh, 326	chlorogenic acid*
4	12.04	15.20	353	191, 179, 135	ud	4-caffeoylquinic acid*
5	17.52	25.65	515	353,191,179,135	240, 300sh, 326	1,3-dicaffeoylquinic acid*
6	31.22	40.18	515	353,191,179,135	ud	1,4-dicaffeoylquinic acid*
7	31.22	41.50	515	353,191,179,135	240, 300sh, 326	3,4-dicaffeoylquinic acid*
8	32.57	42.45	515	353,191,179,135	240, 300sh, 326	1,5-dicaffeoylquinic acid*
9	32.04	43.25	515	353,191,179,135	240, 300sh, 326	3,5-dicaffeoylquinic acid*
10	35.51	45.65	515	353,191,179,135	240, 300sh, 326	4,5-dicaffeoylquinic acid*
11	40.67	49.97	677	515,353,191,179	240, 300sh, 326	1,3,5-tricaffeoylquinic acid
12	42.10	50.97	677	515,353,191,179	240, 300sh, 326	1,4,5-tricaffeoylquinic acid*
13	47.72	54.20	691	529,515,353,191,179	ud	feruloyldicaffeoylquinic acid
14	48.46	53.96	677	515,353,191,179	240, 300sh, 326	3,4,5-tricaffeoylquinic acid*

- s= Symmetry column; ss= SymmetryShield column; ud=undetected
- *****directly checked with standard or reference compound

Table 2: Peak assignment for aqueous methanol extracts of *Russelia equisetiformis* whole plant.

Peak No.	t_R (min)	$[M+H]^+$/ $[M-H]^-$ (*m/z*)	PI/NI, aglycone, and other ions (*m/z*)	UV λ_{max}(nm)*	Identification
1	7.43	---/487	---/191, 179, 135	240, 300sh, 326	caffeoyl acid- rutinoside
2	15.18	595/593	577, 475, 355/---	ud	6,8-diglucosylapigenin*
3	18.52	---/639	ud	242, 300sh, 324	un-identified phenylethanoid
4	19.05	---/639	ud	242, 300sh, 324	un-identified phenylethanoid
5	19.46	565/563	547, 475, 445/---	ud	6-arabinosyl-8-glucosylapigenin*
6	21.28	449/447	329/327	360, 270, 348	orientin*
7	21.94	---/639	ud	ud	un-identified phenylethanoid
8	23.40	---/639	ud	ud	un-identified phenylethanoid
9	24.97	433/431	313/311	268, 332	vitexin*
10	26.94	799/797	287/---	268, 334	un-identified flavonoid
11	27.50	---/623	ud	240sh, 300sh, 328	verbascoside*
12	29.55	463/461	287/285	256, 266, 344	luteolin 7-*O*-glucuronide*
13	30.84	---/623	ud	240sh, 300sh, 328	isoverbascoside*
14	33.91	---/637	ud	300sh, 328	un-identified phenylethanoid
15	36.32	447/445	271/269	268, 336	apigenin *O*-glcuronide
16	42.25	813/811	ud	240sh, 300sh, 328	ud-identified
17	50.26	461/459	285/283	268, 334	hydroxymethoxyflavone glycoside
18	52.44	271/269	ud	268, 336	apigentin

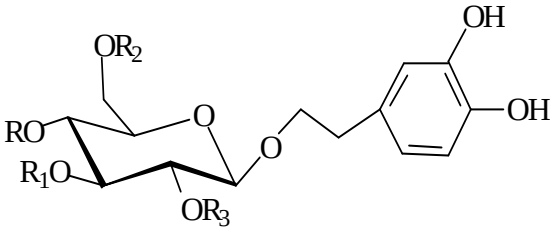
- *****directly checked with standard or reference compound
- ud=undetected



Flavones

Apigenin: R_1=H, MW=270

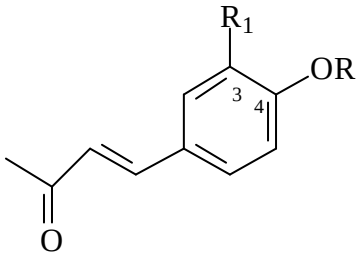
Luteolin: R_1=OH, MW=286



Phenylethanoids

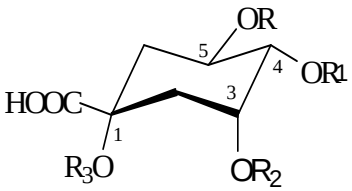
Verbascoside: R=caffeoyl, R_1=rhamnosyl, R_2=R_3=H, MW=624

Isoverbascoside: R_2=caffeoyl, R_1=rhamnosyl, R=R_3=H, MW=624



E-Caffeoyl: R=H, R_1=OH

E-feruloyl: R=H, R_1=OMe



Quinic acid, MW=192

Caffeoylquinic acids

R or R_n=caffeoyl for the position as its name indicates, and the remaining R or R_n=H

Figure- 1: Structures of the phenolic components of the two plants.

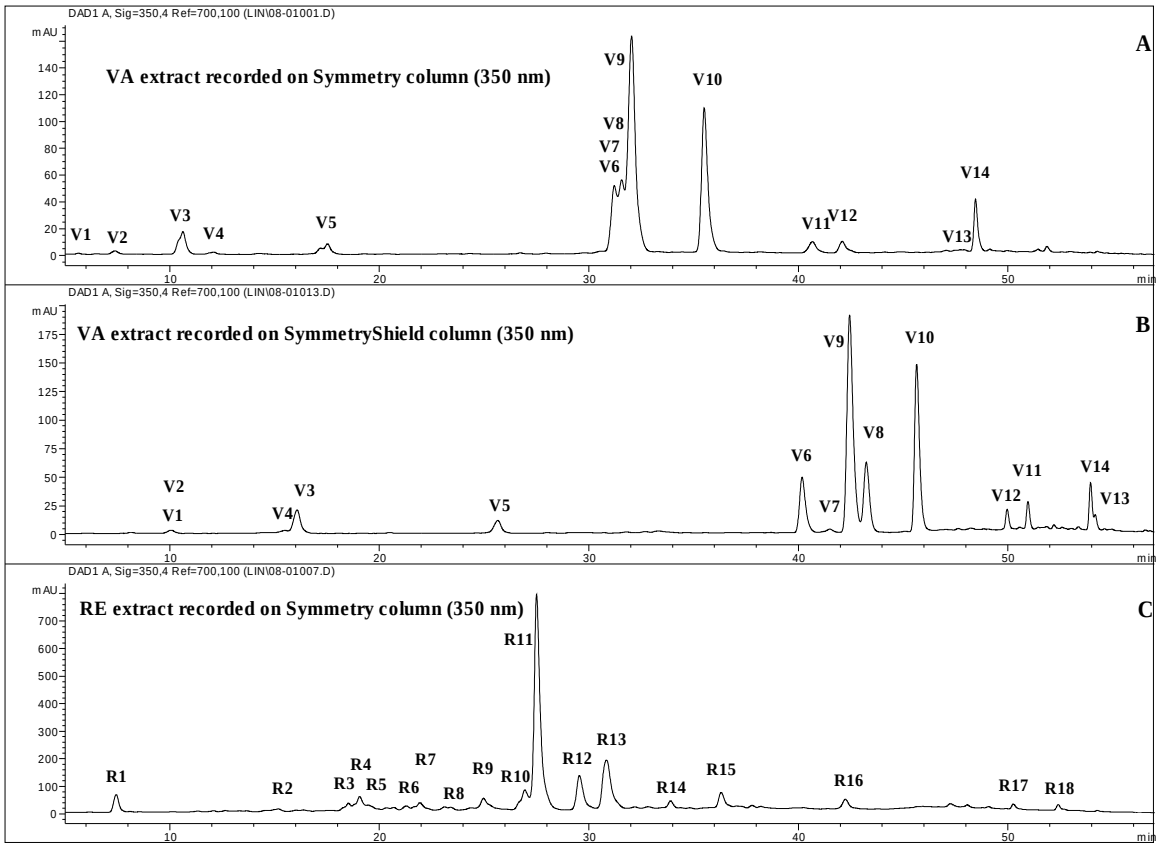


Figure-2: LC chromatograms of the extracts of *Vernonia amygdalina* (VA) leaves and *Russelia equisetiformis* (RE) whole plants. A and C were recorded with the Symmetry column and B was recorded with the SymmetryShield Column. Peak identifications are listed in Tables 1 and 2, respectively.