Journal of Chromatography A, 1217 (2010) 6388–6393

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Comparative study of the flavonoids of some *Verbena* species cultivated in Egypt by using high-performance liquid chromatography coupled with ultraviolet spectroscopy and atmospheric pressure chemical ionization mass spectrometry

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ARTICLE INFO

Article history:
Received 25 April 2010
Received in revised form 5 August 2010
Accepted 6 August 2010
Available online 13 August 2010

Keywords:
Verbenaceae
Flavon aglycones
Flavon glycosides
HPLC
LC–UV/APCI-MS
Shift reagents

ABSTRACT

Verbena rigida L., *Verbena tenera* Spreng. and *Verbena venosa* L. were investigated for their flavonoid content. Analysis was carried out by high-performance liquid chromatography coupled to diode array UV detection (LC–UV), using different techniques, also using post-column addition of shift reagents, afforded precise structural information about the position of the free hydroxyl groups in the flavonoid nucleus. LC–MS using atmospheric pressure chemical ionization (APCI) in the positive mode provided the molecular weight, the number of hydroxyl groups, the number of sugars and an idea about the substitution pattern of the flavonoid. On-line UV and MS data demonstrated the presence of orientin, vitexin, isovitexin, luteolin, luteolin 7-O-glucoside, apigenin 7-O-glucoside in addition to luteolin, chryseriol and apigenin aglycones in the three *Verbena* species with different concentrations. Quantitative determination of flavonoid content revealed the presence of 69.84 mg/g dry sample, 88.26 mg/g dry sample and 85.82 mg/g dry sample total flavonoid compounds in *V. rigida* L., *V. tenera* Spreng. and *V. venosa* L., respectively. The method developed for identification is useful for further chromatographic fingerprinting of plant flavonoids.

1. Introduction

Several species of genus *Verbena* (Verbenaceae) are traditionally used in many countries to treat diarrhea, fever, gastrointestinal disorders and some sexually transmitted diseases [1] and as anti-inflammatory topical applications [2]. Numerous *Verbena* species were previously investigated and found to have different flavonoids [3–9], In addition to phenolic acids [10], iridoids [11–16], phenylethanoids [14,17], phenylpropanoids [15,16], verbenachalcons [18], essential oils [19,20] and triterpenes [21]. Some of these compounds, specially the flavonoids have been shown to exhibit anti-inflammatory and anticancer properties [22–26], as well as antibacterial, antiviral, antiallergic, antithrombotic, vasodilator, antimutagenic and neoplastic [27,28]. Thus development of a method for comprehensive and accurate identification and determination of flavonoids in *Verbena* species would be useful for the study of other functional aspects of this group of compounds. LC hyphenated techniques are playing increasingly important roles in support of phytochemical investigations, such as targeting the isolation of new active compounds, for the dereplication of known plant constituents and for metabolic profiling studies [29,30]. For identification of flavonoids, liquid chromatography/mass spectrometry (LC–MS) was considered as the most powerful approach [31,32]. It is ascribed that LC–MS not only provides the molecular mass of the different constituents but also could be used to differentiate *O*-glycosides, *C*-glycosides and *O*,*C*-glycosides [31–33] as well as, the position of some functional groups such as hydroxyl and carboxylic groups in flavonoids [34–36]. Ionization was achieved through an atmospheric pressure chemical ionization (APCI) interface. APCI is a soft ionization technique which generates mainly protonated molecular ions in the positive mode and deprotenated molecular ions in the negative mode. It is widely applied and especially suitable for relatively small molecules with molecular weights up to 2000 Da and medium polarity, such as polyphenols [37]. HPLC–UV with post-column addition of UV shift reagents provides rapid information about the flavonoid aglycone and its substitution pattern [38]. Shift reagents induce a displacement of the absorption maxima, which can be used to determine the position of free hydroxyl groups [38]. On the other hand, the chromatographic fingerprint have become a pivotal tool in the quality control of complex herbal plants extracts [39].

In this study we attempt to develop a liquid chromatography-diode array detector coupled with atmospheric pressure chemical

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doi:10.1016/j.chroma.2010.08.025

ionization/mass spectrometry (LC–UV/APCI-MS) method using different techniques for identification, quantification and chromatographic fingerprinting of various flavonoids in three different *Verbena* species cultivated in Egypt.

2. Experimental

2.1. Plant material

Verbena rigida L., *Verbena tenera* Spreng. and *Verbena venosa* L. plants were collected from the Garden of Faculty of Agriculture, Al-Azhar University, Cairo (Egypt) at its flowering stage (April 2008). The identification of the plants was kindly done by Dr. Adel Okeal, Director of El-Orman Gardens, Giza, Egypt. A voucher herbarium specimen had been deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt. The collected plants were air-dried, powdered and kept from light in tightly closed amber coloured glass containers at low temperature as possible.

2.2. Chemicals

HPLC grade acetonitrile, methanol and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). HPLC grade water was prepared from distilled water using a Milli-Q plus system (Millipore, Bedford, MA, USA). 0.45 μm PTFE membrane filter was purchased from Water Co. (Bedford, MA). Sodium hydroxide (NaOH), aluminium chloride ($AlCl_3$) and sodium acetate (NaOAc) were purchased from Sigma–Aldrich Chemie (Steinheim, Germany). All laboratory chemicals used in this study were of reagent grade.

2.3. Flavonoid standards

Luteolin 7-O-glucoside and apigenin 7-O-glucoside were from Extrasynthase (Genay Cedex, France), luteolin and apigenin were from Indofine Chemical Co. (Somerville, NJ, USA), vitexin, isovitexin, orientin and chrysoeriol were from Sigma–Aldrich GmbH (Steinheim, Germany). Flavonoid standards were kindly supplied by Dr. Mkrauza Baranowska, Department of Pharmacognosy, Medical University of Gdansk, 80-416 Gdansk-Wrzeszcz, Al. Gen. J. Hallera, 107, 80-416 Gdansk, Poland.

2.4. Sample preparation and extraction

Air-dried powdered whole plants (*V. rigida* L., *V. tenera* Spreng. and *V. venosa* L.), 50 g each, were defatted with petroleum ether. Methanol–water (1:1, v/v) was proved to be the solvent of choice for extraction of flavonoids from several *Verbena* species [2,3,9]. So the defatted powdered *Verbena* species under investigation were extracted with methanol–water (1:1, v/v), filtered, evaporated under reduced pressure at 40 °C in a rotavapor (Rotavapor Büchi RE 111, Büchi 461 waterbath, VB-171 vacobox, Flawil, Switzerland). 50 mg of each extract was dissolved in 2 mL methanol, centrifuged and the supernatants were used for HPLC–UV/MS analysis.

2.5. Sample preparation for quantitative analysis

Samples were extracted as described above for HPLC–UV/MS study but only diode array detection was used. Each of the three extracts was prepared in triplicate and each preparation was analyzed in triplicate.

2.6. LC–UV/APCI-MS analysis

Analyses were performed using a Hewlett-Packard series 1100 system (Waldbronn, Germany) with symmetry C_{18} column (250 mm × 4.6 mm, i.d. 4 μm) and guard column (10 mm × 3.9 mm, i.d. 4 μm) from Waters (Barcelona, Spain), equipped with a vacuum degasser, a binary pump and a photodiode array detector (HP1050), connected to HP ChemStation software (Hewlett-Packard) and APAL autosampler (CTC analytics) controlled by its own software. Eluted with gradient of acetonitrile (solvent B): 0.05% TFA solution in water (solvent A), elution conditions applied were as follows: 0–60 min, linear gradient 5–50% B, 60–70 min, linear gradient 50–75% B, 70–80 min 75–100% B and 80–90 min 100% B isocratic. Flow rate was 0.7 mL/min and injection volume, 50 μl, the system operated at room temperature.

Mass spectra were obtained on a Finnigan-MAT model TSQ 700 (San Jose, CA, USA) triple quadrupole mass spectrophotometer equipped with an APCI interface. LC/APCI-MS analyses were performed in the positive ion mode. The APCI parameters of the source were: capillary temperature, 200 °C; vaporizer temperature, 450 °C; corona needle current, 5 μA; sheath gas, nitrogen; collision gas, helium; collision energy, 50%.

2.7. UV shift reagents

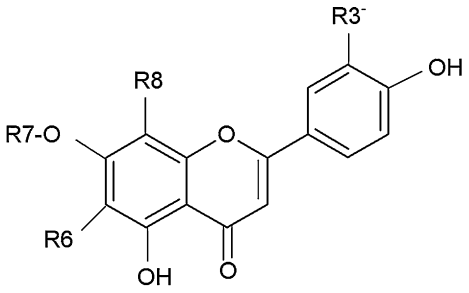
The classical shift reagents were prepared according to the literature [40]. The reagents used in post-column derivatization system were as follows: strong base, aqueous sodium hydroxide (0.01 M); aqueous weak base, 0.5 M aqueous sodium acetate solution (basified with a 0.01 M NaOH solution to pH 8); 0.3 M aqueous aluminium chloride solution (with this reagent, the reaction coil was heated to 90 °C and the eluent was previously neutralized with a 0.02 M NaOH solution); 0.3 M aqueous aluminium chloride solution with 0.1% TFA.

V. venosa L. extract was analyzed after post-column addition of a weak base, $AlCl_3$ and acidic $AlCl_3$. A weak base (sodium acetate) deprotenated only the more acidic phenolic groups; hydroxyl groups in positions 3, 7 and 4^-. Aluminium chloride in neutral solution forms complexes with *ortho*-dihydroxy groups and/or with keto functions having a hydroxyl group in an α- or *peri*-position. The former complexes are unstable in strong acid solutions. UV band shift interpretation took into account Markham's studies on flavonoid compounds [38].

2.8. LC–UV analysis with post-column addition of UV shift reagents

Chromatographic analyses were performed on a Hewlett-Packard Series 1050 system equipped with a quaternary pump, a photodiode array high speed spectrophotometer detector (HP 1050), all controlled by HP ChemStation software. Solvents were degassed on-line with helium. Mobile phase and elution conditions were as under LC–UV/APCI-MS analysis. Flow rate was 0.6 mL/min and injection volume, 50 μl, the system operated at room temperature.

The method used for post-column addition of UV shift reagents was based on the protocol described by Wolfender and Hostettmann [41]. The post-column device consisted of a Gilson 303 pump, for post-column neutralization of eluent; a shimadzu LV-9A pump, for addition of UV shift reagents; Derivatization reactions were performed in a reaction coil (1 m × 0.5 mm, i.d. 0.2 μm). Shift reagents were added to the eluent at a flow rate 0.3 mL/min.



No.	Compound	$R_{3'}$	R_6	R_7	R_8
1	Orientin	OH	H	H	Glucose
2	Vitexin	H	H	H	Glucose
3	Isovitexin	H	Glucose	H	H
4	Luteolin-7-O-glucoside	OH	H	Glucose	H
5	Apigenin-7-O-glucoside	H	H	Glucose	H
6	Luteolin	OH	H	H	H
7	Chrysoeriol	OCH_3	H	H	H
8	Apigenin	H	H	H	H

Fig. 1. Chemical structure of detected flavonoid compounds.

3. Results and discussion

3.1. General aspects

Efficient separation of flavonoids of the extracts of *V. rigida* L., *V. tenera* Spreng. and *V. venosa* L. were achieved with a reversed phase RP-18 column with gradient elution systems consisting of acetonitrile–0.05% trifluoroacetic acid in water for LC/APCI-MS and LC–UV with post-column addition of UV shift reagents. A total of eight flavones (Fig. 1) were identified by the combination of LC/UV and LC/MS data. The chromatographic profiles of the different *Verbena* species under investigation were characteristic (Fig. 2). Photodiode array detection allowed the on-line recording of UV spectra and a rapid attribution of peaks which corresponding to flavonoids in the chromatograms. Fig. 2 revealed that the chromatographic profiles were very similar and the flavonoid constituents of those species are similar, differing in concentration which represented by the peak area of each constituent.

3.2. LC–UV photodiode array detection

All the detected compounds displayed UV spectra characteristic of flavones or flavonols with maximum between 251–271 nm (band II) and 335–350 nm (band I).

3.3. LC–APCI/MS detection

Mass spectra of the flavonoids analysed in *V. rigida* L., *V. tenera* Spreng. and *V. venosa* L. extracts provided data about their molecular weight and their constituents units. APCI mass spectra of flavonoids in the positive ion mode usually show a weak protonated molecular ion $[M+H]^+$ and a main peak corresponding to the protonated aglycone moiety $[A+H]^+$. The mass difference between $[M+H]^+$ and the protonated aglycone $[A+H]^+$ ions gives important information on the nature of the sugar or acid moiety [42,43]. In Table 1 mass spectral data of the flavonoids detected in *Verbena* species extracts are summarized.

MS spectra of compounds **1**–**3** displayed fragment ions with loss of 90 and 120 characteristic of *C*-glycoside derivatives. While MS spectra of compound **4** and **5** showed fragment ions at *m*/*z* 287 $[M-162+H]^+$ and 271 $[M-162+H]^+$ respectively, indicating the loss of hexosyl or uronic acid residues which were characteristic of flavonoid *O*-glycosides.

MS spectra of compounds **6**–**8** showed protonated molecules with 100% relative abundance at *m*/*z* 287 $[M+H]^+$, 301 $[M+H]^+$ and 271 $[M+H]^+$, respectively, revealing the presence of flavonoid aglycones. The fragment ion at *m*/*z* 286 $[M-15+H]^+$ observed in MS spectra of compound **7** was produced by the loss of methyl group.

Table 1
Results of LC–ACPI/MS of flavonoid compounds.

No.	MS *m*/*z* (%)
1	329 (100), 359 (38), 395 (13), 413 (23), 449 (71, $M+H)^+$
2	313 (100), 343 (13), 379 (13), 397 (8), 415 (16), 433 (36, $M+H)^+$
3	313 (100), 343 (13), 379 (13), 397 (8), 415 (16), 433 (36, $M+H)^+$.
4	287 (100), 449 (47, $M+H)^+$
5	271 (100), 443 (70, $M+H)^+$
6	287 (100, $M+H)^+$
7	286 (18), 301 (100, $M+H)^+$
8	271 (100, $M+H)^+$

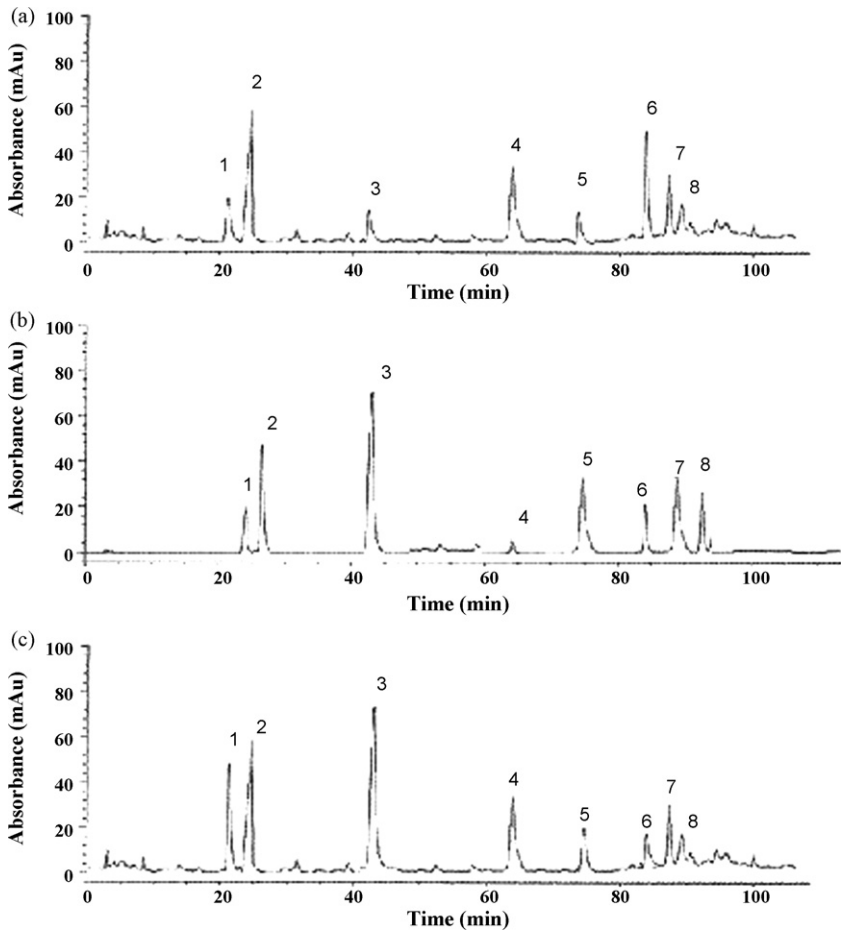


Fig. 2. HPLC–UV chromatograms of flavonoids extracts of *Verbena rigida* L. (a), *Verbena tenera* Spreng. (b) and *Verbena venosa* L. (c) at 350 nm. Chromatographic conditions: column symmetry C_{18} (250 mm × 4.6 mm, i.d.; 4 μm); linear gradient of acetonitrile (solvent B), 0.05% TFA solution in water (solvent A), 0–60 min: linear gradient 5–50% B, 60–70 min: 50–75% B, 70–80 min: 75–100% B and 80–90 min: 100% B isocratic, at a flow rate of 0.7 mL/min.

3.4. LC–UV photodiode array detection with post-column addition of shift reagents

The LC/UV data (Table 2 and Fig. 3) in combination with shift reagents added post-column allows the determination of the hydroxylation pattern and the position of the sugar or other component linked on the aglycone. A strong base reacts with all the phenolic groups except those in the *peri* position to the keto function. Al^{3+} forms a complex with *ortho*-dihydroxy groups and keto groups having a hydroxyl in the *peri* position. NaOAc deprotonated the more acidic phenolic groups (3,7 and 4⁻-OH groups) affecting mainly the UV band I; the shift of band II shows the presence of a free –OH group at position 7, and this 7-OH group influences the shift and the presence of a shoulder on band I, which indicates a free –OH group at position 3⁻ or/and 4⁻. Analyses and measurements of UV spectra in HPLC with post-column addition of reagents are normally performed in methanol [43,44]. However, the conditions described here involved HPLC with acetonitrile–water system. Therefore, the shift reagents were tested on different reference flavones under the same chromatographic conditions.

Bathochromic shift of band I (28–45 nm) with acidic $AlCl_3$ showed the presence of a hydroxyl group at C-5 in all detected flavonoids, Moreover, bathochromic shift of band I obtained after addition of NaOH revealed the presence of free hydroxyl group at C-4⁻ for all the detected flavonoids. Further bathochromic shift of band I was observed upon addition of $AlCl_3$ relative to that with $AlCl_3/H^+$ indicated the presence of *ortho*-dihydroxy groups in ring B (C-3⁻ and C-4⁻) in compounds **1**, **4** and **6**. The absence of UV shift

Table 2
UV data of *Verbena venosa* L. flavonoids.

No.	UV spectra (nm)	Shifted UV spectra (nm)			
	CH_3OH	NaOH	$AlCl_3$	$AlCl_3/H^+$	NaOAc
1	255, 270, 353	274, 414	276, 427	278, 362, 389	275, 390
2	272, 334	281, 332 sh, 391	278, 305, 340, 385	278, 305, 340, 385	281, 305 sh, 391
3	272, 332	281, 333 sh, 399	278, 305, 340, 385	278, 305, 340, 385	281, 305 sh, 391
4	225, 267, 346	264, 300, 398	272, 300, 330,430	271, 294 sh, 361, 388	259, 265, 360, 398
5	268, 332	266, 300 sh, 388	274, 298, 347, 379	275, 297, 340, 377	266, 390
6	253, 268, 290 sh, 348	265, 330 sh, 402	274, 300 sh, 329 sh, 425	275, 296, 356, 388	271, 325 sh, 390
7	255, 270, 345	264, 275 sh, 326, 406	262, 297, 362 sh, 389	276, 296, 354, 390	266, 275 sh, 325, 402
8	268, 335	276, 325, 393	276, 302, 346, 383	277, 301, 343, 382	275, 382

sh: shoulder.

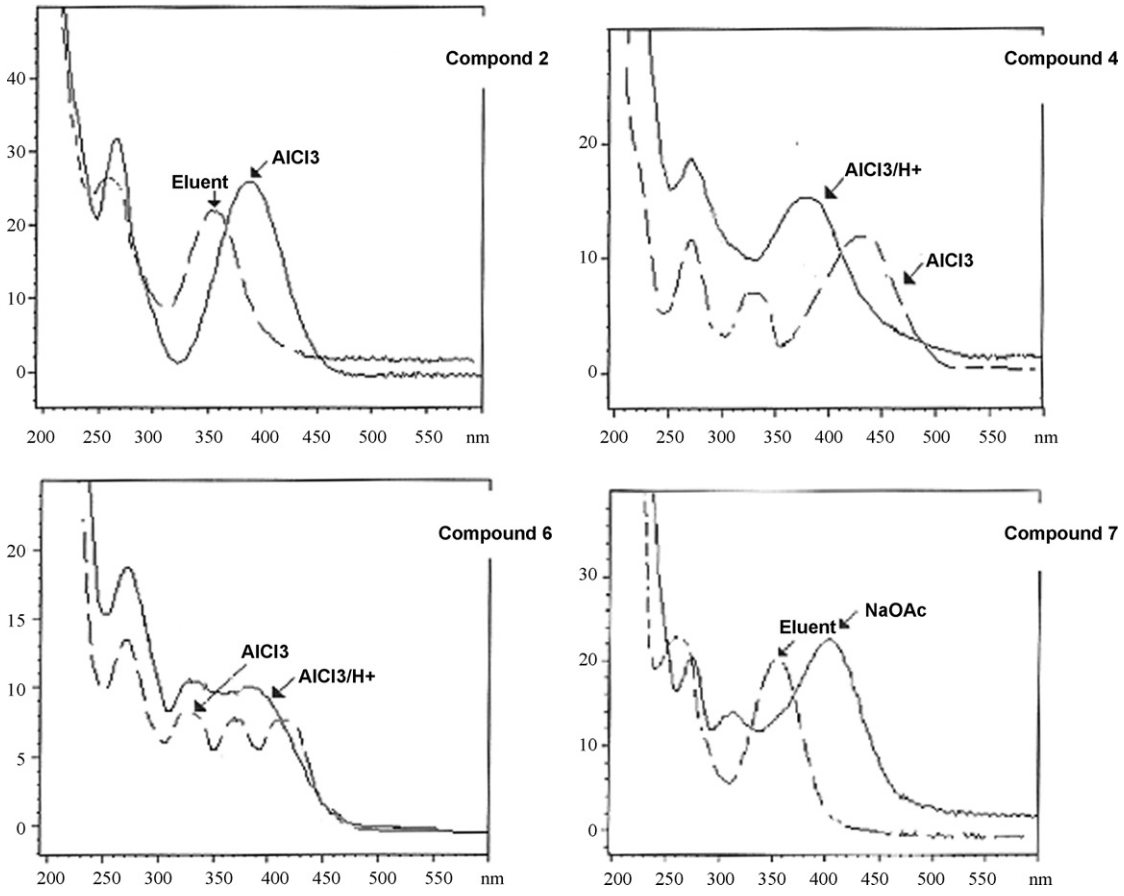


Fig. 3. Representative UV spectra obtained after post-column addition of shift reagents on compounds **2**, **4**, **6** and **7**.

in band II after addition of NaOAc of compounds **4** and **5** indicated the glycosidation at C-7, in contrast, a bathochromic shift of band II was observed in UV spectra of compounds **1**–**3** and **6**–**8** indicating a free hydroxyl group at C-7.

3.5. On-line structure information

LC/APCI-MS and LC–UV together with LC–UV with post-column addition of shift reagents provide a powerful tool for flavonoid analysis in plant extracts. Regarding the results obtained by the on-line analysis of the three *Verbena* extracts by means of these techniques and by comparing retention times and UV–vis spectra with standards. HPLC–UV/APCI-MS provided the molecular mass, the number of hydroxyl groups, the number of sugars and an idea about the substitution pattern of flavonoids found in the extracts of *Verbena* species under investigation. HPLC–UV with post-column addition of shift reagents gave more precise structural information on the position of the free hydroxyl groups in the flavonoid nucleus.

Compound (**1**) showed *m*/*z* 449 $[M+H]^+$ and 329 $[M+H-120]^+$, indicating *C*-glycoside with molecular weight 448 and one hexosyl moiety. A bathochromic shift with $AlCl_3/H^+$ relative to that with $AlCl_3$ indicated 3^-, 4^- dihydroxy substitution in ring B and C-5 OH group, free OH group at C-7 was proved by the bathochromic shift in band II with NaOAc.

Compounds (**2** and **3**) showed *m*/*z* 433 $[M+H]^+$ and 313 $[M+H-120]^+$, indicating *C*-glycosides with molecular weight 432 and one hexosyl moiety. A bathochromic shift with $AlCl_3$ similar to that with $AlCl_3/H^+$ indicated the presence of C-5 OH group and absence of O-dihydroxy groups in ring B.

Compound (**4**) showed *m*/*z* 449 $[M+H]^+$ and 287 $[M-162+H]^+$, indicating *O*-glycoside with molecular weight 448 and one hexosyl moiety. A bathochromic shift with $AlCl_3/H^+$ relative to that with $AlCl_3$ indicated 3^-, 4^- dihydroxy substitution in ring B and C-5 OH group, no bathochromic shift in band II with NaOAc proved the absence of free OH group at C-7.

Compound (**5**) showed *m*/*z* 443 $[M+H]^+$ and 271 $[M-162+H]^+$ (100%), indicating *O*-glycoside with molecular weight 442 and one hexosyl moiety. A bathochromic shift with $AlCl_3/H^+$ equivalent to that with $AlCl_3$ indicated absence of 3^-, 4^- dihydroxy substitution in ring B and occurrence of C-5 OH group, no bathochromic shift in band II with NaOAc proved the absence of free OH group at C-7.

Compound (**6**) showed *m*/*z* 287 $[M+H]^+$ (100%), indicating free aglycone with molecular weight 286. A bathochromic shift with $AlCl_3/H^+$ relative to that with $AlCl_3$ indicated 3^-, 4^- dihydroxy substitution in ring B and C-5 OH group, a bathochromic shift in band II with NaOAc proved the occurrence of free OH group at C-7.

Compound (**7**) showed *m*/*z* 301 $[M+H]^+$ (100%) and 286 $[M-CH_3+H]^+$ (18%), indicating free monomethoxylated aglycone with molecular weight 300. A bathochromic shift with $AlCl_3/H^+$ equivalent to that with $AlCl_3$ indicated absence of 3^-, 4^- dihydroxy substitution in ring B and occurrence of C-5 OH group, a bathochromic shift in band II with NaOAc proved the presence of free OH group at C-7.

Compound (**8**) showed *m*/*z* 271 $[M+H]^+$ (100%), indicating free aglycone with molecular weight 270. A bathochromic shift with $AlCl_3/H^+$ equal to that with $AlCl_3$ indicated absence of 3^-, 4^- dihydroxy substitution in ring B and occurrence of C-5 OH group, a bathochromic shift in band II with NaOAc proved the presence of free OH group at C-7.

Depending on the previous data and in accord with their molecular weights, the fragments observed in their mass spectra and their retention times, eight flavonoid compounds detected in the

Table 3
Results of quantitative analysis of flavonoid content of *Verbena rigida* L., *Verbena tenera* Spreng. and *Verbena venosa* L. extracts.

No.	Compound	Concentration (mg/g dry sample)		
		Verbena rigida L.	*Verbena tenera* Spreng.	*Verbena venosa* L.
1	Orientin	3.44 ± 0.67	3.96 ± 1.03	9.16 ± 0.3
2	Vitexin	22.70 ± 0.24	18.59 ± 0.86	20.86 ± 1.24
3	Isovitexin	2.05 ± 0.65	20.50 ± 0.39	18.84 ± 0.86
4	Luteolin-7-O-glucoside	15.43 ± 0.25	2.57 ± 0.28	16.20 ± 0.38
5	Apigenin-7-O-glucoside	3.36 ± 0.94	16.85 ± 0.07	4.23 ± 1.31
6	Luteolin	10.24 ± 0.64	4.52 ± 0.36	3.81 ± 1.03
7	Chrysoeriol	8.24 ± 0.82	14.85 ± 0.31	7.96 ± 0.48
8	Apigenin	4.38 ± 0.77	6.42 ± 0.85	4.76 ± 0.62
	Total	69.84 ± 0.32	88.26 ± 0.15	85.82 ± 0.36

Average (mean) content ± standard deviation of triplicate.

extracts of *Verbena* species under investigation could be identified as, orientin (**1**), vitexin (**2**), isovitexin (**3**), luteolin 7-O-glucoside (**4**), apigenin 7-O-glucoside (**5**), luteolin (**6**), chrysoeriol (**7**) and apigenin (**8**), which were previously detected in several *Verbena* species. El-Hela et al. [3] have isolated orientin, vitexin and isovitexin from *Verbena bonariensis*, while luteolin 7-O-glucoside and apigenin 7-O-glucoside were detected in *Verbena officinalis* and *V. supine* [6], as well as, luteolin, chrysoeriol and apigenin aglycones were isolated from *V. officinalis* [7].

3.6. Quantification of flavonoids in three Verbena species

Each of the three samples was prepared in triplicate and each preparation was analyzed in triplicate. Standard flavonoids were used for the calibration.

Quantitative determination of flavonoid content in *Verbena* species under investigation (Table 3) revealed the presence of 69.84 mg/g dry sample, 88.26 mg/g dry sample and 85.82 mg/g dry sample total flavonoid compounds in *V. rigida* L., *V. tenera* Spreng. and *V. venosa* L., respectively. Vitexin (22.70 and 20.86 mg/g dry sample) was the major flavonoid detected in *V. rigida* L. and *V. venosa* L., respectively, followed by luteolin-7-O-glucoside (15.43 mg/g dry sample) in *V. rigida* L. and isovitexin (18.84 mg/g dry sample) in *V. venosa* L., isovitexin (20.50 mg/g dry sample) was the predominant flavonoid compound in *V. tenera* Spreng. followed by vitexin (18.59 mg/g dry sample).

4. Conclusion

The combination of data obtained by LC/UV with shift reagents and LC/techniques allowed the on-line structure elucidation of eight flavone compounds, as well as, their quantitative determination in three *Verbena* species. This study proved the presence of orientin, vitexin, isovitexin, luteolin 7-O-glucoside, apigenin 7-O-glucoside in addition to luteolin, chryseriol and apigenin aglycones, in *V. venosa* L., *V. tenera* Spreng. and *V. rigida* L. with different concentrations. Although these flavonoids were detected in several *Verbena* species, they are detected for the first time in *V. venosa* L., *V. tenera* Spreng. and *V. rigida* L. plants.

In this study, we attempt to develop a liquid chromatography–diode array detector–atmospheric pressure chemical ionization/mass spectroscopy (LC/UV–APCI/MS) method for comprehensive identification and characterization of the flavonoids in *Verbena* species. The proposed method and flavonoids detected are useful for further chromatographic fingerprinting of plant flavonoids.

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