Chemical composition, mineral content and antioxidant activity of *Verbena officinalis* L

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Abstract

Aqueous and hydroalcoholic extracts from *Verbena officinalis* L were obtained and characterized. The analysis by HPLC-DAD and LC-MS allowed the detection and identification of three iridoids, fifteen flavonoids and four phenolic acid derivatives. Four flavonoids, scutellarein 7-diglucuronide (**9**), scutellarein 7-glucuronide (**13**), pedalitin 6-galactoside **(15**) and scutellarein 7-glucoside (**19**) are reported for the first time from this plant. In addition, three new flavonoids have been isolated: scutellarein 7-*O*-(2-*O*-feruloyl)-diglucuronide (**5),** pedalitin 6-*O*-diglucuronide (**6**) and pedalitin 6-*O*-(2-*O*-feruloyl)-diglucuronide (**13**). To our knowledge, these flavonoids have not been reported as natural products. Both extracts showed significant antioxidant activity using three *in vitro* model systems and the results have been correlated with total phenolic and total flavonoid contents. The results have allowed establishing an important relation structure-activity and significant correlations have also been found between the mineral content and the flavonoids present in both extracts.

Keywords:

Verbena officinalis; flavonoid; phenolic acid; antioxidant activity; mineral composition

1. Introduction

There is nowadays a growing interest in searching natural antioxidants for three main reasons: (i) numerous clinical and epidemiological studies have demonstrated that consumption of fruits and vegetables rich in this type of compounds is associated with reduced risks of developing chronic diseases such as cancer, cardiovascular disorders and diabetes; (ii) safety consideration regarding the potential harmful effects of the chronic consumption of synthetic antioxidants, such as butylhydroxyanisole and butylhydroxytoluene, in foods and beverages; and (iii) the public's perception that natural and dietary antioxidant are safer than synthetic analogues (Dastmalchi, Dorman, Kos-ar, & Hiltunen, 2007). Therefore, the food industry is making a great effort to find out new sources of safe and inexpensive antioxidants of natural origin.

Verbena officinalis L. grows in all temperature regions of the globe and is cited in the traditional medicine of many countries. It has been one of the most mentioned plants in a recent ethnobotanic study carried out in Navarre (northern Iberian region) (Akerreta, Cavero, & Calvo, 2007) in which 108 informants (20% of the total) have indicated 175 different uses belonging to 9 therapeutic categories. It is also listed in the Chinese Pharmacopoeia and the British Herbal Pharmacopoeia. Several scientific studies have demonstrated the anti-inflammatory (Calvo, San Julián, & Fernández, 1998; Deepak & Handa, 2000), antibacterial (Hernández, Tereschuk, & Abdala, 2000), neuroprotective (Lai, Yu, Yuen, & Chang, 2006), analgesic (Calvo, 2006), antifungal and antioxidant (Bilia et al., 2008; Casanova, Garcí-Mina, & Calvo, 2008) properties of this plant.

The main components of *Verbena officinalis* L. are iridoids, phenylpropanoids, flavonoids, luteolin and terpenoids (Calvo, San Julián, & Fernández, 1997; Deepak & Handa, 2000; Bilia et al., 2008).

From the nutritional point of view, other valuable components in herbs are also contained in considerable amounts. It is quite well understood that mineral constituents, particularly macrominerals and trace elements perform an essential role for the activation of enzymatic systems or the involvement in the metabolism of biomolecules. The presence of these micronutrients in plants is largely dependent on

growing conditions including cultivation techniques, abiotic or biotic stress and nutrient status. In fact, it has been established that deficiency of several minerals (nitrogen, phosphorous, boron and manganese) induce flavonoids accumulation, whereas potassium, calcium, cobalt and nickel supplementation increase flavonoid production and as a consequence, antioxidant enzymatic activity (Dixon & Paiva, 1995; Stewart et al., 2001; Vyn et al., 2002; Scheible et al., 2004; Lillo et al., 2007; Eman et al., 2007; Jayakumar et al., 2007; Kováčik & Bačkor, 2007; Whittaker et al., 2009).

In the course of our interest in chemical and biological investigations of *Verbena officinalis*, this study reports a total phytochemical characterization (major and minor compounds), mineral composition, and antioxidant activity of the polar extracts obtained from *Verbena officinalis.* As a result, four flavonoids and two phenolic acid derivatives are reported for the first time from this plant, and three new flavonoids have been isolated and structurally characterised using NMR and MS methods. Furthermore, correlations between mineral content and flavonoids content are evaluated.

2. Materials and methods

2.1. Chemicals

All chemicals and solvents, such as DPPH, xantine, ABTS, BHA, BHT, methanol, ethanol, ethyl acetate, were of the highest analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Verbenalin (**2**), aucubin (**8**), luteolin 7-O-glucoside (**14**), apigenin 7-O-glucoside (**18**), verbascoside (**20**) and apigenin (**22**) were supplied by Extrasynthese (Genay Cedex, France). Hastatoside (**1**), luteolin 7-O-diglucuronide (**3**), 1,5-dicaffeoylquinic acid (**10**), 4,5-dicaffeoylquinic acid (**11**), luteolin 7-O-glucuronide (**12**), pedalitin 6-O-glucoside (**16**), apigenin 7-O-galactoside (**17**) and isoverbascoside (**21**) were previously isolated in our laboratory. Sephadex LH-20 (Sigma) was used for all column chromatography separations (CC), while silica gel 60 PF_{254} (Merck) was used for analytical (0.50 mm) and preparative TLC (1.0 mm). Mineral standard solutions (1000 mg L-1) were supplied by Merck (Darmstad, Germany).

2.2. Preparation of extracts

50% ethanolic (HAE) and aqueous (AQ) extracts of dry aerial parts from *Verbena officinalis* were prepared according to the published procedure (García-Íñiguez de Cirano et al., 2010). Both extracts presented a very similar percentage of extraction (HAE: 11.92%; AQE: 11.48% dry weight base).

2.3. Fractionation of extracts and isolation

A multi-step fractionation procedure was carried out in order to separate the different constituents of the extracts. The stationary phase bed was firstly prepared by equilibrating Sephadex LH-20 beads for at least 24 h in 50% ethanol. After transferring the slurry to the column (glass, Afora, 55×3.5 cm) the bed was allowed to settle during 2 h. The same column was used to separate both extracts.

In the case of 50% ethanolic extract, 3.5 g was dissolved in 2 mL of 50% ethanol and chromatographied, with 50% ethanol as mobile phase, yielding five fractions (HAE1: 0.58 g; HAE2: 1.68 g; HAE3: 0.20 g; HAE4: 0.3 g; HA5: 0.45 g). Fraction HAE2 was further loaded on a column of silica gel and eluted with $CHCl_3$–MeOH (95:5 (100 mL); 70:30 (300 mL); 55:45 (250 mL)) to give five fractions (HAE2.1 to HAE2.5). Fraction HAE2.2 was separated by Sephadex LH-20 CC with 70% methanol to give compound **3** (13 mg) and compound **7** (9 mg). Fraction HAE3 was fractionated by silica gel CC $CHCl_3$–MeOH (9:1 (100 mL); 7:3 (100 mL); 5:5 (100 mL); 3:7 (50 mL); 1:9 (250 mL)) to give six fractions (HAE3.1 to HAE3.6). Fraction HAE3.3 was separated by Sephadex LH-20 CC with 80% MeOH to afford compound **6** (22 mg) and compound **7** (18 mg). Fraction HAE3.4 was separated by Sephadex LH-20 CC with 50% MeOH to afford compound **13** (16 mg), compound **14** (21 mg) and compound **16** (16 mg). Similar purification of fraction HA3.5 yielded compound **14** (9 mg) and **20** (11 mg). Purification of fraction HAE4 on prep. TLC using mobile phase EtOAc-MeOH-H_2O (65:15:5) yielded **20** (18 mg) and **21** (7 mg).

In the case of the aqueous extract (AQ), 3.5 g was dissolved in 2 mL of 50% ethanol and elution was carried out with the same solvent until no more components were detected. Six fractions were collected (AQ1: 2.35 g; AQ2: 0.17 g; AQ3: 0.03 g; AQ4: 0.02 g; AQ5: 0.6 g; AQ6: 0.02 g). Fractions AQ1 and AQ2 were chromatographied over Sephadex LH-20 column with methanol to afford compound **12** (25 mg) and **17** (13 mg). Fraction AQ3 was chromatographied over Sephadex LH-20 column with methanol

80% to afford compound **15** (7 mg) and **19** (13 mg). Separation of fraction AQ5 through silica gel column (i.d. 2.5×30 cm) with a gradient $CHCl_3$–MeOH ((9:1 (100 mL); 7:3 (100 mL); 5:5 (100 mL); 3:7 (50 mL); 1:9 (250 mL)), afforded four subfractions (AQ5.1→AQ5.4). Purification of subfractions AQ5.3 and AQ5.4 on prep. TLC using mobile phase MeOH–EtOAc (1:1) yielded **4** (11 mg) and **5** (12.1 mg), respectively.

The purity of peaks, >95%, was checked by a Diode Array Detector coupled to the HPLC system. Identification of all constituents was performed by HPLC-DAD and LC-MS comparing the retention time, UV and MS spectra of the peaks with authentic reference sample or data reported in the literature, acid or basic hydrolysis and NMR analysis.

2.4. HPLC-DAD and HPLC-MS analysis

The extracts and fractions were analyzed by HPLC using a Waters (Milford, MA, USA) 600E multi-solvent delivery system, a Waters U6K sampler and a Waters 991 photodiode-array detector. Chromatography was performed on a C18 reversed-phase column (Nova-Pak, 150 mm x 3.9 mm., 4 μm, Waters) at 25ºC. Detection was a range between 210 and 500 nm. The dried sample (10 mg) was dissolved in 1 mL of a corresponding solvent using subsonic bath followed by filtration of the solution over a Millipore® filtration unit type HV 0.45 μm. The gradient was formed by varying the proportion of acetonitrile (A) and twice distilled water adjusted to pH 3 with phosphoric acid (B). The elution system was: 0-0.6 min, 93-90% of B; 0.6-5 min, 90% of B; 5-8 min, 90-85% of B; 8-13 min, 85% of B; and 13-30 min, 85-40% of B. The flow rate employed was 1.5 mL/min throughout the run. The injection volume was 10 μl (Calvo, San Julián, & Fernández (1997)).

HPLC-MS analysis was performed using a HP 1100L liquid chromatgraph linked to a HP 100 MSD mass spectrometer with a API/electrospray interface (Agilent Technologies, Palo Alto, CA, USA). The column, time period and flow rate were similar to those used during the HPLC-DAD analysis. The mass spectrometer operating conditions were: gas temperature, 350 ºC; nitrogen flow rate, 10 L/min; nebulizer pressure 30 psi; quadrupole temperature, 25 ºC; capillary voltage, 3500 V. The mass spectrometer was operated in positive and negative mode at 12 eV.

2.5. NMR analysis

^{1}H and ^{13}C NMR spectra were measured on a Bruker Avance 300 at 300 MHz and 75 MHz, respectively, with TMS as an internal standard and $CDCl_3$ (Aldrich, CA, USA) as solvent. Chemical shifts were reported in δ units (ppm) and coupling constants (J) in Hz. 2D experiments ^{1}H-^{1}H double quantum filtered-direct chemical shift correlation spectroscopy (DQF-COSY), inverse detected ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond connectivity (HMBC) were obtained using UX-NMR software.

2.6. Determination of total phenol content (TPC) and total flavonoid content (TFC)

Total phenolics composition of samples was quantified spectrophotometrically by the Price method (Prince & Butler, 1977) with slight modifications, using gallic acid as standard (Uriarte & Calvo, 2009).

Total flavonoids were estimated as rutin equivalents per gram of dry weight plant. The assay was carried out using 96 well plates and absorbance was recorded in the Power Wave XS Microplate Reader (KcJunior Biotek program) at 25 ºC. 1 mL of sample in methanol (10 g/L) was mixed with 1 mL aluminium trichloride in ethanol (20 g/L) and diluted with methanol to 25 mL. After 30 min at 25 ºC, the absorption was read at 415 nm. Blank samples were prepared from 1 mL samples and diluted to 25 mL. The rutin calibration curve was prepared in methanolic solutions using the same procedure. The concentrations of the samples were adjusted taking into account their total flavonoid content in order to obtain comparable results in the subsequent experiments. All determinations were carried out in triplicate and the mean values were used.

2.7. Antioxidant assessment

Three different methods were used for the evaluation of the antioxidant activity of the extracts: DPPH radical-scavenging assay (López et al., 2008), 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid ($ABTS^{\bullet+}$) assay (adapted from Re et al. (1999) reported in López et al. (2010)) and superoxide radical-scavenging activity (enzymatic assay) systems (López et al., 2010).. The results were compared with BHT

and BHA, two of the most usually used synthetic antioxidant. The antioxidant activity was expressed as IC50 values, which were calculated by non-linear regression with a one phase exponential association equation using GraphPad Prism version 4.0.

2.8. Mineral and trace element analysis

A Perkin-Elmer Model AAnalyst 800 atomic absorption spectrometer equipped with flame and graphite furnace atomizers with auto sampler AS90 was used (Perkin-Elmer, Norwalk, CT, USA). Appropriate working standard solution was prepared for each element. The calibration curves were obtained for concentration vs. absorbance. The data were statistically analyzed by using fitting of straight line by least square method. The analytical procedure for mineral and trace elements determination in extracts samples was performed according to Ražić, Onjia, & Potkonjak (2003).

2.9. Statiscal analysis

Results were expressed as mean ± standard error. Correlations between total phenol contents, total flavonoid contents, mineral content and antioxidant activity were established by regression analysis.

3. Results and discussion

3.1. Analysis of extracts and fractions by HPLC-DAD-MS

The solubility profiles of the hydroalcoholic and aqueous extract were different, as checked by HPLC-DAD (Figure 1). However, in both extracts the main detected constituents belong to three classes of natural compounds, iridoids, flavonoids and phenolic acid, in agreement with literature data (Bilia et al., 2008; Calvo, San Julián, & Fernández, 1997). HAE and AQ extracts were fractionated by applying them to a Shephadex column chromatography and eluting with 50% EtOH as mobile phase. The analysis of all the fractions by HPLC-DAD and LC-MS allowed the detection of 22 compounds, 3 iridoids (**1**, **2**, **8**), 15 flavonoids (**3**-**7**, **9**, **12**-**19, 22**) y 4 phenolic acid derivatives (**10**, **11, 20, 21**) (Table 1).

3.2. Identification of iridoids, phenolic acids and flavonoids

The identification of the three iridoids hastatoside (**1**), verbenalin (**2**) and aucubin (**8**), was carried out by comparison with reference substances, UV spectra and mass analyses in negative and positive mode (Bilia et al., 2008).

Phenolic acid derivatives (λ_{max} = 325 nm) were also found and amongst them, verbascoside (**20**) was the most abundant in both extracts. The compound **21** was also identified as isoverbascoside. From the fraction HAE4 we had isolated and identified 1',5'-*O*-dicaffeoylquinic acid (**10**) and 4',5'-*O*-dicaffeoylquinic acid (**11**), present in very low proportion.

The 15 identified flavonoids were luteolin (**3**, **4**, **12, 14**), apigenin (**5**, **7**, **17, 18**, **22**), scutellarein (**9**, **13**, **19**) and pedalitin (**6**, **15**, **16**) derivatives.

Flavonoids luteolin 7-diglucuronide (**3**), apigenin 7-diglucuronide (**7**), luteolin 7-glucuronide (**12**), luteolin 7-glucoside (**14**), apigenin 7-galactoside (**17**), apigenin 7-glucoside (**18**), apigenin (**22**) and pedalitin 6-glucoside (**16**) have been previously reported in *Verbena officinalis* (Calvo, San Julián, & Fernández, 1997; Bilia et al., 2008). Nevertheless, scutellarein 7-diglucuronide (**9**), scutellarein 7-glucuronide (**13**), pedalitin 6-galactoside (**15**) and scutellarein 7-glucoside (**19**) have been described in other species but there are no previous reports on the isolation of these flavonoids from *Verbena officinalis* (Moore, Williams, & Yates, 1973; Rao & Rao, 1980; Harborne, 1993; Meng et al., 2006). The identification of these compounds was carried out by comparison with reference substances, UV spectra and mass analyses in negative and positive mode (Table 1).

After the separation and identification of the total components of verbena, we isolated flavonoid **6**, a finely crystalline light yellow powder, mp 184-186 ºC (dec.), $[\alpha]_D^{20}$ -32.5.º(*c* 0.05, MeOH), *m/z* = 668.1 $[M]^+$.

The UV spectrum of **6** displayed two strong absorption bands at 280 nm and 343 nm, typical of flavones with -OH group in C-6. Analysis of the ^{1}H-MNR date (Table 2) revealed characteristic resonances of aromatic and glycosidic protons (including two anomeric protons) and one methoxy group. These include the C-3 proton that was observed as a singlet at *δ* 6.67 ppm, the chelate hydroxyl (5-OH) at *δ* 13.0 ppm, and two hydroxyls groups at *δ* 9.88 and 9.37 ppm (4'-OH and 3'-OH), respectively (Table 2). The singlet at *δ* 6.79 was assigned to H-8 of the flavone A-ring on the basis of its characteristic ^{13}C NMR

resonance at δ 96.5. The position of the methoxy group was assigned at C-7 on the basis of observed gHMBC (Figure 2) and NOESY correlations The NOESY ^{1}D spectrum showed a strong interaction between the methoxy proton signal at δ 3.88 and H-8 where as a gHMBC correlation was observed between the signals at δ 161.0 (C-7) and δ 3.88, which confirmed the position of the methoxy group at C-7. Signals at δ 5.44 and 5.18 ppm were consistent with the presence of two anomeric protons. The absorption band at 1740 cm^{-1} in the IR spectrum was consistent with glucuronic acid in the flavone. Furthermore, a 1H doublet at 4.95 and 4.75 ppm (J = 9.0 Hz each) was characteristic of H-5' and H-5'' of D-glucuronic acids.

Hydrolysis of **6** gives an aglycon of formula $C_{16}H_{12}O_7$ and D-glucuronic acid. Judging from the yield of genin (~53%) and the ^{1}H-NMR data for the glycoside, there should be two molecules of glucuronic acid. The physicochemical properties of the aglycon were compared with an authentic sample and identified as 5,6,3',4'-tetrahydroxy-7-methoxyflavone, pedalitin. These observations were confirmed by the LC-MS analyses of compound **6**, where the most important ion fragmentations were *m/z* = 667.1 [M-H]$^-$, 351 [glucuronide acidx2] and 315.0 [aglycone-H]$^-$. A correlation between H-1'' and C-6 confirmed the position of the glycosylation. The coupling constant of the anomeric proton at δ 5.44 (1H, d, J=7.5Hz) indicated the β configuration of the glucuronide (Harborne, 2000).

Assignment of the inner sugar resonances was achieved by using the anomeric proton resonance as a starting point for the interpretation of ^{1}H–^{1}H-COSY and gHMQC data sets. The chemical shifts of C-1 and C-2 of the carbohydrate part was similar to luteolin diglucuronide (**3**), which confirms that the second molecule of glucuronic acid is located at the C-2' position. Therefore, the compound **4** was identified as 5,6,3',4'-tetrahydroxy-7-methoxy-6-*O*-β-D-glucuronopiranosyl-(1$\rightarrow$2)-*O*-β-D-glucuronopyranosyde or pedalitin 6-diglucuronide. This compound has not been previously reported.

Two novel luteolin and apigenin diglucuronides acylated with ferulic acid (**4** and **5**, respectively) were isolated from the leaves of *Verbena officinalis*. The position of the acyl group was determined by the ^{13}C NMR data.

Flavonoid **4** was isolated as an amorphous yellow powder; mp 205-206 °C $[\alpha]_D^{20}$ -36.5 º(*c* 0.05, MeOH) and *m/z* = 844.2 $[M]^+$. Acid hydrolysis of **4** gave the same aglycon as **6**, pedalitin, D-glucuronic and ferulic acid. Alkaline hydrolyses gave ferulic acid and compound **6**. All the hydrolyzed compounds were identified by direct comparison with authentic markers. These observations were confirmed by the LC-MS analyses, where the most important ion fragmentations were *m*/*z* = 843.2 $[M-H]^-$, 813.3 $[M-H-30]^-$, 623.3 [M-H-Ferulic acid]$^-$, 351 [Glucuronide acidx2], 315.0 [Aglycone-H]$^-$. The ^1H and ^{13}C NMR spectra of **4** also suggested the occurrence of pedalitin, two glucuronic acid units, and a ferulic acid moiety. HSQC experiments allowed the identification of the glycosilation sites by comparison of the observed carbon chemical shifts with those of the corresponding methylpiranosides. Glycosidation shifts were observed for C-2'' (δ 84.1) and C-3''' (δ 77.2). The structure of **4** was definitely determined by HMBC experiment which showed long-range correlations between H-1''' (δ 5.05) and C-6 of the aglycons (δ 158.1), H-1''' (δ 5.05) and C-2'' (δ 84.1), and H-2'' (δ 4.88) and C=O of the ferulic moiety (δ 168.8). Thus, **4** was determined as pedalitin 6-*O*-[2-*O*-feruloyl-β-D-glucuronopyranosyl(1$\rightarrow$2)-*O*-β-D-glucuronopyranoside.

Compound **5,** amorphous yellow powder; mp 210-211 °C; $[\alpha]_D^{20}$ -50.8° (MeOH, *c* 0.1), showed a molecular ion at *m/z* = 813 $[M-H]^-$ and additional intensive peaks at *m/z* = 607.3 [M-H-Ferulic acid]$^-$, 351 [Glucuronide acidx2]$^-$ and 285.0 [Aglycone-H]$^-$ corresponding to the loss of ferulic acid and two glucuronic acid molecules and to the aglycon part (scutellarein as in **9**), respectively. Analysis of ^1H and ^{13}C NMR data of **5** in comparision to the **4** clearly suggested that the two compounds possessed and identical sugar portion, and differed only for the aglycons, being scutellarein in **5** instead of pedalitin as in **4**. Thus, the structure of **5** was established as scutellarein 6-*O*-[2-*O*-feruloyl-β-D-glucuronopyranosyl(1$\rightarrow$2)-*O*-β-D-glucuronopyranoside.

The chemical structures of the main flavonoids present in verbena leaf extracts are present in Figure 3.

3.3. Evaluation of antioxidant activity

Both DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radicals have been widely used to investigate the ability of plant extracts and fractions and/or pure compounds of those, to act as free radical-scavengers or hydrogen donors. Both extracts tested in this work were capable of scavenging DPPH$^{\bullet}$ radicals. Activity of 50% ethanolic (IC_{50} = 21.04±1.61 µg/mL) and aqueous (IC_{50} = 33.8±0.43 µg/mL) extracts demonstrated a higher scavenging capacity compared to other medicinal plants previously analyzed (López et al., 2007; López et al., 2008). The ABTS$^{\bullet+}$ assay, revealed similar results as the DPPH$^{\bullet}$ assay. Superoxide anions were generated *in vitro* enzymatically by hypoxanthine/xanthine oxidase system that reduces NBT and forms a chromophore, diformazan. In this system, there are two possibilities either the plant extracts may scavenge the $O_2^{\bullet-}$ or it may inhibit the xanthine oxidase activity. In this study, effect of plant extracts on xanthine oxidase activity was evaluated. The 50% hydroalcoholic and aqueous extracts were inhibitors of xanthine oxidase (12.77±1.65 µg/mL and 18.05±3.80 µg/mL, respectively) (Table 3). Significant (*P<0.05) negative correlation was observed between total phenolic content and the IC_{50} for DPPH$^{\bullet}$ radical scavenging activity (r^2=-0,885), ABTS$^{\bullet+}$ radical scavenging activity (r^2=-0,848) and superoxide anion radical inhibition activity (r^2=-0,855). Results showed that total flavonoid content showed a correlation extremely high and statistically significant (**P<0.01) with DPPH$^{\bullet}$ radical scavenging activity (r^2=-0,946), ABTS$^{\bullet+}$ radical scavenging activity (r^2=-0,967) and superoxide anion radical inhibition activity (r^2=-0,958). Therefore, good correlation suggests that phenolic compounds, flavonoids mainly, presents in *Verbena officinalis* leaves play an important role as antioxidants.

The antioxidant activity evaluation tests of the fractions showed that in the hydroalcoholic extract, the most active fractions were HAE3 (10.25±1.25 µg/mL) and HAE4 (11.53±3.07 µg/mL) against DPPH radical. The HPLC analysis of HAE4 showed that verbascoside (**20**) was the main compound and it could be the responsible of the activity. However, there were also present, in less proportion, luteolin 7-glucoside (**14**), 1,5-dicaffeoylquinic acid (**10**), 4,5-dicaffeoylquinic acid (**11**) and isoverbascoside (**21**), whose antioxidant activity has been previously demonstrated (Bilia et al., 2008). A fraction HAE3 had different chemical composition that HAE4 and also possessed a high antioxidant activity. The compounds

in this fraction were flavonoids mainly, luteolin 7-*O*-diglucuronide (**3**), pedalitin 6-*O*-diglucuronide (**6**), apigenin 7-*O*-diglucuronide (**7**), scutellarein 7-*O*-glucuronide (**13**), luteolin 7-*O*-glucoside (**14**) and apigenin 7 *O*-glucoside (**18**).

In the aqueous extract, four fractions presented high antioxidant activity, AQ5 (11.30±1.15 μg/mL) > AQ4 (13.33±1.84 μg/mL) > AQ3 (16.80±1.65 μg/mL) > AQ2 (29.87±2.54 μg/mL). The main compounds identified in these fractions also were flavonoids, pedalitin 6-*O*-(2-*O*-feruloyl)-diglucuronide (**4**), scutellarein 7-*O*-(2-*O*-feruloyl)-diglucuronide (**5**), luteolin 7-*O*-glucuronide (**12**), pedalitin 6-*O*-galactoside (**15**), scutellarein 7-*O*-glucoside (**19**) together with two phenylpropanoids verbascoside (**20**) and isoverbascoside (**21**).

The antioxidant activity of individual flavonoid against DPPH radical was also evaluated (Table 4). The results had evidenced the importance of the catechol system on rings A and/or B and their substituent for increased antioxidant activity. Pedalitin, luteolin and scutellarein derivatives had better radical scavenging activity than apigenin derivatives, due to presence of the *ortho*-dihydroxy group, which is responsible for enhanced free radical stabilization after one hydrogen radical donation for the antioxidant capacity (Fernandes et al., 2008). Additionally, among pedalitin derivatives **6** and **4** showed higher scavenging activity towards DPPH radical (1.23±0.11 and 0.79±0.03 μg/mL, respectively), than **5** (7.9±0.2 μg/mL), evidencing an important role of the glucuronide and feruloyl acid moiety in the structures. The same profile was observed for scutellarein derivatives, increased activities were observed for **5** (0.8 ±0.04 μg/mL) >**9** (1.41±0.19 μg/mL) >**13** (2.58±0.21 μg/mL) >**19** (8.2±0.2 μg/mL), evidencing the influence of the substituent in the structure and the presence of hydroxy group at C-6, to generate a catechol group (on ring A).

3.4. Mineral composition of verbena extract

Mineral and trace element content in aqueous and hydroalcoholic extracts of verbena is listed in Table 5. Thereby, the concentration of minerals in plant extracts had the same profile in both, and quantitative differences had been detected according to the different solvent of extraction.

The most abundant macroelement was potassium in both extracts, followed by phosphorus, calcium, and magnesium; whereas sodium was found at the lowest concentration in both extracts. The content of potassium was especially high in comparison to sodium, and, in consequence, the Na/K ratio was very low. This is considered to be an advantage from the nutritional point of view, since the intake of sodium chloride and diets with a high K/Na ratio have been related to a lower incidence of hypertension. In addition it is highlighted the high value of calcium found in both extracts which can be considered appropriate feeding source for the maintenance of biological role in mediating vascular contraction and vasodilatation, muscle contraction, nerve transmission and glandular secretion (NCR, 1989). Both extracts are also rich in magnesium. Inadequate magnesium intake leads to biochemical abnormalities and clinical manifestations, mainly linked with ischaemic heart disease, deficient skeletal growth and osteoporosis and diabetes mellitus (NCR, 1989)

The trace elements content exhibits identical pattern than macroelement content in assayed extracts. AQ contained higher levels of micronutrient than HAE, except for the zinc. It reveals the chemical differences in those organic-metal binding compounds contained by AQ and HAE extracts, mainly in iron and manganese, and in a lesser extend for copper. These micronutrients are involved in a high number of biological processes, as a component of proteins or essential components of numerous enzymes required for oxidative, amino acid, lipid or carbohydrate metabolism (NCR, 1989).

Globally, mineral levels supplied by analysed verbena extracts were higher when compared to the other Verbenaceae plants (Montiel, Camachoi, Ríos, & Delgado, 2004).

In our study, significant positive correlations were observed between total flavonoid content in both extracts and their mineral content. Results showed that total flavonoid content showed a correlation extremely high (**P<0.01) with K (r^2=0.900) and Zn (r^2=0.866), and significant (**P<0.05) with Cu (r^2=0.800), Ca (r^2=0.800) and Mg (r^2=0.800). These findings are related with the ability to chelate ion metals by polyphenols compounds and to retain more effectively the complex into the macromolecular structure formed by condensed flavonoids reported in other extracts of medicinal plants (Pohl, & Prusisz, 2007; Whittaker, Vazzana, Vecchio, & Benedettelli, 2009; Weber, & Konieczynski, 2003). Thus, these

chelating role observed in verbena extracts might be of potential interests as dietary antioxidants in food industry, preventing or delaying metal-catalized initiation and decomposition of lipid hidroperoxides. In this sense, and with the aim to establish more precise correlations between mineral elements and and antioxidant polyphenols activity, it would be interesting to analyze the mineral profile of the different fractions and the isolated flavonoids.

4. Conclusions

Hydroalcoholic and aqueous extracts of *Verbena officinalis* represent a good source of antioxidant and mineral compounds (except for iron content, the verbena aqueous extract provides between 11–27 % of RDA recommendations for mineral and trace elements studied). These results may be attractive for various commercial purposes as food supplements and herbal medicine products. From the antioxidant point of view, these fractions enriched in glucuronide based flavonoids are also worth mentioning due to their obtaining in a single-step column separation is feasible.

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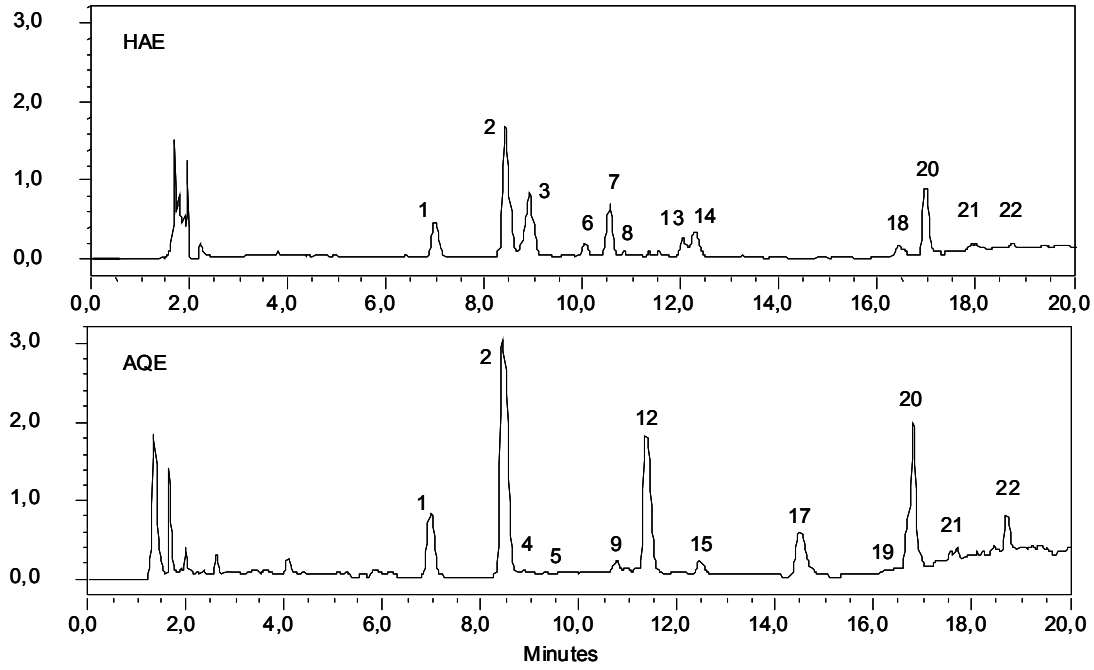


Figure 1. HPLC chromatograms of *Verbena officinalis* extracts. HAE: 50% hydroalcoholic extract; AQE: aqueous extract. Column: C18 reversed-phase column (Nova-Pak, 150 mm x 3.9 mm., 4 μm, Waters). Solvent: Gradient of acetonitrile and twice distilled water adjusted to pH 3 with phosphoric acid. Detection: 240 nm.

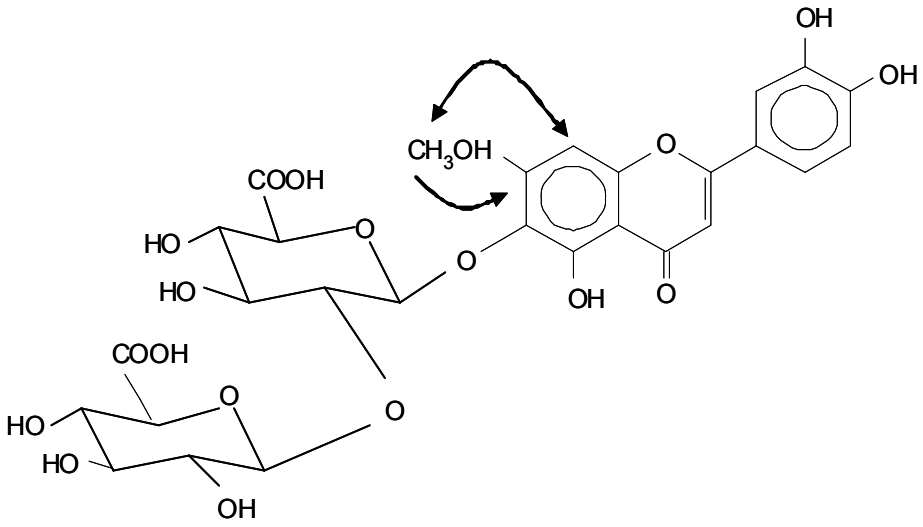
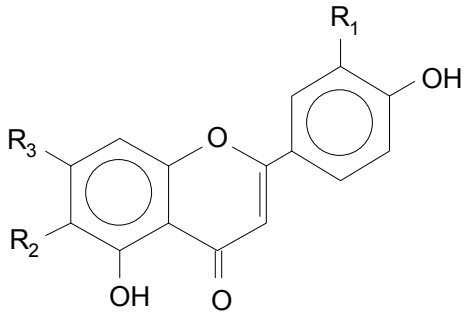


Figure 2. gHMBC of compound **6**.



No.	R_1	R_2	R_3	Compound
3	-OH	-H	-OGluA-GluA	Luteolin 7-diglucuronide
6	-OH	-OGluA-GluA	$-OCH_3$	Pedalitin 6-diglucuronide
7	-H	-H	-OGluA-GluA	Apigenin 7-diglucuronide
13	-H	-OH	-OGluA	Scutellarein 7-glucuronide
14	-OH	-H	-OGlu	Luteolin 7-glucoside
18	-H	-H	-OGlu	Apigenin 7-glucoside
22	H	H	OH	Apigenin
4	-OH	-OGluA-GluA-Feruloyl	$-OCH_3$	Pedalitin 6-(2-feruloyl)-diglucuronide
5	-H	-OH	-OGluA-GluA-Feruloyl	Scutellarein 7-(2-feruloyl)-diglucuronide
9	-H	-OH	-OGluA-GluA	Scutellarein 7-diglucuronide
12	-OH	-H	-OGluA	Luteolin 7- glucuronide
15	-OH	-OGal-	$-OCH_3$	Pedalitin 6-galactoside
17	-H	-H	-OGal	Apigenin 7-galactoside
19	-H	-OH	-OGlu	Scutellarein 7-glucoside
16	-OH	-Glu	$-OCH_3$	Pedalitin 6-glucoside

GluA = glucuronic acid; Glu = glucose; Gal = galactose

Figure 3. Structures of flavonoids in leaves of verbena (identified from the current and previous studies)

Table 1. Iridoids, flavonoids and phenolic acid identified in extracts from leaves of *Verbena officinalis*.

No.	t_R (min)	UV λ_{Max} (nm)	$[M+H]^+$ (*m/z*)	$[M-H]^-$ or diagnostic fragments (*m/z*)	Compound
1	6.83	235	405	807.1 $[2M-H]^-$, 403 $[M-H]^-$, 241 [M-H-Glucose]$^-$	Hastatoside
2	8.35	238	389	775.3 $[2M-H]^-$, 387.0 $[M-H]^-$, 225 [M-H-Glucose]$^-$	Verbenalin
3	8.80	254, 346	639	637.1$[M-H]^-$, 351 [Glucuronide acid x2], 285.0 [Aglycone-H]$^-$	Luteolin 7-*O*-diglucuronide
4	8.89	289, 329	845	843.2 $[M-H]^-$, 813.3 [M-H-30]$^-$, 623.3 [M-H-Ferulic acid]$^-$, 351 [Glucuronide acidx2], 315.0 [Aglycone-H]$^-$	Pedalitin 6-*O*-(2-*O*-feruloyl)-diglucuronide
5	9.42	285, 322	815	813 $[M-H]^-$, 607.3 [M-H-Ferulic acid], 351 [Glucuronide acidx2], 285.0 [Aglycone-H]$^-$	Scutellarein 7-*O*-(2-*O*-feruloyl)-diglucuronide
6	10.01	280, 343	669	667.1 $[M-H]^-$, 351 [Glucuronide acidx2], 315.0 [Aglycone-H]$^-$	Pedalitin 6-*O*-diglucuronide
7	10.6	266, 336	623	621.1 $[M-H]^-$, 351.1 [Glucuronide acidx2], 269.1 [Aglycone-H]$^-$	Apigenina 7-*O*-diglucuronide
8	10.72	238	347	691.0 $[2M-H]^-$, 345.0 $[M-H]^-$, 183.0 [M-H-Glucose]$^-$	Aucubin
9	10.83	281, 334	639	637.1 $[M-H]^-$, 351 [Glucuronide acidx2], 285.0 [Aglycone-H]$^-$	Scutellarein 7-*O*-diglucuronide
10	10.91	244, 326	517	537 $[M+Na-2H]^-$, 515 $[M-H]^-$, 353 [M-163]$^-$, 191 [M-325]$^-$, 179 [M-337]$^-$, 161 [M-355]$^-$	1,5-*O*-dicaffeoylquinic acid
11	11.10	244, 326	517	537 $[M+Na-2H]^-$, 515 $[M-H]^-$, 353 [M-163]$^-$, 191 [M-325]$^-$, 179 [M-337]$^-$, 161 [M-355]$^-$	4,5-*O*-dicaffeoylquinic acid
12	11.46	254, 346	463	461.1 $[M-H]^-$, 285.0 [Aglycone-H]$^-$	Luteolin 7-*O*-glucuronide
13	12.15	281, 334	463	461.1 $[M-H]^-$, 285.0 [Aglycone-H]$^-$	Scutellarein 7-*O*-glucuronide
14	12.24	254, 346	450	448.0 $[M-H]^-$, 285.0 [Aglycone-H]$^-$	Luteolin 7-*O*-glucoside
15	12.56	280, 343	480	478.1 $[M-H]^-$, 315.0 [Aglycone-H]$^-$	Pedalitin 6-*O*-galactoside

16	12.86	280, 343	480	478.1 $[M-H]^-$, 315.0 $[Aglycone-H]^-$	Pedalitin 6-*O*-glucoside
17	14.55	266, 336	434	432.1 $[M-H]^-$, 269.0 $[Aglycone-H]^-$	Apigenin 7-*O*-galactoside
18	16.20	266, 336	434	432.1 $[M-H]^-$, 269.0 $[Aglycone-H]^-$	Apigenin 7-*O*-glucoside
19	16.40	281, 334	450	448.1 $[M-H]^-$, 285.0 $[Aglycone-H]^-$	Scutellarein 7-*O*-glucoside
20	16.52	328	625	623.0 $[M-H]^-$, 461.1 $[M-H-Glucose]^-$	Verbascoside
21	17.53	328	625	623.0 $[M-H]^-$, 461.1 $[M-H-Glucose]^-$	Isoverbascoside
22	18.61	266, 336	271	269.1 $[M-H]^-$	Apigenin

Table 2. ^{1}H-(300 MHz) and ^{13}C-NMR (125 MHz) Data for Flavonoid **6**, **4** and **5** in DMSO-*d6*.

	6		**4**		**5**	
	δ ^{1}H (*J* in Hz)	δ ^{13}C	δ ^{1}H (*J* in Hz)	δ ^{13}C	δ ^{1}H (*J* in Hz)	δ ^{13}C
Aglycons						
2	-	164.4	-	164.1	-	164.3
3	6.67 (s)	102.4	6.48 (s)	105.3	6.57(s)	103
4	-	181.8	-	183.1	-	181.9
5	-	160.8	12.90 (s)	158.7	-	161.1
6	-	158.0	-	158.1	-	159.3
7	-	161.0	-	164.8	-	162.6
8	6.79 (s)	96.5	6.50 d (*J*=2.0)	95.7	6.77 d (*J*=1.5)	95.1
9	-	162.66	-	162.0	-	156.7
10	-	105.5	-	105.5	-	105.4
1'	-	121.1	-	121.1	-	121
2'	7.50 (d. *J*=2.0)	114.1	7.77 (br s)	114.1	7.80 d (*J*=8.0)	128.5
3'	-	145.9	-	145.9	6.89 d (*J*=8.0)	115.9
4'	-	149.7	-	149.7		161.2
5'	6.88 (d. *J*=8.5)	115.7	6.86 d (*J*=8.4)	115.7	6.89 d (*J*=8.0)	115.9
6'	7.42 (dd. *J*=8.5, 2.0)	118.4	7.41 dd (*J*=8.4, 2.3)	118.4	7.80 d (*J*=8.0)	128.5
7-OMe	3.88 (s)	56.4	3.90 (s)	56.3	-	-
5-OH	13.0 (s)	-	13.0 (br s)	-	13.0 (s)	-
6-OH	-	-	-	-	12,9 (s)	-
3'-OH	9.37 (s)	-	9.37 (s)	-	-	-
4'-OH	9.88 (s)	-	9.88 (s)	-	9.88 (s)	-
Sugars						
1''	5.44 d (*J*=7.5)	100.31	5.35 d (*J*=7.5)	100.0	5.42 d (*J*=7.5)	99.9
2''	3.85 dd (*J*=7.5, 9.5)	84.14	3.73 dd (*J*=7.5, 9.5)	84.1	3.75 dd (*J*=7.5, 9.5)	83.9
3''	3.80 dd (*J*=8.5, 8.5)	76.98	3.65 dd (*J*=8.5, 8.5)	76.3	3.70 dd (*J*=9.5, 9.5)	75.7
4''	3.70 dd (*J*=8.5, 8.5)	72.58	3.60 dd (*J*=8.5, 8.5)	72.4	3.65 dd (*J*=9.5, 9.5)	72.2
5''	4.03 d (*J*=8.5)	77.55	4.03 d (*J*=8.5)	76.7	4.10 d (*J*=9.5)	76.5
6''	-	171.91	-	172.3	-	172
1'''	5.18 d (*J*=7.5)	106.97	5.05 d (*J*=7.5)	104.1	5.12 d (*J*=7.5)	103.9
2'''	5.12 dd (*J*=7.5, 9.0)	76.2	4.88 dd (*J*=7.5, 9.5)	75.5	5.10 dd (*J*=7.5, 9.0)	75.4
3'''	4.01 dd (*J*=9.5, 9.5)	77.81	3,89 dd (*J*=8,5)	77.2	3.99 dd (*J*=9.5, 9.5)	77
4'''	3.84 dd (*J*=9.5, 9.5)	73.32	3,70 dd (*J*=8.5, 8.5)	73.3	3.75 dd (*J*=9.5, 9.5)	73
5'''	4.05 d (*J*=9.5)	78.19	3.92 d (*J*=8.5)	76.8	4.00 d (*J*=9.5)	76.7
6'''	-	172.49	-	172.3	-	172.2
Ferulic acid						
1	-	-	-	127.8	-	127.6
2	-	-	7.14 d (*J*=1.7)	111.7	7.11 d (*J*=1.2)	111.3
3	-	-	-	149.5	-	149.4
4	-	-	-	150.5	-	150.4
5	-	-	6.78 d (*J*=8.3)	116.4	6.75 d (*J*=8.0)	116.6
6	-	-	7.04 dd (*J*=1.7, 8.3)	124.1	7.00 dd (*J*=8.0, 1.2)	124.3
α	-	-	6.38 d (*J*=15.9)	115.5	6.33 d (*J*=16.0)	115.3

β	-	-	7.74 d (*J*=15.9)	147	7.57 d (*J*=16.0)	147
C=O	-	-	-	168.8	-	168.5
OCH_3	-	-	3.83 (s)	56.4	3.91 (s)	56.3

Table 3. Total phenol and flavonoids, antioxidant activity of *Verbena officinalis* extracts.

	% TPC (g/100g)	% TFC (g/100g)	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	X/XO IC_{50} (µg/mL)
HAE	1.25 ± 0.01	0.76 ± 0,01	21.04 ± 1.61	99.27 ± 4.73	12.77 ± 1.65
AQE	1.70 ± 0.02	0.69 ± 0.02	33.87 ± 0.43	301.11 ± 24.11	18.05 ± 3.80
BHA	-	-	4.05 ± 0.73	2.79 ± 0.13	0.26 ± 0.02
BHT	-	-	5.05 ± 0.63	5.22 ± 0.75	2.25 ± 0.01

TPC: total polyphenol content; TFC: total flavonoid content; HAE: hydroalcoholic extract; AQE: aqueous extract.

Table 4. DPPH Radical Scavenging Activity of flavonoids isolated from *Verbena officinalis*

	IC50 (μg/mL)			
	-feruloyl-diglucuronide	-diglucuronide	-glucuronide	-glucoside/galactoside
Pedalitin	0.79 ± 0.03 (**4**)	1.23 ± 0.11 (**6**)	n.d.	7.9 ± 0.2 (**15**)
Scutellarein	0.81 ± 0.04 (**5**)	1.41 ± 0.19 (**9**)	2.58 ± 0.21 (**13**)	8.2 ± 0.2 (**19**)
Luteolin	n.d.	1.54 ± 0.10 (**3**)	2.62 ± 0.74 (**12**)	8.6 ± 0.6 (**14**)
Apigenin	n.d.	10.9 ± 0.5 (**7**)	n.d.	> 1000 (**18**)

n.d.: no detected in *Verbena officinalis*

Table 5. Macroelements and microelements of verbena extracts (mg element/kg plant dry)

	HAE extract	AQE extract
Mg	689 ± 8	979 ± 1
Ca	926 ± 3	2560 ± 7
Na	107 ± 7	72 ± 1
K	7934 ± 319	10798 ± 18
P	2307 ± 48.	1795 ± 4
Fe	1.24 ± 0.12	4.63 ± 0.05
Zn	6.43 ± 0.13	3.57 ± 0.01
Cu	1.57 ± 0.01	2.06 ± 0.02
Mn	2.29 ± 0.05	6.74 ± 0.02