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HPLC–DAD–ESI–MS analysis of the constituents of aqueous preparations of verbena and lemon verbena and evaluation of the antioxidant activity

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Abstract

Verbena and lemon verbena aqueous preparations were investigated for their content of constituents, especially polyphenols by HPLC/DAD/ESI/MS analysis because they are used worldwide as herbal teas. The main class of compounds of these plants were phenylpropanoids (from 16 to 120 mg/g of dried extract), being verbascoside the most abundant in all the preparations up to 97% of the total phenylpropanoids. Also iridoids, hastatoside and verbenalin together with flavonoids, mono- and di-glucuronidic derivatives of luteolin and apigenin were found. These simple preparations, especially that obtained from infusion of lemon verbena, could be lyophilized to obtain a powder having interesting technological properties to be used as ingredients of cosmetics, food supplements and herbal medicinal products do to the many biological properties of verbascoside. In addition, the antioxidant property of the lemon verbena infusion was evaluated by the DPPH test using Trolox as the reference compound.

Keywords: Lemon verbena and verbena (*Lippia citriodora* and *Verbena officinalis*); Infusions and decoctions; Polyphenols; Phenylpropanoids; DPPH test

1. Introduction

As a continuation of our studies on herbal preparations [1–4], we now report the HPLC/DAD/ESI/MS analysis of infusions and decoctions of verbena (*Verbena officinalis* L.) and lemon verbena (*Lippia citriodora* Humb. B. et Kwidely), plants used for food preparations but also largely employed in folk medicines [5,6]. The two plant species are characterised by the presence of polyphenols, namely verbascoside and correlated molecules and derivatives of luteolin [7,8], but very little [9] or nothing is known about the constituents of the aqueous preparations, even if numerous activities are attributed to these preparations, e.g. the antigenotoxic and protection against genetic damage of lemon verbena infusion [10,11], the neuroprotective effects [12] and hypnotic effects of the aqueous extract of verbena [13].

Indeed, it is well known that the use of antioxidant nutrients may decrease or prevent the risk of many diseases caused by oxidative stress, such a chronic inflammation and cardiovascular diseases, and recently their use has also been associated with a reduced risk of cancer because they may prevent damage to DNA caused by free radicals [14].

Thus, many plants have now been identified as having potential antioxidant activities and their consumption is recommended, particularly those containing carotenoids, ascorbic acid, tocopherols, and polyphenols focusing on some commonly consumed beverages such as coffee, tea, wine, and fruit juices [15,16]. However, recently interest in investigating the antioxidant properties of herbal infusions has increased, especially those traditionally used in folk medicine. Indeed, some epidemiologic studies have indicated that coffee or tea is not associated with a risk for cancer [17–19] and in a large pooled analysis of eight cohort studies there was a modest inverse association between fruit consumption and breast cancer risk [20]. Moreover, a recent paper concerning the consumption of antioxidant-rich beverages and risk for breast cancer in French women [21] confirmed similar observations. The authors concluded that the risk for breast cancer does not seem to be related to consumption of antioxidant beverages in general, but perhaps to some particular compounds found, for example, in herbal teas widely used by the women involved in the investigations. Even if this result must be interpreted cautiously, the authors suggested

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more research, both epidemiologic and experimental, to establish this association.

In view of these new perspectives, verbena and lemon verbena aqueous preparations were selected for the present investigation because they are used worldwide as herbal teas and for their high content of polyphenols in the plant materials, especially the water soluble phenylpropanoid glycosides [7–9].

The HPLC/DAD/MS profiles of decoctions obtained by 5 and 20 min boiling and the infusions are here reported and compared with those obtained with the ethanolic (EtOH) extracts and a commercial aqueous extract (1:4, D/E) of verbena.

In addition, the antioxidant property of the lemon verbena infusion was evaluated by the DPPH test according to Son and Lewis [22].

2. Experimental

2.1. Chemicals

All solvents used were HPLC grade; CH_3CN and MeOH for HPLC were purchased from Merck (Darmstadt, Germany). Eighty-five percentage of formic acid was provided by Carlo Erba (Milan, Italy). Water was purified by a Milli-Qplus system from Millipore (Milford, MA, USA).

0.45 mm PTFE membrane filter was purchased from Waters Co. (Milford, MA). All laboratory chemicals used in this study were of reagent grade.

2.2. Standards

Verbascoside reference standard (purity 98%) was an isolated compound from IRB (Istituto di Ricerche Biotecnologiche) of Altavilla Vicentina.

2.3. Samples

Dried leaves of cultivated *L. citriodora* HBK and wild *V. officinalis* L., harvested in 2004, were from Aboca (Pistrino di Citerna, PG, Italy), lot n. 4L0466 and 4F2505, respectively. The commercial dried extract of verbena (1:4, D/E) was from Galeno (Prato, Italy), lot n. 34938.

2.4. Herbal preparations

To prepare the decoctions of lemon verbena and verbena leaves, two procedures were used: 100 g of lemon verbena (Dec1-LV) and 200 g of verbena dried leaves (Dec1-V) were put in 2 and 2.5 L, respectively, of hot water and boiled for 20 min. Within the second procedure 5 g of each herbal drug was put in 100 mL of water and boiled for 5 min (Dec2-LV and Dec2-V). The decoctions were then lyophilized to obtain a solid residue.

The infusions (Tea-LV and Tea-V) were prepared with 5 g of leaves and 100 mL of boiling water. The mixture was cooled for 20 min before filtration.

All the aqueous preparations were freeze-dried.

EtOH extracts were obtained using 200 g of each herbal drug (HD) and 7.5 L of EtOH (3× 2.5 L). A total of 10.52 g of extract was obtained from verbena (EtEx-V) (5.26% with respect to the HD), while 13.92 g was obtained from lemon verbena (EtEx-LV) (6.96% with respect to the HD). The analysis was also performed on a commercial aqueous extract (1:4) of verbena. For the HPLC–DAD–MS analysis samples were obtained by dissolving and filtrating the dried residues (about 6 mg exactly weighed) with 1 mL of MeOH.

2.5. HPLC apparatus

2.5.1. HPLC–DAD analysis instrumentation

The HPLC system consisted of a HP 1100 L instrument with a Diode Array Detector and managed by a HP 9000 workstation (Agilent Techologies, Palo Alto, CA, USA).

The column was a Varian Polaris TM C18-E (250 mm × 4.6 mm i.d., 5 (m) maintained at 26 °C with a pre-column of the same phase.

The eluents were H_2O at pH 3.2 by formic acid (A) and acetonitrile (B). The following multi-step linear gradient was applied: from 87% A to 85% A in 10 min, in 10 min to 75% B and then a plateau for 3 min; 2 min to 95% CH_3CN and a final plateau of 3 min. Total time of analysis was 28 min, equilibration time 10 min, and flow rate was 0.8 mL min^{-1}, oven temperature 26 °C.

The UV–vis spectra were recorded between 220–500 nm and the chromatographic profiles were registered at 240, 330 and 350 nm.

2.5.2. HPLC–MS analysis instrumentation

The HPLC system described above was interfaced with a HP 1100 MSD API-electrospray (Agilent Techologies, Palo Alto, CA, USA). The interface geometry, with an orthogonal position of the nebulizer with respect to the capillary inlet, allowed use of analytical conditions similar to those used for HPLC–DAD analysis in order to achieve the maximum sensitivity of ESI values.

The same column, time period and flow rate were used during the HPLC–MS analyses.

Mass spectrometry operating conditions were optimised in order to achieve maximum sensitivity values: negative and positive ionisation mode, scan spectra from *m/z* 100 to 800, was used with a gas temperature of 350 °C, nitrogen flow rate of 10 L min^{-1}, nebulizer pressure 30 psi, Quadrupole temperature 30 °C, Capillary voltage 3500 V. The applied fragmentors were in the range 80–180 V. Identification of constituents was carried out by HPLC/DAD and HPLC/ESI/MS analysis, and/or by comparison and combination of their retention times, UV–vis and mass spectra of the peaks with those of authentic standards when possible, or isolated compounds or characterised extracts as well as based on literature data.

2.6. Identification of peaks and peak purity

Identification of all constituents was performed by HPLC–DAD and –MS analysis and/or by comparing the retention time, the UV and MS spectra of the peaks in the samples with those of authentic reference samples or data reported in

the literature. The purity of peaks was checked by a Diode Array Detector coupled to the HPLC system, comparing the UV spectra of each peak with those of authentic references samples and/or by examination of the MS spectra.

2.7. Linearity and repeatability

The linearity range of responses was determined on five concentration levels with three injections for each level. Calibration graphs for HPLC were recorded with sample amounts ranging from 1 μg mL^{-1} to 0.5 mg mL^{-1} ($r > 0.9996$).

To evaluate the repeatability, six samples of each residue were analyzed by HPLC. The contents of each constituent were evaluated to calculate the relative standard deviation (R.S.D.) which ranged between 1.50 and 2.35%.

To evaluate the repeatability of the injection integration, the standard solutions and each sample were injected six times and the relative standard deviation values were calculated. A range of 1.00–1.75% was obtained.

2.8. Quantitative determination of phenylpropanoids

Quantitative evaluation of verbascoside and its analogues was performed by means of a four-point regression curve ($r^2 \geq 0.999$) in a concentration range between 0 and 0.65 mg mL^{-1}, using verbascoside as reference external standard, and evaluated at 330 nm.

LOD (Limit of Detection) was 0.6 μg mL^{-1} and LOQ (Limit of Quantitation) was 1 μg mL^{-1}.

2.9. Antioxidant test

The lyophilized lemon verbena tea was solubilised in EtOH and submitted to a DPPH test according to the published procedure [4]. Four solutions at different concentrations (100, 75, 50 and 25 μM) were tested and compared with pure verbascoside and Trolox, used as reference compounds. The decrease of DPPH· adsorbance, in presence of the antioxidant solutions, was measured at 517 nm.

3. Results and discussion

In continuing our studies on the constituents of herbal drug preparations, the present paper report on the qualitative profiles of different extracts of verbena and lemon verbena. In addition phenyl propanoids, namely verbascoside and its analogues, the major constituents were also quantified.

The two plant species were submitted to different extraction procedures in order to recover the phenolic fraction: two decoctions obtained with different boiling times, an infusion and an EtOH extraction were considered, both for verbena and lemon verbena leaves.

Decoctions and infusions were tested because they represent the most widely used forms of preparation of herbal drugs, even if decoction is a more aggressive treatment and can cause a partial degradation of some of the constituents. The EtOH extraction procedure was selected due to its very high capacity of recovery of organic constituents compared to water.

As described in Section 2, aliquots of the obtained dried samples were submitted to HPLC/DAD/ESI/MS analysis to quali-quantitatively evaluate the compounds of interest.

The constituents of the three extracts for the two considered plants were identified by UV and MS spectral data. The qualitative profiles of the three extracts, within the same plant, were quite similar. Figs. 1 and 2 report the HPLC/DAD profiles from infusion of lemon verbena and verbena at different wavelengths: 240 nm for monitoring iridoids, 330 nm for phenylpropanoids and 350 nm for the detection of flavonoids.

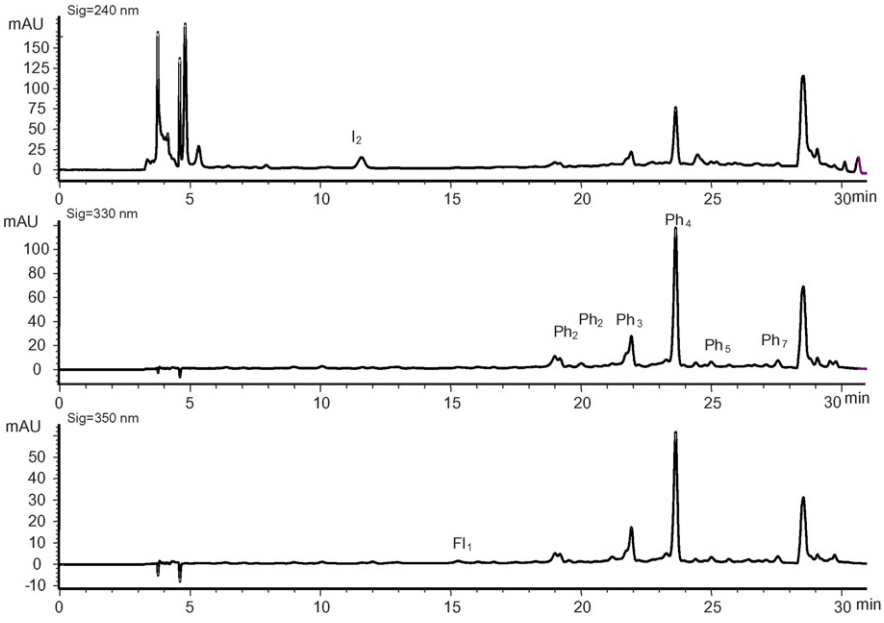

Fig. 1. Chromatographic profiles at 240, 330 and 350 nm of a representative sample of lemon verbena leaf extracts. I = iridoid compounds; Fl = flavonoids derivatives; Ph = phenylpropanoids derivatives.

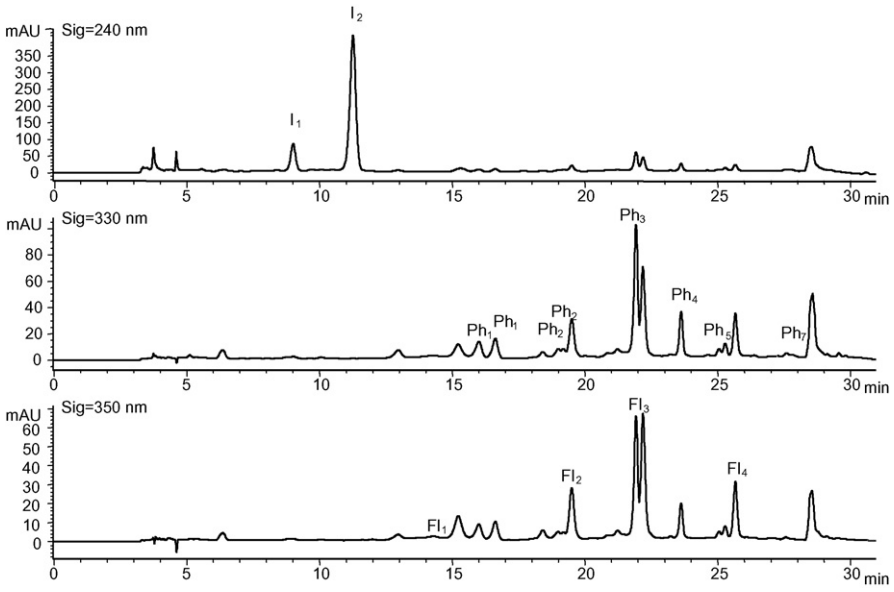

Fig. 2. Chromatographic profiles at 240, 330 and 350 nm of a representative sample of verbena leaf extracts. I = iridoid compounds; Fl = flavonoids derivatives; Ph = phenylpropanoids derivatives.

In Table 1 all the identified compounds in the different extracts for both the investigated plants are listed.

The constituents of the extracts of the two considered plants mainly belong to three classes of natural compounds, iridoids, flavonoids and phenylpropanoids, in agreement with literature data [7–9,23].

The chemical structures of the main compounds present in the lemon verbena and verbena leaf extracts are presented in Fig. 3.

Two iridoids, identified as verbenalin and hastatoside, were detected in verbena extracts, in accordance with previous data [23], while only the less polar iridoid, verbenalin, was evidenced in lemon verbena.

In Fig. 4a and b the mass spectra, in negative ion mode and at fragmentor 80 V, of hastatoside and verbenalin are reported with the relative UV spectra which evidence a typical characteristic maximum near 240 nm.

As shown, the following ions are present: the quasi-molecular ion $[M-H]^-$ at 403 *m/z*; the ion due to the loss of glucose, $[M-H\text{-}162]^-$ at 241 *m/z*; and finally an ion species at 449 *m/z* related to the formation of an adduct with formic acid ($[M-H+HCOOH]^-$), the organic acid used to modify the pH of the mobile phase for mass analysis. The same fragmentation pattern was observed for verbenalin (Fig. 4b) together with another ion at 775 *m/z* related to the formation of dimer of verbenalin $[2M-H]^-$.

The mass analyses were also carried out in positive ion mode to confirm these structures: in fact they show the presence of the adducts with sodium and potassium to the quasi-molecular ion $[M+H]^+$ for both the iridoids, as often happens when working in positive polarity.

Regarding the class of flavonoids, mono- and di-glucuronidic derivatives of luteolin were detected in the extracts of both the plants, in accordance with literature data, while

Table 1
List of the identified compounds in the three extracts from the two plants

	Composition	LV dec1	V dec1	LV EtEx	V EtEx	LV tea	V tea
I_2	Verbenalin	+	+	+	+	+	+
I_1	Hastatoside	−	+	−	+	−	+
Fl_1	Luteolin 7-*O*-diglucuronide	+*	+	−	−	+	−
Fl_3	Luteolin 7-*O*-glucuronide	−	+	−	−	−	+
Fl_2	Apigenin 7-*O*-diglucuronide	−	+	−	−	+	−
Fl_4	Apigenin 7-*O*-glucuronide	−	+	−	−	−	+
Ph_3	Verbascoside	+	+	+	+	+	+
Ph_4	Isoverbascoside	+	+	+	+	+	+
Ph_1	β-OH verbascoside	+*	+	−	−	Traces	−
Ph_2	β-OH isoverbascoside	+	+	−	−	Traces	−
Ph_5	Eukovoside or isomer	+	+	+	+	+	+
Ph_6	Eukovoside or isomer	−	−	+	+	Traces	−
Ph_7	Eukovoside or isomer	+	+	+	Traces	Traces	−

I = iridoid compound; Fl = flavonoid derivatives; Ph = phenylpropanoids derivatives; LV = lemon verbena; V = verbena; dec = decoction; EtEx = EtOH extract; + = presence of the compound; − = absence of the compound. *Compound present only in dec2

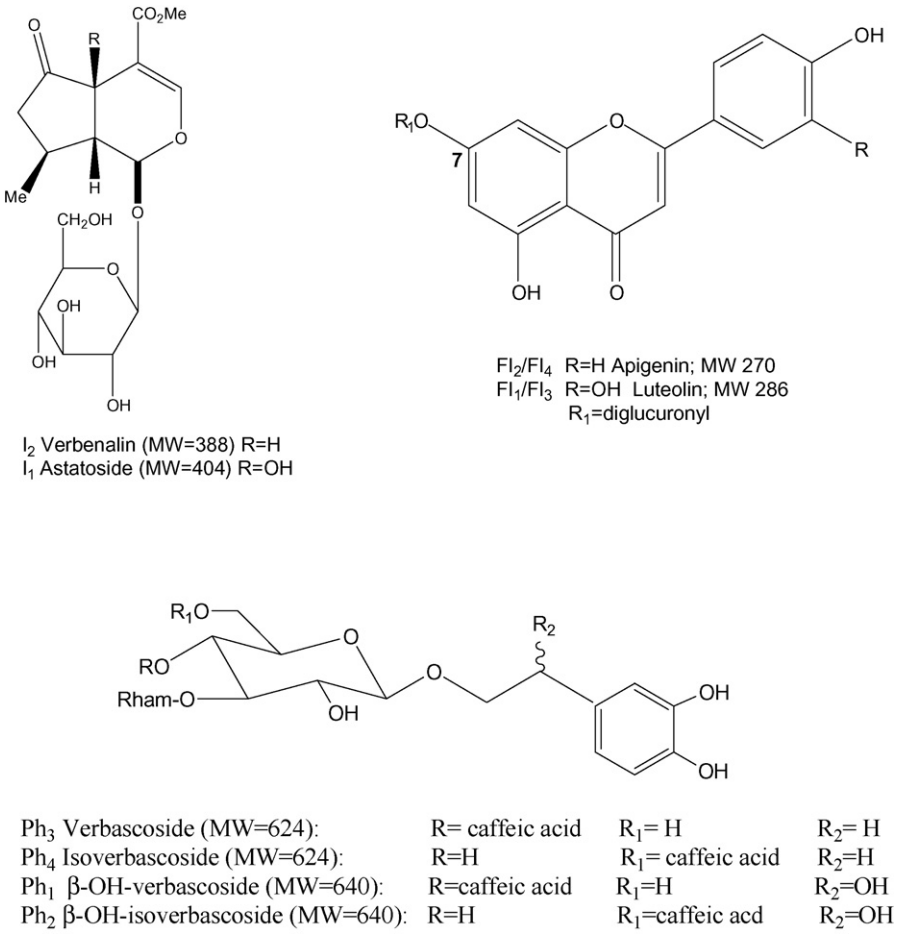

Fig. 3. Chemical structures of the main constituents of the leaves of lemon verbena.

the same derivatives of apigenin were evidenced only in verbena.

To the best of our knowledge, this is the first report on the presence of apigenin derivatives in the aerial part of verbena.

Fig. 5 reports the mass spectra, in negative ion mode and with fragmentor 180 V, of the diglucuronidic derivatives of luteolin, MW = 638, (a) and apigenin, MW = 622, (b). They evidenced the presence of the quasi-molecular ion $[M-H]^-$, at 637 *m/z* and 621 *m/z*, the most intense ions related to the aglycone $[M-H-(176\times 2)]^-$, at 285 *m/z* and 269 *m/z*, for luteolin and apigenin, respectively, and finally the ion due to the loss of the aglycone at 351 *m/z*.

As expected, the main class of compounds of these plants were phenylpropanoids, and amongst them verbascoside was the most abundant in all the extracts and in both the plants.

In addition, other verbascoside derivatives were also detected. In particular, isoverbascoside, an isobaric isomer of verbascoside, and two couples of diasteroisomeric forms of β-OH-acteoside and β-OH-isoacteoside (MW = 640), analogous of verbascoside and isoverbascoside, respectively, with the presence of a hydroxyl group in the β position of the 3,4-dihydroxy-phenyl-ethanol (see Fig. 3) were also revealed. The first two diasteroisomers of β-OH-acteoside were identified by the comparison of their R_{ts}, UV and MS data with those of isolated compound from a solid olive residue in which they were recently characterised [24].

Fig. 6 reports, as an example of these four phenylpropanoidic compounds, the MS spectra in negative ionisation mode. The quasi-molecular ion $[M-H]^-$, with 639 Th, together with another diagnostic species, the ion with 621 *m/z*, due to the loss of a water molecule, can be noted. The fragment with 459 Th, relating to the losses of caffeic acid and a water molecule, was evidenced together with two other species correlated with the fragmentation of caffeic moiety (179 *m/z* and 161 *m/z*). This fragmentation patterns is typical of verbascoside derivatives as reported in literature [25].

Three other phenylpropanoidic derivatives (MW = 638), with the presence of a ferulic moiety instead of a caffeoyl group, were identified as eukovoside, previously reported in literature in these plants [7] and its isomers.

The different extraction procedures applied were selective for the recovery of the different classes of compounds. In fact decoctions and tea were richer in more polar compounds and showed a more complex profile than EtOH extracts that presented mainly lypophilic molecules.

The complete absence of the flavonoidic derivatives in the EtOH extract of both the plants is worth noting. On the contrary, they are all present in verbena decoction while in lemon verbena decoction only luteolin 7-*O*-diglucuronide was detected. For the

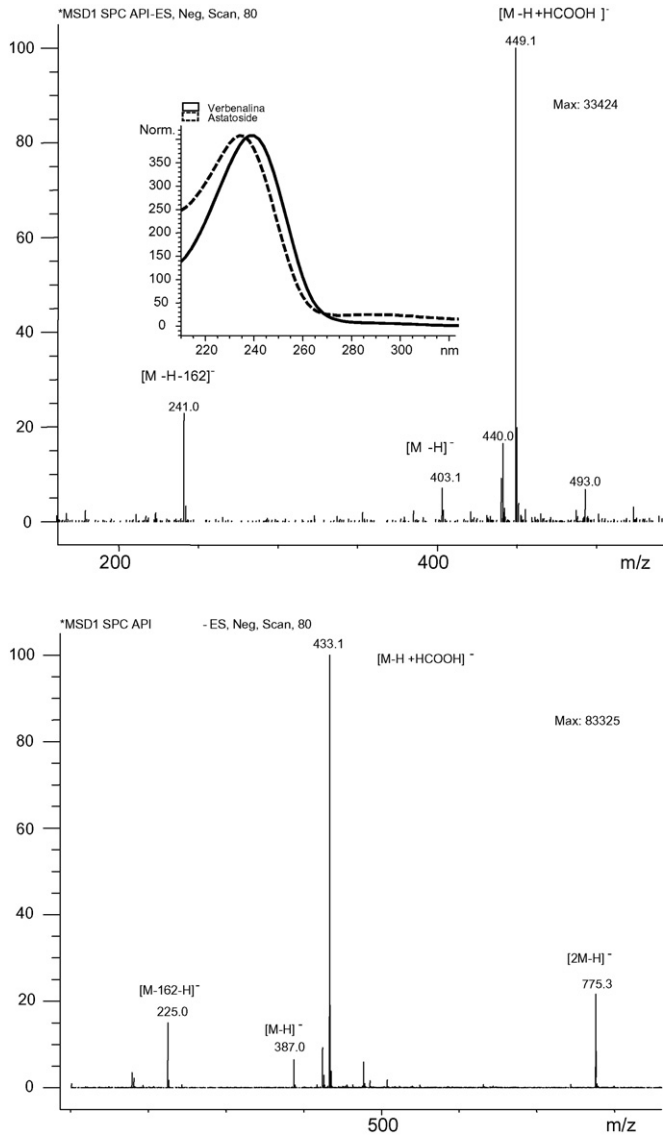

Fig. 4. MS spectra of verbenalin and hastatoside in negative ion mode at 80 eV of fragmentor.

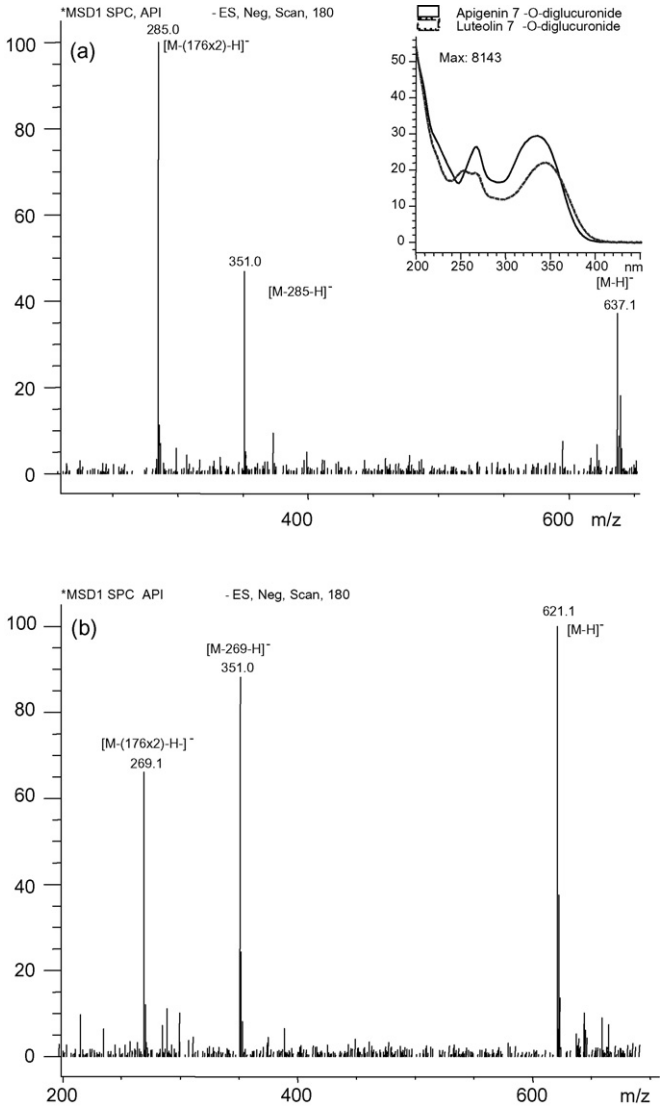

Fig. 5. MS spectra in negative polarity with fragmentor 180 eV of the luteolin (a) and apigenina (b) 7-*O*-diglucuronides, and their relative UV–vis spectra.

infusion we can observe an opposite behaviour of the flavonoidic content between the two plants: the diglucuronidic derivatives of both luteolin and apigenin were absent in verbena but present in lemon verbena.

Even for phenylpropanoids there was a variability of their content in the two investigated matrices. In particular verbascoside and isoverbascoside were detected in all the extracts, while their β-hydroxilated derivatives were found only in the samples obtained with the use of water as extractive solvent due to their higher polarity; they are completely absent in the EtOH extracts.

The eukovoside and its isomers are phenylpropanoids with an higher lypophilicity and, as expected, were detected only in the EtOH extracts.

Table 2 reports the yields of the dried extracts with respect to the dried leaves for lemon verbena and verbena.

Fig. 7 presents, as example, the HPLC/DAD profiles at 330 nm of the phenylpropanoid derivatives, the molecules on which a quantitative estimation was performed, for the lemon

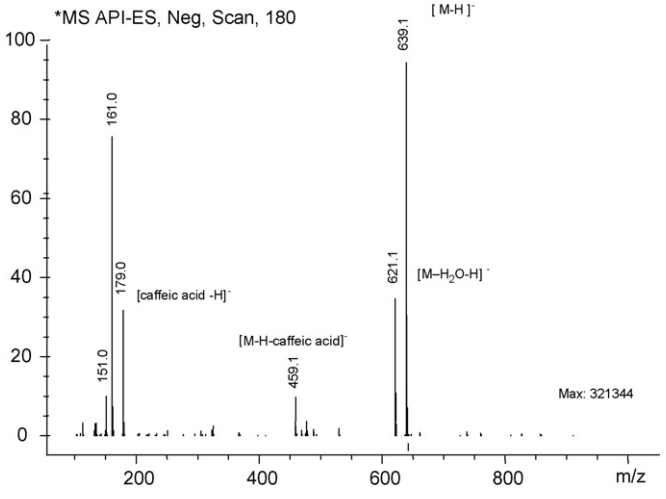

Fig. 6. MS spectrum of one of the two diasteroisomers of β-OH acteoside in negative ion mode and with fragmentor 180 eV.

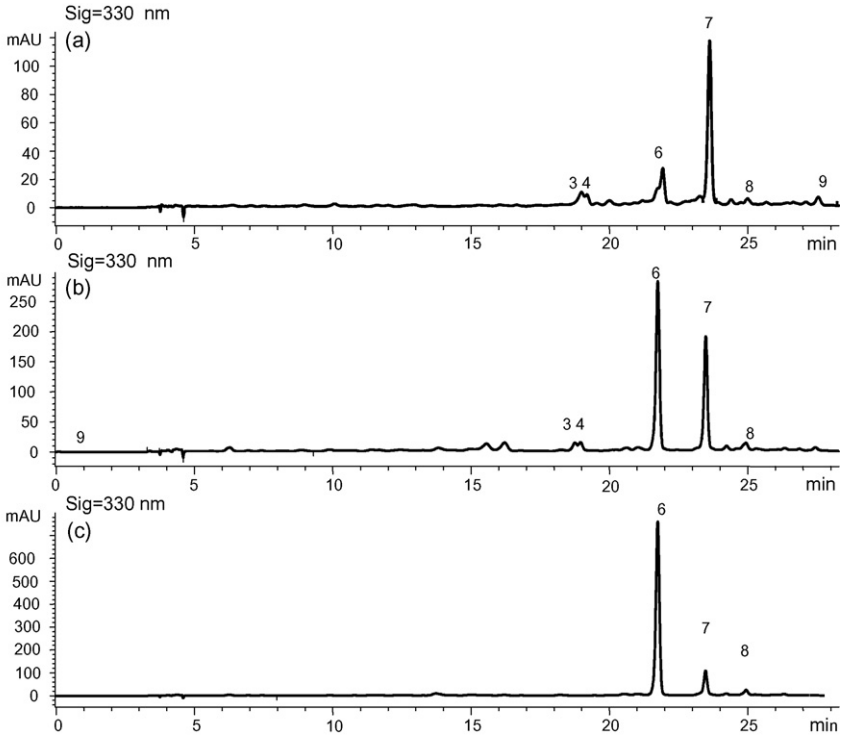

Fig. 7. Comparison of the HPLC/DAD profiles at 330 nm of the 20-min decoction (a), 5-min decoction (b) and tea (c) of lemon verbena.

verbena and verbena extracts. The yield of phenylpropanoids was also evaluated and resulted very different between the different extractions: for lemon verbena and verbena, respectively, 8.3 and 3.24% in infusions, from 1.7 to 4.4% and from 1.59 to 1.87% in decoctions and finally 12 and 5.52% for EtOH extracts.

Fig. 8 shows the histogram of the quantitative data of verbascoside and the other phenylpropanoids in the aqueous and EtOH extracts for the two investigated plants. The lowest content of phenylpropanoids in the lyophilized decoctions was probably due to the heat instability of verbascosides and analogues which can be totally degraded after 20 min boiling.

The EtOH extracts showed the highest percentages of verbascoside and its analogues: 5.5% in verbena and 12% in lemon verbena; infusions had intermediate values with a content of phenylpropanoids of 3.24 and 8.3% for verbena and lemon verbena, respectively.

Table 2
Yields (in %) of the dried extracts with respect to the dried weights of leaves for verbena and lemon verbena

	Yields in % of dried weight
LV dec1	7.13
V dec1	3.7
LV dec2	17.1
V dec2	9.02
LV tea	15.85
V tea	12.27
LV EtEx	6.96
V EtEx	5.26

Dec1: 20-min decoction; dec2: 5-min decoction; EtEx = EtOH extract.

It is very interesting to note that the commercial aqueous extract of verbena contained less than 0.5% of total verbascoside and its analogues, a similar or lower content of phenylpropanoids compared to our decoctions, the extracts with the minor amount of these compounds.

On the basis of the obtained quantitative results, the best phytocomplex was represented by the tea of lemon verbena for both its concentration of phenylpropanoids and its better technological characteristics for use in cosmetic or herbalistic applications. In light of the great evidence regarding the antioxidant activities of phenylpropanoids, also a DPPH test was performed on this selected lemon verbena tea and the results were compared

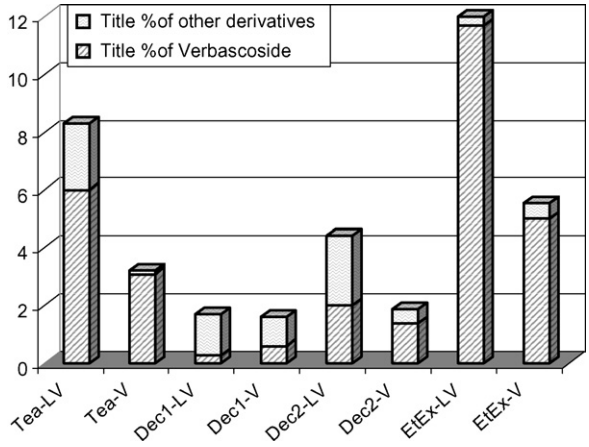

Fig. 8. Histogram of the title % of verbascoside and the other phenylpropanoids in the different lemon verbena (LV) and verbena (V) extracts. Dec1: 20-min decoction; dec2: 5-min decoction; EtEx = EtOH extract.

with pure verbascoside and Trolox used as positive reference compound.

The inhibition percentage was calculated according to the following formula where A is the absorbance measured at 517 nm:

$$\% \text{ of Inhibition} = \frac{A_{\text{DPPH}} - A_{\text{sample}}}{A_{\text{DPPH}}} \times 100$$

The most important observation is that both verbascoside and the infusion are more active than Trolox. In addition, pure verbascoside is less active with respect to the infusion (considering an amount containing the same verbascoside concentration) and this behaviour is probably due to a synergistic effect of the phenylpropanoid compounds present in the phytocomplex.

4. Conclusions

A rapid and simple HPLC assay was developed and validated for both verbena and lemon verbena preparations. The analytical method provided a satisfactory accuracy, specificity and reproducibility, together with a good separation of the different classes of constituents such as iridoids, flavonoids and phenylpropanoids. Good linearity of the calibration curves was achieved between 0.1 and 2.5 μg ($r > 0.99$); the repeatability and reproducibility of the methods were satisfactory.

From the quantitative analyses, herbal teas of verbena and lemon verbena represent a good source of antioxidant compounds, mainly represented by verbascoside. These simple tea preparations, especially that obtained from lemon verbena, could be lyophilized to obtain powders having interesting technological properties and valuable phytocomplex to be used as ingredients of cosmetics, food supplements and herbal medicinal products due to the many biological properties of verbascoside. The commercial product and decoctions have a very low content of antioxidant compounds due to their degradations during the prolonged heating. The EtOH can exaustively extract verbascoside and analogs but it cannot be used as such because of the presence of chlorophill and other lipofilic constituents.

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