# Phenylpropanoids and their metabolites are the major compounds responsible for blood-cell protection against oxidative stress after administration of *Lippia citriodora* in rats

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## ABSTRACT

*Lippia citriodora* (lemon verbena) has been widely used in folk medicine for its pharmacological properties. Verbascoside, the most abundant compound in this plant, has protective effects associated mostly with its strong antioxidant activity. The purpose of this study was to test the effect of *Lippia citriodora* extract intake on the antioxidant response of blood cells and to correlate this response with the phenolic metabolites found in plasma. For this purpose, firstly the *Lippia citriodora* extract was characterized and its radical scavenging activity was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Then, catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GRed) activities were determined in lymphocytes, erythrocytes, and neutrophils isolated from rats after acute intake of *Lippia citriodora*. Phenolic metabolites were analysed in the same plasma samples by HPLC-ESI-TOF-MS. Myeloperoxidase (MPO) activity in neutrophils, which has been proposed as a marker for inflammatory vascular damage, was also determined. After *Lippia citriodora* administration, the antioxidant enzymes activities significantly accelerated ($p < 0.05$) while MPO activity subsided, indicating that the extract protects blood cells against oxidative damage and shows potential anti-inflammatory and antiatherogenic activities. The main compounds found in plasma were verbascoside and isoverbascoside at a concentration of 80 ± 10 and 57 ± 4 ng/mL, respectively. Five other metabolites derived from verbascoside and isoverbacoside were also found in plasma, namely hydroxytyrosol, caffeic acid, ferulic acid, ferulic acid glucuronide, and homoprotocatechuic acid, together with another eight phenolic compounds. Therefore, the phenylpropanoids verbascoside and isoverbascoside, as well as their metabolites, seem to be the responsible for the above-mentioned effects, although the post-

transcriptional activation mechanism of blood-cell antioxidant enzymes by these compounds needs further investigation.

**Keywords:** *Lippia citriodora*, DPPH assay, antioxidant enzymes, phenolic compounds, verbascoside, anti-inflammatory.

**Abbreviations:** ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; GRed, glutathione reductase; GPx, glutathione peroxidise; MPO, myeloperoxidase; DPPH, 2,2-diphenyl-1-picrylhydrazyl

## INTRODUCTION

*Lippia citriodora* (lemon verbena), a shrub indigenous to South America, was introduced into Europe at the end of the 17th century and has been widely used in infusions for its antispasmodic, antipyretic, sedative, and digestive properties (Carnat et al., 1999, Pascual et al., 2001, Valentao et al., 1999). *Lippia citriodora* leaves contain a large number of polar compounds such as phenylpropanoids, flavonoids, phenolic acids, and iridoid glycosides, verbascoside being the most abundant (Quirantes-Pine et al., 2009). Several properties have been described for this compound, such as anti-inflammatory (Deepak and Handa, 2000, Diaz et al., 2004), antimicrobial (Avila et al., 1999), and antitumor (Ohno et al., 2002) activity. These protective effects have been attributed, among other factors, to its antioxidant activity (Valentao et al., 2002, Wong et al., 2001).

Reactive oxygen species (ROS) have been associated with the mediation of several pathological processes, including inflammatory diseases, cancer, and atherosclerosis. Phenolic compounds can help to limit the oxidative damage caused by ROS either by acting directly on ROS or by stimulating endogenous defence systems. These defence systems include antioxidant enzymes, namely catalase (CAT), glutathione reductase (GRed), and glutathione peroxidase (GPx), which act as scavengers of the ROS. CAT catalyses the conversion of $H_2O_2$ to water, preventing the generation of hydroxyl radicals, GRed reduces glutathione disulfide to the sulfhydryl form, and GPx reduces lipid hydroperoxides to their corresponding alcohols and free hydrogen peroxide to water (Aymoto Hassimotto et al., 2008).

Other studies in humans have reported that the intake of *Lippia citriodora* extract promotes the protection of blood cells by activating GRed and CAT in erythrocytes and lymphocytes, and by decreasing sport-induced oxidative damage in neutrophils

(Carrera-Quintanar et al., 2012, Funes et al., 2011). Nevertheless, these studies have not reported any metabolite derived from *Lippia citriodora* in the plasma of human volunteers. Therefore, there is a lack of knowledge about the effects of *Lippia citriodora* metabolites on the antioxidant defences of white and red blood cells.

The *in vivo* antioxidant activity of *Lippia citriodora* depends on its absorption and metabolism in the gut. Although the pharmacokinetics of verbascoside have been investigated (Funes et al., 2009, Wu et al., 2006), little is known about its metabolism as well as the bioavailability of the other compounds present in this plant.

Therefore, the aim of this study was to test the effect of *Lippia citriodora* extract intake on the antioxidant response of lymphocytes, erythrocytes, and neutrophils, and to correlate it with the phenolic metabolites found in plasma. In this way, the metabolites present in plasma samples and probably related to blood-cell protection activity against oxidative stress by *Lippia citriodora* were determined.

## MATERIALS AND METHODS

### Chemicals

All chemicals were of analytical reagent grade and used as received. Verbascoside and taxifolin were from Sigma-Aldrich (St. Louis, MO, USA). The stock solutions containing these analytes were prepared in methanol at a concentration of 100 µg/mL and stored at -20°C until used. Acetonitrile, methanol, and ammonia were from Panreac (Barcelona, Spain), hydrochloric acid from Scharlau (Barcelona, Spain), and formic acid as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH) from Sigma-Aldrich. The Ficoll reagent was obtained from GE Healthcare (Sweden). Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA).

The *Lippia citriodora* (lemon verbena) extract (20% verbascoside, w/w) was kindly provided by Monteloeder (Elche, Spain).

**Animals and experimental design**

Nine male Wistar rats (250-300 g) from 10 to 12 weeks old were housed in standard cages at room temperature with free access to food and water for two weeks. Throughout the experiments, animals were processed according to the suggested ethical guidelines for the care of laboratory animals (Morton et al., 2001).

Rats were orally treated with *Lippia citriodora* extract (1440 mg/kg) via gastric gavage (n=6). For the administration, the extract was dissolved in saline serum (2.5 mL). The control group (n=3) received only saline serum. Rats were subjected to ketamine/xylazine anaesthesia.

**Erythrocytes, lymphocytes and neutrophils purification**

Blood samples were withdrawn via cardiac puncture into heparinized tubes at 20 min after dosing. They were used to purify erythrocytes, lymphocytes and neutrophils following an adaptation of the method described by Boyum (Boyum, 1964), and plasma was stored at -80ºC for further analysis of metabolites.

**Enzymatic determinations**

CAT activity was measured by the spectrophotometric method of Aebi (Aebi, 1984) based on the decomposition of $H_2O_2$. GRed activity was measured by a modification of the Goldberg and Spooner spectrophotometric method (Goldberg and Spooner, 1983). This assay required oxidized glutathione as the substrate. GPx activity was measured by an adaptation of the spectrophotometric method of Flohé and Gunzler using $H_2O_2$ as the

137 substrate (Flohe and Gunzler, 1984). Myeloperoxidase (MPO) activity of neutrophils
138 was measured by guaiacol oxidation (Capeillere-Blandin, 1998). All activities were
139 determined with a SPECTROstar Omega microplate reader at 37ºC.
140
141 **DPPH radical scavenging assay**
142 The antioxidant capacity of the *Lippia citriodora* extract was determined by the *in vitro*
143 DPPH radical scavenging method, based on a procedure described by Brand-Williams
144 et al. (Brand-Williams et al., 1995). Briefly, a solution was prepared dissolving 19.7 mg
145 of DPPH in 100 mL of methanol. This stock solution was further diluted 1:10 with
146 methanol. Both solutions were stored at 4 ◦C until use. Different concentrations of
147 extracts were tested (250-2000 µg/mL). 20 µL of these extracts solutions were added to
148 980 µL of DPPH diluted solution to complete the final reaction medium (1 mL). After 1
149 h at room temperature in the dark, 200 µL of the mixture was transferred into a well of
150 the microplate, and the absorbance was measured at 516 nm in a microplate
151 spectrophotometer reader (BioTek). DPPH–methanol solution was used as a reference
152 sample. The DPPH concentration remaining in the reaction medium was calculated
153 from a calibration curve. The percentage of remaining DPPH against the extract
154 concentration was then plotted to obtain the amount of antioxidant necessary to decrease
155 the initial DPPH concentration by 50% or $EC_{50}$. Measurements were done by triplicate.
156
157 **Plasma treatment for HPLC-ESI-TOF-MS analyses**
158 First, 1 mL of plasma was spiked with 10 µL of the taxifolin stock solution used as
159 internal standard. Afterwards, the plasma was treated with 5 mL of HCl 200 mM in
160 methanol, vortex-mixed, kept for 2.5 h at 50ºC and centrifuged at 14800 g for 5 min.
161 The supernatant was neutralized at pH 7.0 by ammonia addition, evaporated in a

vacuum concentrator and then dissolved in 1 mL of aqueous formic acid 0.5% (v/v) at pH 2.5. Subsequently, a solid phase extraction of phenolic compounds was performed on Discovery DSC-18 cartridges (50 mg, 1 mL) Supelco, Sigma-Aldrich (Bellefonte, PA, USA). Prior to use, the cartridge was conditioned with 2 mL of methanol/formic acid 0.5% (v/v) and equilibrated with 2 mL of water/formic acid 0.5% (v/v). The plasma solution previously prepared was loaded into the cartridge, followed by a washing with 1 mL of water/formic acid 0.5% (v/v). Finally, the phenolic fraction was eluted with 1 mL of methanol, dried in a vacuum concentrator, and then, resolved in 100 µL of mobile phase A.

**HPLC-ESI-TOF-MS analyses**

Analyses were performed using an Agilent 1200 Series Rapid Resolution Liquid Chromatography system (Agilent Technologies, Palo Alto, CA, USA), including a standard autosampler and a diode array detector. The HPLC column used was a Phenomenex Gemini C18 (5 µm, 4.6 x 250 mm). The separation was carried out at room temperature with a gradient elution program at a flow rate of 0.2 mL/min. The mobile phases consisted of water:acetonitrile (90:10, v/v) with 1% of formic acid (A) and acetonitrile (B). The following multi-step linear gradient was applied: 0 min, 5% B; 35 min, 20% B; 45 min, 40% B; 50 min, 5% B. The initial conditions were maintained for 10 min. The injection volume in the HPLC system was 10 µL.

The HPLC system was coupled to a microTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with an ESI interface operating in negative ion mode using a capillary voltage of +4 kV. The other optimum values of the ESI-TOF-MS parameters were drying gas temperature, 190ºC; drying gas flow, 7 L/min, and

nebulizing gas pressure, 1.5 bar. The detection was conducted considering a mass range of 50-1000 m/z.

During the execution of the method, the mass spectrometer was externally calibrated with a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA) directly connected to the interface and injected with a sodium formate cluster solution containing 5 mM sodium hydroxide and 0.2% formic acid in water:isopropanol (1:1, v/v). The calibration solution was injected at the beginning of each run and all the spectra were calibrated prior to compound identification. This method gave an exact calibration curve based on numerous cluster masses, each differing by 68 Da ($NaCHO_2$). Due to the compensation of temperature drift in the TOF-MS, this external calibration provided accurate mass values for a complete run.

The accurate mass data of the molecular ions were processed through the software Data Analysis 4.0 (Bruker Daltonik, Bremen, Germany), which provided a list of possible elemental formulas by using the Smart Formula Editor. This editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula.

**Validation of HPLC-ESI-TOF-MS method**

The accuracy of the method was further assessed with recovery studies by spiking verbascoside into control plasma in triplicates. The linearity range of the method was determined on five concentration levels from 0.5 to 10 µg/mL with three injections for each level. Limits of detection (LOD) and quantification (LOQ) were, respectively, set at S:N = 3 and S:N = 10, where S:N is the signal-to-noise ratio. Repeatability of the

method was measured as relative standard deviation (RSD %) in terms of concentration. A plasma sample was injected (n=3) on the same day (intraday precision) and 3 times on the 2 consecutive days (interday precision, n=9).

**Statistical analysis**

Statistical analysis was performed using the software OriginPro v 7.5. The results were expressed as the mean and standard deviation (Mean ± SD). The means of quantitative variables were analysed using a one-way ANOVA test. All determinations were performed in triplicate. Differences between the groups were compared using non-parametric tests and were considered statistically significant when $p < 0.05$.

## RESULTS AND DISCUSSION

### *Lippia citriodora* extract composition and radical scavenging activity

The *Lippia citriodora* extract administered to the rats was analysed by the HPLC-ESI-TOF-MS method described above. Figure 1 shows the base peak chromatogram of an aqueous solution of the extract at a concentration of 1 mg/mL where the peaks have been numbered according to their elution order. The compounds were identified by interpretation of their MS spectra obtained by TOF–MS combined with the data provided in the literature and their relative retention time values, and they are summarised in Table 1.

As can be observed, phenylpropanoids are the major class of metabolites found in this extract, being verbascoside the most abundant one which is in agreement with the data previously reported (Bilia et al., 2008, Funes et al., 2009). The presence of several verbascoside derivatives is also noteworthy including the decaffeoyl-derivative

verbasoside, two hydroxylated derivatives and the isomer isoverbascoside. Several flavonoids have been also identified, all of them as diglucuronide conjugates.

Iridoids have been widely described as one of the main families of compounds in *Lippia citriodora* (Bilia et al., 2008, Quirantes-Pine et al., 2009, Quirantes-Pine et al., 2010). In this way, shanzhiside, gardoside and theveside have been identified in the extract. In addition to these compounds, other components of the extract such as organic acids or a tuberonic acid derivative have been characterized.

After the HPLC-MS characterization of the extract, its scavenging effect on DPPH radical was examined. The *Lippia citriodora* extract showed a high radical scavenging activity with an $EC_{50}$ value of 13 ± 1 µg/mL. This antioxidant effect was stronger than other strong antioxidant extracts such as olive leaf (Hayes et al., 2011), white and green teas (Unachukwu et al., 2010), and other extracts belonging to *Lippia* genus non enriched in verbascoside (Shikanga et al., 2010) but also higher than the reported for verbascoside pure standard (Georgiev et al., 2011, Shikanga et al., 2010) which is in agreement with the previously reported data (Bilia et al., 2008). Therefore, a synergistic effect of the phenylpropanoids, flavonoids and iridoids compounds present in the extract could be responsible for the strong *in vitro* radical scavenging activity of the extract.

**Blood-cell antioxidant response in rats after oral administration of** *Lippia citriodora* **extract**

Once revealed the *in vitro* antioxidant activity of the extract, the *ex vivo* activity of several antioxidant enzymes (CAT, GPx and GRed) was determined in erythrocytes, lymphocytes, and neutrophils of rats 20 min after the acute intake of *Lippia citriodora* extract since previous studies in rats showed the maximum plasma antioxidant capacity

261 at 20 min (Funes et al., 2009). MPO activity, which has been proposed as a marker for
262 inflammatory vascular damage (Faith et al., 2008, Koelsch et al., 2010), was also
263 determined in neutrophils of the same animals. Mean values of antioxidant enzyme and
264 MPO activities in different circulating cell types are listed in Fig. 2 (see also
265 supplementary information).
266 As shown, CAT activity was significantly stimulated in lymphocytes and erythrocytes,
267 although no significant effect was detected in neutrophils. GPx activity considerably
268 accelerated in all three cell fractions studied. The results also showed a significant
269 activation of GRed both in lymphocytes and in neutrophils, this increase being
270 especially significant in neutrophils. Finally, MPO activity significantly declined in
271 neutrophils, indicating a reduction in the release of damaging ROS in this cell type
272 (Faith et al., 2008).
273
274 **Characterization of metabolites in plasma by HPLC-ESI-TOF-MS**
275 For an understanding of how the phenolic compounds from the *Lippia citriodora* extract
276 affect the antioxidant and anti-inflammatory processes, it is necessary to determine
277 which compounds from the extract effectively reach the target tissues and whether they
278 do so in their native form or as metabolites. Therefore, plasma samples were analysed
279 by HPLC-ESI-TOF-MS to determine the phenolic compounds and their metabolites
280 present in plasma.
281 The method applied was previously validated and its analytical parameters are shown in
282 Table 2. The verbascoside recovery was established at 76 ± 8%, and LOD and LOQ
283 were 7.5 and 25 ng/mL, respectively. Intraday repeatability of the method developed
284 was 1.95%, whereas the interday repeatability was 4.04%.

285 This method enabled the identification of several compounds in plasma samples, which
286 are shown in Table 3 together with their retention time and MS data, including
287 experimental and calculated m/z for the molecular formulas provided, error and mSigma
288 value. The compounds were identified by comparison of retention times from plasma
289 samples and *Lippia citriodora* extract, in the case of the compounds previously detected
290 in the extract, as well as by using molecular formulas provided by the software
291 according to the accurate mass and isotopic pattern. Fig. 3 shows the extracted ion
292 chromatograms (EIC) of some of the compounds detected in plasma.
293 The main compounds found in plasma were verbascoside and isoverbascoside. These
294 compounds were quantified showing a mean concentration of 80 ± 10 and 57 ± 4
295 ng/mL, respectively. The detection of high levels of intact verbascoside and
296 isoverbascoside in plasma indicated that these glycosylated phenylpropanoids can be
297 absorbed in their native forms, in agreement with previous studies where verbascoside
298 was found in plasma after oral administration of verbascoside-enriched extracts (Funes
299 et al., 2009, Wu et al., 2006).
300 Five metabolites deriving from verbascoside and isoverbacoside were also found in
301 plasma due to the metabolism of the rats, namely hydroxytyrosol, caffeic acid, ferulic
302 acid, ferulic acid glucuronide, and homoprotocatechuic acid. Polyphenol glycosides are
303 absorbed mainly through the gut barrier after deglycosylation by the action of lactase
304 phloridzin hydrolase in the brush border of the small-intestine epithelial cells, cytosolic
305 ß-glucosidase within the epithelial cells, or colonic microbiota in the large intestine
306 (Crozier et al., 2010). Therefore, hydroxytyrosol and caffeic acid may be formed mostly
307 by the hydrolysis of glycosydic bonds of verbascoside and isoverbascoside. Moreover,
308 caffeic acid may also derive from the hydrolysis of other complex polyphenols from the
309 extract such as cistanoside F and campneoside I which in their structures contain a

caffeoyl group bound to glycoside moieties. Similarly, hydroxytyrosol may also be formed by hydrolysis of verbasoside and eukovoside.

The catechol-like structure of caffeic acid makes it predictably prone to *O*-methylation by soluble catechol-*O*-methyltransferase, resulting in ferulic acid structure, which may undergo subsequent glucuronidation due to the action of uridine-5´-diphosphate glucuronosyltransferases. Indeed, ferulic acid has been described as a metabolite of caffeic acid after coffee ingestion by humans (Stalmach et al., 2009). Furthermore, the hydrolysis of eukovoside and martynoside may contribute to the formation of ferulic acid. Homoprotocatechuic acid has been also described as a metabolite of caffeic acid (Gonthier et al., 2003, Olthof et al., 2003).

Furthermore, flavone derivatives have been detected in the plasma samples, mainly, acacetin diacetate, luteolin diglucuronide, and chrysoeriol diglucuronide. Acacetin diacetate probably arises from the metabolism of acacetin diglucuronide, which may cleave the glucuronic bond and then conjugate with two acetate groups as a result of the phase-II metabolism. Luteolin diglucuronide and chrysoeriol diglucuronide may come from the absorption of the intact compounds from the extract as well as further conjugation with two glucuronic moieties after the absorption of the free flavones in the gut.

Other compounds from the extract have also been detected intact in plasma, such as gardoside, cistanoside F, theveside, eukovoside, and martynoside. As mentioned above, polyphenol glycosides are absorbed mainly through the gut barrier after deglycosylation and, subsequently, they are conjugated to form *O*-glucuronides, sulphate esters and *O*-methyl ethers (Scalbert et al., 2002). However, some studies have suggested that intact glycosides of quercetin may be absorbed in the small intestine by a mechanism involving the glucose-transport pathway (Gee et al., 1998, Hollman et al., 1995), and

non-conjugated polyphenols have also been detected in studies administering pharmacological doses, indicating a possible saturation of the conjugation pathways (Das, 1971).

**Correlation between plasmatic phenolic metabolites and antioxidant enzyme modulation**

The major metabolites found in rat plasma were the phenylpropanoids verbascoside and isoverbascoside. Some other metabolites, probably deriving from verbascoside and/or isoverbascoside metabolism, were also found in plasma but at much lower concentrations, mainly hydroxytyrosol and phenolic acid derivatives such as caffeic, ferulic, and homoprotocatechuic acids. In previous studies, the antioxidant effect of *Lippia citriodora* extracts has been attributed entirely to verbascoside. However, isolated phenolic acids such as gentisic, gallic, ferulic, and p-coumaric acids, as well as coffee rich in caffeic, ferulic, and p-coumaric acids have shown to increase hepatic SOD, GPx, and CAT activities (Valadao Vicente et al., 2011, Yeh and Yen, 2006). Likewise, other study has reported increased CAT and SOD activities in the liver after hydroxytyrosol supplementation in rats (Jemai et al., 2008). Therefore, the enhancement of the antioxidant defences may not be exclusively due to the direct modulation of enzymes activity by verbascoside but the combined action of its metabolites.

Moreover, low levels of flavone derivatives (acacetin, luteolin and chrysoeriol) and phenylpropanoids different from verbascoside were also found. Many flavonoids, including flavones, have been shown to modulate CAT activity by binding to the heme group or a protein region of CAT structure, helping to stimulate activity (Doronicheva et al., 2007). However, little is known about the effect of the other phenylpropanoids found in plasma on antioxidant enzymes.

Therefore, verbascoside, isoverbascoside and their metabolites seem to be the compounds most plausibly responsible for the observed enzymatic activation in the different blood cells. Currently, the precise mechanism by which antioxidant enzymes are activated after the administration of the extract is unknown, although the gene-expression regulation must be ruled out due to the short time of the observed effect. Recently, the *in vitro* activation of GSH-reductase by verbascoside and *Lippia citriodora* extract has been reported (Carrera-Quintanar et al., 2012). It is postulated that this activation may occur at the post-translational level, a situation that may deserve further attention.

## CONCLUSIONS

In conclusion, these findings demonstrate that the consumption of *Lippia citriodora* extract protects blood cells by powering endogenous antioxidant defences of the different cell types, especially in lymphocytes, and shows potential anti-inflammatory and antiatherogenic activities through the inhibition of MPO in neutrophils. This finding may indicate a protective effect of these metabolites in neutrophils against free radicals which may compromise immune function. As derived from the metabolites detected in plasma by HPLC-ESI-TOF-MS, the phenylpropanoids verbascoside and isoverbascoside, as well as their metabolites appear to be the responsible for the above-mentioned effects. In any case, further research is needed to elucidate the post-transcriptional activation mechanism of blood-cell antioxidant enzymes.

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518 **Table 1.** Compounds identified in lemon verbena extract classified by families.
519 Numbers designing compounds correspond to peaks as depicted in Fig. 1.
520

| Peak | $t_R$ (min) | Proposed compound |
|---|---|---|
| Organic acids | | |
| 1 | 7.5 | Gluconic acid |
| 2 | 8.5 | Malic acid |
| 3 | 8.9 | Citric acid |
| Iridoids | | |
| 5 | 10.0 | Shanzhiside |
| 6 | 10.2 | Gardoside |
| 9 | 15.1 | Theveside |
| Phenylpropanoids | | |
| 7 | 10.8 | Verbasoside |
| 8 | 12.3 | Cistanoside F |
| 12 | 24.0 | β-hydroxy-verbascoside/ β-hydroxy-isoverbascoside |
| 13 | 24.6 | |
| 15 | 30.7 | Campneoside I or isomer |
| 16 | 31.0 | |
| 17 | 32.7 | Verbascoside |
| 19 | 36.0 | Isoverbascoside |
| 20 | 38.9 | Eukovoside |
| 22 | 46.8 | Martynoside |
| Flavonoids | | |
| 14 | 27.0 | Luteolin-7-diglucuronide |
| 18 | 34.3 | Chrysoeriol-7-diglucuronide |
| 21 | 45.2 | Acacetin-7-diglucuronide |
| Other compounds | | |
| 10 | 20.7 | Tuberonic acid glucoside/ 5´-hydroxyjasmonic acid 5´-O-glucoside |
| Not identified | | |
| 4 | 9.4 | Unknown |
| 11 | 21.2 | Unknown |

521 **Table 2.** Analytical parameters of the HPLC-ESI-TOF-MS method

| Analyte | Verbascoside |
|---|---|
| Recovery | 76 ± 8 % |
| Calibration range (ng/mL) | 500 - 10000 |
| Calibration equation | y = 0.062x – 0.030 |
| $r^2$ | 0.991 |
| LOD (ng/mL) | 7.5 |
| LOQ (ng/mL) | 25 |
| RSD intraday (n=3) | 1.95% |
| RSD interday (n=9) | 4.04% |

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525 **Table 3.** Compounds identified in rat plasma by HPLC-ESI-TOF-MS after oral administration of the *Lippia citriodora* extract.

| $t_r$(min) | m/z experimental | Molecular formula | m/z calculated | Error (ppm) | mSigma | Proposed compound |
|---|---|---|---|---|---|---|
| 10.3 | 373.1133 | $C_{16}H_{21}O_{10}$ | 373.1140 | 2.0 | 90.2 | Gardoside |
| 10.9 | 461.1665 | $C_{20}H_{29}O_{12}$ | 461.1664 | -0.2 | 25.1 | Verbasoside |
| 12.5 | 487.1474 | $C_{21}H_{27}O_{13}$ | 487.1457 | -3.4 | 34.1 | Cistanoside F |
| 12.9 | 367.0893 | $C_{20}H_{15}O_{7}$ | 367.0823 | -18.9 | 58.0 | Acacetindiacetate |
| 14.4 | 153.0551 | $C_{8}H_{9}O_{3}$ | 153.0557 | 4.0 | 50.1 | Hydroxytyrosol |
| 14.8 | 167.0331 | $C_{8}H_{7}O_{4}$ | 167.0350 | 11.1 | 95.4 | Homoprotocatechuic acid |
| 15.5 | 389.1060 | $C_{16}H_{21}O_{11}$ | 389.10894 | 7.6 | 90.6 | Theveside |
| 17.80 | 193.0482 | C10H9O4 | 193.0506 | 12.8 | 62.9 | Ferulic acid |
| 19.9 | 179.0321 | C9H7O4 | 179.0350 | 16.3 | 56.7 | Caffeic acid |
| 27.6 | 637.1076 | $C_{27}H_{25}O_{18}$ | 637.1046 | -4.7 | 154.9 | Luteolin diglucuronide |
| 28.1 | 369.0836 | $C_{16}H_{17}O_{10}$ | 369.0827 | -2.3 | 90.2 | Ferulic acid glucuronide |
| 33.1 | 623.1950 | $C_{29}H_{35}O_{15}$ | 623.1981 | 5.1 | 10.5 | Verbascoside |
| 34.7 | 651.1147 | $C_{28}H_{27}O_{18}$ | 651.1203 | 8.5 | 55.6 | Chrysoeriol diglucuronide |
| 36.4 | 623.1928 | $C_{29}H_{35}O_{15}$ | 623.1981 | 8.5 | 31.5 | Isoverbascoside |
| 39.1 | 637.2104 | $C_{30}H_{37}O_{15}$ | 637.2138 | 5.3 | 110.2 | Eukovoside |
| 46.7 | 651.2230 | $C_{31}H_{39}O_{15}$ | 651.2294 | 9.8 | 178.5 | Martynoside |

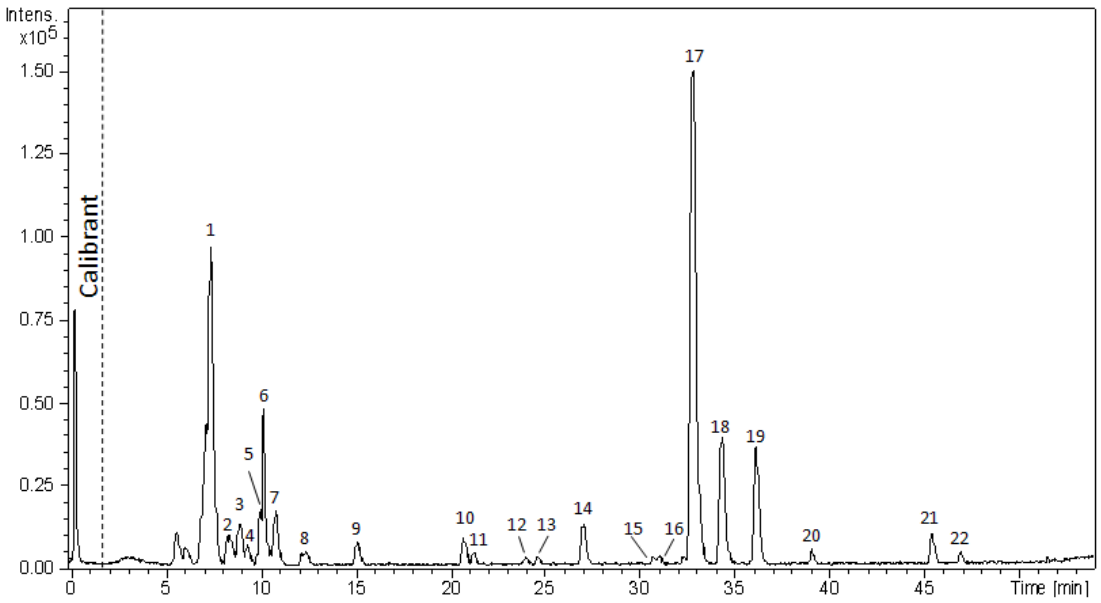


Figure 1. Base peak chromatograms (50–1000 m/z) of the *Lippia citriodora* extract in which the peaks are identified with numbers 1–22 according to the order of elution (see Table 1).

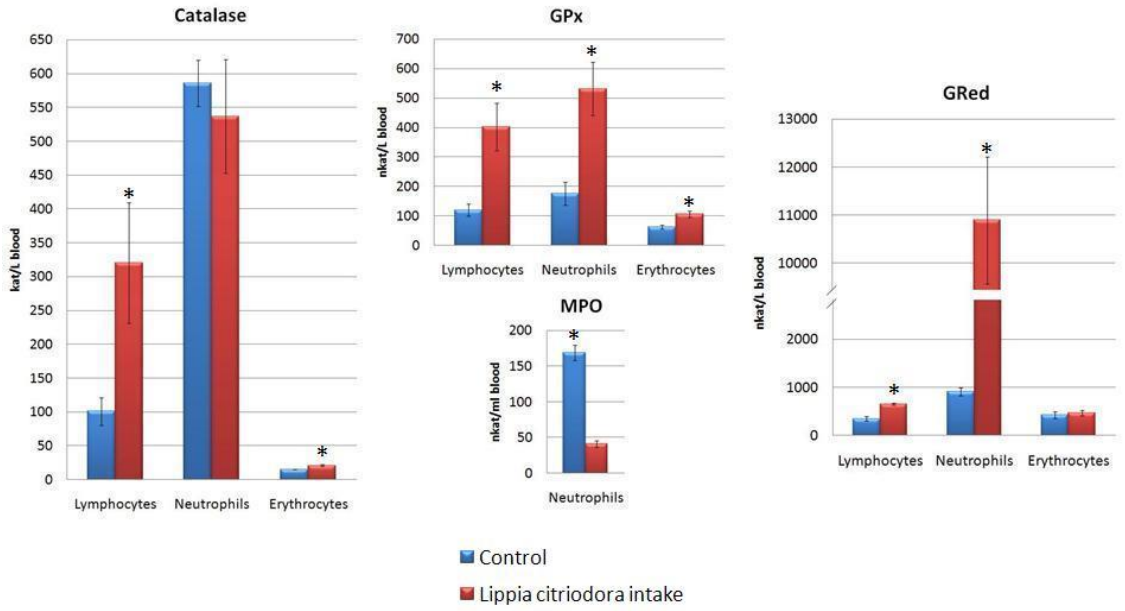


Figure 2. Effect of *Lippia citriodora* administration on the activity of antioxidant enzymes of several blood cells and myeloperoxidase activity in neutrophils. *Significant differences between control group and *Lippia citriodora* administered rats ($p < 0.05$).

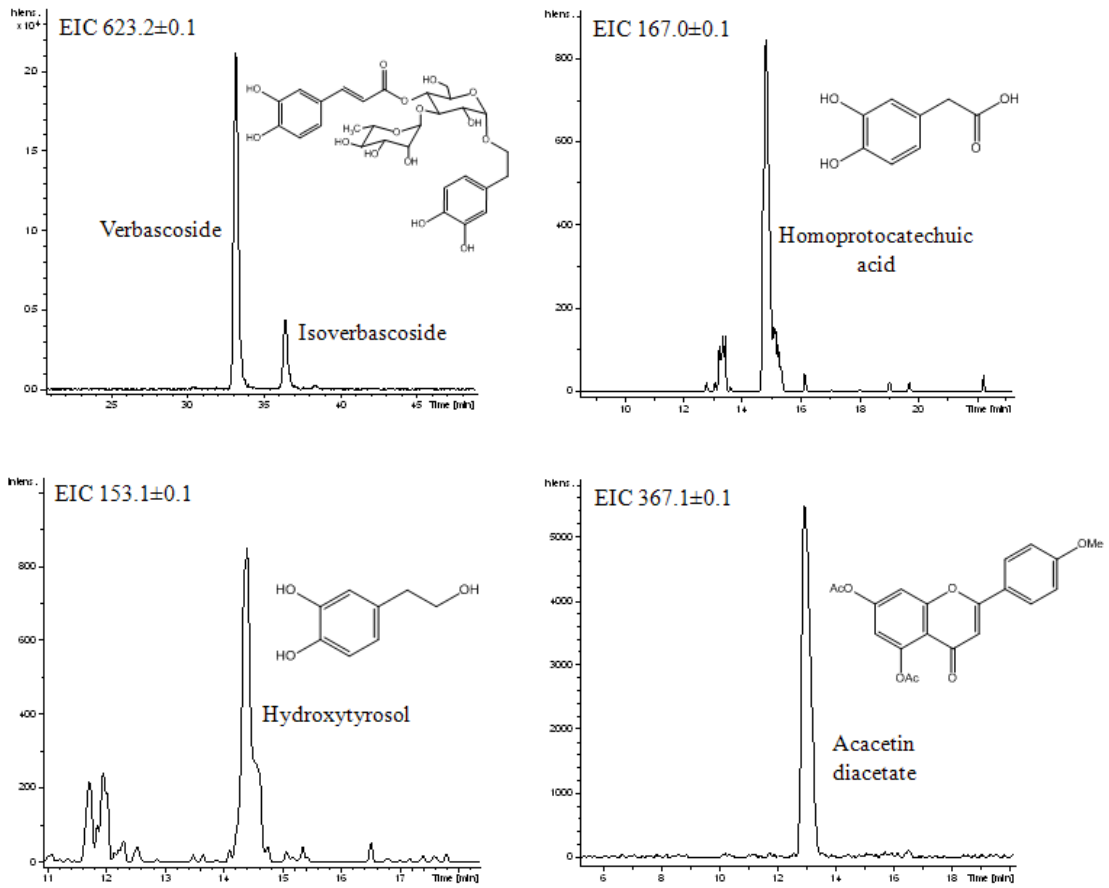


Figure 3. Extracted ion chromatograms of the main compounds found in rat plasma 20 min after acute *Lippia citriodora* intake.

**Supplementary Table 1**

**Table 1.** Effect of *Lippia citriodora* administration on the activity of antioxidant enzymes of several blood cells and neutrophil myeloperoxidase activity in rats.

| Blood-cell fraction | Enzymatic activity | Control | *L. citriodora* supplemented |
|---|---|---|---|
| **Lymphocytes** | Cat (kat/l blood) | 100 ± 20 | 320 ± 90 (*$p$ = 0.014) |
| | GPx (nkat/l blood) | 120 ± 20 | 400 ± 80 (*$p$ = 0.002) |
| | GRed (nkat/l blood) | 350 ± 50 | 650 ± 20 (*$p$ = 0.00003) |
| **Neutrophils** | Cat (kat/l blood) | 590 ± 30 | 540 ± 80 |
| | GPx (nkat/l blood) | 180 ± 40 | 530 ± 90 (*$p$ = 0.0015) |
| | GRed (nkat/l blood) | 910 ± 80 | 11000 ± 1000 (*$p$ = 0.00016) |
| | MPO (nkat/ml blood) | 170 ± 10 | 41 ± 4 (*$p$ = 0.00000) |
| **Erythrocytes** | Cat (kat/l blood) | 15.0 ± 0.2 | 21 ± 1(*$p$ = 0.00014) |
| | GPx (nkat/l blood) | 62 ± 6 | 110 ± 10(*$p$ = 0.00098) |
| | GRed (nkat/l blood) | 420 ± 70 | 470 ± 60 |

*Significant differences between control and supplemented with extract (one-way ANOVA). *P* values are indicated.

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## FIGURE CAPTIONS

**Figure 1.** Base peak chromatograms (50–1000 m/z) of the *Lippia citriodora* extract in which the peaks are identified with numbers 1–48 according to the order of elution (See Table 1).

**Figure 2.** Effect of *Lippia citriodora* administration on the activity of antioxidant enzymes of several blood cells and myeloperoxidase activity in neutrophils. *Significant differences between control group and *Lippia citriodora* administered rats ($p < 0.05$).

**Figure 3.** Extracted ion chromatograms of the main compounds found in rat plasma 20 min after acute *Lippia citriodora* intake.