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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 *L. 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 *L. 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 *L. citriodora*. Phenolic metabolites were analyzed 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 *L. 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 isoverbascoside 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.

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). *L. 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

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

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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 *L. 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 *L. citriodora* in the plasma of human volunteers. Therefore, there is a lack of knowledge about the effects of *L. citriodora* metabolites on the antioxidant defences of white and red blood cells.

The *in vivo* antioxidant activity of *L. citriodora* depends on its absorption and metabolism in the gut. Although the pharmacokinetics of verbascoside has 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 *L. 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 *L. 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 *L. 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 *L. 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 (1964), and plasma was stored at −80 °C for further analysis of metabolites.

Enzymatic determinations

CAT activity was measured by the spectrophotometric method of 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 substrate (Flohe and Gunzler 1984). Myeloperoxidase (MPO) activity of neutrophils was measured by guaiacol oxidation (Capeillere-Blandin 1998). All activities were determined with a SPECTROstar Omega microplate reader at 37 °C.

DPPH radical scavenging assay

The antioxidant capacity of the *L. citriodora* extract was determined by the *in vitro* DPPH radical scavenging method, based on a procedure described by Brand-Williams et al. (1995). Briefly, a solution was prepared dissolving 19.7 mg of DPPH in 100 mL of methanol. This stock solution was further diluted 1:10 with methanol. Both solutions were stored at 4 °C until use. Different concentrations of extracts were tested (250–2000 μg/ml). 20 μl of these extracts solutions were added to 980 μl of DPPH diluted solution to complete the final reaction medium (1 ml). After 1 h at room temperature in the dark, 200 μl of the mixture was transferred into a well of the microplate, and the absorbance was measured at 516 nm in a microplate spectrophotometer reader (BioTek). DPPH–methanol solution was used as a reference sample. The DPPH concentration remaining in the reaction medium was calculated from a calibration curve. The percentage of remaining DPPH against the extract concentration was then plotted to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% or EC_{50}. Measurements were done by triplicate.

Plasma treatment for HPLC–ESI-TOF-MS analyses

First, 1 ml of plasma was spiked with 10 μl of the taxifolin stock solution used as internal standard. Afterwards, the plasma was treated with 5 ml of HCl 200 mM in methanol, vortex-mixed, kept for 2.5 h at 50 °C and centrifuged at 14,800 × *g* for 5 min. 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 mm × 250 mm). The separation was carried out at room temperature with a gradient elution programme 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