Studies on the Antioxidant Activity of *Lippia citriodora* Infusion: Scavenging Effect on Superoxide Radical, Hydroxyl Radical and Hypochlorous Acid

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*Lippia citriodora* is an herbal species which contains several flavonoids and phenolic acids. In view of the pharmacological interest in natural phenolic compounds as antioxidants, this study examined the superoxide radical, hydroxyl radical and hypochlorous acid scavenging activities of *L. citriodora* infusion. Superoxide radical was generated either in an enzymatic or in a chemical system, and scavenging ability was assessed by the inhibition of nitroblue tetrazolium reduction. Hydroxyl radical was generated by the reaction of an iron-EDTA complex with H₂O₂ in the presence of ascorbic acid, and was assayed by evaluating deoxyribose degradation. Hypochlorous acid scavenging activity was tested by measuring the inhibition of 5-thio-2-nitrobenzoic acid oxidation. The results demonstrate that this infusion has a potent superoxide radical scavenging activity and a moderate scavenging activity of hydroxyl radical and hypochlorous acid. The chemical composition of the lyophilized infusion was also determined in an attempt to establish its relationship with the antioxidant activity found in the present study.

Key words *Lippia citriodora*; phenolic compound; superoxide radical; hydroxyl radical; hypochlorous acid

In the past few years, natural antioxidants have generated considerable interest in preventive medicine and in the food industry. For the replacement of conventional synthetic antioxidants in food by natural products, spices are considered to be a promising source.1) In consequence, attention has been focused on the characterization of the antioxidant properties of extracts from several plant materials, or on the isolation and identification of the constituents responsible for those activities.2–4) Within these compounds, flavonoids and phenolic acids, phytochemicals with a large distribution in nature, have been the object of a great number of studies of their antioxidative activity, which is mainly due to their capacity to act as free radical scavengers and/or as metal chelators.5)

*Lippia citriodora* (Ort.) HBK (Verbenaceae) is a herbal species mainly used as a spice and medicinal plant. It grows spontaneously in South America and is cultivated in North Africa and Southern Europe. The leaves of this species are reported to possess digestive, antispasmodic, antipyretic, sedative and stomachic properties. It has traditionally been used in infusions for the treatment of asthma, cold, fever, flatulence, colic, diarrhoea and indigestion.6) Previous studies on *L. citriodora* characterized its chemical composition, and revealed the presence of several flavonoids and phenolic acids.7–11) However, there is no available information relating to the antioxidant properties of this species.

The aim of the present study was to evaluate the antioxidant capacity of *L. citriodora* infusion, since this is the common form of usage of the plant. Thus, the scavenging effect on O₂⁻ generated by either an enzymatic or chemical system was studied. The scavenging capacity of the lyophilized infusion on ·OH generated by a Fenton system, in the presence and absence of EDTA or ascorbic acid, and on HOCl, was also evaluated. The antioxidant activity exhibited by the lyophilized infusion is the resulting sum effect of the several compounds present, probably belonging to different chemical classes. Certainly, the phenolic constituents of the extract should contribute to this antioxidant capacity to an important extent; in order to characterize the phenolic fraction we performed HPLC/diode array detection (HPLC/DAD) analysis, and the correlation between the observed effects and phenolic composition was made.

MATERIALS AND METHODS

**Chemicals** Xanthine (X), xanthine oxidase (XO) grade I from buttermilk (EC 1.1.3.22), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), anhydrous ferric chloride (FeCl₃), ethylenediaminetetraacetic acid disodium salt (EDTA), ascorbic acid, trichloroacetic acid, thiobarbituric acid (TBA), deoxyribose, sodium hypochlorite (NaOCl), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), lipoic acid and sodium borohydride were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). All other reagents were of analytical grade. Ultrapure Milli Q water was used throughout.

**Plant Material** *L. citriodora* leaves were collected in Vila da Feira (Portugal), in August 1997, and identified by Prof. Dr. Rosa Seabra. A voucher specimen was deposited at Laboratory of Pharmacognosy, Faculty of Pharmacy, Porto University.

**Plant Infusion Preparation** *L. citriodora* leaves were dried at room temperature. The infusion was made by pouring 200 ml of boiling water on 5 g of plant material. The mixture was left to stand for 15 min and then filtered. The obtained infusion was freeze-dried (Modulyo 4K Freeze Dryer Edwards). The yield of the lyophilized infusion was 1.18 g.

**HPLC Analysis of Phenolic Compounds** Separation and identification of phenolic compounds was performed as previously reported.8–10)
**Superoxide Radical Scavenging Activity**  The antiradical activity of *L. citriodora* lyophilized infusion was determined spectrophotometrically in a 96-well plate reader (Ceres 900) by monitoring its effect on the reduction of NBT by superoxide radical at 560 nm.

**Non-enzymatic Assay**  Superoxide radicals were generated by the PMS/NADH system according to a described procedure.12,13) The reaction mixtures in the sample wells consisted of NADH (166 μM), NBT (43 μM), lyophilized infusion (5.2, 10.4, 20.8, 41.7, 83.3, 166.7 μg/ml) and PMS (2.7 μM), in a final volume of 300 μl. All components were dissolved in phosphate buffer 19 mM, pH 7.4. The reaction was conducted at room temperature for 2 min, and initiated by the addition of PMS.

**Enzymatic Assay**  Superoxide radicals were generated by the X/XO system, following a described procedure.12,13) The reaction mixtures in the sample wells consisted of xanthine (44 μM), XO (0.29 U/ml), NBT (50 μM), and lyophilized infusion (0.5, 1.0, 2.1, 4.2, 8.3, 16.7 μg/ml), in a final volume of 300 μl. Xanthine was dissolved in NaOH 1 μM, and subsequently in phosphate buffer 50 mM with EDTA 0.1 mM, pH 7.8, xanthine oxidase in EDTA 0.1 mM, and the other components in phosphate buffer 50 mM with EDTA 0.1 mM, pH 7.8. The reaction was conducted at room temperature for 2 min, and initiated by the addition of XO.

**Effect on XO Activity**  The effect of the lyophilized infusion on xanthine oxidase activity was evaluated by measuring the formation of uric acid from xanthine in a double beam spectrophotometer (Shimadzu 2600), at room temperature. The reaction mixtures contained the same proportion of components as in the enzymatic assay for superoxide radical scavenging activity, except NBT, in a final volume of 600 μl. The absorbance was measured at 295 nm for 2 min. The effect of the lyophilized infusion on xanthine oxidase activity was determined spectrophotometrically at 295 nm for 2 min.

**Hydroxyl Radical Scavenging Effect**  The sugar deoxyribose is degraded by exposure to hydroxyl radicals generated by Fenton systems. If the resulting complex mixture is heated under acid conditions, fragments of deoxyribose may be detected by its ability to react with TBA to form a pink chromogen.14) The deoxyribose method for determining the scavenging effect of the lyophilized infusion on hydroxyl radical was performed according to a described procedure.15) Reaction mixtures contained, in a final volume of 1 ml, ascorbic acid (50 μM), FeCl₃ (20 μM), EDTA (2 mM), H₂O₂ (1.42 mM), deoxyribose (2.8 mM) and lyophilized infusion (0.03, 0.16, 0.80, 4.00, 20.0, 100, 500 μg/ml). All components were dissolved in KH₂PO₄–KOH buffer, 10 mM, pH 7.4. After incubation at 37 °C for 1 h, 1 ml of 2.8% trichloroacetic acid (w/v) and 1 ml of 1% TBA (w/v) were added, and the mixture was heated in a water bath at 100 °C for 15 min. The absorbance of the resulting solution was measured at 532 nm. This assay was also performed without ascorbic acid or EDTA.

**Hypochlorous Acid Scavenging Activity**  Synthesis of Hypochlorous Acid  For the assay, 75 μM HOCl was prepared immediately before use by adjusting a solution of NaOCl to pH 6.2 with diluted sulfuric acid. The concentration of HOCl was further determined spectrophotometrically at 235 nm using the molar absorption coefficient of 100 M⁻¹ cm⁻¹.

**Synthesis of 5-Thio-2-nitrobenzoic Acid (TNB)**  TNB was prepared according to a described procedure.16) Briefly, to a 1 mM solution of DTNB in a 50 mM potassium phosphate buffer (pH 6.6) containing 5 mM EDTA, 20 mM sodium borohydride was added. The solution was incubated at 37 °C for 30 min. The concentration of TNB was determined by measuring the absorbance at 412 nm and using the molar absorption coefficient of 13600 M⁻¹ cm⁻¹.

**Assay for Hypochlorous Acid Scavenging Activity**  The assay was performed at room temperature in a cuvette containing 40 μM TNB solution, with or without the lyophilized infusion (0, 62.5, 125, 250, 500, 1000 μg/ml). The absorbance at 412 nm was measured before and 5 min after the addition of hypochlorous acid (40 μM). A positive control was performed with lipoic acid, which inhibited TNB oxidation in a concentration dependent manner.

**RESULTS AND DISCUSSION**

Reactive oxygen species (ROS) relevant to human disease are derived from three sources: (a) those generated via normal intracellular biological processes, but in an exaggerated, inappropriate fashion, or in a milieu where the normal defenses that serve to protect tissues are inadequate; (b) those released by inflammatory cells into their local environment; and (c) those secondary to xenobiotics, either because the xenobiotic includes oxidants (e.g., inhaled oxidant gases) or because the xenobiotic induces oxidant generation within cells (e.g., drugs that injure tissues through oxidant mechanisms).17) Recently, considerable attention has been given to the involvement of ROS in several pathological situations, including cancer, aging and atherosclerosis.14) ROS produced in vivo include superoxide radical (O₂⁻), hydrogen peroxide (H₂O₂) and hypochlorous acid (HOCl). H₂O₂ and O₂⁻ can interact in the presence of certain transition metal ions to yield a highly-reactive oxidizing species, the hydroxyl radical (·OH).18) *L. citriodora* lyophilized infusion strongly scavenged X/XO-generated superoxide radical in a concentration dependent manner, as shown in Fig. 1A, with an IC₅₀ at 3.3 μg/ml. A control experiment was performed to determine whether the lyophilized infusion might inhibit XO, since an inhibitory effect on the enzyme itself would also lead to a decrease in NBT reduction.21) However, it was observed that the lyophilized infusion had no effect on XO (data not shown). The capacity of the lyophilized infusion to scavenge superoxide radicals was confirmed when this radical was generated by a chemical system composed of PMS, NADH and oxygen (Fig. 1B), which indicated an IC₅₀ at 22.8 μg/ml. The different IC₅₀ values found with the two systems might be due to the higher production of superoxide radical in the non-enzymatic system, as judged by the faster rate of NBT reduction and the need for more superoxide dismutase for effective scavenging activity in the NADH/PMS (data not shown).

The *L. citriodora* lyophilized infusion also exhibited scavenging activity for the hydroxyl radical in a concentration dependent manner (Table 1). If we omit ascorbate in the reaction mixture, and if prooxidant compounds are present, they will be able to redox cycle the metal ion required for hydroxyl generation, and thus increase the radical production.14) In order to evaluate the pro-oxidant potential of the infusion, we omitted ascorbic acid, and we found that the *L. citriodora* lyophilized infusion was a very effective substitute for ascorbic acid.19)
bic acid when the concentration was higher than 4.00 mg/ml (Table 1). Thus, for concentrations above 4.00 mg/ml, this infusion may act as a pro-oxidant. Some compounds inhibit deoxyribose degradation in this assay, not by reacting with hydroxyl radicals, but because they present ion-binding capacity and can withdraw the iron ions and render them inactive or poorly active in Fenton reactions.15) Attending to this fact, the assay was also performed in the absence of EDTA in order to check the ability of the infusion to chelate iron ions. The assay performed in these conditions showed that the lyophilized infusion was able to chelate iron ions (Table 1).

Table 1. Absorbances and Scavenging Effect Obtained in the Deoxyribose Assay in the Presence and Absence of Ascorbic Acid (-AA) or EDTA (-EDTA)

<table>
<thead>
<tr>
<th>Concentration of <em>L. citriodora</em> lyophilized infusion (mg/ml)</th>
<th>ABS</th>
<th>Scavenging ratio (%)</th>
<th>ABS (-AA)</th>
<th>ABS (-EDTA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.374</td>
<td>—</td>
<td>0.158</td>
<td>0.250</td>
</tr>
<tr>
<td>0.03</td>
<td>0.364</td>
<td>2.9</td>
<td>0.140</td>
<td>0.225</td>
</tr>
<tr>
<td>0.16</td>
<td>0.334</td>
<td>11.0</td>
<td>0.125</td>
<td>0.221</td>
</tr>
<tr>
<td>0.80</td>
<td>0.281</td>
<td>24.9</td>
<td>0.120</td>
<td>0.248</td>
</tr>
<tr>
<td>4.00</td>
<td>0.231</td>
<td>38.3</td>
<td>0.146</td>
<td>0.244</td>
</tr>
<tr>
<td>20.00</td>
<td>0.249</td>
<td>33.6</td>
<td>0.252</td>
<td>0.226</td>
</tr>
<tr>
<td>100.00</td>
<td>—</td>
<td>—</td>
<td>0.470</td>
<td>0.152</td>
</tr>
<tr>
<td>500.00</td>
<td>—</td>
<td>—</td>
<td>0.446</td>
<td>0.113</td>
</tr>
</tbody>
</table>

Under the experiment conditions, lipoic acid used as a reference compound effectively scavenged HOCI in a concentration dependent manner, with a protective effect of 95% at 500 μM (Fig. 2B). HOCI is produced *in vivo* by the oxidation of Cl⁻ ions catalyzed by neutrophil-derived myeloperoxidase in the presence of H₂O₂ at sites of inflammation.18) One of the major extracellular targets of HOCI is α₁-antiproteinase, the major circulating inhibitor of serine proteases such as elastase.18) Thus, this infusion may have protective effects *in vivo* during inflammation processes.

In order to estimate a putative correlation between the observed antioxidant effect and some of its constituents, an aliquot of *L. citriodora* lyophilized infusion was subjected to HPLC-DAD analysis. This analysis revealed that, concerning UV absorbing compounds, the lyophilized infusion is mainly characterized by the presence of verbascoside, a phenyl-ethanoid compound widely distributed in medicinal plants, despite the existence of other phenolic compounds, namely luteolin derivatives (Fig. 3). The antioxidant activity of verbascoside has been thoroughly studied, as this compound has been reported to have strong protective effects in several experimental models, such as the suppression of NADPH/CCl₄-induced lipid peroxidation in rat liver microsomes,19) the inhibition of Cu²⁺-induced low-density lipoprotein oxidation,20) scavenging of O₂⁻ and 'OH using spin trap methodology21) and scavenging of O₂⁻ generated in the NADH/PMS22) or in the X/XO systems.23) The protective activity may be attributed either to the caffeoyl residue in the molecule or to the phenylethyl moiety.19) Free radical scavenging properties
of luteolin and luteolin derivatives have also been examined by assessing their ability to prevent Cu^{2+}-mediated low-density lipoprotein peroxidation. The position at which glucuronidation occurs is important for the resulting antioxidant potential, especially since the reduction potential of the B-ring of these phenolics is lower than that of the A-ring. However, in this case, glucuronidation occurs at the hydroxyl group in the A-ring of the flavonoid, and the antioxidant potential is less influenced.

Considering the results obtained, it may be anticipated that *L. citriodora* lyophilized infusion has potent antioxidant activity, achieved by the scavenging abilities observed against superoxide, hydroxyl radicals and hypochlorous acid, although a pro-oxidant effect was obtained for higher concentrations of the lyophilized infusion. Regarding its phenolic composition, the protective effects observed in this study are due, most probably, to the presence of phenolic compounds, namely verbascoside and luteolin derivatives.

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