Oxygen and Nitrogen Reactive Species Are Effectively Scavenged by *Eucalyptus globulus* Leaf Water Extract

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**ABSTRACT** *Eucalyptus globulus* Labill. (Family Myrtaceae) is a plant of Australian origin, with a reported therapeutic use in airway inflammatory diseases. Considering that reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been implicated in the pathogenesis of airway inflammatory diseases such as asthma and chronic obstructive pulmonary disease, an effective scavenging activity against these reactive species may contribute for the therapeutic effect of this plant. In the present study, a water extract of *E. globulus* leaves was evaluated for its putative *in vitro* scavenging effects on ROS (HO·, O2·−, ROO·, and H2O2) and RNS (‘NO and ONOO−) and on 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). Qualitative and quantitative analyses of the extract’s phenolic composition were also performed. The *Eucalyptus* leaf water extract presented a remarkable capacity to scavenge all the reactive species tested, with all the 50% inhibitory concentrations being found at the µg/mL level. Phytochemical analysis showed the presence of polyphenols such as flavonoids (rutin and quercitrin) and phenolic acids (chlorogenic acid and ellagic acid), which may be partially responsible for the observed antioxidant activity. These observations provide further support, beyond the well-known antibacterial and antiviral activities of the *Eucalyptus* plant, for its reported use in traditional medicine such as in the treatment of airway inflammatory diseases, considering the important role of ROS and RNS in the inflammatory process, although further studies are needed to prove the bioavailability of the antioxidants/antibacterial compounds of the extract as well as the ability of the active compounds to reach specific tissues and to act in them.

**KEY WORDS:** • airway inflammatory diseases • antioxidant activity • *Eucalyptus globulus* • reactive nitrogen species • reactive oxygen species

**INTRODUCTION**

*Eucalyptus globulus* Labill. (Family Myrtaceae) is an Australian native plant that is cultivated in subtropical regions of the world, including Africa, North and South America, Asia, and Southern Europe. According to the World Health Organization, *Eucalyptus* leaves have medicinal use as an expectorant for symptomatic treatment of mild inflammation of the respiratory tract and bronchitis and for the symptomatic treatment of asthma, fever, and inflammation of the throat.

Research data have demonstrated that this plant exhibits several biological effects, such as antibacterial,1,2 antihyperglycemic,3 analgesic, and anti-inflammatory.4 Antioxidant activity has also been reported for the essential oil,5 wood samples,6 or stem bark.7 The antioxidant activity of a commercial *Eucalyptus* leaf extract was previously evaluated by the linoleic acid peroxidation assay, the superoxide radical (O2·−) generation assay (with the xanthine–xanthine oxidase system as the source of superoxide radical), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.8 The nitric oxide (‘NO) scavenging activity of the *Eucalyptus* leaf extract was also reported before.9 No data related to scavenging of other reactive oxygen species (ROS) and reactive nitrogen species (RNS) were found in the literature for the leaf extract. The scientific acquisition of these data is considered of utmost importance since ROS and RNS are known to play a prominent role in the pathogenesis of various airway disorders such as adult respiratory distress syndrome, cystic fibrosis, idiopathic fibrosis, chronic obstructive pulmonary disease, and asthma.10,11

ROS, in accordance with their name, derive and present higher redox reactivity than molecular oxygen. The ROS designation encompasses not only free radicals, such as
O$_2$•−, hydroperoxyl radical (HO$_2$•), hydroxyl radical (HO•), peroxyl radical (ROO•), and alkoxyl radical (RO•), but also non-radicals, namely, hydrogen peroxide (H$_2$O$_2$), singlet oxygen (1O$_2$), and hypochlorous acid (HOCl). When sustainably overproduced, these ROS may strongly contribute for the aggravation of inflammatory diseases.$^{12}$

The RNS designation comprises NO and its derivatives. NO is an important endogenous messenger molecule but when generated at high concentrations can initiate a cascade of tissue damage effects. NO toxicity is not only related to levels of NO generation but is also highly dependent upon its reactivity with other species, by conversion to toxic oxidants and nitrating agents. It is well known that NO reaction with O$_2$•− produces peroxynitrite anion (ONOO$^−$), which is a strong pro-oxidant and deleterious compound.$^{13}$

The objective of the present study is thus to assess the scavenging activity for ROS and RNS exhibited by the E. globulus Labill. leaf water extract, considering the possible relevance of these effects on the plant’s therapeutic use. The evaluation of the extract’s phenolic composition was also carried out.

MATERIALS AND METHODS

Plant material

E. globulus leaves were collected during the summer season, July 2003, in Mirandela, Northern Portugal, a highland with high thermal amplitude. The leaves were dried at room temperature. Voucher specimens were preserved in our laboratory for further reference (LTF13573).

Chemicals

Dihydrorhodamine-123 (DHR-123), 4,5-diaminofluorescein (DAF-2), 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene, nitro blue tetrazolium chloride (NBT), lucigenin, diethylenetriaminepentaacetic acid, reduced form of nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), ferric chloride anhydrous (FeCl$_3$), thio-barbituric acid, mannitol, manganese dioxide, deoxyribose (DR), Folin Ciocalteu reagent, gallic acid, DPPH, chlorogenic acid, rutin, and quercitrin were purchased from Sigma (St. Louis, MO). Azodisobutryramidine dihydrochloride (AAPH), histidine, and Trolox were obtained from Fluka Chemie GmbH (Steinheim, Germany). Ellagic acid was obtained from Extrasyntèse (Genay, France). Fluorescein sodium salt and dimethylformamide were obtained from Aldrich (St. Louis). Trichloroacetic acid was obtained from Panreac (Barcelona, Spain). Ethanol (96%) was purchased from Aga (Prior Velho, Portugal). Dimethyl sulfoxide was obtained from Riedel-de Haën (Seelze, Germany). Hydrogen peroxide (30%), sodium phosphate, potassium phosphate, sodium bicarbonate, sodium nitrite, sodium hydroxide, sodium chloride, potassium chloride, EDTA disodium salt, hydrochloric acid, sodium carbonate, formic acid, and ascorbic acid were obtained from Merck (Darmstadt, Germany).

Preparation of water extracts

The dried leaves (2 g) were grounded (500 µm) and extracted five times (10 minutes, 500 rpm, 40°C with deionized water (5 × 50 mL). The extracts were gathered and then lyophilized (model 4.5 Freezone apparatus, Labconco, Kansas City, MO).

Determination of total phenolics

The amount of total phenolics in the extract was determined using the Folin-Ciocalteu colorimetric method, according to a previously described procedure.$^{14}$ In brief, 1 mL of Folin Ciocalteu reagent was added to 300 µL of extract dissolved in ethanol/water (7:3 vol/vol) solution, followed by addition of 5 mL of 20% sodium carbonate solution. The mixture was made up to 10 mL with water and thorough shaking, and the absorbance was read after 20 minutes, at 735 nm (model V 530 ultraviolet [UV]-visible spectrophotometer, Jasco Corp., Tokyo, Japan).

The contents are expressed as milligrams of gallic acid equivalents/gram of dry extract. The measurements were performed in triplicate.

Qualitative analysis

Identification of phenolic compounds was achieved with an analytical high-performance liquid chromatography unit (Gilson Medical Electronics, Villiers le Bel, France) using a reversed-phase Spherisorb ODS2 column (250 × 4.6 mm, 5 µm particle size, Merck) with a C18 guard column. Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the 200–400 nm range, and chromatograms were recorded at 280, 320, and 350 nm.

The phenolic compounds were identified by their UV spectra and their retention times. For identity confirmation, co-injection with the standards was carried out. The solvent system was a gradient of water/formic acid (19:1 vol/vol) (solvent A) and methanol (solvent B), starting with 5% methanol and installing a gradient to obtain 15% B at 3 minutes, 25% B at 13 minutes, 30% B at 25 minutes, 45% B at 39 minutes, 45% B at 42 minutes, 50% B at 44 minutes, 55% B at 47 minutes, 70% B at 50 minutes, 75% B at 56 minutes, and 80% B at 60 minutes, at a solvent flow rate of 0.9 mL/minute.$^{15}$ The lyophilized extract was dissolved in deionized water and filtered (pore size 0.22 µm) before being analyzed. Data were processed on Unipoint® system software (Gilson Medical Electronics).

Quantitative analysis

The extract (20 µL) was analyzed on an analytical high-performance liquid chromatography unit (Varian, Palo Alto, CA) using a reversed-phase Spherisorb ODS2 column (250 × 4.6 mm, 5 µm particle size, Merck) with a C18 guard column and with the same solvent system described previously. The extract was analyzed in triplicate. Phenolic com-
pound quantification was carried out using linear calibration graphs obtained from the correspondent standard solutions. The unidentified flavonol glycoside was quantified as rutin equivalents. The quantification was conducted at 320 nm for chlorogenic acid and at 350 nm for the other polyphenols. Regression equations for chlorogenic acid, ellagic acid, rutin, and quercitrin were: 

\[ y = 2.71 \times 10^{-5} x - 28888, R^2 = 0.9999; \]

\[ y = 1.49 \times 10^{-5} x + 458, R^2 = 0.9987; \]

\[ y = 1.74 \times 10^{-7} x - 7713, R^2 = 1.0000; \]

\[ y = 2 \times 10^{-7} x - 45823, R^2 = 0.9994, \]

respectively. Standards were tested in five concentrations ranging from 0.0039 to 0.5 mg/mL, except for ellagic acid (concentration range from 0.000977 to 0.125 mg/mL). Data were processed on Star Chromatography workstation software version 6.3 (Varian).

**Free radical scavenging assays**

**DPPH scavenging assay.** The scavenging effect of the stable DPPH free radical was measured by monitoring its reduction reflected in the decrease in absorbance at 515 nm, according to a described procedure, with modifications. Reaction mixtures contained DPPH dissolved in ethanol/water (7:3 vol/vol) solution (190 μM) and the extract at different concentrations dissolved in ethanol/water (7:3 vol/vol) solution, in a final volume of 200 μL. After 20 minutes, the absorbance was measured at 515 nm in a microplate reader (model ELX 808 IU, Bio-Tek, Winooski, VT). The effects are expressed as the percentage reduction of DPPH. Ascorbic acid was used as the positive control. Each study corresponded to three independent experiments performed in duplicate.

**Superoxide radical scavenging assay.** Superoxide radicals were generated by the NADH/PMS system, and the O$_2^{-}$ scavenging activity was determined spectrophotometrically in a microplate reader (model Synergy HT, Bio-Tek), by monitoring the effect of the tested extract on the O$_2^{-}$-induced reduction of NBT at 560 nm for 2 minutes. The assay was performed at 25°C. Reaction mixtures contained the following reagents, dissolved in 19 mM phosphate buffer (pH 7.4) at the indicated final concentrations (in a final volume of 300 μL): NADH (166 μM), NBT (43 μM), and tested extract at different concentrations dissolved in buffer and PMS (2.7 μM). The effects are expressed as the percentage inhibition of O$_2^{-}$-induced NBT reduction to formazan. Trolox and ascorbic acid were used as positive controls. Each study corresponded to four independent experiments, performed in triplicate.

**Hydroxyl radical scavenging assay.** Hydroxyl radicals were generated by a Fenton system (ascorbic acid/FeCl$_3$-EDTA/H$_2$O$_2$). Deoxyribose is degraded to malonaldehyde when exposed to hydroxyl radicals, which generates a pink compound with thiobarbituric acid, at low pH under heating.

Reaction mixtures contained the following reagents, at the indicated final concentrations (in a final volume of 1 mL): potassium phosphate buffer, pH 7.4 (10 mM), deoxyribose (2.8 mM), H$_2$O$_2$ (1.42 mM), the tested extract, FeCl$_3$-EDTA (20 and 100 μM), and ascorbic acid (50 μM). The iron salt was premixed with the chelator dissolved in water before addition to the reaction mixture. All other components were dissolved in potassium phosphate buffer, pH 7.4 (10 mM). After incubation at 37°C for 1 hour, 1 mL of 2.8% (wt/vol) trichloroacetic acid and 1 mL of 1% (wt/vol) thiobarbituric acid were added, and the mixture was heated in a water bath at 100°C for 15 minutes. The absorbance of the resulting solution was measured at 532 nm. This assay was also performed in the absence of ascorbic acid or EDTA to evaluate a possible pro-oxidant and/or iron chelating activity. Mannitol was used as the positive control. The effects are expressed as the percentage inhibition of DR degradation to malonaldehyde. Each study corresponded to four independent experiments performed in duplicate.

**Peroxyl radical scavenging assay.** Peroxyl radicals were generated by thermodecomposition of AAPH in a microplate reader at 37°C. The ROO$^·$ scavenging activity was measured by monitoring the decay in fluorescence due to the oxidation of fluorescein, according to a described procedure, termed the oxygen radical absorbance capacity (ORAC). Reaction mixtures contained the following reagents dissolved in 75 mM potassium phosphate buffer (pH 7.4) at the indicated final concentrations (in a final volume of 200 μL): fluorescein (81.6 nM), the tested extract at different concentrations, and AAPH (19.1 mM). Fluorescence measurements were performed on a Synergy HT microplate reader, with excitation and emission wavelengths of 485 and 528 nm, respectively.

The effects are expressed as the relative Trolox equivalent ORAC value, which is calculated by the following equation, where AUC represents the area under the curve:

Relative ORAC value = \[
\frac{[\text{AUCsample} - \text{AUCblank}]}{(\text{AUCtrolox} - \text{AUCblank})} \times \text{Trolox molarity/extract concentration (mg/mL)}
\]

Ascorbic acid was used as the positive control. Each study corresponded to four independent experiments performed in triplicate.

**Hydrogen peroxide scavenging assay.** The hydrogen peroxide scavenging activity was measured using a chemiluminescence methodology, by monitoring the H$_2$O$_2$-induced oxidation of lucigenin, according to a previously described procedure. Reaction mixtures contained the following reagents, dissolved in potassium phosphate buffer (pH 7.4) (50 mM) at the indicated final concentrations (in a final volume of 250 μL): lucigenin (3 mM) and the tested extract dissolved in buffer at different concentrations. Blank values were measured in the absence of H$_2$O$_2$. Ascorbic acid was used as the positive control. H$_2$O$_2$-elicited lucigenin oxidation results in a chemiluminescence signal that was detected using a Synergy HT microplate reader. The resulting chemiluminescence was monitored for 10 minutes. The effects are
expressed as the percentage inhibition of H$_2$O$_2$-induced lucigenin oxidation. Each study corresponded to four independent experiments performed in triplicate.

'NO scavenging assay. The 'NO scavenging activity was measured by monitoring the oxidation of the nonfluorescent DAF-2 to the fluorescent triazolofluorescein by 'NO, using the method of Nagata et al.,$^{21}$ with modifications. A stock solution of 2.073 mM DAF-2 in dimethyl sulfoxide was purged with nitrogen and stored at −20°C. Working solutions of DAF-2 diluted with buffer (50 mM Na$_2$HPO$_4$, pH 7.4) to 1/368-fold from the stock solution were prepared immediately before the determinations and placed on ice in the dark. The reaction mixtures contained the following reagents at the indicated final concentrations (in a final volume of 300 μL): DAF-2 (5 μM), 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (10 μM), and the tested extract dissolved in buffer, at different concentrations. The mixtures were incubated for 30 minutes at 37°C in a Synergy HT microplate reader. The fluorescence signal caused by the reaction of DAF-2 with 'NO was measured using the microplate reader with excitation and emission wavelengths of 485 and 528 nm, respectively. The effects are expressed as the percentage inhibition of the 'NO-induced DAF-2 oxidation. Rutin was used as the positive control. Each study corresponded to four independent experiments performed in triplicate.

Peroxynitrite scavenging assay. Synthesis of ONOO$^-$ was carried out according to a previously described procedure.$^{22}$ In brief, an acidic solution (0.7 M HCl) of 0.6 M H$_2$O$_2$ was mixed with 0.66 M NaNO$_2$ on ice for 1 second, and the reaction was quenched with ice-cold 3 M NaOH. Residual H$_2$O$_2$ was removed by mixing with granular MnO$_2$ prewashed with 3 M NaOH. The stock ONOO$^-$ solution was filtered and then frozen (−80°C), and the top layer of the solution was collected for the experiment. The concentration of ONOO$^-$ was determined by measuring the absorbance at 302 nm (ε = 1.670 M$^{-1}$ cm$^{-1}$). The typical concentration of freshly prepared ONOO$^-$ ranged from 60 to 80 mM.

The ONOO$^-$ scavenging activity was measured by monitoring the oxidation of the nonfluorescent DHR-123 to the fluorescent rhodamine-123 by ONOO$^-$ using the method of Kooy et al.,$^{23}$ with modifications. A stock solution of 2.89 mM DHR-123 in dimethyl formamide was purged with nitrogen and stored at −20°C. Buffer (90 mM NaCl, 50 mM Na$_2$HPO$_4$, and 5 mM KCl with pH 7.4) was purged with nitrogen and placed on ice before use. At the beginning of the experiments, 100 μM diethylenetriaminepentaacetic acid was added to the buffer. Working solutions of DHR-123 were diluted with the buffer from the stock solution immediately before the determinations and placed on ice in the dark. Reaction mixtures contained the following reagents at the indicated final concentrations (in a final volume of 300 μL): DHR-123 (5 μM), the tested extract dissolved in buffer at different concentrations, and ONOO$^-$ (600 nM). The mixtures were incubated for 5 minutes at 37°C in a Synergy HT microplate reader. The fluorescence signal caused by the reaction of DHR-123 with ONOO$^-$ was measured using the microplate reader with excitation and emission wavelengths of 485 and 528 nm, respectively. Ascorbic acid and cysteine were used as positive controls. The effects are expressed as the percentage inhibition of ONOO$^-$-induced DHR oxidation.

In another set of experiments, the assays were performed in the presence of 25 mM NaHCO$_3$, in order to simulate the physiological conditions with high CO$_2$ concentrations in vivo. Under those conditions, the reaction of ONOO$^-$ with CO$_2$ is predominant, with a very fast rate constant of reaction.$^{24}$ Thus, the reactivity of the putative scavengers for ONOO$^-$ should be able to match or exceed that of CO$_2$, in order to avoid any loss in its effectiveness. On the other hand, a putative high scavenging effect on the reactive species that are produced from ONOO$^-$/CO$_2$ reaction such as the nitrogen dioxide radical ('NO$_2$) and the carbonate radical anion (CO$_3$$^{2-}$), may increase its efficacy.

Each study corresponded to four independent experiments performed in triplicate.

**RESULTS**

The *Eucalyptus* leaf water extract presented a remarkable capacity to scavenge all the tested reactive species, with all the 50% inhibitory concentration (IC$_{50}$) values being found at the μg/mL level. The IC$_{50}$ values (mean ± SE) for ROS and DPPH are presented in Table 1. IC$_{50}$ values for O$_2$$^{•−}$, H$_2$O$_2$, HO$^•$, and DPPH were 8.39 ± 0.44, 389 ± 36, 104 ± 9, and 12.0 ± 0.9 μg/mL, respectively. Ascorbic acid presented IC$_{50}$ values of 4.93 ± 0.36 μg/mL for DPPH,

| Table 1. DPPH, O$_2$$^{•−}$, H$_2$O$_2$, and HO$^•$ Scavenging Activities |
|---------------------------------|----------------|----------------|------------------|
| Extract/compound                | IC$_{50}$ (μg/mL) | IC$_{50}$ (μg/mL) | IC$_{50}$ (μg/mL) |
| E. globulus                     | 12.0 ± 0.9       | 8.39 ± 0.44     | 389 ± 36         | 104 ± 9          |
| Trolox                          | —               | 452 ± 9         | —                | —                |
| Ascorbic acid                   | 4.93 ± 0.36      | 0.028 ± 0.005   | 200 ± 22         | 913 ± 48         |

Data are mean ± SEM values.
0.028 ± 0.005 μg/mL for O_2^•−, and 200 ± 22 μg/mL for H_2O_2. Trolox yielded an IC_{50} of 452 ± 9 μg/mL in the O_2^•− scavenging assay. Mannitol was the positive control for the HO• scavenging assay and exhibited an IC_{50} of 913 ± 48 μg/mL. The assay performed in the absence of ascorbic acid did not indicate any pro-oxidant activity, since no increased malonaldehyde formation was observed in the presence of the Eucalyptus extract. When the assay was performed without EDTA, iron is added as ferric chloride instead of ferric-EDTA. Some of the Fe^{3+} binds directly to deoxyribose, and generated hydroxyl radicals attack the sugar immediately. Only compounds that are able to interfere with the ion-binding capacity of sugar and withdraw the ions and render them inactive or poorly active in the Fenton reaction can bind iron ions strongly enough to inhibit deoxyribose degradation in the absence of EDTA.²⁵ A decrease of malondialdehyde formation was observed in the absence of EDTA which is an indicator of iron-chelating activity.

The Eucalyptus leaf extract promoted the DPPH discoloration (Fig. 1) and prevented the O_2^•−-induced reduction of NBT (Fig. 2), the H_2O_2-induced oxidation of lucigenin (Fig. 3), and the hydroxyl-induced degradation of deoxyribose (Fig. 4) in a concentration-dependent manner. The ORAC value obtained was 1.43 ± 0.02 μmol of Trolox equivalents/mg of extract, which represents around 35% of the Trolox value (4). Ascorbic acid presented an ORAC value of 1.24 ± 0.42 μmol of Trolox equivalents/mg (Table 2).

The Eucalyptus leaf extract also exerted a potent scavenging activity against RNS. IC_{50} values for NO and ONOO^− were 3.36 ± 0.15 and 1.27 ± 0.06 μg/mL, respectively. The positive controls presented IC_{50} values of 0.86 ± 0.06 μg/mL (rutin) for NO and 0.46 ± 0.03 (cys-

### Table 2. ROO• Scavenging Activity Evaluated by the ORAC Assay

<table>
<thead>
<tr>
<th>Extract/compound</th>
<th>ORAC (μmol of Trolox/mg of extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. globulus</td>
<td>1.43 ± 0.02</td>
</tr>
<tr>
<td>Trolox</td>
<td>4</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>1.24 ± 0.42</td>
</tr>
</tbody>
</table>

Data are mean ± SEM values.

### Table 3. NO and ONOO^− Scavenging Activities

<table>
<thead>
<tr>
<th>Extract/compound</th>
<th>IC_{50} (μg/mL)</th>
<th>NOO^− in absence of NaHCO₃</th>
<th>NOO^− in presence of 25 mM NaHCO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. globulus</td>
<td>3.36 ± 0.15</td>
<td>1.27 ± 0.06</td>
<td>1.57 ± 0.14</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.86 ± 0.06</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>—</td>
<td>0.21 ± 0.01</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td>Cysteine</td>
<td>—</td>
<td>0.46 ± 0.03</td>
<td>0.42 ± 0.06</td>
</tr>
</tbody>
</table>

Data are mean ± SEM values.
teine) and 0.21 ± 0.01 μg/mL (ascorbic acid) for ONOO⁻ (Table 3). For the latter reactive species a slight reduction of the scavenging activity of the Eucalyptus extract was observed in the presence of NaHCO₃ (IC₅₀ = 1.57 ± 0.14 μg/mL). No meaningful difference was found for cysteine (IC₅₀ = 0.42 ± 0.06 μg/mL), while ascorbic acid showed a significant decrease in activity (IC₅₀ = 0.34 ± 0.04 μg/mL). The Eucalyptus leaf extract inhibited the ·NO-induced oxidation of DAF-2 to triazolofluorescein (Fig. 5) and the ONOO⁻-induced oxidation of DHR-123 (Fig. 6) in a concentration-dependent manner.

The yield of extract obtained from E. globulus leaves was 23.8% (wt/wt). The total phenolic content of the Eucalyptus leaf extract was 311 ± 20 mg of gallic acid equivalents/g of lyophilized extract (mean ± SD). Four phenolic compounds were identified in the extract: chlorogenic acid, ellagic acid, rutin, and quercitrin (Fig. 7). The major component was recognized as a flavonol derivative according to
its UV spectrum, which presented two absorption maxima at 256 and 356 nm. The concentrations of the identified phenolic compounds are presented in Table 4.

The overall results showed that the Eucalyptus leaf extract was more active in scavenging RNS, compared to the other studied reactive species.

**DISCUSSION**

The results obtained in the present in vitro study clearly demonstrated that the Eucalyptus aqueous leaf extract is a very effective scavenger against all the assayed ROS and RNS as well for DPPH. The present studies corroborate and reinforce previous findings showing that pretreatment of the lipopolysaccharide/interferon-

- Production of ROS and RNS in the lungs can also take place via cellular pathways, for instance, by the reaction of O2
- NO with other strong oxidizing RNS, such as ONOO
- The results of the present study may be expected that the Eucalyptus aqueous leaf extract is also an effective scavenger for 'NO2 and CO3
- Besides the generation of ROS via cellular pathways, formation of ROS and RNS in the lungs can also take place after inhalation of exogenous compounds like ozone, nitrogen dioxide, cigarette smoke, and other chemicals and dust particles.
- Superoxide dismutase activity is lower in the lungs of asthmatic patients compared to healthy individuals.

**Table 4. Phenolic Composition of E. globulus Leaf Water Extract**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount (mg/g of lyophilized extract, mean ± SD)</th>
<th>Linearity</th>
<th>Linear range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorogenic acid</td>
<td>4.49 ± 0.16</td>
<td>y = 2.71 \times 10^x - 28888</td>
<td>0.0039–0.5 mg/mL</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>2.68 ± 0.27</td>
<td>y = 2.71 \times 10^x - 458</td>
<td>0.000977–0.125 mg/mL</td>
</tr>
<tr>
<td>Rutin</td>
<td>4.44 ± 0.33</td>
<td>y = 2.71 \times 10^x - 7713</td>
<td>0.0039–0.5 mg/mL</td>
</tr>
<tr>
<td>Flavonol glycoside(^a)</td>
<td>9.85 ± 0.07</td>
<td>y = 2.71 \times 10^x - 45823</td>
<td>0.0039–0.5 mg/mL</td>
</tr>
<tr>
<td>Quercitin</td>
<td>2.41 ± 0.09</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Quantified as rutin equivalents.
in the *Eucalyptus* leaf extract were rutin, an unidentified flavonol glycoside or a mixture of flavonol glycosides with the same genin, and chlorogenic acid. Scavenging activity of $O_2^{•−}$, $HO^•$, $ROO^•$, and $ONOO^•$ has been reported for rutin and chlorogenic acid. Rutin is also an 'NO scavenger, which was confirmed in this study. The observed antioxidant activity for the *Eucalyptus* leaf extract could be, therefore, partially attributed to the identified phenolic compounds.

In conclusion, the *Eucalyptus* aqueous leaf extract studied was shown to be very effective against ROS and RNS that have been implicated in airway inflammatory diseases, and thus these observations provide further support for some of the reported uses of the *Eucalyptus* plant in traditional medicine, beyond its well-known antibacterial and antiviral activities. Further studies are needed to prove the bioavailability of the antioxidants/antibacterial compounds of the extract as well as the ability of the active compounds to reach specific tissues and to act in them. More studies are also required for assessing a rational and safe use of the extract, especially for the determination of anti-inflammatory effects of the aqueous extract of *E. globulus*.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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