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In Vitro Activity of the Essential Oil of *Cinnamomum zeylanicum* and Eugenol in Peroxynitrite-Induced Oxidative Processes

SILVIO CHERICONI,* JOSÉ M. PRIETO, PATRIZIA IACOPINI, PIERLUIGI CIONI, AND IVANO MORELLI

Dipartimento di Chimica Bioorganica e Biofarmacia, Università di Pisa, via Bonanno 33, 56126 Pisa, Italy

The essential oil obtained from the bark of *Cinnamomum zeylanicum* Blume (Lauraceae) and three of its main components, eugenol, (*E*)-cinnamaldehyde, and linalool (representing 82.5% of the total composition), were tested in two in vitro models of peroxynitrite-induced nitration and lipid peroxidation. The essential oil and eugenol showed very powerful activities, decreasing 3-nitrotyrosine formation with IC_{50} values of 18.4 μg/mL and 46.7 μM, respectively (reference compound, ascorbic acid, 71.3 μg/mL and 405.0 μM) and also inhibiting the peroxynitrite-induced lipid peroxidation showing an IC_{50} of 2.0 μg/mL and 13.1 μM, respectively, against 59.0 μg/mL (235.5 μM) of the reference compound Trolox. On the contrary, (*E*)-cinnamaldehyde and linalool were completely inactive.

KEYWORDS: Peroxynitrite; lipid peroxidation; 3-nitrotyrosine; *Cinnamomum zeylanicum*; essential oil; eugenol

INTRODUCTION

The importance of peroxynitrite ($ONOO^-$) in biological systems is based on its powerful ability to react with almost all classes of biomolecules due to the radicals formed from its degradation ($NO_2^\bullet$ and $OH^\bullet$) in physiological conditions. These radicals induce lipid peroxidation, disruption of cellular structures, inactivation of enzymes and ion channels through protein oxidation and nitration, and DNA damage (*1*), contributing to the onset and maintenance of many pathologies such as chronic inflammation (*2*), atherosclerosis (*3*), neurodegenerative diseases (*4*), hepatopathies (*5*), and cardiovascular disorders (*6*).

Scavengers of those deleterious radicals and/or compounds able to prevent the consequences of their reactivity can contribute to the maintenance of health or healing processes. It has been recognized that food can be a constant source of such kinds of compounds and, from this point of view, variable amounts of spices are present every day in our diet (*7*). It is well-known that many of their secondary metabolites are endowed with antioxidant, antimicrobial, antifungal, and anti-inflammatory properties (*8*), among others, when used in proper concentrations.

The aim of the present study was to evaluate the ability of the essential oil obtained from dried and powdered barks of *Cinnamomum zeylanicum* Blume (Lauraceae), one of the most widely used spices, to prevent in vitro peroxynitrite-induced formation of 3-nitrotyrosine (3-NT) and malondialdehyde (MDA), two biomarkers of the oxidative stress (*9*, *10*), and to determine how its main constituents can contribute to this activity.

MATERIALS AND METHODS

Chemicals. Dried and powdered barks of *C. zeylanicum* were purchased in the local market, and the essential oil was obtained by hydrodistillation in a Clevenger apparatus for 2 h as described in the Italian Pharmacopea XI Ed. Eugenol and linalool were from Fluka Chemie, whereas (*E*)-cinnamaldehyde was purchased from Merck. The essential oil and pure compounds were diluted in methanol (Baker, HPLC grade) to the required concentration just before use.

Peroxynitrite was synthesized from sodium nitrite/H_2O_2 acidified with HCl as described by Beckman et al. (*11*), and the residual H_2O_2 was removed by passing the solution through granular MnO_2. The yellowish stock solution was stored at −80 °C, and its concentration was evaluated immediately before use by measuring the absorbance at 302 nm (ϵ = 1670 M^{-1} cm^{-1}).

3-Nitrotyrosine was synthesized and purified according to the literature (*12*, *13*). Standard solutions were prepared by successive dilutions with phosphate buffer (50 mM, pH 7.4). Malondialdehyde was synthesized as reported by Fenaille et al. (*14*) and the complex with thiobarbituric acid (TBA) as previously described by Brannan and Decker (*15*). Standard solutions of MDA−2TBA complex were obtained by dilutions with 100 mM Tris buffer (pH 7.3).

Ascorbic acid (Riedel de Haën) and Trolox (Sigma-Aldrich) were taken as reference compounds. All other chemicals were of analytical or higher grade, and the aqueous solution were prepared by using freshly deionized, ultrafiltered water further purified by using a Milli-Q system.

Gas Chromatography−Mass Spectrometry. GC-EIMS analyses were performed with a Varian CP-3800 gas chromatograph equipped with a DB-5 capillary column (30 m × 0.25 mm; coating thickness = 0.25 μm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions were as follows: injector and transfer line temperatures, 220 and 240 °C, respectively; oven temperature programmed from 60 to 240 °C at 3 °C/min; carrier gas, helium, at 1 mL/min; injection of

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0.2 μL (10% hexane solution); split ratio, 1:30. Identification of the constituents was based on the comparison of the retention times with those of authentic samples, comparing their linear retention indices relative to the series of *n*-hydrocarbons, and on computer matching against commercial (NIST 98 and ADAMS) and homemade library mass spectra built from pure substances and components of known oils and MS literature data (*16*−*21*). Moreover, the molecular weights of all the identified substances were confirmed by GC-CIMS, using methanol as CI ionizing gas.

HPLC Analyses. The HPLC system consisted of a Waters 600E pump system coupled with a Waters UV 486 detector and a Waters 717 Plus autosampler. Data processing was performed by using the software Millenium v. 3.2 (Waters) running on a PC coupled with the HPLC system. The column was a Lichrosphere 100 RP-18, 5 μm, 250 × 4.6 mm (Merck).

Elution conditions for the detection of 3-NT were as follows: 20 mM phosphate buffer (pH 3.2)/methanol (92:8); flow, 1 mL/min in isocratic mode (*22*); UV detection, 356 nm. For the detection and quantification of the MDA−2TBA complex we used 20 mM phosphate buffer (pH 6)/methanol (6:4); flow of 1.1 mL/min in isocratic mode, and UV detection at 532 nm according to the method of Fenaille et al. (*14*).

Liver Microsomal Preparations. Wistar rat livers were kindly donated by the Department of Psychiatry, Neurobiology, Pharmacology and Biotechnologies of the University of Pisa. Animal handling was according to the guidelines established by the European Union on Animal Care (CEE Council 86/609) as required by the Ethical Committee of the Faculty of Pharmacy, University of Pisa (Italy).

Liver microsomes were prepared by standard differential centrifugation techniques as described by Slater and Sawyer (*23*). Briefly, under diethyl ether anesthesia, male Wistar rats (200−250 g) were killed by exsanguination from the abdominal aorta. The liver was exhaustively perfused with ice-cold saline through the portal vein until uniformly pale, immediately removed, and weighed. After being trimmed and minced, the pieces of liver were homogenized with 4 volumes of ice-cold 0.1 M potassium phosphate buffer (pH 7.4) containing 1.15% (w/v) KCl. The homogenate was spun at 10000*g* for 30 min. The supernatant was collected and further centrifuged at 105000*g* for 60 min. The resultant microsomal fraction was washed and resuspended in the same buffer as above. Aliquots of microsomal suspensions were stored at −80 °C for a maximum of 2 months. Protein contents were quantified according to Lowry's method (*24*) using bovine serum albumin as standard.

Tyrosine Nitration Induced by Peroxynitrite. This method is based on the determination, by HPLC-UV analyses, of the quantity of 3-NT formed from the reaction between free tyrosine and peroxynitrite at a physiological pH. The reaction was carried out by adding, under vigorous vortexing, peroxynitrite (5−40 μL, 1 mM final concentration) to a solution containing the essential oil or pure compounds at the desired concentrations, tyrosine (2 mM), and HCO_3^- (50 mM), all dissolved in 50 mM phosphate buffer (pH 7.4). Test compounds were dissolved in methanol (final concentration = 0.5%) and kept on ice until used. Blanks, with or without methanol, were always performed to detect any interference of the solvent with the tests. Quantitative determination of the formed 3-NT was performed by HPLC-UV using an external standard calibration curve ($r^2 = 0.999$).

Lipid Peroxidation Induced by Peroxynitrite. Peroxynitrite (5−30 μL, final concentration of 1 mM) was added to a solution of microsomes (5 mg/mL) in 50 mM phosphate buffer solution. Test compounds and essential oil were prepared and added as described above. The reaction mixture was incubated at 37 °C; after 20 min, 1 mL of TCA 10% (p/v) and 1 mL of TBA 0.7% (p/v) were added, and then the solutions were kept at 90 °C for 1 h to allow the formation of the complex MDA−2TBA. Quantitative determination of this complex by HPLC-UV was performed by using an external standard calibration curve ($r^2 = 0.999$).

Statistics. The inhibitory concentration 50% (IC_{50}) was calculated from concentration/effect regression; an appropriate range of four to five concentrations was used.

Table 1. Percentage Composition of the Essential Oil from *C. zeylanicum* Barks

compound	%	RI[a]	compound	%	RI[a]
benzaldehyde	0.2	971	eugenol	46.5	1356
p-cymene	0.4	1030	α-copaene	0.7	1377
1,8-cineole	0.2	1039	β-caryophyllene	2.8	1420
linalool	3.3	1101	(*E*)-cinnamyl acetate	2.8	1449
camphor	tr[b]	1154	α-humulene	0.9	1457
terpinen-4-ol	0.3	1184	eugenol acetate	2.2	1512
α-terpineol	0.7	1197	caryophyllene oxide	0.3	1584
(*Z*)-cinnamaldehyde	tr	1223	benzyl benzoate	1.4	1769
(*E*)-cinnamaldehyde	32.7	1278			
carvacrol	1.6	1300	% identified	98.5	

[a] Kovats retention index. [b] Traces.

RESULTS

Chemical Composition of the Essential Oil. A total of 18 compounds, representing 98.5% of the total oil, were identified (**Table 1**). The main constituents were eugenol (46.5%) and cinnamaldehyde (32.7%), followed by linalool (3.3%), β-caryophyllene (2.8%), cinnamyl acetate (2.8%), and eugenol acetate (2.2%).

HPLC Analyses. The essential oil and three of its major compounds, namely, eugenol, cinnamaldehyde, and linalool, accounting for 82.5% of the total composition, were tested for their scavenger activity. Qualitative analyses were previously performed to avoid any interference of the tested products with the detection of 3-NT and MDA−2TBA. No coelution or other problems were detected.

Tyrosine Nitration Induced by Peroxynitrite. The essential oil of *C. zeylanicum* and its main compound, eugenol, inhibited significantly the formation of 3-NT with IC_{50} values of of 18.4 μg/mL ($r^2 = 0.996$; $P \leq 0.0001$) and 7.7 μg/mL (46.7 μM) ($r^2 = 0.998$; $P \leq 0.0013$), respectively. The reference compound, ascorbic acid, exhibited an IC_{50} of 71.3 μg/mL (405.0 μM) ($r^2 = 0.998$; $P \leq 0.0001$). (*E*)-Cinnamaldehyde and linalool did not show any inhibitory activity in this model.

Lipid Peroxidation Induced by Peroxynitrite. The total essential oil and eugenol showed significant activities with IC_{50} values of 2.0 μg/mL ($r^2 = 0.995$; $P \leq 0.0001$) and 2.2 μg/mL (13.1 μM) ($r^2 = 0.995$; $P \leq 0.0001$), respectively. The reference compound, Trolox, had an IC_{50} of 59.0 μg/mL or 235.5 μM ($r^2 = 0.997$; $P \leq 0.0001$). (*E*)-Cinnamaldehyde and linalool did not show any inhibitory activity in this model.

DISCUSSION AND CONCLUSION

Cinnamomum is a large genus, many species of which yield a volatile oil on distillation. The composition of the oil, and therefore its value and the use to which it is put, depends very much on the species that is distilled as well as the part of the plant that is utilized. The most important *Cinnamomum* oils in world trade are those from *C. verum* (cinnamon bark and leaf oils), *C. cassia* (cassia oil), and *C. camphora* (sassafras and Ho leaf oils). The latter species provides oils that are utilized as sources of chemical isolates. However, a number of other *Cinnamomum* species are distilled on a much smaller scale, and the oils are used either locally or exported to regional markets (*25*).

C. zeylanicum is one of the world's finest spices, mainly exported as "cinnamon quills." From a phytochemical viewpoint, the volatile oils obtained from the bark, leaf, root bark, and buds vary significantly in chemical composition. The classic primary constituents are cinnamaldehyde in the bark oil, eugenol

in the leaf oil, camphor in the root-bark oil (*26*), and α-bergamotene and α-copaene in bud essential oil (*27*). More detailed phytochemical studies on the general composition of the essential oil extracted from barks of *C. zeylanicum* report that this oil is typically rich in (*E*)-cinnamaldehyde, *β*-caryophyllene, and linalool (*28*). In our sample, the content of (*E*)-cinnamaldehyde was high (32.7%) but was surpassed by eugenol (46.5%). Linalool and *β*-caryophyllene were also present but in lower percentages (3.3 and 2.8%, respectively). Other compounds above 2% are cinnamyl acetate and eugenol acetate.

Variability in the composition of essential oils from the same *Cinnamomum* species part is a common trend and depends on the provenance; thus, numerous chemotypes can be differentiated (*29*). Consequently, there is no international standard for cinnamon bark oil, although batches containing cinnamaldehyde at the higher end of the range fetch the higher price (*25*).

Oxygenated compounds represent up to 93.7% of the essential oil studied here. Three of them, eugenol, carvacrol, and eugenol acetate, representing 50.3% of the total, have a phenolic structure conferring potential antioxidant activity.

Cinnamon essential oil and its constituents have been studied in different systems for their antioxidant activity (*30*, *31*), but none of them deals with the protection of the essential oil and its main components against tyrosine nitration or lipid peroxidation induced by peroxynitrite. This radical can promote oxidative damage to blood vessels, skin, heart, lung, kidney, and brain. One sign of damage left by peroxynitrite is nitration of amino acids in proteins, contributing to acute injury and chronic diseases (*1*). Our in vitro results show that *C. zeylanicum* essential oil is able to prevent 3-NT formation and lipid peroxidation better than reference compounds such as ascorbic acid and Trolox.

When three of the major compounds were assayed, only eugenol was found to be active. This result could imply that essential oils from this species with low content of eugenol may be less active in preventing peroxynitrite-induced damage in vitro, but it is not possible to discard the role of other constituents in these models.

Eugenol was the first component of an essential oil proved to be a significant germicide and sedative used in dentistry, and today is still in use (*32*). It is also an important flavoring agent in cosmetic and food products (*7*). Its scavenging properties against different radicals such as DPPH (*33*), ABTS (*34*), superoxide (*35*), and azide, hydroxyl, and haloperoxyl radicals (*36*) have been reported. The ability of this compound to inhibit lipidic peroxidation induced by ferric ion, ferrous ion, and cumene hydroperoxide has been studied, and its mechanism was related with the efficiency with which it can transfer one electron to these oxidizing radicals (*36*). Two further studies on the protective effect on metal-mediated lipid peroxidation of eugenol and its isomer isoeugenol showed that it can be explained by their methoxyphenolic structure (*37*) and their reducing properties and by forming inactive metal−eugenol complexes that cannot react with oxygen (*38*). Eugenol, an active principle of clove, was shown to offer in vivo protection against CCl_4-induced hepatotoxicity in rats (*7*). In humans, eugenol is rapidly absorbed and metabolized after oral administration and was almost completely excreted in the urine within 24 h (*39*), reaching blood plasma concentrations of 5 μM just 2 h after the administration of 150 mg of eugenol (*40*). This concentration of eugenol can prevent peroxynitrite-induced lipidic peroxidation effectively as demonstrated in this study, indicating that this compound could be active in vivo on similar processes.

In conclusion, this work establishes the ability of *C. zeylanicum* essential oil and eugenol in preventing in vitro oxidative processes induced by peroxynitrite, a radical of pathological relevance.

ABBREVIATIONS USED

$ONOO^-$, peroxynitrite; 3-NT, 3-nitrotyrosine; MDA, malondialdehyde; TBA, thiobarbituric acid.

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