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Supercritical CO_2 Extract of *Cinnamomum zeylanicum*: Chemical Characterization and Antityrosinase Activity

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The volatile oil of the bark of *Cinnamomum zeylanicum* was extracted by means of supercritical CO_2 fluid extraction in different conditions of pressure and temperature. Its chemical composition was characterized by GC-MS analysis. Nineteen compounds, which in the supercritical extract represented >95% of the oil, were identified. (*E*)-Cinnamaldehyde (77.1%), (*E*)-β-caryophyllene (6.0%), α-terpineol (4.4%), and eugenol (3.0%) were found to be the major constituents. The SFE oil of cinnamon was screened for its biological activity about the formation of melanin in vitro. The extract showed antityrosinase activity and was able to reduce the formation of insoluble flakes of melanin from tyrosine. The oil also delayed the browning effect in apple homogenate. (*E*)-Cinnamaldehyde and eugenol were found to be mainly responsible of this inhibition effect.

KEYWORDS: Supercritical CO_2 extraction; volatile oil; *Cinnamomum zeylanicum*; tyrosinase; melanin; inhibition

INTRODUCTION

Cinnamon (*Cinnamomum zeylanicum* Blume syn. *C. verum* J.S. Presl, Lauraceae) is a medium-sized tree native to Sri Lanka and tropical Asia. It is also known as Ceylon cinnamon, true cinnamon, Ceylon-Zimtbaum, and cannelle de Ceylan (*1*). The term cinnamon is also used to indicate other species: *Cinnamomum loureirii* Nees syn. *C. obtusifolium* Nees var. *loureirii* Perr et Eb. and *C. burmanii* (Nees & T. Nees) Blume syn. *C. pedunculata* J. S. Presl.

Trees of *C. zeylanicum* are cultivated in Sri Lanka and India to obtain leaves, bark, and roots. The essential oils, obtained by steam distillation from different parts of this species, are dissimilar, and the oil obtained from the bark, called oil of cinnamon Ceylon, is considered to be superior for its aroma. Essential oils obtained from twigs, pedicels, buds, flowers, and fruits are characterized by a very low content (<4.0%) of (*E*)-cinnamaldehyde and larger and variable amounts of (*E*)-cinnamyl acetate, (*E*)-β-caryophyllene, and linalool (*2*). Cinnamon leaf oil has been reported to show a notable antifungal activity against *Botrytis cinerea* (*3*). The chemical composition of the volatile oil has been also determined in fruits of *C. zeylanicum* (*4*). Simic et al. reported that a commercial oil, chiefly constituted by (*E*)-cinnamaldehyde (62.79%), limonene (8.31%), and linalool (7.09%), showed a very strong antifungal activity against 17 different micromycetes (*5*). Pawar and Thaker, who tested 75 essential oils against the fungus *Aspergillus niger*, found that the highest inhibitory effect was exhibited by the oil obtained from the bark of cinnamon (*6*). The antioxidant activity of this oil is also known (*7, 8*).

Hydrodistillation or steam distillation, even when it does not induce extensive phenomena of hydrolysis and thermal degradation, gives in any case a product with a characteristic off-odor (*9*). Solvent extraction can give oil, but due to a high content of waxes and/or other high molecular mass compounds, often gives rise to a concrete with a scent very similar to the material from which it was derived. However, small amounts of organic solvents can pollute the extraction product.

Supercritical fluid extraction (SFE) is a good technique for the production of flavors and fragrances from natural materials and can constitute a valid alternative to both of the above-mentioned processes. In fact, compressed carbon dioxide, CO_2, is able to solubilize hydrocarbons and oxygenated mono- and sesquiterpenes (*10*), the main essential oil constituents. The separation of the extractant is easy, hydrolysis and thermal degradation are practically absent, and the extract retains the organoleptic features of the starting material. Possible residues do not cause a risk for human health. Indeed, CO_2, besides being

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safe, noncombustible, and inexpensive, is nontoxic. No paper concerning the SFE from *C. zeylanicum* bark was found in the literature.

Tyrosinase, a copper enzyme belonging to type-3 copper proteins, is involved in the initial step of melanin biosynthesis, and it is widespread throughout microrganisms, plant and fungal species, invertebrates, and vertebrates. This enzyme catalyzes the ortho-hydroxylation of monophenols to the corresponding catechols, monophenolase or cresolase activity, and the oxidation of such catechols to the corresponding *o*-quinones, diphenolase or catecholase activity (*11–15*). Tyrosinase, also known as a polyphenol oxidase (PPO), is involved in important biological processes; among them, enzymatic browning observed in vegetables (*16*), fruits (*17*), and crustaceans (*18*). This browning from enzymatic oxidation of phenols has therefore been of great concern, and tyrosinase inhibitors could have wide interest (*19, 20*). In addition, tyrosinase inhibition has drawn increasing interest in both medicinal and cosmetic fields in relation to the development of products for hyperpigmentation treatment (*21–23*).

The aim of the present study was the chemical characterization of a supercritical carbon dioxide extract of cinnamon (Cz-oil). This oil was compared with the extracts from other extraction techniques. Moreover, this product was tested for its effects on tyrosinase activity and on melanin formation in vitro, in view of future applications in food processing.

MATERIALS AND METHODS

Extraction Phase. *Materials.* Vegetable matter had been cultivated in Sri Lanka and marketed by A. Minardi & Figli (Bagnacavallo-Ravenna, Italy). We examined the bark of *C. zeylanicum* Blume from lot B-041005111005 (2005). The material was not ground and had been treated by a blanching agent (SO_2). Before extraction, cinnamon was ground to obtain particles sizes in the range of 300–800 μm. The experimentally determined moisture content, by weight on dry basis, was 10.0%. Carbon dioxide (purity = 99%) was supplied by Air-Liquide, Cagliari, Italy. Some reference compounds used in the identification process (borneol, eugenol, linalool, γ-terpinene, and *p*-cymene) were obtained from Aldrich (Sigma Italia, Milan, Italy). Myrcene, (*E*)-cinnamaldehyde, and (*E*)-β-caryophyllene were from Fluka (Sigma Italia).

SFE Apparatus. Supercritical CO_2 extractions were performed in a laboratory apparatus equipped with a 320 cm^3 extraction vessel operating in the single-pass mode of CO_2 through the fixed bed of ground material. About 200 g of cinnamon was charged at each run. A single separator of 200 cm^3 connected in series allowed discharge of the volatile oil at desired time intervals. In this section, the temperature was maintained at the wanted value, by means of a water-thermostated system and by using a heating ribbon wrapped to the pipe exiting the separator. A high-pressure diaphragm pump (Lewa, model EL 1) with a maximum capacity of 6 kg/h, pumped liquid CO_2 at the desired flow rate. Carbon dioxide was heated to extraction temperature in a thermostated oven controlled by a PID controller, model 2116 (Eurotherm). The extraction was carried out in a semibatch mode: batch charging of vegetable matter and continuous flow solvent. Carbon dioxide flow was monitored by a calibrated rotameter (Sho-rate, model 1355) located after the separator. Total CO_2 delivered during an extraction test was measured by a dry test meter. Temperatures and pressures along the extraction apparatus were measured by thermocouple and Bourdon-tube test gauges, respectively. High-pressure valves, under manual control, regulated pressure. Experiments were carried out at different conditions. No repeated runs were carried out.

Hydrodistillation (HD). HD was performed in a circulatory Clevenger-type apparatus, for 4 h, up to the point at which the oil contained in the matrix was exhausted. About 100 g of material belonging to the same batch employed in SFE were charged. No duplicate distillations were performed.

Solvent Extraction (SE). SE was performed in a Soxhlet apparatus on about 10 g of matrix using n-hexane/acetone for 4 h. No duplicate extractions were performed.

GC-MS Analysis. A Hewlett-Packard 5890 series II gas chromatograph (GC) was employed. It was equipped with a split–splitless injector and a DB5-MS fused silica column: 5% phenyl-methylpolysiloxane, 30 m × 0.25 mm i.d., film thickness = 0.25 μm. GC conditions used were as follows: programmed heating from 60 to 280 °C at 3 °C/min followed by 30 min under isothermal conditions. The injector was maintained at 250 °C. Helium was the carrier gas at 1.0 mL/min; the sample (1 μL) was injected in the split mode (1:20). The GC was fitted with a quadrupole mass spectrometer (MS), model HP 5989 A. MS conditions were as follows: ionization energy, 70 eV; electronic impact ion source temperature, 200 °C; quadrupole temperature, 100 °C; scan rate, 1.6 scan/s; mass range, 40–500 u. Software adopted to handle mass spectra and chromatogram was ChemStation. NIST98 (*24*), FLAVOUR, and LIBR(TP) (*25*) mass spectra libraries were used as references. Samples were run diluted in chloroform with a dilution ratio of 1:100. In **Table 1**, chromatographic results, expressed as area percentages calculated without any response factor, are reported as a function of retention indices, I_R, relative to the series of n-hydrocarbons. Identifications were done by matching their mass spectra and I_R with those reported in the literature. Moreover, whenever possible, identification has been confirmed by injection of authentic sample of the compounds.

Enzyme Extraction and Purification. The mushroom tyrosinase (EC 1.14.18.1) used for the present investigation was partially purified as follows: freshly packed white mushrooms (*Agaricus bisporus*) were supplied by a local mushroom grower, stored at 4 °C, and treated within 6 h from harvest. In a typical extraction step, the sporocarps (250 g) were gently cleaned to remove earthy residue and then washed with 20 mM ascorbic acid maintained at 4 °C. After they had been dried with blotter, the sporocarps were immediately immersed in liquid nitrogen and then stored at −80 °C until use.

Just before use, the frozen sporocarps were homogenized in a blender in the presence of 250 mL of cold (−50 °C) acetone. The homogenate was filtered through a Buchner funnel equipped with fast-flow filter paper and washed with 2 L of cold acetone until the filtrate was colorless. The solid residue was dried by an air flow and ground with a mortar and pestle. The obtained powder was divided in 5 g aliquots and stored at −20 °C.

When necessary, one aliquot was suspended in 100 mL of 20 mM potassium phosphate (KPs) buffer, pH 6.5, under gentle stirring for 15 min. The insoluble matter was separated by centrifugation (20 min at 20000*g*) and discarded. The supernatant was subjected to fractional precipitation with ammonium sulfate between 45 and 65% of saturation. The final precipitate was resuspended in the KPs buffer and dialyzed overnight against the same buffer. The dialysate was centrifuged and the precipitate discarded. The supernatant was then passed through a 2.6 × 60 cm column of Sephacryl S-200 HR (Amersham Biosciences) previously equilibrated with 10 mM KPs buffer, pH 6.5. Fractions showing tyrosinase activity were collected, dialyzed, and concentrated by means of Vivaflow 200 (Vivascience AG, Hannover, Germany) equipped with a Hydrosart membrane (10.000 MWCO). The resulting enzyme solution contained 3610 units/mL of tyrosinase with a specific activity of 3240 units/mg of protein. It was carefully analyzed to rule out any laccase contamination that could alter the activity data of the enzyme (*26, 27*). When not specifically indicated, all of the purification steps were run at 4 °C. Double-distilled water was used to prepare solutions during the purification procedure.

Biological Assays. As the oil is only sparingly water-soluble, it was diluted 1:1 with dimethyl sulfoxide (DMSO) before biological assays.

Enzyme Assay. One tyrosinase unit corresponds to the amount of enzyme that causes an absorbance increase of 0.001 per minute in KPs buffer at pH 6.5 and 25 °C (L-tyrosine as substrate, 3.0 mL reaction volume). Because tyrosinase catalyzes a reaction between two substrates, molecular oxygen and a phenolic, the assay was carried out in air-saturated solutions.

In Vitro Melanin Formation from Tyrosine. This process was catalyzed by tyrosinase purified as described under Materials and

Table 1. Retention Indices, I_R, and Chromatographic Area Percentage of Compounds Found in Cinnamon Oil Extracted by Different Methods: Supercritical Fluid Extraction, Solvent Extraction, and Hydrodistillation

I_R	SFE (90 bar, 50 °C)	SFE (90 bar, 40 °C)	SFE (120 bar, 40 °C)	SE	HD	compound	identification[a]
906	–	–	–	–	0.3	α-thujene	MS, I_R
913	Tr[b]	–	0.1	–	2.2	α-pinene	MS, I_R
929	–	–	–	–	1.0	camphene	MS, I_R
955	–	–	–	–	0.6	sabinene	MS, I_R
968	–	–	–	–	Tr	myrcene	MS, I_R, Inj
983	0.4	0.2	0.3	–	3.6	α-phellandrene	MS, I_R
991	–	–	0.2	–	2.2	α-terpinene	MS, I_R
998	0.5	0.2	0.5	Tr	5.4	*p*-cymene	MS, I_R, Inj
1005	1.7	1.0	1.5	Tr	11.3	β-phellandrene	MS, I_R
1031	–	–	–	–	Tr	γ-terpinene	MS, I_R, Inj
1055	–	–	–	–	Tr	isoterpinolene	MS, I_R
1069	2.6	1.2	1.5	0.4	9.4	linalool	MS, I_R, Inj
1136	–	–	–	–	Tr	borneol	MS, I_R, Inj
1146	0.7	0.2	0.5	Tr	2.2	terpinen-4-ol	MS, I_R
1163	0.7	4.4	0.7	0.3	2.3	α-terpineol	MS, I_R
1200	–	–	Tr	–	–	(*Z*)-cinnamaldehyde	MS, I_R
1253	72.6	77.1	79.0	69.4	19.7	(*E*)-cinnamaldehyde	MS, I_R, Inj
1264	–	–	–	–	1.7	(*E*)-anethole	MS, I_R
1297	–	–	–	Tr	–	(*E*)-cinnamyl alcohol	MS, I_R
1330	4.2	3.0	4.1	3.3	2.0	eugenol	MS, I_R, Inj
1349	0.9	0.7	0.8	0.3	1.4	α-copaene	MS, I_R
1375	0.1	–	0.1	–	–	NI[c]	–
1375	–	–	–	–	Tr	(*Z*)-caryophyllene	MS, I_R
1390	7.6	6.0	5.1	2.2	13.6	(*E*)-β-caryophyllene	MS, I_R, Inj
1420	1.9	0.8	–	3.4	2.1	(*E*)-cinnamyl acetate	MS, I_R
1424	2.3	2.3	1.3	–	4.0	α-humulene	MS, I_R
1487	0.1	–	–	–	Tr	δ-cadinene	MS, I_R
1505	0.4	0.8	2.2	3.0	–	(*E*)-*o*-methoxycinnamaldehye cinnamaldehyde	MS, I_R
1538	0.1	Tr	0.3	Tr	1.1	caryophyllene alcohol	MS, I_R
1543	0.5	0.7	0.3	0.7	1.8	caryophyllene oxide	MS, I_R
1559	–	–	–	0.7	Tr	NI	–
1568	0.2	0.1	0.2	Tr	0.5	humulene epoxide II	MS, I_R
1579	–	–	–	0.5	0.8	tetradecanal	MS, I_R
1645	0.1	–	–	–	–	NI	–
1731	1.6	1.0	1.1	2.8	4.0	benzyl benzoate	MS, I_R
1822	–	–	–	0.4	0.9	NI	–
1919	–	–	–	–	Tr	NI	–
1933	–	–	–	4.5	3.3	n-hexadecanoic acid	MS, I_R
1939	0.2	–	0.2	–	1.5	NI	–
1948	–	–	–	0.4	–	NI	–
2000	–	–	–	0.4	–	NI	–
2066	–	0.2	–	0.5	1.0	NI	–
2073	–	–	–	–	Tr	NI	–
2083	–	–	–	1.3	–	NI	–
2093	–	–	–	1.3	–	NI	–
2106	–	3.8	–	2.0	–	NI	–
2124	–	–	–	0.7	–	n-octadecanoic acid	MS, I_R
3161	–	–	–	0.3	–	NI	–
3550	–	–	–	0.7	–	NI	–
3587	–	–	–	0.6	–	NI	–
total identified (%)	**99.2**	**95.7**	**99.7**	**91.4**	**96.5**	–	–
yield of extraction (%)	**0.63**	**0.78**	**0.80**	**–**	**0.24**	–	–

[a] Identification has been realized by comparing mass spectra (MS), retention indices (I_R), and injection of authentic compound (Inj). [b] Tr = trace, i.e., percentage lower than 0.1%. [c] NI = not identified compound.

Methods; the product was extracted as already described (*28*) with minor modifications. In brief, 722 units of tyrosinase reacted with 7.5 mL of 2 mM tyrosine (previously dissolved in hot water), 1 mL of 0.5 M KPs buffer, pH 6.5, 1 mL of DMSO, and 300 μL of water (final volume = 10 mL). This control experiment was compared to similar reactions in which 100 or 200 μL of Cz-oil had been respectively added. Water volumes were lowered accordingly to leave concentrations unchanged. All samples were incubated at room temperature under very gentle stirring for 24 h. The reactions were then stopped by adding 0.5 mL of 6 M HCl, and the obtained melanin was recovered as follows. After centrifugation for 15 min at 20000*g*, the precipitate was resuspended in 0.8 mL of 0.5 M NaOH, heated in a water bath at 95 °C for 30 min, and neutralized with 3 mL of 1 M Tris HCl buffer, pH 7.0. Melanin was collected after filtration at reduced pressure on 0.45 μm filter membranes.

Effect of C. zeylanicum Extract on Apple Homogenate Browning. A yellow apple (golden delicious cultivar) was split into two halves at room temperature; these were quickly and aseptically peeled and stoned. Sixty grams samples, coming from each half, were rapidly weighed and then homogenized in two Braun PowerBlend MX2000 blendors for 20” in distilled water, both in the presence or in the absence of Cz-oil. The test mixture contained 58.2 mL of distilled water, Cz-oil (0.3 mL) and DMSO (1.5 mL). In the control mixture, Cz-oil was omitted. The two homogenates were poured in two beakers and kept at room temperature, and photographed after one hour.

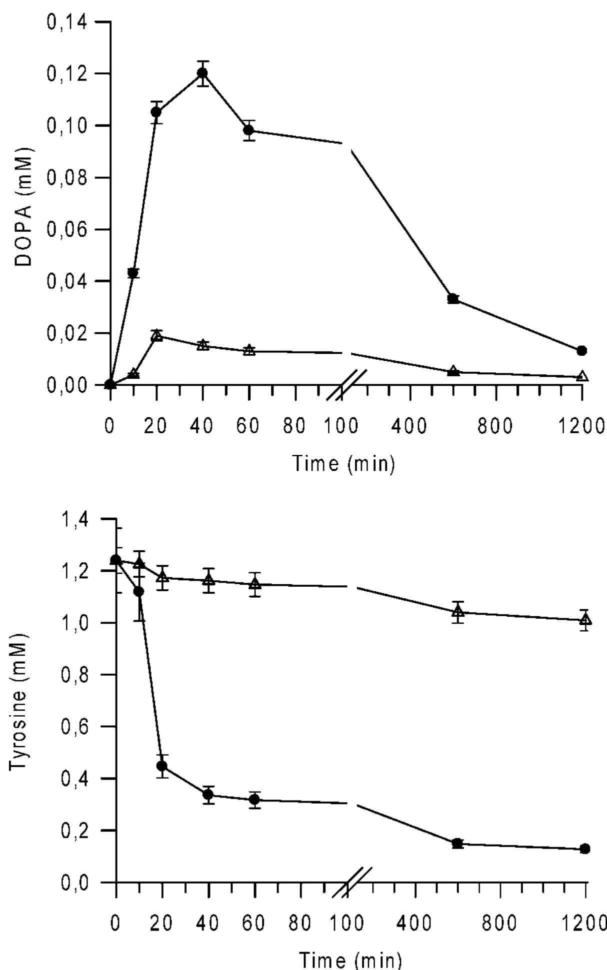

Figure 1. HPLC measurement of the time course of tyrosine and DOPA concentration. The reaction mixture contained 72.2 units/mL of mushroom tyrosinase, 1.25 mM tyrosine, 50 mM KPs buffer (pH 6.5), 10% DMSO. The control sample (●) also contained distilled water up to a final volume of 10 mL. The test sample (△) contained 1% Cz-oil (v/v). Reported values represent the mean of two HPLC injections from duplicate experiments.

Spectrophotometric assay of catecholase activity of tyrosinase. Six samples (both the control and the test were carried out in triplicate) were assayed as follows: 200 μL of a 15 mM L-3,4-dihydroxyphenylalanine (DOPA) solution, 190 μL of DMSO, 10 μL of Cz-oil, 500 μL of 100 mM KPs, pH 6.5, and 90 μL of water were mixed. The mixture was preincubated for 3 min. Then, 10 μL (36.6 units) of tyrosinase solution was added to start the reaction. At fixed times, 1 mL of acetone was added to stop the reaction and to clear the mixture. The catecholase activity was measured at 475 nm with an Ultrospec 2100 Pro UV–vis (Amersham Biosciences) spectrophotometer. A control reaction was conducted by adding 10 μL of DMSO in place of Cz-oil. Absorbance increase was linear at least within the first 3 min from the start of the reaction. The percentage of inhibition (I%) of the enzyme activity was calculated as follows: $I\% = (\Delta A - \Delta B)/\Delta A \times 100$, where ΔA is the difference in the absorbance of the control sample between the incubation times of 1.5 and 2.5 min and ΔB is the difference in the absorbance of the test sample calculated in the same range of time. The results were the mean of three readings. The same conditions were used in the experiments carried out with (E)-cinnamaldehyde or eugenol in place of Cz-oil.

DOPA stock solutions were made in 10 mM acetic acid to prevent autoxidation. The addition of DMSO was necessary to obtain an acceptable solubilization of the substrate.

Experiments were carried out in triplicate without changes among the trials.

RESULTS AND DISCUSSION

Extraction of *C. zeylanicum* Bark and Chemical Characterization of the Extract. Operative conditions of extraction were chosen on the basis of previous results (*29–31*) by SFE from similar matrices. The cinnamon volatile oil was obtained by employing a single depressurization stage because the starting material, a bark, was deprived of cuticular waxes. SFEs reported in this paper were conducted by maintaining a flow rate of 1.0 kg/h and 20 bar and 10 °C in the separation section where the oil was recovered at the desired time intervals. The water, which may be coextracted with the volatile oil, when present, was removed from the samples by means of a syringe.

We performed a preliminary run, at 90 bar and 40 °C in the extraction section, drawing the oil, in separate vials, after each hour of extraction for 5 h. The oil collected after the first hour of extraction was the richer in volatile compounds (data not shown). The amount of (E)-cinnamaldehyde increased regularly from 72.1 to 95.3% from the first to the last sample. Jointly, the percentages of the other constituents showed a decreasing trend. The yield of the process is expressed as the percentage of total mass of oil recovered, m_{oil}, with respect to the mass of material loaded onto the apparatus, m_0

$$Y\% = (m_{oil}/m_0) \times 100$$

The yields of oil and (E)-cinnamaldehyde, the main constituent of the extract, were, respectively, 0.78 and 0.63%.

In **Table 1** [SFE (90 bar, 40 °C) column] are reported the analytical results concerning the sample obtained, at the above-mentioned conditions, putting together all fractions in a single vial. The main constituents were (E)-cinnamaldehyde, 77.1%; (E)-β-caryophyllene, 6.0%; α-terpineol, 4.4%; and eugenol, 3.0%.

SFE was conducted also at two other different conditions for 4 h. The oil obtained at higher CO_2 densities was richer in (E)-cinnamaldehyde and gave higher yields (**Table 1**). Indeed, the densities of CO_2 were 0.287, 0.479, and 0.728 g cm^{-3} at 90 bar, 50 °C; 90 bar, 40 °C; and 120 bar, 40 °C respectively. Greater density caused an increase of the solvent power.

In **Table 1**, the compositions of the extracts obtained by SE and HD are also reported. Thirty-four compounds were identified in total. As expected, the oil obtained by SE was very similar to the extract obtained by SFE. Its content of (E)-cinnamaldheyde was quite high (69.4%), but it also contained a relevant amount of unidentified high molar mass compounds.

The oil obtained by HD is more complex with respect to those already considered. In particular, higher amount of p-cymene (5.4%), β-phellandrene (11.3%), linalool (9.4%), and (E)-β-caryophyllene (13.6%) were present. On the other hand, the percentage of (E)-cinnamaldehyde was very low: 19.7%. The loss of this compound from the oil may be ascribed to a noticeable solubility in water: 1 part in 700 parts of water (*32*). Indeed, in the HD process about 100 g of plant material was treated by employing about 2.0 L of water. The GC-MS analysis of this water at the end of the experiment confirmed this treatment. No traces of (E)-o-methoxycinnamaldehyde, typically found in the extracts obtained by SFE and SE, were present in the hydrodistilled oil.

Effect of *C. zeylanicum* Extract on Tyrosinase Activity and Melanin Formation. The action of tyrosinase activity in vitro, either on tyrosine or on DOPA, leads to the formation of melanin. This appears as a black, flocculent, and insoluble polymer, which easily sediments at the bottom of the test tube and can be recovered by filtering. On the other hand, when a similar experiment was carried out in the presence of increasing amounts of Cz-oil, a quite different behavior occurred. The

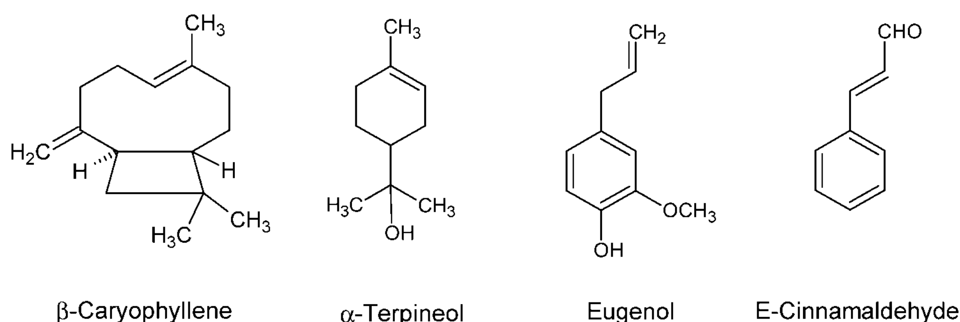

Figure 2. Molecular structures of the most abundant compounds found in Cz-oil.

reaction mixture did not turn reddish as quickly as the control experiment lacking Cz-oil. Besides, after the precipitation/solubilization procedure, the samples containing Cz-oil showed a reduced formation of melanin flakes.

This effect was even more evident when the samples were filtered through 0.45 μm membranes. Only from the control experiments could melanin be recovered; by contrast, the recovery was limited or almost absent in the presence of Cz-oil.

Effect of *C. zeylanicum* Extract on the Browning of Apple Homogenate. The enzymatic browning in fruits and vegetables is often undesirable; hence, the discovery and application of "safe" compounds to prevent this phenomenon are drawing steadily increasing attention. From this point of view, we have tested the capability of Cz-oil to inhibit browning of apple homogenates. Apples are generally suited to test browning and its inhibition as they contain both the phenolase activity and the phenolic substrates of that enzyme (*33*). The latter are more or less rapidly oxidized at the expense of molecular oxygen to the corresponding quinones, responsible for the browning. Accordingly, an apple homogenate turned brown within a few minutes, whereas in the presence of 0.3% Cz-oil the effect was substantially hindered after 1 h, and it seemed to persist for a longer time. Further experiments were then planned to establish whether the observed antibrowning effect was caused by a chemical inhibition of the melanization process or rather by an enzymatic inhibition of at least one step of the biosynthetic enzymatic pathway leading to melanin and related compounds.

A series of tyrosinase activity measurements using DOPA as the substrate showed that 0.5% (v/v) Cz-oil caused an inhibition of DOPA oxidation as high as 72.2% at pH 6.5. Moreover, when tyrosinase action on tyrosine was checked by means of HPLC analysis, to study both tyrosine consumption and DOPA formation, a substantial inhibition of tyrosine utilization by the enzyme became evident in the presence of Cz-oil (**Figure 1**, bottom), whereas in the control experiment tyrosine was mostly transformed within a few minutes. Conversely, DOPA concentration was only ¼ of the seen in the control experiment (**Figure 1**, top), suggesting that Cz-oil is active against both tyrosinase activities, that is, cresolase and catecholase.

Cz-oil analysis (**Table 1**) showed that its main constituents are (*E*)-β-caryophyllene, α-terpineol, eugenol, and (*E*)-cinnamaldehyde (**Figure 2**). (*E*)-β-Caryophyllene is a sesquiterpenoid hydrocarbon, having biological activities as a local anesthetic (*34*), anti-inflammatory agent (*35*), and antimicrobial (*36*) that have been already described. However, its chemical nature conceivably rules out any significant involvement in the observed tyrosinase inhibition. It has been reported that α-terpineol shows antifungal and antibacterial properties, but also in this case any involvement in the observed tyrosinase inhibition by Cz-oil seems unlikely.

On the contrary, eugenol is already known as a tyrosinase inhibitor, although a less potent one than other vanilloids (*37*). Therefore, a series of experiments measuring the catecholase activity of tyrosinase were carried out under the same conditions described above and with a final concentration of eugenol identical to that found in Cz-oil. In the presence of a 3% eugenol solution (v/v), further diluted 1:1 with DMSO, a 4% inhibition of catecholase activity of tyrosinase was seen in comparison to a control without eugenol. This small effect is clearly related to the modest concentrations of eugenol found in Cz-oil and cannot explain the noticeable inhibition exerted by Cz-oil and shown in **Figure 1**.

(*E*)-Cinnamaldehyde is the most abundant compound among those typical for Cz-oil; its inhibitory activity was tested as already described for both Cz-oil and eugenol, but starting from a 77% solution (v/v), that is, the same concentration of (*E*)-cinnamaldehyde found in Cz-oil. Under these conditions a 40% inhibition of catecholase activity was seen. (*E*)-Cinnamaldehyde is not only the main flavor of Cz-oil, but also it is among cinnamic acid derivatives and analogues already known as mushroom tyrosinase inhibitors (*38, 39*). Lee (*38*) has compared the inhibitory activities of 3,4-dihydroxycinnamic (caffeic) and 4-hydroxy-3-methoxycinnamic (ferulic) acids found in methanolic extracts of *Pulsatilla cernua* roots, a medicinal plant used in East Asia, and other structurally related compounds. Among these, (*E*)-cinnamaldehyde showed a noncompetitive inhibition toward the catecholase activity of mushroom tyrosinase when DOPA was used as the substrate. This noticeable inhibitory activity is, in all likelihood, due to the aldehyde group of this relatively simple aromatic compound. The same conclusion has been reached by Kubo and Kinst-Hori for cuminaldehyde (4-isopropylbenzaldehyde), a strong tyrosinase inhibitor found in *Cuminum cyminum* seeds (*40*). The aldehyde function could be responsible for an electrophilic attack on a Lewis base in the active site of the enzyme, important for the catalytic activity and therefore explaining the observed noncompetitive behavior.

It is worth noting that when considered separately, eugenol and (*E*)-cinnamaldehyde showed inhibitory effects toward mushroom tyrosinase lower than those they exerted when present together in the reaction mixture. In other words, the presence of the two compounds in Cz-oil seemed to lead to synergistic rather than an additive inhibitory effect.

In conclusion, we demonstrated that the use of compressed CO_2 as an extraction fluid is, as usual, the best method to obtain a safe extract deprived of pollutants and substances bearing off-odors. Moreover, in this case, it demonstrated the additional advantage of giving rise to a product that better reproduces the aroma of the starting material; that is, it is able to extract the volatile oil contained, avoiding the loss of relevant amounts of (*E*)-cinnamaldehyde, the main constituent of bark cinnamon oil. In addition, eugenol was successfully extracted with this technique. Both compounds inhibited mushroom tyrosinase (mainly its monophenolase/cresolase activity), thus significantly slowing melanin formation in vitro and browning of apple

homogenates. It has to be pointed out that the use of cinnamon extracts is already permitted (*41*). Obviously, a significant change of the original flavors, if any, of the treated foods could be expected. Therefore, Cz-oil is a very promising candidate (also together with other techniques such as storage under modified atmosphere) for food storage and preservation when prevention of enzymatic browning is important.

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