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Potential Food Additives from *Carex distachya* Roots: Identification and *in Vitro* Antioxidant Properties

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In the present study, the chemical composition and antioxidant properties of root methanol extract of *Carex distachya* Desf. (Cyperaceae) were assessed to use this plant as sources of food additives and nutraceuticals. The IC_{50} of the extract (4.2 μg/mL), derived from the DPPH radical scavenging capacity assay, was similar to those of ascorbic acid, α-tocopherol, and BHT. These results revealed a strong antioxidant activity because of the presence of an extraordinary quantity of bioactive phytochemicals. The phytochemical study of the root extract led to the isolation and identification of new and known polyphenols, most of them common constituents of plant foods. A total of 16 polyphenols, identified on the basis of spectroscopic data as 7 lignans, 4 phenylethanoids, 3 resveratrol derivatives, a monolignol, and a secoiridoid glucoside, were isolated. The tentative structural elucidation of the new metabolites 5′-*O*-β-D-glucopyranosyloxy-3,3′-dimethoxy-7,9′-epoxylignan-4,8′,9-triol and 3,5-*bis*-*O*-β-D-glucopyranosyloxy-3′-methoxy-*trans*-stilben-4′-ol have been performed by a combined approach using ESI/TQ/MS techniques and 1D and 2D NMR experiments. All of the compounds have been tested for their antioxidant activity using six different antioxidant and radical scavenging tests. Interestingly, the extract contained high quantities of polyphenols, most of them reported as constituents of edible plants, such as grape and olive, suggesting that the methanol root extract of this plant could be used as a source of natural antioxidants useful as potential food additives.

KEYWORDS: ***Carex distachya*** **Desf.; Cyperaceae; lignans; phenyletanoid glycosides; resveratrol derivatives; food additives; antioxidant; spectroscopic analysis**

INTRODUCTION

Food oxidation by atmospheric oxygen and free radicals is a destructive process, causing the loss of nutritional value and changes in chemical composition. It is known that polyunsaturated fatty acids react with oxygen to produce peroxide derivatives, leading to rancidity (*1*), while oxidation of fruits, such as apples, pears, or their transformation products, as fruit juice, jams, jelly, etc., produces compounds that compromise their organoleptic and nutritional properties (*2*). The natural content of antioxidants in foods, especially fruits and vegetables, in processed foods, as well as in industrial manufactured feeds is reduced or wholly absent. Air-tight packaging, using inert gases, such as nitrogen, vacuum packing, and refrigeration can be used to reduce the oxidation processes. To prevent this inconvenience, antioxidants are added to foods to slow the rate of oxidation, and if used properly, they can extend the shelf life of the food products in which they have been used. There may be health benefits from the use of antioxidants. Oxidation reactions in the body could be linked to the build-up of fatty deposits that, causing blockages in arteries, can induce heart attacks. Furthermore, antioxidants may be important in heart diseases and the prevention of certain cancers, arthritis, and other pathologies induced by the free-radical exposure.

The number of antioxidants available to food technologies is small. Synthetic and natural antioxidants give similar performances, and they are often used in combination. Ascorbic acid (vitamin C) is present in beers, jams, and cut fruits and prevents the discoloration caused by oxidation reactions; tocopherols (vitamin E), generally obtained from soya beans and maize, reduce oxidation of fatty acids and vitamins; citric acid, added to jams, tinned fruits, biscuits, alcoholic drinks, cheese, etc., increases the antioxidant effects of other compounds; finally, the synthetic antioxidants, butylated hydroxytoluene (BHT) and butylated hydroxyani-

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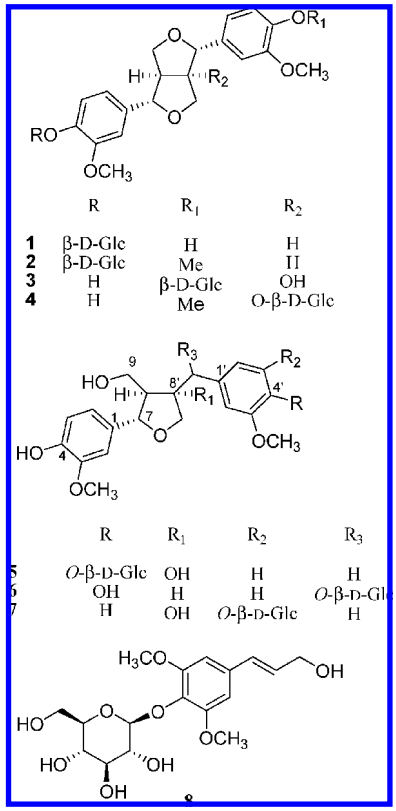

Figure 1. Structures of lignans **1**−**7** and monolignol **8** from *C. distachya* roots.

sole (BHA), are added to oils, margarine, cheese, and crisps to prevent food rancidity.

In recent years, many studies are demonstrating that, besides nutrients and vitamins, there are additional substances in foods, particularly in plant foods, that may have antioxidant activity (*3*−*5*). Some foods, enriched with phytochemicals, herbal supplements, and/or vitamins and minerals, are sold as functional foods, in accordance with the Food and Drug Administration (FDA) structure/function provisions (*6*). Nowadays, the research in this field is focused on the discovery from natural sources of new antioxidant molecules, to be used in alternative to synthetic compounds. Many potential candidates have been isolated and characterized from plant foods (quercetin, resveratrol, ellagic acid, chlorogenic acid, etc), as well as not edible plants are usually studied as sources of antioxidant and radical scavenging molecules (*7*).The aim of the present study is the investigation of the antioxidant power and the phenol content of the root alcoholic extract of *Carex distachya* to identify and characterize new natural compounds useful as food additives.

The phytochemical investigation of leaf extracts of *C. distachya*, an herbaceous plant characteristic of the Mediterranean *macchia*, led to the identification of new secondary metabolites with a strong antioxidant activity (*8*−*10*).

MATERIALS AND METHODS

General Experimental Procedures. Nuclear magnetic resonance (NMR) spectra were recorded at 300 MHz for ^{1}H and 75 MHz for ^{13}C on a Varian Mercury 300 Fourier transform NMR spectrometer, in CD_3OD and $CDCl_3$ solns at 25 °C. Proton-detected heteronuclear correlations were measured using heteronuclear single-quantum coherence (HSQC) (optimized for $^1J_{HC}$ = 140 Hz) and heteronuclear multiple-bond correlation (HMBC) (optimized for $^nJ_{HC}$ = 8 Hz). Optical rotations were measured on a model 141 Perkin-Elmer (Perkin-Elmer Co., Norwalk, CT) in MeOH solution. The high-performance liquid chromatography (HPLC) apparatus consisted of a pump (Shimadzu LC-10AD), a refractive index detector (Shimadzu RID-10A), and a Shimadzu Chromatopac C-R6A recorder. Preparative HPLC was performed using a 250 × 10 mm i.d., 10 μm, Luna RP-8 column (Phenomenex, Torrance, CA). Analytical thin-layer chromatography (TLC) was performed on Merck Kieselgel 60 F_{254} or RP-18 F_{254} plates with 0.2 mm layer thickness. Spots were visualized by UV light or spraying with H_2SO_4/AcOH/H_2O (1:20:4). The plates were then heated for 5 min at 110 °C. Preparative TLC was performed on Merck Kieselgel 60 F_{254} plates, with 0.5 or 1 mm film thickness. Flash column chromatography (FCC) was performed on Merck Kieselgel 60 (230−400 mesh) at medium pressure. Column chromatography (CC) was performed on Merck Kieselgel 60 (70−240 mesh), Baker Bond phase C18 (0.040−0.063 mm), Fluka reversed phase silica gel 100 C8 (0.040−0.063 mm), Amberlite XAD-4 (Fluka, Buchs, Switzerland), or Sephadex LH-20 (Pharmacia, Piscataway, NJ).

Electrospray mass spectra were acquired using a QuattroLC (Micromass, Manchester, U.K.) triple quadrupole instrument operating in the negative- and positive-ion modes. Nitrogen was used as nebulization and desolvation gas at flow rates of 70 and 700 L/h, respectively. Source and desolvation temperatures were set at 120 and 350 °C. Potential applied on the electrospray capillary and the cone ranged from 2.7 to 3.0 kV and from 15 to 55 V, respectively, and were optimized for each molecule and the ionization mode. MS/MS experiments were performed using argon as collision gas at a pressure of 3.5×10^{-3} mbar, with collision energy varying from 20 to 60 eV. All mass spectra were performed by direct introduction of purified compounds 10^{-4} M solutions in MeOH/water (1:1, v/v), with a 10 mL/min flow rate. Full-scan mass spectra were acquired from *m*/*z* 50−1500 in MS and from 20 to the *m*/*z* ratio of the precursor ion in MS/MS experiments, with a scan time of 2 s.

Plant Material. Plants of *C. distachya* Desf. (Cyperaceae) were collected in June 2004, in the vegetative state, in the Natural Reserve of Castel Volturno (Caserta, Italy), and identified by Dr. Assunta Esposito of the Second University of Naples. A voucher specimen has been deposited in the Herbarium of the Dipartimento di Scienze della Vita of Second University of Naples.

Extraction and Isolation. Fresh roots of *C. distachya* (5.4 kg) were extracted in a refrigerated chamber at 4 °C first with MeOH, then in ethyl acetate (EtOAc), and finally in hexane, each one for 3 days. After the removal of the solvents by Rotavapor, we obtained the methanolic (264.6 g), the EtOAc (99.7 g), and hexane (20.8 g) crude extracts. Crude residues were stored at −80 °C until purification.

Organic Extract Fractionation. The methanolic crude extract was dissolved in water (1.0 L) and shaken with EtOAc (1.5 L) to obtain an organic (133.8 g) and aqueous (42.7 g) fraction. The aqueous fraction was chromatographed on Amberlite XAD-4 with MeOH/H_2O solutions as eluents to give one fraction that was chromatographed on SiO_2 under N_2 pressure eluting with the lower organic phase of a $CHCl_3$/MeOH/H_2O (from 13:7:3 to 13:9:3, v/v/v) and $CHCl_3$/MeOH/EtOH/H_2O (10:7:5:5, v/v/v/v) biphasic solutions to obtain five fractions A−E.

Fraction A, rechromatographed on TLC (1.0 mm) eluting $CHCl_3$/MeOH (9:1), furnished pure compounds **1** (142.1 mg) and **2** (68.5 mg).

Fraction B was chromatographed by SiO_2 FCC eluting with the lower organic phase of a $CHCl_3$/MeOH/H_2O (13:9:3) biphasic solution to obtain one fraction that was purified by RP-8 CC eluting with MeOH/MeCN/H_2O (1:1:8) to give fractions I−II. Fraction I was purified by TLC (1.0 mm) eluting with biphasic solution $CHCl_3$/MeOH/H_2O (13:9:3). One of the obtained fractions, purified by RP-8 HPLC eluting with MeOH/MeCN/H_2O (1:1:8), gave compounds **5** (24.7 mg), **6** (23.9 mg), and **7** (11.1 mg). Data for 5′-*O*-*β*-D-glucopyranosyloxy-3,3′-dimethoxy-7,9′-epoxylignan-4,8′,9-triol (**7**). ESI−MS, in the negative-ion mode, $[M - H]^-$ *m*/*z* 537; in the positive-ion mode, $[M + Na]^+$ *m*/*z* 561. CAD $[M - H]^-$ (collision energies of 20 eV): *m*/*z* 375 (100%), 345 (12%), 327 (5%), 195 (19%), and 179 (28%). $[\alpha]^{25}_D$ −20.0 (*c* 0,12, MeOH). ^{1}H NMR (CD_3OD) *δ*: 7.22 (d, *J* = 1.8 Hz, H-2), 7.11 (d, *J* = 8.4 Hz, H-5), 6.98 (dd, *J* = 8.4 and 1.8 Hz, H-6), 6.90 (brs, H-4′), 6.72 (s, H-2′), 6.71 (s, H-6′), 4.86 (ob, H-1″), 4.72 (os, H-7), 3.89 (m, H-9*β*), 3.81 (ov, H-9′α), 3.86 (s, OCH_3), 3.84 (s, OCH_3), 3.65 (ov, H-9α), 3.64 (dd, *J* = 11.7 and 6.0 Hz, H-6″α), 3.43 (m, H-9′*β*), 3.41 (ov, H-6″*β*), 3.23 (m, H-2″, H-3″, H-5″), 3.14 (m, H-4″), 3.04 (d, *J* = 21.0 Hz, H-7′*β*), 2.95 (d, *J* = 21.0 Hz, H-7′α), 2.27 (dt, *J* =

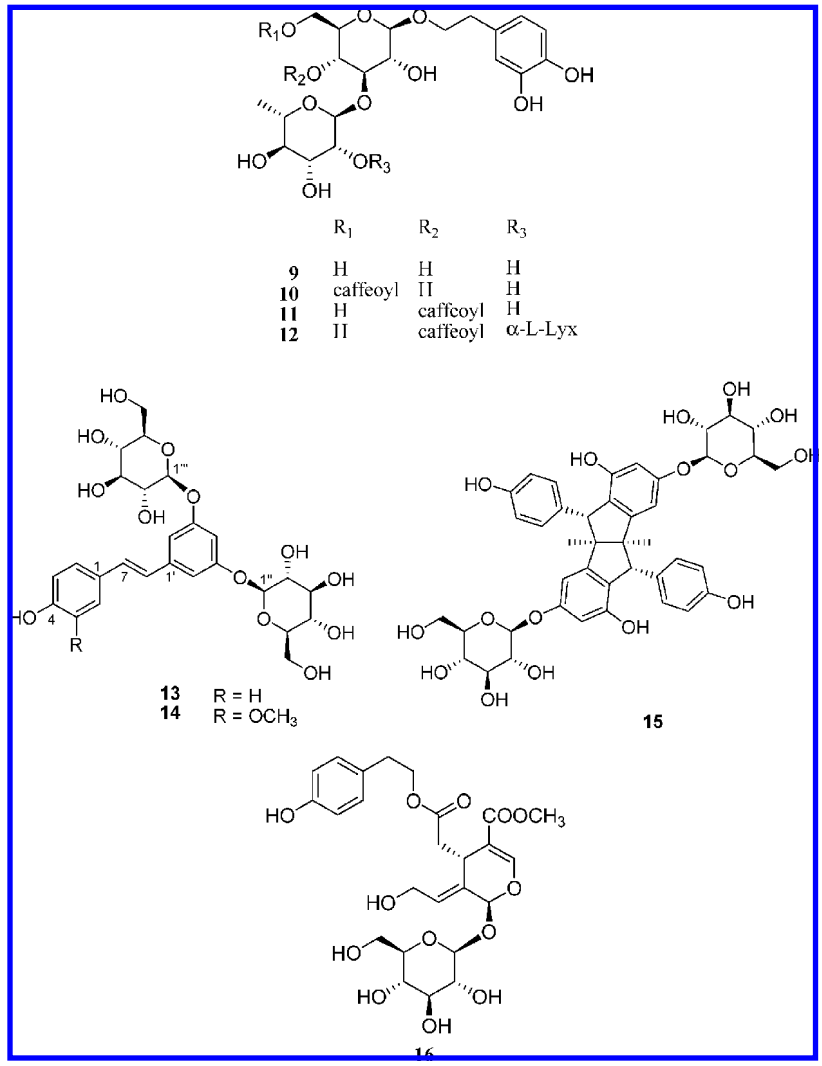

Figure 2. Chemical structures of phenylethanoid glycosides **9**−**12**, resveratrol derivatives **13**−**15**, and secoiridoid glucoside **16** from *C. distachya* roots.

6.6 and 6.3 Hz, H-8). ^{13}C NMR (CD_3OD) δ: 149.6 (C-3), 147.2 (C-3′), 146.1 (C-4), 144.9 (C-5′), 137.0 (C-1), 137.0 (C-1′), 119.4 (C-6), 115.1 (C-5), 114.7 (C-2′ and C-6′), 111.1 (C-2), 109.5 (C-4′), 103.0 (C-1″), 84.4 (C-7), 81.3 (C-8′), 77.9 (C-5″), 77.8 (C-3″), 76.7 (C-9′), 74.9 (C-2″), 71.3 (C-4″), 60.8 (C-8), 62.0 (C-6″), 59.9 (C-9), 55.3 (OCH_3), 55.2 (OCH_3), 49.3 (C-7′). Fraction II purified by RP-8 HPLC eluting with MeOH/MeCN/H_2O (1:1:8) gave compound **9** (58.8 mg).

Fraction C was chromatographed by SiO_2 FCC eluting with a $CHCl_3$/EtOH/H_2O (24:4:1) to obtain one fraction that was rechromatographed by RP-8 CC eluting with MeOH/MeCN/H_2O (1:1:38), to furnish pure compounds **3** (157.2 mg), **4** (144.6 mg), **8** (29.1 mg), and **16** (21.2 mg).

Fraction D purified by Sephadex LH-20 eluting with MeOH/H_2O, increasing the amount of MeOH, gave pure metabolites **10** (78.9 mg), **11** (37.4 mg), and two fractions that were rechromatographed by RP-8 HPLC eluting with MeOH/MeCN/H_2O (1:1:8), to give pure compounds **13** (37.0 mg) and **14** (7.1 mg). Data for 3,5-bis-4β-D-glucopyranosyloxy-3′-methoxy-trans-stilbene-4′-ol (**14**). Amorphous powder. ESI−MS, in the negative-ion mode: $[M - H]^-$ m/z 581; in the positive-ion mode, $[M + Na]^+$ m/z 605. CAD $[M - H]^-$ (collision energies of 25 eV): m/z 419 (100%), 257 (43%). CAD $[M + Na]^+$ (collision energies of 40 eV): m/z 443 (100%), 281 (18%), 428 (9%). ^{1}H NMR (CD_3OD) δ: 7.14 (d, J = 2.1 Hz, H-2′), 7.08 (d, J = 16.2 Hz, H-8), 6.98 (dd, J = 8.1 and 2.1 Hz, H-6′), 6.96 (s, H-2), 6.95 (s, H-6), 6.92 (d, J = 16.2 Hz, H-7), 6.76 (s, H-4), 6.75 (d, J = 8.1 Hz, H-5′), 4.94 (d, J = 7.5 Hz, H-1″, H-1‴), 3.93 (dd, J = 12.0 and 2.1 Hz, H-6″α, H-6‴α), 3.90 (s, OCH_3), 3.50 (m, H-2″, H-3″, H-5″, H-2‴, H-3‴, H-5‴), 3.49 (m, H-4″, H-4‴), 3.69 (dd, J = 12.0 e 6.0 Hz, H-6″β, H-6‴β). ^{13}C NMR (CD_3OD) δ: 159.1 (C-3), 159.1 (C-5), 154.8 (C-4′), 148.1 (C-3′), 140.1 (C-1), 129.8 (C-8), 129.4 (C-1′), 126.2 (C-7), 120.4 (C-6′), 116.8 (C-5′), 112.0 (C-2′), 108.5 (C-6), 108.5 (C-2), 103.8 (C-4), 102.2 (C-1″, C-1‴), 78.2 (C-3″, C-3‴), 78.0 (C-5″, C-5‴), 74.9 (C-2″, C-2‴), 71.6 (C-4″, C-4‴), 62.7 (C-6″, C-6‴).

Fraction E purified by Sephadex LH-20 eluting with MeOH/H_2O, increasing the amount of MeOH, gave pure metabolites **12** (15.3 mg) and **15** (35.6 mg).

Preparation of the Alditol Acetate of 14. A sample of **14** (2 mg) was hydrolyzed with 2 N TFA (250 μL) for 1 h at 120 °C. A total of 250 μL of *iso*-PrOH were added to the mixture and kept under nitrogen for 1 h. To the dried residue, dissolved in deionized water (200 μL), 2 mg of $NaBH_4$ was added in magnetic stirring. After 1 h, two drops of AcOH were added to eliminate the excess of hydride and the solution was dried under N_2. The residue was kept overnight on P_2O_5 at room temperature. Successively, 250 μL of pyridine dry and 100 μL of Ac_2O were added, and the mixture was kept in magnetic stirring for 30 min at 120 °C. After the removal of the solvent, the residue was dissolved in water (0.5 mL), extracted with CH_2Cl_2 (0.5 mL), and analyzed by gas liquid chromatography (GLC).

Antioxidant Activities. *Total Phenol Determination.* The amount of total phenols in crude extracts was determined according to the Folin−Ciocalteu procedure reported by Kähkönen et al. (*11*). Crude extracts (100 μL, 10 mg/mL) were introduced into test tubes: 0.5 mL of Folin-Ciocalteu's reagent (Fluka Chemie, Buchs, Switzerland) and 4 mL of Na_2CO_3 (7.5%) were added. The tubes were mixed and allowed to stand for 3 h. Then, each aliquot (750 μL) were poured into 750 μL of deionized water. Absorption at 765 nm was measured. The total

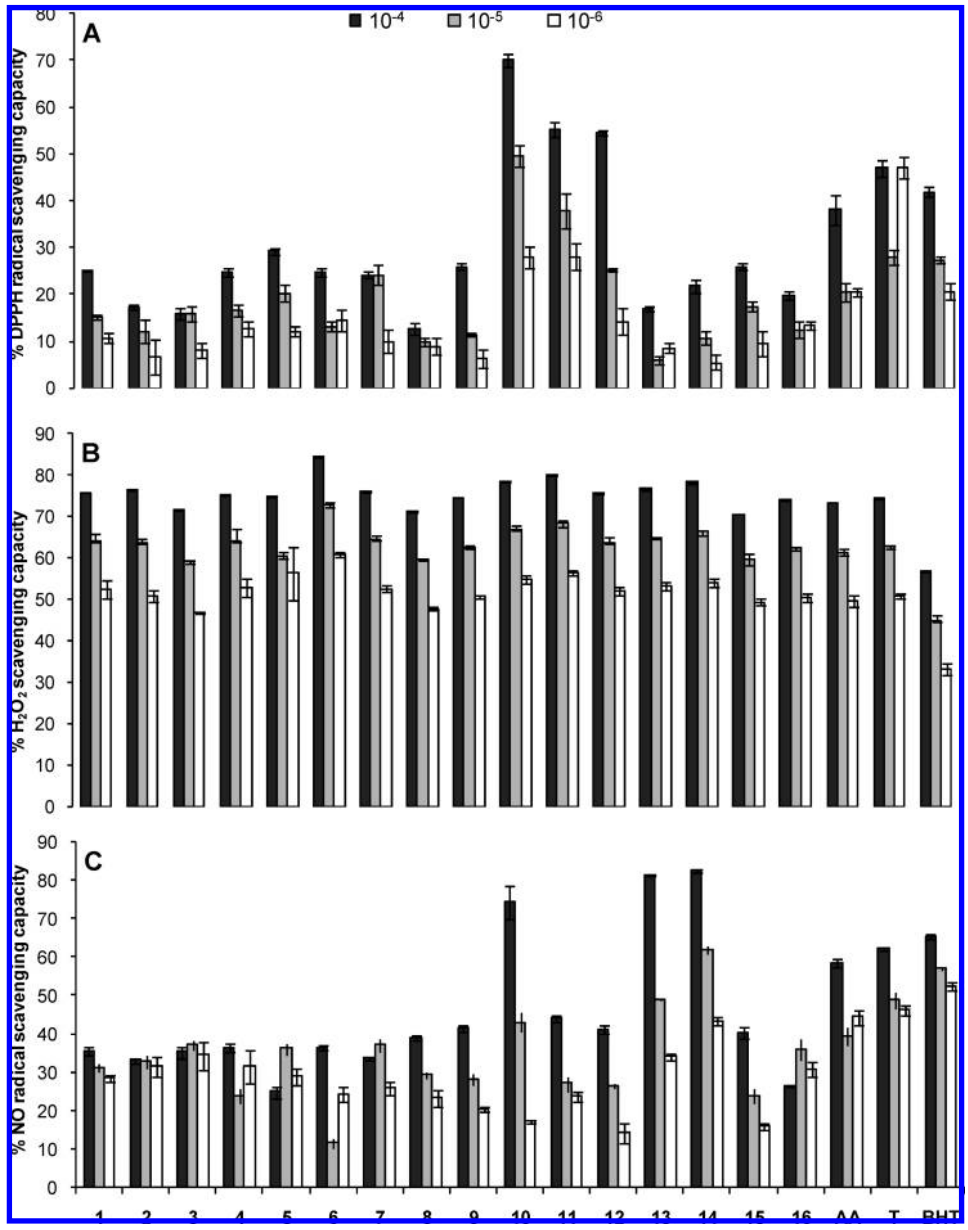

Figure 3. Radical scavenging capacity of metabolites **1**−**16** on (A) DPPH radical, (B) H_2O_2, and (C) NO radical. Values are reported as percentage versus a blank ± SD. AA, ascorbic acid; T, α-tocopherol.

phenols of the samples are expressed as milligrams of gallic acid equivalents (GAE)/100 g of fresh material and reported as phenol mg/g of fresh product.

DPPH Radical Scavenging Capacity. The DPPH radical scavenging method was based on the reduction of methanolic DPPH radical in the presence of an hydrogen-donating antioxidant (*12*).

To evaluate the crude extracts scavenging capacity, their rates (12.5, 25, 50, 100, 150, 200, and 300 μg) were dissolved in 1.0 mL of MeOH. An aliquot (250 μL) were combined to a methanolic DPPH• solution (1.25 mL, 9.4×10^{-5} M) (Fluka Chemie, Buchs, Switzerland) at room temperature. The absorbance at 515 nm was measured by a Shimadzu UV-1700 spectrophotometer at 30 min versus a blank (250 μL of MeOH in 1.25 mL of DPPH• solution).

To determine the DPPH• scavenging capacity of pure isolated metabolites, 250 μL of each sample solution (10^{-4}−10^{-6} M) containing the compound was added to a DPPH• solution (1.25 mL, 9.4×10^{-5} M) at room temperature. The absorbance at 515 nm was measured by a Shimadzu UV-1700 spectrophotometer at 30 min versus blank. The analyses were carried out in triplicate. The results are expressed in terms of the percentage reduction of the initial DPPH radical adsorption by the test samples. The DPPH radical scavenging activity has been compared to those exercised by ascorbic acid, α-tocopherol, and BHT used as standards.

H_2O_2 Scavenging Capacity. The H_2O_2 scavenging capacity evaluation was carried out as reported from Sroka and Cisowski (*13*), with some modifications. The isolated metabolite in H_2O (250 μL, 10^{-4}−10^{-6} M) was added to a H_2O_2 (100 μL, 0.002%) and NaCl (400 μL, 0.1 M) solution. After incubation at 37 °C for 30 min, to each sample, 700 μL of Red Phenol-HRP (Red Phenol, 1.5 mg/mL; HRP, 1.0 mg/mL; Fluka Chemie, Buchs, Switzerland) in buffer phosphate (0.2 M, pH 7.8) was added. After incubation at room temperature for 15 min, the sample was transferred in a policarbonate cuvette. NaOH (0.1 M, 150 μL) was added. The absorbance at 560 nm was measured by a Shimadzu UV-1700 spectrophotometer versus blank. The analyses were carried out in triplicate. The scavenging capacity was expressed as a reduction percentage of the red phenol absorbance sample induced and compared to those exercised by ascorbic acid, α-tocopherol, and BHT used as standards.

NO Radical Scavenging Capacity. The NO radical scavenging efficacy determination was performed in accordance with the method reported by Yen et al. (*14*), with some modifications. The reaction mixture containing sodium nitroprusside (SNP, 0.1 M) (Fluka Chemie, Buchs, Switzerland) in buffer phosphate (400 μL, pH 7.4) and the sample test in H_2O (250 μL, 10^{-4}−10^{-6} M) was incubated at 37 °C for 3 h. Then, 600 μL of buffer phosphate (0.2 M, pH 7.4) and 150 μL

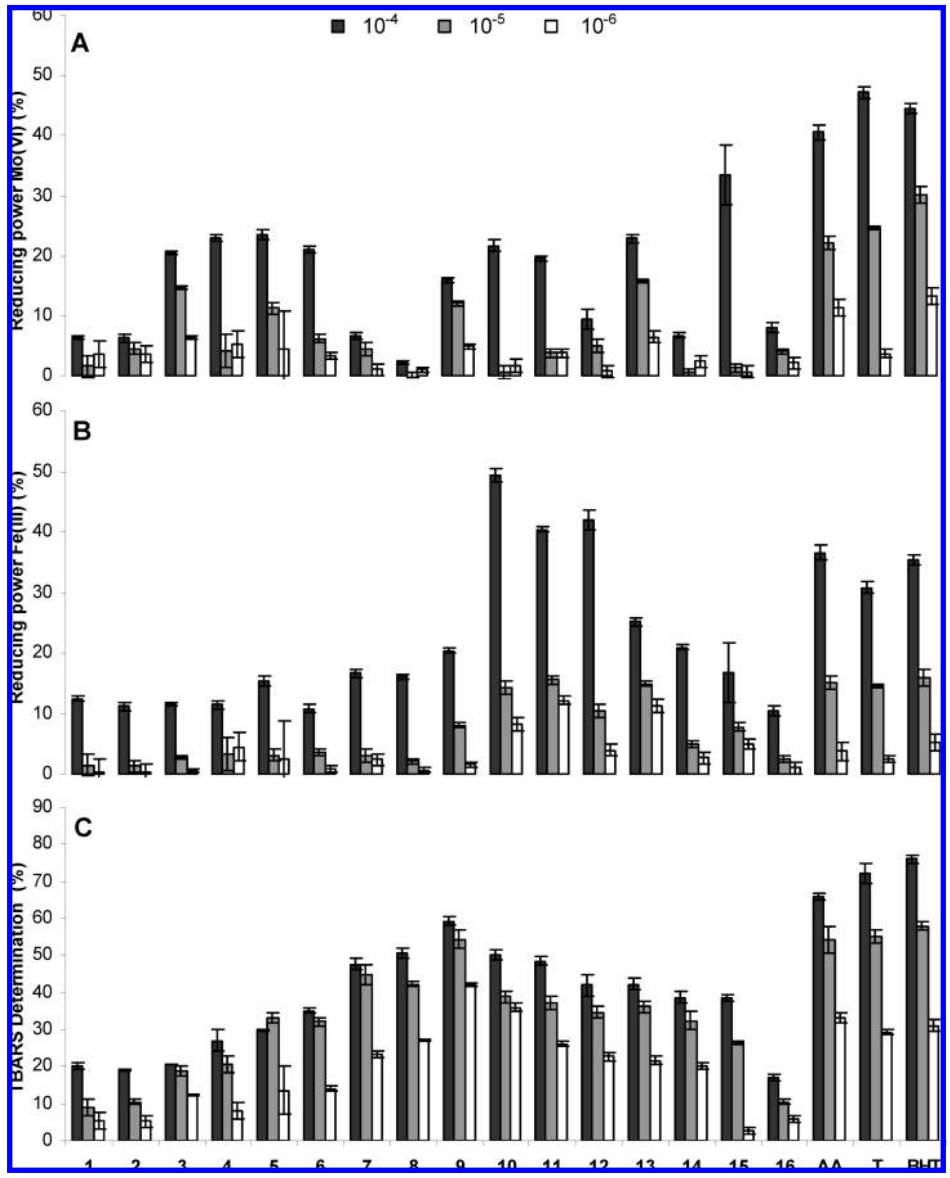

Figure 4. Antioxidant capacity of metabolites **1**−**16**. (A) Reducing power Mo^{VI}. (B) Reducing power Fe^{III}. (C) TBARS synthesis determination. Values are reported as percentage versus a blank ± SD. AA, ascorbic acid; T, α-tocopherol.

of Griess reagent (1% sulfanilamide and 0.1% naphthylethylendiamine dihydrochloride in 5% H_3PO_4) (Fluka Chemie, Buchs, Switzerland) were added to the reaction mixture. The absorbance of the chromophore formed during the diazotination of nitrite with sulfanilamide, and subsequent coupling with naphthylethylendiamine was read at 540 nm and referred to the absorbance of standard solution of sodium nitrite treated in the same way with Griess reagent. The analyses were carried out in triplicate. The radical scavenging capacity was expressed as the reduction percentage of nitrite absorbance sample induced and compared to those exercised by ascorbic acid, α-tocopherol, and BHT used as standards.

Reducing Power. The isolated metabolite reducing power was determined according to the method of Oyaizu (*15*), with some modifications. Each metabolite (10^{-4}−10^{-6} M) in phosphate buffer (250 μL, 0.2 M, pH 6.6) was added to potassium ferricyanide (250 μL, 10 mg/mL) (Riedel-de Haën, Buchs SG, Switzerland) and trichloroacetic acid (TCA, 250 μL, 100 mg/mL). The mixture was incubated at 50 °C for 30 min. Then, ferric chloride (250 μL, 1.0 mg/mL) and deionized water (0.5 mL) were added. The absorbance was read spectrophotometrically at 700 nm. A higher absorbance of the reaction mixture indicated greater reducing power. The analyses were carried out in triplicate, and the registered activities were compared to those exercised by α-tocopherol and ascorbic acid used as standards in the assay.

Evaluation of Antioxidant Capacity. The quantitative determination of the antioxidant capacity was carried out according to the method of Prieto et al. (*16*) Sample metabolite (250 μL, 10^{-4}−10^{-6} M in 1:1 DMSO/H_2O) was combined in a Eppendorf tube with 1.25 mL of reagent solution (0.6 M solforic acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) (Fluka Chemie, Buchs, Switzerland). The tubes were capped and incubated in a thermal block at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance was measured at 650 nm versus a blank. The absorption value increase blank related is the antioxidant capacity estimate. The analyses were carried out in triplicate, and the registered activities were compared to those exercised by α-tocopherol and ascorbic acid used as standards in the assay.

TBARS Determination. Determination of thiobarbituric acid reactive substances (TBARS) was measured by the method reported from Sroka and Cisowski (*13*), with some modifications. Vegetal fat (10.0 μL) (Riedel-de Haën) was emulsified with 30.0 mg of Tween-40 (Fluka Chemie, Buchs, Switzerland) initially dissolved in 1.5 mL of 0.2 M Tris-HCl buffer at pH 7.4. The emulsion was stirred for 24 h and then irradiated with UV light at 254 nm at room temperature for 60 min. Then, test compound (250 μL, 10^{-4}−10^{-6} M) was added, and the reaction mixture was exposed to UV light for 60 min again. TBA reagent (2.0 mL), prepared by dissolving 375 mg of thiobarbituric acid, 30 mg of tannic acid, and 15 mg of

trichloroacetic acid in hydrogen chloride aqueous solution (0.1 L, 0.2 M), was added. Test tubes were placed into a boiling water bath for 60 min and then centrifuged for 3 min at 3500*g*. The supernatant was measured at 532 nm by a Shimadzu UV-1700 spectrophotometer. Inhibition of test lipid peroxidation was measured as a percentage against a blank containing no test compounds. The analyses were carried out in triplicate, and the registered activities were compared to those exercised by α-tocopherol and ascorbic acid used as standards in the assay.

RESULTS AND DISCUSSION

The antioxidant activity of the crude root extracts of *C. distachya* was evaluated by measuring its capability to scavenge the DPPH radical and measuring their phenol content using the Folin−Ciocalteu method. The radical scavenging activities have been compared to those showed by known antioxidant standards, as ascorbic acid, α-tocopherol, and butylated hydroxytoluene (BHT). The scavenging capacity of the MeOH crude extract increased massively with the amount of the samples, and the addition of 150 μg completely reduced the DPPH radical. The measured IC_{50} value of the methanolic crude extract (4.2 μg/mL) is comparable to those of ascorbic acid, α-tocopherol, and BHT, showing an IC_{50} of 4.3, 5.1, and 3.9 μg/mL, respectively. This high antioxidant power of the extract should be correlated to its high phenol contents (2.26 ± 0.19 mg/g). Organic crude extracts resulted less active than the alcoholic one. The measured IC_{50} values were equal to 13.9 and 36.6 μg/mL for ethyl acetate and hexane extracts, respectively. The phenol content decreased to 1.42 ± 0.04 mg/g in ethyl acetate extract and 1.06 ± 0.07 mg/g in the hexane one.

Chemical Characterization of Phytochemicals. The most antioxidant crude methanolic root extract of *C. distachya* was dissolved in water and shaken with EtOAc. From the aqueous fraction, after various chromatographic steps, we isolated two new metabolites 5′-*O*-β-D-glucopyranosyloxy-3,3′-dimethoxy-7,9′-epoxylignan-4,8′,9-triol (**7**) and 3,5-*bis*-4-*O*-β-D-glucopyranosyloxy-3′-methoxy-*trans*-stilbene-4′-ol (**14**), besides 14 already known phytochemicals, which structures have been elucidated on the basis of their one- and two-dimensional NMR data and mass spectrometry. We identified seven lignans (**1**−**7**), the phenylpropane glucoside synapic alcohol 4-*O*-β-D-glucopyranoside (**8**), four phenylethanoid glycosides (**9**−**12**), three resveratrol derivatives (**13**−**15**), and a secoiridoid **16** (**Figures 1** and **2**).

Lignans **1** and **2** were identified as (+)-pinoresinol 4-*O*-β-D-glucopyranoside and phylliroside, respectively, already isolated from *Forsythia* leaves (*17*). Compound **3** was identified as (+)-1-hydroxypinoresinol 4′-*O*-β-D-glucopyranoside by comparison of our spectroscopic data to those reported for the metabolite isolated from *Eucommia ulmoides* (*18*). Lignan **4** was already isolated from the bark of *Olea europea* and *Olea africana* (*19*), while compound **5** was characterized by field desorption mass spectrometry, from *Ligustrum japonicum* (*20*). Finally, tetrahydrofuranoid-lignan glycoside **6**, tanegosides A, was previously isolated from *Trachelospermum liukiuense* (*21*).

Lignan **7** was tentatively identified as 5′-*O*-β-D-glucopyranosyloxy-3,3′-dimethoxy-7,9′-epoxylignan-4′,8′,9-triol. Its molecular formula was $C_{26}H_{34}O_{12}$, as suggested by the ^{13}C NMR spectrum and ESI−MS data. In fact, on the basis of the ^{13}C NMR/DEPT experiments, we identified 26 carbons, as 2 methyls, 4 methylenes, 13 methines, and 7 quaternary carbons. In the downfield part of the ^{1}H NMR spectrum, signals of a 1,2,4-trisubstituted aromatic ring as two doublet at δ 7.22 and 7.11 were evident, besides a double doublet at δ 6.98 and three singlet signals at δ 6.90, 6.72, and 6.71, each integrated for one proton. The two-dimensional NMR data were in agreement with the presence of a glucosylated furolignan. In particular, the correlations in the HMBC between the H-7 proton and the C-2, C-6, and C-8 carbons, between the H-8 and C-1, C-7, and C-8′ carbons and between the Glc-1 proton and the C-5′ carbon were in accordance with the presence of a C_6C_3 unit, bonding a glucopyranosyloxy moiety at C-5′ and a methoxy group at C-3 carbon. In the aliphatic region of the ^{1}H NMR, two diasterotopic methylene protons were evident as two doublets at δ 2.95 and 3.04 attributed to the C-7′. These protons showed correlations, in the HMBC experiment, with the aromatic carbons at δ 137.0 and 114.7 and with a tetrasubstituted carbinol at δ 81.3. This latter was heterocorrelated with the H-8 proton and the H-9′ protons. Further correlations were in agreement with the presence on the second aromatic ring of a methoxyl group at the C-3 carbon and the hydroxyl at the C-4 carbon. Supporting this structural hypothesis, the positive ESI−MS spectrum showed the ions at *m/z* 561 and 577 corresponding to the $[M + Na]^+$ and $[M + K]^+$ adducts and the negative ESI−MS spectrum displayed the ion at *m/z* 537 corresponding to the $[M - H]^-$ ion. The $[M - H]^-$ CAD mass spectra showed the ion at *m/z* 375 arising from the loss of a 162 Da unity that corresponds to a hexose residue. Likely, this ion fragments into the ions at *m/z* 195 and 179 reasonably, following the opening and cleavage of the tetrahydrofuran ring. Minor intensity ions, corresponding to the loss of a CH_2O moiety giving the ion at *m/z* 345 and a water molecule giving the ion at *m/z* 357, are observed. In-source CAD of the ion at *m/z* 179 showed the loss of a methyl group followed by the loss of a water molecule leading the ions at *m/z* 164 and 146, respectively, or the loss of water molecule followed by the loss of a methyl group leading the ions at *m/z* 161 and 146, respectively. In-source CAD of the ion at *m/z* 195 showed the loss of a methyl group and a water molecule leading to the ions at *m/z* 180 and 177, respectively.

Compound **8** was identified as 3-(4-*O*-β-D-glucopyranosyloxy-3,5-dimethoxy)phenyl-2*E*-propenol, already isolated from *Zantedeschia aethiopica* (*22*).

Metabolites **9**−**12** were identified as phenylethanoid glycosides (**Figure 2**). Compounds **9**−**11** were identified as decaffeoylverbascoside (*23*), isoverbascoside, and verbascoside (*24*). All of these glycosides were also isolated from *Markhama stipulate* (*25*), while teucrioside **12** was reported as a constituent of *Teucrium chamaedris* (*26*).

Compounds **13**−**15** have been identified as resveratrol derivatives. Compound **13** was recently isolated from *Vitis vinifera* cell-suspension cultures (*27*).

Compound **14** has been tentatively characterized for the first time. It showed a molecular formula $C_{27}H_{34}O_{14}$, in agreement with ^{1}H and ^{13}C NMR and MS data. Its ^{1}H NMR spectrum showed, in the downfield region, two olefinic protons as AB spin system at δ 7.08 and 6.92 ($J = 16.2$ Hz), three protons of a 1,2,4-trisubstituted aromatic ring as two doublets at δ 7.14 ($J = 2.1$ Hz) and 6.75 ($J = 8.1$ Hz), and a double doublet at δ 6.98 ($J = 8.1$ and 2.1 Hz), besides three broad singlets at δ 6.96, 6.95, and 6.76. The region of protons geminal to oxygens displayed a doublet because of two coincident homotopic protons at δ 4.94 ($J = 7.5$ Hz), a methyl as singlet at δ 3.90, two double doublets, each because of two protons, at δ 3.93 and 3.69, besides other eight overlapped protons ranging from 3.60 to 3.30 ppm.

The ^{13}C NMR spectrum showed 19 signals identified, on the basis of a DEPT experiment, as 1 methyl, 1 methylene, 12 methines, and 5 tetrasubstituted carbons. The HMBC experiment showed correlations between the olefinic proton at δ 7.08 with the carbons 140.1, 129.4, 120.4, and 112.0. The carbon at δ 129.4 was correlated with the protons of the aromatic ring at δ 7.14,

6.75, and 6.98, which were, in turn, correlated with the carbons at δ 154.8 and 148.1. Finally, the carbon at δ 148.1 showed correlations with the methoxyl protons at δ 3.93. The carbon at δ 140.1 showed correlations with both the olefinic protons and the aromatic singlets at δ 6.96 and 6.95, which showed cross-peaks with the carbon at δ 103.8 bonded, in the HSQC experiment, to the proton at δ 6.76.

These data led us to hypothesize the presence of a stilbenic unit with a methoxyl and hydroxyl on the C-3 and C-4 carbons of the A ring and with two hydroxyl groups at the C-3 and C-5 carbons of the B ring. The correlation between the anomeric proton at δ 4.94 with the carbon at δ 159.1 as well as ESI−MS mass spectrometric fragmentations suggested the presence of two sugar units on the B ring. The ^{13}C NMR values were in good agreement with the presence of a glucose moiety, which was definitively characterized by comparison of the alditol acetate of compound **14** to authentic samples. The coupling constant value of the anomeric proton indicated a β configuration for both sugars.

The positive ESI mass spectrum of compound **14** displayed the peaks at *m/z* 605 and 621, corresponding to the $[M + Na]^+$ and $[M + K]^+$ adducts. In the negative ESI mass spectrum, the peaks at *m/z* 581 and the peaks at *m/z* 617/619 corresponding to the $[M - H]^-$ ions and the $[M + Cl]^-$ adducts are present. The $[M + Na]^+$ CAD spectra showed, as the main fragmentation channel, the ion at *m/z* 443 corresponding to the loss of a 162 Da moiety. The $[M - H]^-$ CAD spectra showed the ion at *m/z* 581, besides the ion at *m/z* 419 (M − H−162), the ion at *m/z* 257 arising from to the loss of a second sugar unit. All attempts to induce fragmentation of this ion, generated by in-source CAD, were unsuccessful.

Compound **15** was identified as pallidol diglucoside, a resveratrol dimer glucoside isolated from Riesling wine (*36*). Finally, compound **16** is known as 10-hydroxyligustroside, a seco-iridoid glucoside reported as a constituent of *Ligustrum* spp. (*28*).

To evaluate their antioxidant and radical scavenging capacities, all of the compounds were tested at three different concentrations (10^{-4}, 10^{-5}, and 10^{-6} M) in triplicate. Several activity tests were useful to define the antioxidant capacity of the isolated substances. The detected activities were compared to those carried out by the natural standards α-tocopherol and ascorbic acid and the synthetic standard BHT. Three radical scavenging capacity assays, each one involving a specific redox reaction with the oxidant, were performed. The results of the DPPH radical (**Figure 3A**), the prooxidant hydrogen peroxide (**Figure 3B**), and the nitric oxide (**Figure 3C**) radical scavenging capacity assays showed that all of the metabolites scavenged the radical target species; polyhydroxylated compounds exhibit an activity comparable or superior to those standard species determined. The furolignan glucoside **6** produced a strong reduction (84.0%) of the red phenol, used as a probe in the hydrogen peroxide scavenging capacity test.

Phenylethanoid glucosides **10**−**12** showed a peculiar antioxidant efficacy. The isoverbascoside (**10**) was able to induce a DPPH radical reduction by 70.0%. The registered activity was strongly higher than those registered by equal concentrations of ascorbic acid (38.2%), α-tocopherol (47.0%), and BHT (41.9%). The same glycoside inhibited massively the nitrite ions formation in the reaction media, showing an activity of 76.0%.

Resveratrol derivatives, **13** and **14**, showed a strong nitric oxide radical scavenging capacity. In particular the stilbenic metabolite **14**, which skeleton is characterized by a methoxyl function and an hydroxyl on C-3 and C-4 carbons of the A ring, demonstrated a reducing capacity by 82.7%. The registered capacities characterized these metabolites as effective NO scavengers; it should be noted that ascorbic acid and α-tocopherol induced a reduction by 58.5 and 62.2%, respectively.

The results of the antioxidant activities are reported in **Figure 4**. Spectrophotometric evaluation of antioxidant capacity through the formation of a phosphomolybdenum complex showed that the tested metabolites were able to reduce Mo^{VI} to Mo^{V} (**Figure 4A**). The results demonstrated that resveratrol derivatives **13** and **16** and lignans **3**−**6** were responsible for a significant antioxidant capacity. The reducing efficacy of diepoxylignan and epoxylignan metabolites decreased when a β-D-glucose is present on the C-4 carbon.

Phenylethanoid glucosides **10**−**11** exhibit a high reducing power on Fe^{III} (**Figure 4**B). All of these compounds are characterized by the presence of two catechols of the tyrosol and caffeic acid moieties. Malondialdehyde (MDA), an end product of the oxygenation of polyunsaturated fatty acids, is commonly used as a biomarker for assessing lipid peroxidation. MDA reacts with thiobarbituric acid to form a pink/red adduct. The test results (**Figure 4**C) evidenced that the incubation of glycosides **9**−**12**, in a system containing rape seed oil as lipoperoxidation substrate, determined an average inhibition by 60.0% in the formation of thiobarbituric-acid-reactive species. Stilbenic derivatives **14** and **16** resulted also in antilipoperoxidative agents.

The abundance of antioxidant metabolites in *C. distachya* MeOH root extract emphasizes the extraction procedure importance as previously reported (*29*), and it is strongly related to the polarity and solubility features of the isolated molecules. Lignans are phenylpropane oligomers present in plant foods. Some plant lignans can be converted by intestinal bacteria into the enterolignans (*30*). Hu et al. (*31*) have been recently reported the antioxidant activity of flax-seed lignin glucosides, showing that secoisolariciresinol diglucoside and mammalian lignans enterodiol and enterolactone were effective antioxidants against DNA damage and lipid peroxidation. Veselova et al. (*32*) investigated the antioxidant capacity of lignans isolated from *Taxus cuspidata* and concluded that the use of natural antioxidants from plants, mostly of polyphenolic nature, are promising for the creation of new effective drugs for the treatment and prophylaxis of pathologies involving free radicals. Furthermore, many antioxidant lignans have been isolated from alimentary sources. Furofuran lignans with an antioxidant capacity were isolated from beverages, vegetables, fruits, nuts, seeds, bread, etc. (*33*). Lignan compounds were also extracted from roasted sesame oil, and their effects on the autoxidation of methyl linoleate were studied (*34*). These compounds have been identified as major components of the phenolic fraction of olive oil.

The strong antioxidant activity of phenylethanoids has been recently reported for some compounds isolated from *Lippia triphylla*, a plant used in Peru as a spice and herb tea for the prevention of arteriosclerosis (*35*). Among these compounds, together with other polyphenols and secoiridoids, verbascoside **11** has been isolated from *Olea europaea* L. leaves (*35*). Because resveratrol and its oligomers from *Vitis vinifera* have been demonstrated to be powerful antioxidants and bone-health phytochemicals, their discovery in other sources has been extensively investigated. Kim et al. (*36*) showed that resveratrol dimers and trimers, together with resveratrol from seeds of *Paeonia lactiflora* might be useful as potential sources of natural antioxidants.

In conclusion, the methanol extract of roots of *C. distachya* showed a potent antioxidant activity because of the presence of an extraordinary quantity of bioactive phytochemicals. Because the most active and abundant phenols are common constituents of plant foods, the results of this study showed that the methanol root extract of this plant could be used as a source of natural antioxidants useful as potential food additives. Resveratrol derivatives, lignans, and phenylethanoids, the

phenolic compounds isolated from *C. distachya*, appear to be the most responsible compounds for the antioxidant activity.

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