Nitrification Inhibitors from the Roots of *Leucaena leucocephala*

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The nitrification inhibition (NI) bioassay guided fractionation of the methanol extract of lyophilized and milled roots of *Leuceana leucocephala* resulted in the isolation of four compounds, **1—4**, as confirmed from their ^{1}H and ^{13}C NMR spectral data. Compound **1**, gallocatechin, was the most active NI inhibitor at 12 μg/mL. Epigallocatechin, **2**, and epicatechin, **4**, isolated as mixtures, were not assayed individually for their NI inhibitory activities against the nitrification bacterium *Nitrosomonas europaea*.

Keywords: *Leuceana leucocephala; Nitrosomonas europaea; gallocatechin; epigallocatechin; catechin; epicatechin; nitrification inhibitors*

INTRODUCTION

Nitrification is carried out by bacteria whereby ammonia is converted to nitrate. It occurs in almost all soils and also in marine and freshwater sediments. The process is three step and is caused by two sets of bacteria. In the first set, consisting of *Nitrosomonas europaea*, *Nitrosolobus multiformis*, and several other species, ammonia is oxidized to hydroxylamine and then to nitrite (eqs I and II). The second set consists of

$$NH_3 + O_2 + 2e^- + 2H^+ \rightarrow NH_2OH + H_2O \quad (I)$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \quad (II)$$

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \quad (III)$$

Nitrobacter agilis, which converts nitrite to nitrate (eq III). Nitrification is important because much fertilizer is applied as ammonia, and the nitrification process reduces fertilizer efficiency.

There is considerable evidence to show that plants produce secondary metabolites that inhibit nitrification. Early work demonstrated that grasslands displayed low nitrification rates (Theron, 1951; Stiven, 1952; Munro, 1966). Further research showed that specific plant compounds inhibited nitrification, and tannins and gallotannins were reported to inhibit nitrification (Basabara, 1964; Rice, 1965, 1969). A large body of research provided evidence that phenolic acids and some flavonoids inhibit nitrification, including chlorogenic acid, gallic acid, caffeic acid, quercetin, and karanjin (Rice, 1964, 1965; Rice and Pancholy, 1974; Sahrwat and Mukerjee, 1977).

However, there are published reports questioning the nitrification inhibition activity of naturally occurring compounds. Gallic and caffeic acids were tested for nitrification inhibition in both soil and pure *Nm. europaea*, *Nl. multiformis*, and *Nitrospira* cultures but were found to be inactive (McCarty and Bremner, 1986; McCarty et al., 1991). It was also shown that phenolic acids inhibited nitrification only at levels much higher than those found in the soil (Turtura et al., 1989).

Although questions remain as to whether plants produce secondary compounds that influence the nitrification process, there is much evidence indicating that plant secondary compounds play a variety of roles in protecting plants from a variety of stresses. The present research describes the isolation and characterization of nitrification inhibiting compounds from the roots of *Leucaena leucocephala*, using an in vitro nitrification inhibition (NI) assay.

MATERIALS AND METHODS

General Experimental Procedures. NMR spectra (^{1}H and ^{13}C) were recorded on a Varian INOVA 300 spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C) or a Varian VXR 500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). Chemical shifts were recorded in DMSO-d_6 or CD_3OD and the values are in δ (ppm) based on δ residuals of DMSO-d_6 2.49 and 39.5 for ^{1}H and ^{13}C NMR or δ residuals of CD_3OD 3.30 and 49.0 for ^{1}H and ^{13}C NMR, respectively. Coupling constants, *J*, are in hertz. All positive controls and chemicals used in the antioxidant assay were purchased from Sigma Chemical Co. unless otherwise stated. All organic solvents were of ACS reagent grade (Aldrich Chemical Co., Inc., Milwaukee, WI).

Plant Material. *L. leucocephala* seeds, obtained from Nigeria, Africa, were germinated in the Plant and Soil Sciences Building greenhouses in 12-in. plastic pots. Plants were later transferred to the Bioactive Natural Products Laboratory (BNPL) greenhouses, subjected to a 12-h photoperiod, watered once daily, and maintained at 75 °F. For extractions, whole plants were harvested, and root, stem, and leaves were collected separately and stored at −20 °C. A total of 391 g of fresh root material was collected for extraction. The roots were lyophilized using a tray lyophilizer (model TD-3B, FTS Systems, Inc., Stone Ridge, NY) at 5 °C for 48 h to yield 185 g of dry weight. Dried roots were ground to a fine powder in a Wiley mill (mesh size = 2 mm, Thomas-Wiley, laboratory mill, model 4) prior to extraction.

Extraction and Isolation of NI Compounds. Lyophilized, powdered *L. leucocephala* roots (165 g) were extracted sequentially at room temperature by soaking with hexane (2 × 500 mL; 24 h), ethyl acetate (2 × 500 mL; 24 h), and

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methanol (2 × 500 mL; 24h). Solvents were removed in vacuo on a rotary evaporator to yield the respective extracts. The dried hexane, ethyl acetate, and methanol extracts of the roots yielded 0.33, 0.51, and 7.76 g, respectively, and were stored at −20 °C until analysis.

The NI active crude methanol root extract (7.71 g) was stirred with water (45 mL) at room temperature for ∼1 h and centrifuged, and the supernatant was lyophilized, yielding 5.40 g of powder. This (5.31 g) was fractionated on Amberlite XAD-2 resin (51 g, Supelco Inc., Bellefonte, PA; 23.5 × 3.5 cm glass column), using water, methanol/water (1:1), and methanol, to yield three fractions. Fraction 1 was collected by eluting the column with 75 mL of water, fraction II by eluting the column with 75 mL of 1:1 methanol/water, and fraction III by eluting with 100% methanol. After the methanol elution, the column was flushed with water to prepare for the next fractionation. After fraction III was found to be NI active, four more purifications on the Amberlite column gave a total of 453.6 mg of powder. This powder was purified by preparative HPLC (Japan Analytical Instruments LC-20), using MeOH/H_2O (70:30) as the eluting solvent system and dual Jaigel GS-310 F columns (20 × 300 mm i.d., 3 μm pore size, 40000 MW exclusion), in tandem, at 4 mL/min. For each HPLC purification, 100 mg of fraction III was dissolved in 1.25 mL of methanol, filtered through a 0.2-μm filter, and injected onto the HPLC; seven fractions were collected. A total of 410 mg of the fraction III was purified this way, and the combined HPLC fractions were assayed for NI activity. Fractions 5−7 were found to be active and yielded 25.4, 36.4, and 22.6 mg, respectively. The most active fraction, 6, was further purified by preparative HPLC, using two Jaigel ODS columns (Jaigel S-343-15 ODS, i.d. 20 × 250 mm), in tandem, and yielded a 9:1 mixture of compounds **1** and **2** (24.1 mg). Proton and carbon NMR spectra confirmed the structures of these compounds as gallocatechin and epigallocatechin, respectively (Davis et al., 1996). Fraction 7 was further purified by HPLC and afforded a white solid (12.3 mg). Although the chromatogram of this product showed only a single peak, the proton NMR spectrum indicated that it was a 1:1 mixture of compounds **3** and **4**. Analysis of the ^1H spectrum of this product revealed the identities of **3** and **4** as catechin and epicatechin, respectively (Chien-Chang et al., 1993).

Nitrification Inhibition Bioassay. The *Nm. europaea* strain used in NI bioassays was purchased from American Type Culture Collection (ATCC 19718). Cultures used for bioassays were grown in 2 mL of medium in 12 × 75 mm (4-mL) presterilized polystyrene Falcon tubes. The components of the medium followed ATCC catalog (no. 929), and "nitrifying bacteria" (Schmidt and Belser, 1982). It consisted of 1.32 g of NH_4SO_4, 380 mg of $MgSO_4 \cdot 7H_2O$, 20 mg of $CaCl_2 \cdot 4H_2O$, 87 mg of K_2HPO_4, and 0.5% phenol red solution in 1 L of reverse osmosis (RO) water. The trace elements used consisted of 100 mL of RO water, 10 mg of $NaMoO_4 \cdot 2H_2O$, 20 mg of $MnCl_2$, 0.2 mg of $CoCl_2 \cdot 6H_2O$, 10 mg of $ZnSO_4 \cdot 7H_2O$, and 2 mg of $CuSO_4 \cdot 5H_2O$ (Schmidt and Belser, 1982). The chelated iron solution consisted of 246 mg of $FeSO_4 \cdot 7H_2O$ and 331 mg of disodium EDTA in 100 mL of RO water (Schmidt and Belser, 1982). For the completed medium, the ingredients were mixed in a flask with 1 mL each of the micronutrient solution and chelated iron solution and 0.25 mL of 0.5% phenol red solution. The final volume was then adjusted to 1 L with RO water. The pH of the medium was then adjusted to 7.5 with 0.2 M K_2CO_3. The medium was filter-sterilized through a 0.2 μm Nalgene bottle-top filter (500 mL) into a sterilized Pyrex storage bottle (500 mL).

The phenol red used in the medium was to indicate bacterial growth. The culture tubes containing the bacteria were seeded when the pH was 7.5. The optimum pH range for the bacterial growth is ∼6.0−7.5. The pH of the medium lowered as the bacteria grew, and the growth of *Nm. europaea* was shown by the pH indicator color change. When the medium had become acidic, the pH was adjusted to 7.5 by dropwise addition of 0.5% K_2CO_3 under sterile conditions.

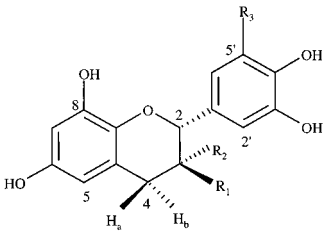
For the bioassay, extracts were filter-sterilized using a presterilized Millex GV 0.22 μm filter unit. Prior to the bioassay, the pH of the bacterial cultures was adjusted to 7.5. The inhibition was measured by lack of color change from pink to yellow. All crude extracts were assayed at 250 μg/mL, and purified fractions were assayed at 100 μg/mL. A 20-μL aliquot of test sample in DMSO was added to each milliliter of bacterial culture. Two controls were employed, the first with 20 μL of DMSO added per milliliter of medium with live cultures; the second control was medium blanks with no bacteria in them. Assays were quantified for nitrite using the Greiss−Ilosvay method (Keeney and Nelson, 1982). The Greiss−Ilosvay method is a two-step colorimetric method to measure nitrite, based on the chemistry of nitrite reacting with primary aromatic amines in acidic solution to form diazonium salts. The salts then couple with aromatic compounds to form colored azo compounds, which are measured spectroscopically. Specifically, nitrite was reacted with sulfanilamide (Sigma Chemical Co., St. Louis, MO) and then was coupled with *N*-(1-naphthyl)ethylenediamine dihydrochloride (Aldrich Chemical Co., Milwaukee, WI) to form reddish purple solutions.

Antioxidant Assay. Antioxidant activities were conducted on the purified compounds by liposome oxidation assay using fluorescence spectroscopy. A mixture containing 5 μmol of 1-steroyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine (Avanti Polar Lipids, Inc., Alabaster, AL) and 5 μmol of the fluorescence probe 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid (Molecular Probes, Inc., Eugene, OR) was evaporated in vacuo on a rotary evaporator at 23 °C. The resulting lipid film was then suspended in 500 μL of buffer solution containing 0.15 M NaCl, 0.1 mM EDTA, and 0.01 M MOPS and subjected to 10 freeze−thaw cycles using a dry ice/ethanol bath. This solution was treated with chelating resin Chelex 100 to remove trace metal ions. The lipid−buffer suspension was then extruded 29 times through a LiposoFast extruder (Avestin, Inc., Ottawa, Canada) containing a polycarbonate membrane with a pore size of 100 nm to produce large unilamellar liposomes (LUVs). A 20-μL aliquot of the LUV suspension was diluted to a final volume of 2 mL in Chelex 100 treated HEPES buffer (100 μL, pH 7.0), 1 M NaCl (200 μL), N_2-sparged water (Millipore, 1.64 mL), and DMSO solution containing the test compound (20 μL), vortexed, and placed in a cuvette. Peroxidation was then initiated by the addition of 20 μL of 2 mM stock $FeCl_2$ solution to achieve a final concentration of 20 μM of Fe^{2+} in the absence or presence of test compounds. The control sample contained neither Fe^{2+} nor the test compounds. The positive controls, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ), and α-tocopherol (vitamin E), were all tested at a final concentration of 10 μM. Fluorescence intensities of these liposome solutions were measured at an excitation wavelength of 384 nm every 3 min over a period of 21 min using a Turner model 450 digital fluorometer (Barnstead Thermolyne, Dubuque, IA). The decrease of relative fluorescence with time indicated the rate of peroxidation (Arora and Strasburg, 1997).

RESULTS AND DISCUSSION

Freshly harvested roots (392 g) of *L. leucocephala* were lyophilized and sequentially extracted with hexane/ethyl acetate. The methanol extract showed activity in the NI bioassay. Fractionation of the methanol extract was accomplished by obtaining the water solubles, which were further separated over Amberlite XAD-2 resin to yield fractions I−III, eluting with water, water/methanol (1:1), and methanol, respectively. Fraction III was found to be biologically active. Further purification of this fraction by preparative HPLC afforded compounds **1—4**. However, compounds **3** and **4** were an inseparable mixture under the HPLC conditions used.

Compound **1** was identified by ^1H and ^{13}C NMR to be gallocatechin, and the structure was unequivocally confirmed by comparison of NMR data reported for



1 R_1 = OH , R_2 = H , R_3 = OH
2 R_1 = H , R_2 = OH , R_3 = OH
3 R_1 = OH, R_2 = H , R_3 = H
4 R_1 = H, R_2 = OH , R_3 = H

Figure 1. Structures of gallocatechin (**1**), epigallocatechin (**2**), catechin (**3**), and epicatechin (**4**).

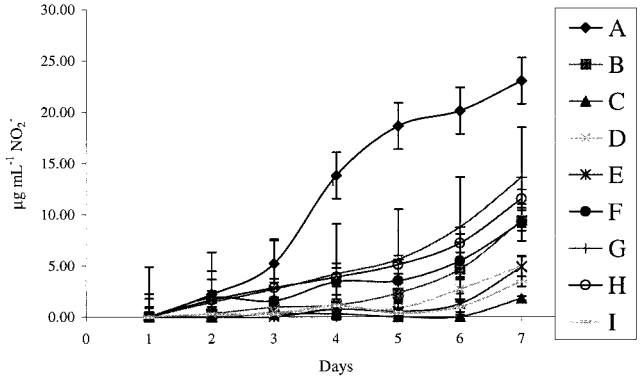


Figure 2. Nitrite levels measured in the NI bioassay for gallocatechin and other test samples: (A) non-DMSO control; (B) DMSO control; (C) 50 μg/mL gallocatechin (g.c.); (D) 12.5 μg/mL g.c.; (E) 6.25 μg/mL g.c.; (F) 3.12 μg/mL g.c.; (G) 10 μg/mL HPLC fraction 5; (H) authentic sample of 10 μg/mL (+)-catechin; (I) 10 μg/mL nitrapyrin. Negative values were normalized to zero. Data were subjected to analysis of variance and found to be highly significant at the 0.01 level.

gallocatechin (Davis et al., 1996). Important peaks in the ^{1}H NMR spectrum of compound **1** (Figure 1) in assigning it as gallocatechin were the doublet at 4.41 ppm, corresponding to the C-2 proton, the multiplet at 3.77 ppm, assigned to the C-3 proton, and the large singlet at 6.23 ppm, which integrated for two protons and was assigned to the C-2′ and C-6′ protons. Compound **2** was identified as epigallocatechin (Figure 1). The structure was confirmed by the presence of a singlet at 4.64 ppm, corresponding to the C-2 proton, and by the peaks of the C-3 multiplet at 4.01 ppm and the C-2′/C-6′ singlet at 6.36 ppm, respectively. The relative chemical shifts of these peaks were identical to the literature values (Chien-Chang et al., 1993; Davis et al., 1996).

Compounds **3** and **4** were isolated as a 1:1 mixture. Two peaks in the ^{1}H NMR spectrum of this mixture at δ 4.56 and 4.81, both assigned to the C-2 proton, gave the strongest evidence for a mixture. The peak at 4.56 ppm was a doublet, which indicated the trans configuration, whereas the singlet at 4.81 ppm suggested the cis (epi) configuration. There were two multiplets at 3.98 and 4.18 ppm, corresponding to the C-3 protons. Also, the multiple peaks at 6.7−7.0 ppm indicated the presence of only two phenolic groups on the B ring, confirming the non-gallo configuration. These NMR values were identical to the reported values for catechin (**3**) and epicatechin (**4**) (Figure 1) (Chien-Chang et al., 1993; Dübeler et al., 1997).

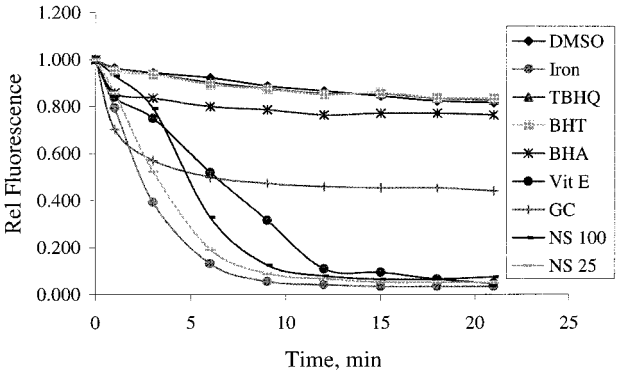


Figure 3. Antioxidant assay results for gallocatechin (GC) and nitrapyrin (NP). Commercial antioxidants TBHQ, BHT, BHA, and vitamin E were used as positive controls at 10 μM concentrations. Gallocatechin and nitrapyrin were assayed at 25 and 100 μM concentrations, respectively. Relative fluorescence (F_t/F_0) was determined by dividing the fluorescence at time t (F_t) by the initial fluorescence (F_0).

In the NI bioassay, 50 μg/mL gallocatechin inhibited the growth of *Nm. europaea* similar to nitrapyrin at 10 μg/mL. Nitrapyrin, 2-chloro-6-(trichloromethyl)pyridine, is a commercial nitrification inhibitor (Figure 2). Gallocatechin also displayed strong activity at 12 μg/mL. The 1:1 mixture of catechin and epicatechin displayed NI activity at 100 μg/mL. However, catechin (Sigma Chemical Co.) gave no activity at 10 μg/mL.

Because the compounds isolated were known to have good antioxidant activities (Plumb et al., 1998; Vinson and Dabbagh, 1998), we have investigated their antioxidant activity, along with nitrapyrin, to see whether the nitrification inhibition was a function of oxygen radical scavenging. The results of the antioxidant assay (Figure 3) showed that while gallocatechin was an effective antioxidant, nitrapyrin was not. Hence, the mechanism of nitrification inhibition by these compounds is not related to their oxygen scavenging properties.

This research presents evidence that gallocatechin is a good nitrification inhibitor. This is the first report of the isolation of these compounds from *L. leucocephala* and the NI activity for gallocatechin.

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