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Cyclooxygenase-2 Inhibitory and Antioxidant Compounds from the Truffle *Elaphomyces granulatus*

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The ethanol extract of fruiting bodies of *Elaphomyces granulatus,* a truffle-like fungus, was evaluated for cyclooxygenase-2 (COX-2) enzyme inhibitory and antioxidant activities. Inhibition of COX-2 activity was evaluated in mouse macrophages (RAW 264.7). The extract of *E. granulatus* caused a 68% inhibition of COX-2 activity at 50 ug/mL. Bioassay-guided investigation led to the isolation and identification of two active compounds, syringaldehyde and syringic acid. Syringaldehyde moderately inhibited COX-2 activity with an IC_{50} of 3.5 ug/mL, while syringic acid strongly inhibited COX-2 activity with an IC_{50} of 0.4 ug/mL. The antioxidant activity of the extract and isolated compounds was evaluated in HL-60 cells by the DCFH-DA method. The extract of *E. granulatus* showed a potent antioxidant effect, with an IC_{50} of 41 ug/mL. Of the pure compounds, syringic acid displayed a strong antioxidant activity, with an IC_{50} of 0.7 ug/mL, while syringaldehyde showed no activity in the assay. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: Elaphomyces granulatus; Ascomycota; hypogeous; truffle; COX-2; RAW 264.7 cells; antiinflammatory; antioxidants; DCFH oxidation; HL-60 cells.

INTRODUCTION

Truffles and truffle-like fungi are characterized by a hypogeous, i.e. underground, fruiting habit, having evolved the use of animal mycophagy for the dispersal of their spores. These fungi produce aromatic compounds by which the animals locate them, dig them up and eat them (Trappe and Claridge, 2005). The animals digest the nutritious tissues of the fruiting bodies, but the spores pass through the digestive tract and are defecated unharmed (Claridge and Trappe, 2005). Because of their underground fruiting habit, truffles are difficult to find, hence research on their chemistry and medicinal properties has been limited and generally focused on the volatile compounds responsible for their unique aromas (Diaz *et al.,* 2002, 2003).

In the course of our program aimed at the investigation of biological activity and chemistry of truffles from North America, a study on *Elaphomyces granulatus* Fr. (Elaphomycetaceae, Ascomycota) was initiated. The most widely distributed and common hypogeous fungus in the Northern Hemisphere, it occurs as a beneficial, mycorrhizal symbiont with feeder rootlets of trees from subarctic and subalpine forests south to the tropics (Trappe, 1971). In Europe, *E. granulatus* has been used as an aphrodisiac and a cheap but illegitimately marketed substitute for more expensive truffles (Arora, 1986). The analysis of the chemical composition of the spore mass and outer layer of *E. granulatus* revealed the occurrence of mixtures of higher aliphatic esters, free fatty acids, hydrocarbons, mannitol, ergosterol, pyrocatechol, protocatechuic acid, salicylic acid, resorcinol, 3-hydroxy- and 4-hydroxy benzoic acids (Solberg, 1976). However, no other studies of its biological activity and/or chemical constituents have been reported.

Cyclooxygenase-2 (COX-2) enzyme plays an important role in the inflammatory process. COX-2 is an inducible isoform of cyclooxygenase enzyme responsible for the production of pro-inflammatory prostaglandins in neoplastic and inflamed tissues. COX-2 inhibitors have a well established role in the treatment of inflammatory disorders, as well as potential application for the prevention and treatment of other diseases, such as cancer (Flower, 2003; Amir and Agarwal, 2005). Reactive oxygen species (ROS) and oxidative stress also play an important role in the etiology and progression of human degenerative diseases. ROS have been implicated in inflammation, aging, cancer, heart disease and other disorders (Pietta, 2000). Antioxidants act as ROS scavengers and are important for protecting against oxidative tissue damage in vital organs. Although, numerous *in vitro* solution-based chemical assay systems, such as 2,2-diphenyl-l-picrylhydrazyl radical (DPPH) assay, have been used for the evaluation of antioxidants (Cuendet *et al.,* 1997), the use of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as a specific probe in a cell based assay provides a better system to evaluate an antioxidant effect in live cells. This method is useful for the direct examination of ROS inhibitory activity of natural products in live human cells (Takamatsu *et al.,* 2003; Choi *et al., 2006).*

As part of our investigation of biologically active compounds from *E. granulatus,* the ethanol extract of

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fruiting bodies of *E. granulatus* was evaluated for COX-2 enzyme inhibitory and antioxidant activities in cellular assays. The bioassay-guided isolation and biological characterization of compounds from *E. granulatus* with antioxidant and COX-2 inhibitory activities were also performed.

MATERIALS AND METHODS

General experimental procedures. The ^{1}H-NMR,^{13}C-NMR, HMBC and HMQC spectra were recorded on a Bruker DRX 400 MHz. The Bruker NMR spectrometer operated at 400 MHz for ^{1}H and 100 MHz for ^{13}C. The NMR spectra were recorded in ppm using the residual solvent peak as an internal standard. The HRESIMS data were acquired on a Bruker BioAPEX 30es mass spectrometer.

Fungal material. *Elaphomyces granulatus* was collected by Mr Adrian Beyerle of the North American Truffling Society (NATS) in Oregon, Wasco Co., Mt Hood National Forest, near 15-Mile Creek on 16 August 2002. All specimens were identified by Dr James M. Trappe. A voucher specimen is deposited in the USDA National Fungus Collections, Beltsville, MD (BPI 864222). Mr Adrian Beyerle subsequently provided additional specimens from the area to supplement the original collection. Additional collections were contributed by other NATS members.

Extraction and isolation. The fruiting bodies of *E. granulatus* were dried for 24 h in a forced air dehydrator at 35°C. Powdered material (482 g) was extracted exhaustively by maceration with 95% EtOH at room temperature, and the combined extracts were concentrated under reduced pressure to yield 12.5 g of residue. The crude extract was divided into EtOH soluble and insoluble fractions. The EtOH soluble fraction (9 g) was subjected to a silica gel gravity column (230 g, 457 mm x 51 mm) and eluted with chloroform, and chloroform-methanol (2%-100%) to yield 16 fractions. The active fractions 4-7 were combined (800 mg) and subjected to another silica gel column (50 g, 450 mm x 17 mm) with hexane-chloroform-ethyl acetate (3:3:1-1:1:1-0:1:1) to give 108 fractions. Fractions 52-74 were combined (61 mg) and separated on preparative TLC (Si gel CF_{254}, 250 um, Uniplate) with hexane-chloroform-ethyl acetate (1:1:1) to yield syringaldehyde (7 mg). Fractions 98-102 were combined (63 mg) and separated on preparative TLC (Si gel CF_{254}, 250 *um*, Uniplate) with chloroform-methanol (2%) to yield syringic acid (10 mg).

Syringaldehyde: pale yellow solid; HRESIMS *m/z* 183.0638 (calcd for $C_9H_{11}O_4$ $[M + H]^+$ 183.0657), 205.0457 (calcd for $C_9H_{10}O_4Na$ $[M + Na]^+$ 205.0477); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 9.81 (1H, s, C*H*O), 7.14 (2H, s, H-2, H-6), 3.95 (6H, s, 2OCH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 190.7 (*C*HO), 147.4 (C-3, C-5), 141.0 (C-4), 128.4 (C-1), 106.8 (C-2, C-6), 56.5 (OCH_3). Spectra corresponded with previously reported data (Ralph *et al.*, 2004).

Syringic acid: pale yellow solid; HRESIMS *m/z* 199.0580 (calcd for $C_9H_{11}O_5$ $[M + H]^+$ 199.0606), 221.0398 (calcd for $C_9H_{10}O_5Na$ $[M + Na]^+$ 221.0426); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.21 (2H, s, H-2, H-6), 3.79 (6H, s, 2OCH_3); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 167.9 (*C*OOH), 148.1 (C-3, C-5), 140.9 (C-4), 121.1 (C-1), 107.6 (C-2, C-6), 56.6 (OCH_3). Spectra corresponded with previously reported data (Ralph *et al.*, 2004).

Cell-based assay **for inhibition of COX-2 activity.** Mouse macrophages (RAW 264.7, ATCC) were cultured in a 75 cm^2 culture flask in RPMI-1640 medium (Gibco) supplemented with 10% bovine calf serum (Hyclone) and 60 *mg/L* amikacin (Sigma), at 37°C in an environment of 95% humidity and 5% CO_2. For the assay, the cells were seeded in the wells of 96-well plates (50,000 cells/well) and incubated at 37°C for 24 h. After washing with RPMI-1640 medium, supplemented with 3% bovine calf serum, the cells were incubated with 5 ug/mL LPS *(Escherichia coli* 055:B5, Sigma) for 16 h to induce the production of COX-2. Induced cells were washed thoroughly with medium to remove LPS completely, and treated with different concentrations of test samples for 2 h. Arachidonic acid (300 *uM,* Sigma) was added and the cells were further incubated for 30 min. The amount of PGE_2 released in the medium was determined with *PGE_2* enzyme immunoassay kit (Cayman Chem. Co.). COX-2 activity was determined by the conversion of exogenous arachidonic acid to PGE_2 and expressed as the percent of the vehicle control. The concentration that caused 50% inhibition of enzyme activity (IC_{50}) was calculated from the dose curves generated by plotting percent COX-2 activity against the test concentrations. NS-398 (Cayman Chem. Co.), a specific inhibitor of COX-2, was included as a positive control in each assay.

Assay **for cytotoxicity to macrophages.** RAW 264.7 cells were cultured as described above. For the assay, cells were seeded to wells of a 96-well plate at a density of 25 000 cells/well and incubated for 24 h. Different dilutions of test compounds were added to the cells and incubated for 48 h. Cell viability was determined by the neutral red assay (Borenfreund *et al.,* 1990). After incubation, the medium was removed and 100 uL, of fresh medium containing 0.2 *mg/mL* neutral red (Sigma) was added to each well and incubated for 90 min. The cells were washed with saline (0.9% NaC1) to remove excess dye. The solution of acidified isopropanol (0.33% HC1) was then added to lyse cells. As a result, the incorporated dye was liberated from viable cells, the absorbance of which was measured at 490 nm using the EL312e plate reader (Bio-Tek instruments).

Assay **for antioxidant activity.** Myelomonocytic HL-60 cells (ATCC) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone) and 60 mg/mL amikacin at 37°C in an environment of 95% humidity and 5% CO_2. For the assay, 125 uL, of the cell suspension (1 x 10^6 cells/mL) was added to the wells of a 96-well plate. After treating with different concentrations of the test samples for 30 min, the cells were stimulated with 100 *ng/mL* phorbol 12-myristate 13-acetate (PMA, Sigma) for 30 min. DCFH-DA (Molecular Probe, 5 ug/mL) was added and the cells were incubated for 15 min. The levels of DCF produced were measured on a PolarStar plate reader with an excitation wavelength at 485 nm and emission at 530 nm as

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described previously (Takamatsu *et al.,* 2003; Choi *et al.,* 2006). The ability of the test materials to inhibit exogenous cytoplasmic ROS-catalysed oxidation of DCFH to fluorescent DCF in HL-60 cells was measured in comparison to PMA treated controls without the test materials. The IC_{50} values were calculated from dose curves of the % DCF production versus test concentrations. Vitamin C (Sigma) was included as a positive control.

Assay for cytotoxicity to HL-60 cells. Cytotoxicity of the test samples to HL-60 cells was determined by the XTT method after incubating the cells with test samples for 48 h as described earlier (Takamatsu *et al.,* 2003). Briefly, 25 uL of XTT-PMS solution (1 mg/mL XTT solution supplemented by 25 uM of PMS) was added to each well. After incubating for 4 h at 37°C, the absorbance at 450 nm was measured on a plate reader (EL312e; Bio-Tek instruments).

RESULTS AND DISCUSSION

The dried fruiting bodies of *E. granulatus* were exhaustively extracted by maceration with 95% EtOH and fractionated by various chromatographic techniques. The bioassay-guided fractionation of *E. granulatus* led to the isolation of two active compounds, syringaldehyde and syringic acid (Fig. 1). Structures of syringaldehyde and syringic acid were determined by using HRESIMS, ^{1}H-NMR and ^{13}C-NMR experiments.

The extract of *E. granulatus* and isolated compounds were evaluated for inhibition of COX-2 activity in a cell-based assay that utilizes the mouse macrophage cell line (RAW 264.7). Unstimulated macrophages express only a small amount of COX-2, while treatment with bacterial lipopolysaccharide (LPS) leads to the induction of COX-2, which converts arachidonic acid to PGE_2 (Chen *et al.,* 2001). LPS-induced RAW 264.7 macrophages were incubated in the presence, Or absence, of test samples for 2 h, followed by the addition of arachidonic acid. The effects of the test samples on COX-2 activity were determined by measuring the PGE_2 produced in the culture medium. NS-398, a specific inhibitor of COX-2, was used as a positive control (IC_{50}:0.2 ug/mL, 0.64 uM).

The extract of *E. granulatus* showed a potent COX-2 inhibitory activity with 68% inhibition at 50 ug/mL, Syringaldehyde inhibited COX-2 activity in a dose-dependent manner (Fig. 2), with an IC_{50} of 3.5 ug/mL, (19.23 uM). Syringic acid showed a stronger inhibition

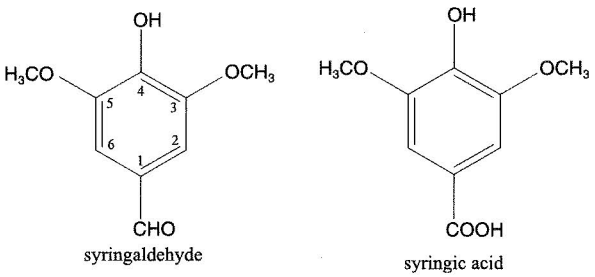

Figure 1. Structures of syringaldehyde and syringic acid.

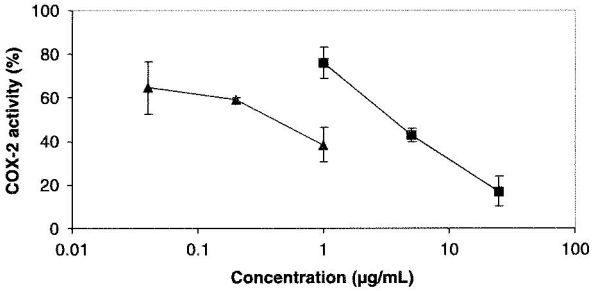

Figure 2. Inhibition of COX-2 enzyme activity by syringaldehyde (■) and syringic acid (▲) in LPS-activated macrophages (RAW 264.7). Each data point represents the mean ± SD of triplicate determination.

of COX-2 activity in a dose-dependent manner (Fig. 2), with an IC_{50} of 0.4 ug/mL (2.02 uM).

The RAW 264.7 cell viability was determined to exclude the possibility that the observed COX-2 inhibitory effect was due to cytotoxicity. Examination of cytotoxicity of syringic acid and syringaldehyde in RAW 264.7 macrophages by the neutral red assay indicated that compounds did not affect the viability of RAW 264.7 cells in concentrations up to 25 ug/mL,

The results of this study demonstrate that syringaldehyde has a moderate COX-2 inhibitory activity, while syringic acid is a strong inhibitor of the COX-2 enzyme. This study is the first report on the occurrence of syringaldehyde and syringic acid in *E. granulatus,* which could account for the potent COX-2 inhibitory activity of the mushroom extract. Although in previous studies syringic acid has been reported to show antiinflammatory activity *in vivo* (Fernandez *et al.,* 1998; Gamaniel *et al.,* 2000), this is the first report on its activity on the COX-2 enzyme. The results of this study suggest that the mechanism responsible for the antiinflammatory activity of syringic acid might be related to COX-2 inhibition.

The antioxidant activity of the extract and isolated compounds was evaluated in HL-60 cells using DCFH-DA. This cell-based method examines directly the ability of test material to penetrate living cells and inhibit ROS catalysed oxidation of DCFH to DCF. DCFH-DA is a non-fluorescent probe that diffuses into cells. Cytoplasmic esterases hydrolyse DCFH-DA to DCFH which is oxidized to DCF (2',7'-dichlorofluorescin) that fluoresces. The antioxidant activity of test samples is determined by measuring the level of DCF produced in treated cells compared with controls.

The extract of *E. granulatus* showed a potent antioxidant effect, with an IC_{50} of 41 ug/mL, The inhibitory effect of syringic acid on DCF production is shown in Fig. 3. Syringic acid displayed a strong antioxidant activity in a dose-dependent manner, with an IC_{50} of 0.7 ug/mL (3.54 uM) which is comparable to the effect of vitamin C, a naturally occurring antioxidant, that showed an IC_{50} of 0.5 ug/mL (2.84 uM) in the same assay. Examination of the cytotoxicity of syringic acid and syringaldehyde in HL-60 cells indicated that compounds were not cytotoxic up to a concentration of 31.25 ug/mL,

The results of this study indicate that syringic acid has a strong antioxidant activity in the cellular-based assay while syringaldehyde was inactive. Most of the previous reports on the antioxidant properties of syringaldehyde

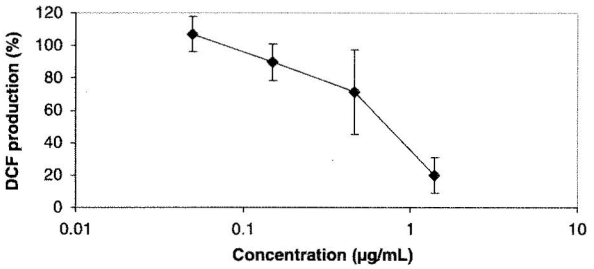

Figure 3. Effect of syringic acid on DCF production in HL-60 cells. Each data point represents the mean ± SD of duplicate determination.

and syringic acid have utilized solution-based chemical assay systems that do not evaluate the antioxidant activity within living cells. One recent study on ROS production by human neutrophils, induced by opsonized zymosan or phorbol 12-myristate 13-acetate (PMA), found ROS inhibitory effects for both syringaldehyde and syringic acid measured as luminol or lucigenin-enhanced chemiluminescence (Worm *et al.*, 2001).

This study is the first report on the potent antioxidant and COX-2 enzyme inhibitory properties of the extract of *E. granulatus.* In addition, COX-2 inhibitory activities of syringaldehyde and syringic acid are reported here for the first time. *E. granulatus* seems to have potential health benefits due to its antioxidant and antiinflammatory effects. Consumption of *E. granulatus* as a dietary supplement or as a food item could contribute to the prevention of cancer and inflammatory disorders.

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