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In-vitro anti-inflammatory activity of *Pinus sylvestris* and *Plantago lanceolata* extracts: effect on inducible NOS, COX-1, COX-2 and their products in J774A.1 murine macrophages

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Abstract

Extracts of the plant species *Pinus sylvestris* L. and *Plantago lanceolata* L. have been used in traditional medicine for the treatment of certain respiratory diseases, but little is known about their precise effects and mechanisms of action. In this study, we investigated the effect of these plant extracts on the production of nitric oxide (NO) and prostaglandin E_2, NO synthase (NOS) type II, cyclooxygenase-1 (COX-1) and COX-2 mRNA expression in the murine macrophage cell line J774A.1. We found that *Pinus sylvestris* and *Plantago lanceolata* extracts inhibited NO production in a concentration-dependent manner in this cell line, without obvious cytotoxic effects as tested by MTT assay. The *Plantago lanceolata* extract at all doses used, and the *Pinus sylvestris* extract at high doses, showed significant scavenging of NO radicals released by the NO donor PAPA-NONOate. Our data also show that pre-treatment with these extracts significantly inhibits inducible NOS (iNOS) mRNA production in this cell line, without affecting COX-1 mRNA expression. COX-2 mRNA levels and PGE_2 levels induced by lipopolysaccharide/interferon-γ were not modified upon pre-treatment with the extracts. Thus, our results suggest that the anti-inflammatory properties of *Pinus sylvestris* and *Plantago lanceolata* extracts may reflect decreased NO production, possibly due to inhibitory effects on iNOS gene expression or to NO-scavenging activity.

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Introduction

Inflammation is a complex process characterized by the contribution of various mediators, including prostaglandins and nitric oxide (NO) (Higgs et al 1984; Nathan 1997). Cyclooxygenase (COX) is one of the main enzymes involved in the metabolism of arachidonic acid, catalysing the synthesis of prostaglandins and thromboxane (Smith et al 1991). COX exists in two isoforms: COX-1 is a ubiquitously and constitutively expressed isoform that is postulated to have housekeeping functions; COX-2 is an inducible isoform that has been implicated in inflammatory responses and the regulation of cell growth and differentiation (Smith et al 1996). Specifically, COX-2 is thought to be the primary generator of the prostanoids that contribute to inflammation, acting in both the inflammation initiation and resolution phases (Gilroy et al 1999). However, several studies have shown that prostanoids formed via COX-1 are also involved in inflammation processes (Langenbach et al 1995; Wallace et al 1998).

The pivotal role of nitric oxide (NO) as a messenger and effector molecule has been demonstrated in a variety of tissues (Palmer et al 1988; Lowenstein et al 1996). NO has been identified as a neurotransmitter in the central nervous system and a potent physiological vasorelaxant that regulates blood pressure by modulating muscular tone (Hibbs et al 1987; Moncada et al 1991). NO is also an important molecule in inflammation and sepsis (Wheeler & Bernard 1999). Exposure to bacterial surface molecules, such as lipopolysaccharide (LPS) and lipoteicholic acid (LTA), stimulates cellular inflammatory responses and induces release of pro-inflammatory factors, including NO, prostaglandin E_2 (PGE_2), cytokines, tumour necrosis factor-α and eicosanoid mediators. At least three types of nitric oxide synthase (NOS) isoform

have been identified in cells. Both the endothelial nitric oxide synthase (eNOS) and neural nitric oxide synthase (nNOS) isoforms are constitutive (cNOS), in that NO produced by cNOS contributes to maintaining the normal active state of vasodilatation through a Ca^{2+}/calmodulin-dependent pathway, and acts as a neurotransmitter in neuron signal transmission. NOS in macrophages and hepatocytes is inducible (the iNOS isoform), and its activation is Ca^{2+}/calmodulin-independent. Exposure to endogenous and exogenous stimulators induces iNOS in various cells, such as macrophages, smooth muscle cells and hepatocytes, triggering various detrimental cellular responses and potentially causing disease, including inflammation, sepsis and stroke (Marletta et al 1988; Nathan 1992; Marletta 1993; Duval et al 1996). NO production induced by iNOS may thus reflect the degree of inflammation, and provides a useful way of assessing the effect of drugs on the inflammatory process. Conversely, inhibition of NO accumulation induced by inflammatory stimuli could be a useful strategy for treatment of inflammatory diseases (Hobbs et al 1999).

Pinus sylvestris L. and *Plantago lanceolata* L. extracts have been used in traditional medicine as anti-inflammatory treatments in bronchitis, asthma and other respiratory diseases (Matev et al 1982; Peris et al 1995; Blumenthal 1998). It has recently been demonstrated that bioflavonoids extracted from the bark of *Pinus maritima* inhibit the expression of the pro-inflammatory cytokine interleukin-1 (IL-1) by regulating redox-sensitive transcription factors, namely nuclear factor-κB (NF-κB) and activating protein-1 (AP-1), in LPS-stimulated RAW 264.7 murine monocyte macrophages (Cho et al 2000). *Pinus maritima* bark extract is also a powerful scavenger of reactive oxygen and nitrogen species (Virgili et al 1998). It has additionally been demonstrated that a hexane extract of *Plantago major* (ursolic acid) inhibits COX-2; this would thus explain the anti-inflammatory effects of *Plantago major* extracts (Ringbom et al 1998) and is in accordance with previous results indicating that *Plantago lanceolata* (probably phenylethanoid components thereof) inhibits arachidonic-acid-induced mouse ear oedema (Murai et al 1995). In this study, to further characterize the anti-inflammatory properties of *Pinus sylvestris* and *Plantago lanceolata* extracts, we investigated their effects at different doses on cell viability, NO production, scavenging activity, iNOS mRNA expression, COX-1 and -2 mRNA expression and PGE_2 levels in the murine macrophage line J774A.1.

Materials and Methods

Materials

The *Pinus sylvestris* extract and the *Plantago lanceolata* extract were obtained from Bioserum Laboratorios S.L. (Malaga, Spain). The *Pinus sylvestris* extract was prepared as follows. Leaf buds were subjected to steam distillation for extraction of essential oils, which were set aside. The same leaf buds were then percolated in ethanol–water (45:55) at 40°C for 10 h, then concentrated in a vacuum concentrator. The essential oils and the percolation concentrate were pooled to give the final extract, to a total essential oils concentration of 1% w/w pinene equivalent and a total flavonoids concentration of 0.3% w/w rutin equivalent. Total essential oils were determined by gas/liquid chromatography (GLC) (Shimazu model GC-14A; 30-cm SP-5 column; detection temp 200°C, injection temp. 200°C; column temp. first 50°C, then linear gradient of 5°C min^{-1} to 110°C; nitrogen carrier 2 kg cm^{-2}; injection volume 3 mL; FID detection). Total flavonoids were determined by reverse-phase high-performance liquid chromatography (HPLC) (Hewlett Packard model HP1050; 150 × 3.20 mm Prodigy 5 mm ODS(3) 100A column; mobile phase methanol–phosphate buffer 5 mM, pH 7 (40:60, v/v); injection volume 2 mL; flow rate 1 mL min^{-1}; detection at 365 nm). Finally, the extract was dried by atomization.

The *Plantago lanceolata* extract was prepared as follows. Whole plants (leaves, flowers and roots) were percolated in ethanol–water (45:55) at 40°C for 10 h, then concentrated in a vacuum concentrator to give the final extract. Tannin concentration in the extract, determined spectrophotometrically at 715 nm in a Beckman DU-40 apparatus, was standardized to 2% w/w. Again, the extract was dried by atomization.

For experiments, the powdered extracts of *Pinus sylvestris* and *Plantago lanceolata* were dissolved in ethanol–water (1:24). Both extracts were used at a stock concentration of 1.68 μg mL^{-1}. For control experiments only the ethanol–water vehicle was used.

The J774.1 cell line was obtained from the European Collection of Cell Cultures (Salisbury, UK). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Gibco-BRL (NY). LPS (*E. coli* 0111:B4), interferon-γ (IFN-γ), dexamethasone, indometacin and PAPA-NONOate were obtained from Sigma (St Louis, MO). 3-(4,5 Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 5 mg mL^{-1}) was purchased from Roche Diagnostics Corporation (Indianapolis, USA). All reagents were of analytical grade. Absorbance was measured using a microplate reader (BioRad 550; Bio-Rad Laboratories, Hercules, CA).

Cell culture and stimulation of macrophages with LPS and IFN-γ

The J774A.1 cell line was maintained in DMEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 130 μg mL^{-1} sodium pyruvate, at 37°C under 5% CO_2 humidified air. Cells were harvested by gentle scraping and passaged every 3–6 days by 1:6 dilution. For stimulation with LPS and IFN-γ, cells were seeded into 24-well plates at a density of 1×10^5 cells/well and allowed to adhere for 12 h at 37°C under 5% CO_2 atmosphere. Culture medium was then replaced with fresh medium without FBS for 5 h, then replaced again with fresh medium containing 10% FBS, 1 μg mL^{-1} LPS and 15 ng mL^{-1} IFN-γ in phosphate-buffered saline (PBS). Doses of LPS

and IFN-γ were chosen on the basis of preliminary findings indicating that they give optimal induction of inducible nitric oxide synthesis in J774A.1 cells. To evaluate the effects of the extracts, cells were first incubated for 5 h with the *Pinus sylvestris* or *Plantago lanceolata* extract (8.5, 16.8, 50.4 or 84 μg mL^{-1}), then for 24 h with LPS plus IFN-γ as above. As reference controls, assays were also performed with the anti-inflammatory steroid dexamethasone (0.01, 0.1 or 1 μM) and the non-steroidal anti-inflammatory drug indometacin (0.01, 0.1 or 0.25 mM).

Measurement of nitrite production

As an indicator of NO production, we determined the nitrite concentration in the culture medium by the Griess reaction (Dirsch et al 1998). One hundred microlitres of each culture supernatant, assayed in triplicate, was reacted with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine HCl in 2.5% phosphoric acid) at room temperature for 10 min. Absorbance was then measured at 540 nm, and nitrite concentration determined using sodium nitrite as standard.

MTT assay for cell viability

Cell viability was assessed by the mitochondrial-respiration-dependent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method. Cells (1×10^4 cells/well) in 96-well plates were incubated with increasing doses of test compound (8.5, 16.8, 50.4 or 84 μg mL^{-1} of *Pinus sylvestris* or *Plantago lanceolata* extract; 0.01, 0.1 or 1 μM of dexamethasone; or 0.01, 0.1 or 0.25 mM of indometacin) at 37°C in 5% CO_2 for 24 h. After treatment, 10 μL of MTT solution was added to each well. After incubation for 4 h at 37°C, the formazan crystals in viable cells were solubilized with 100 μL of lysis buffer (10% sodium dodecyl sulfate in 0.01 M HCl) for 12 h. The absorbance of each well was then read at 540 nm. The optical density of formazan formed in control (untreated) cells was taken as 100% viability.

Assay of scavenging of NO radicals released by PAPA-NONOate

The plant extracts (8.5, 16.8, 50.4 or 84 μg mL^{-1} of *Pinus sylvestris* or *Plantago lanceolata* extract) were dissolved in PBS to a total volume of 250 μL and incubated with 250 μL of PAPA-NONOate dissolved in PBS (pH 7.68, 20 μM) at 37°C for 3 h. After incubation, the concentration of nitrite was measured by the Griess method as described above.

Assay of iNOS, COX-1 and COX-2 mRNA expression: isolation and reverse transcription-polymerase chain reaction (RT-PCR) amplification

The J774A.1 macrophage cell line (1×10^6 cells) was grown in a 90-mm petri dish as described above. The cells were treated with the plant extracts (84 μg mL^{-1} for *Pinus sylvestris* L. and *Plantago lanceolata* L.) and 5 h later LPS plus IFN-γ (1 μg mL^{-1} and 15 ng mL^{-1}, respectively) was added. Total RNA was isolated with TRIzol reagent (Invitrogen, Life Technologies, UK) as previously described (Gil-Puig et al 2002). RNA concentration and purity were determined by spectrophotometry. cDNA synthesis and PCR amplification of GADPH and iNOS were performed as previously described (Vigo et al 2004). For PCR amplification of COX-1 and COX-2, samples were denatured at 94°C for 1 min, annealed at 55°C for 1 min and extended at 72°C for 1 min, for 28 cycles, with an extension step of 10 min at 72°C in the last cycle.

To determine the relative amounts of iNOS, COX-1 and COX-2 mRNAs in each sample, iNOS, COX-1 and COX-2 mRNA amounts were standardized with respect to GADPH mRNA amounts. Specifically, PCR products were separated on 2% agarose gel, stained with ethidium bromide, visualized with UV light and quantified using the Gel Doc 1000 Documentation System (Bio-Rad Laboratories, CA).

Primer sequences for PCR amplification of iNOS were as follows: primer A (5′-GCCTCCCTCTGGAAAGA-3′) was a 17-mer corresponding to residues 1213–1230 of the rat coding sequence, and primer B (5′-TCCATGCAGA CAACCTT-3′) was an antisense 17-mer corresponding to residues 1696–1712 of the coding sequence. The PCR product obtained was 499 bp in length. Primer sequences for COX-1 were as follows: the forward primer 5′-CGGTGCGGTCCAACCTTATCC-3′, corresponding to residues 411–431 of the rat coding sequence, and the reverse primer 5′-CCGCAGGTGATACTGTCGTT-3′, corresponding to residues 774–793 of the coding sequence. The PCR product obtained was 382 bp in length. Primer sequences for COX-2 were as follows: the forward primer 5′-GGGAAGCCTTCTCCAACC-3′, corresponding to residues 498–515 of the rat coding sequence, and the reverse primer 5′-GAACCCAGGTCCTCGCTT-3′, corresponding to residues 725–742 of the coding sequence. The PCR product obtained was 245 bp in length. Primer sequences for rat GADPH were as follows: the forward primer 5′-TGAT GACATCAAG AAGGTGGTGAAG-3′, corresponding to residues 758–782 of the rat coding sequence, and the reverse primer 5′-TCCTTGGAGGCCATGTAGGCCAT-3′ corresponding to residues 974–997 of the coding sequence. The PCR product obtained was 309 bp in length.

PGE_2 immunoassay

The culture medium of control and treated cells was collected, centrifuged and stored at −70°C until tested. The level of PGE_2 released into culture medium was quantified using a specific enzyme immunoassay (EIA) according to the manufacturer's instructions (Amersham Biosciences, UK).

Statistical analysis

Each experiment was performed at least three times with at least 3 replicates: the minimum total number of replicates was 12 (3 experiments × 4 replicates). All values are expressed as means ± s.d. Means were compared by

one-way analysis of variance with the Dunnet's multiple comparison test for post-hoc comparisons. Statistical significance is taken to be indicated by $P < 0.05$.

Results

Effect of dexamethasone, indometacin, *Pinus sylvestris* extract and *Plantago lanceolata* extract on NO production by LPS/IFN-γ-stimulated J774.1 cells

Incubation of the cells with LPS plus IFN-γ resulted in an increase in NO concentration in culture medium ranging from 4.7 to 5.8 μM after 24 h incubation, versus no detectable NO in non-treated cultures. These values (controls) were considered as the maximal increase in NO, for calculation of the reduction in NO release by LPS/IFN-γ-stimulated cells following pre-treatment with *Pinus sylvestris* extract, *Plantago lanceolata* extract, dexamethasone or indometacin (Table 1).

Pre-treatment with dexamethasone at 0.1 μM or higher induced a significant reduction ($P < 0.001$) in NO production. From this concentration up, the observed effect was dose dependent (Table 1). Pre-treatment with indometacin induced a significant reduction ($P < 0.001$) in NO production at concentrations of 0.1 mM or higher (Table 1).

Table 1 Effects of *Pinus sylvestris* extract, *Plantago lanceolata* extract, dexamethasone and indometacin on NO production, cell viability and (plant extracts only) NO-scavenging activity

	Nitrite (μM)	Cell viability (absorbance)	NO scavenging (μM)
Pinus sylvestris L.			
Control	5.20 ± 0.38	0.430 ± 0.777	0.0120 ± 0.0077
8.5 μg mL^{-1}	2.30 ± 0.52***	0.320 ± 0.027***	0.0100 ± 0.0068
16 μg mL^{-1}	2.20 ± 0.68***	0.330 ± 0.033***	0.0090 ± 0.0061
50.4 μg mL^{-1}	1.60 ± 0.41***	0.360 ± 0.041*	0.0090 ± 0.0061
84 μg mL^{-1}	1.10 ± 0.64***	0.380 ± 0.041	0.0080 ± 0.0051*
Plantago lanceolata L.			
Control	4.70 ± 1.21	0.420 ± 0.064	0.0100 ± 0.0066
8.5 μg mL^{-1}	2.70 ± 0.70***	0.310 ± 0.059***	0.0100 ± 0.0062*
16 μg mL^{-1}	2.60 ± 0.83***	0.360 ± 0.058***	0.0090 ± 0.0061**
50.4 μg mL^{-1}	2.10 ± 0.64***	0.380 ± 0.065*	0.0090 ± 0.0058**
84 μg mL^{-1}	2.10 ± 0.90***	0.390 ± 0.064	0.0080 ± 0.0050**
Dexamethasone			
Control	5.80 ± 0.43	0.52 ± 0.24	
0.01 μM	5.20 ± 0.48	0.50 ± 0.22	
0.1 μM	4.70 ± 1.10***	0.49 ± 0.19*	
1 μM	2.90 ± 1.58***	0.47 ± 0.19***	
Indometacin			
Control	5.30 ± 0.56	0.65 ± 0.12	
0.01 mM	4.10 ± 0.18	0.600 ± 0.099	
0.1 mM	3.10 ± 0.64***	0.620 ± 0.046	
0.25 mM	1.90 ± 0.65***	0.580 ± 0.082*	

***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$ with respect to control.

Pre-treatment with the *Pinus sylvestris* or *Plantago lanceolata* extracts (8.5–84 μg mL^{-1}) in all cases significantly reduced NO production, and the effect of both extracts was dose dependent (Table 1).

Effect of dexamethasone, indometacin, *Pinus sylvestris* extract and *Plantago lanceolata* extract on cell viability

To rule out possible cytotoxic effects of the *Pinus sylvestris* and *Plantago lanceolata* extracts in the absence of LPS + IFN-γ, we used an MTT assay. Both the *Pinus sylvestris* and the *Plantago lanceolata* extracts significantly reduced cell viability at the low and intermediate concentrations tested (8.5, 16.8 and 50.4 μg mL^{-1}) (Table 1); however, no significant reduction was caused by the highest concentration tested (84 μg mL^{-1}). Dexamethasone significantly reduced cell viability at the higher concentrations tested (0.1 and 1 μM), while indometacin had no significant effect, except at the highest concentration tested (0.25 mM).

Scavenging of NO by the *Pinus sylvestris* and *Plantago lanceolata* extracts

As noted, pre-treatment of LPS/IFN-γ-stimulated cells with the *Pinus sylvestris* or *Plantago lanceolata* extracts led to a significant reduction in nitrite levels in the medium. To assess whether this reduction was due to NO scavenging, we performed assays of scavenging activity using PAPA-NONOate for NO generation. The *Plantago lanceolata* extract, at all doses tested, had significant NO-scavenging activity, whereas the *Pinus sylvestris* extract showed NO-scavenging activity only at the highest concentration tested (84 μg mL^{-1}) (Table 1).

Effect of the *Pinus sylvestris* and *Plantago lanceolata* extracts and dexamethasone on iNOS mRNA levels

To investigate whether *Pinus sylvestris* and *Plantago lanceolata* extracts affect iNOS gene expression, RT-PCR was carried out using specific primers for the iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. PCR amplification of cDNA from J774A.1 cells yielded a 499-bp product corresponding to mouse iNOS and a 309-bp product corresponding to mouse GAPDH (Figure 1C). Non-stimulated J774A.1 cells showed barely detectable iNOS mRNA levels (0.15 ± 0.08, relative iNOS/GAPDH value; Figure 1C, lane 3). Cells incubated for 24 h with LPS and IFN-γ showed markedly and significantly higher levels (0.80 ± 0.13, $P < 0.001$ with respect to non-stimulated cells). Pre-treatment of cells with the *Pinus sylvestris* or *Plantago lanceolata* extract (84 μg mL^{-1}) significantly reduced ($P < 0.05$) iNOS mRNA levels with respect to the cells treated with LPS and IFN-γ only (0.62 ± 0.07 and 0.7 ± 0.03, respectively; Figure 1C, lanes 5 and 6, and Figure 1D). Dexamethasone treatment likewise significantly reduced ($P < 0.05$) iNOS

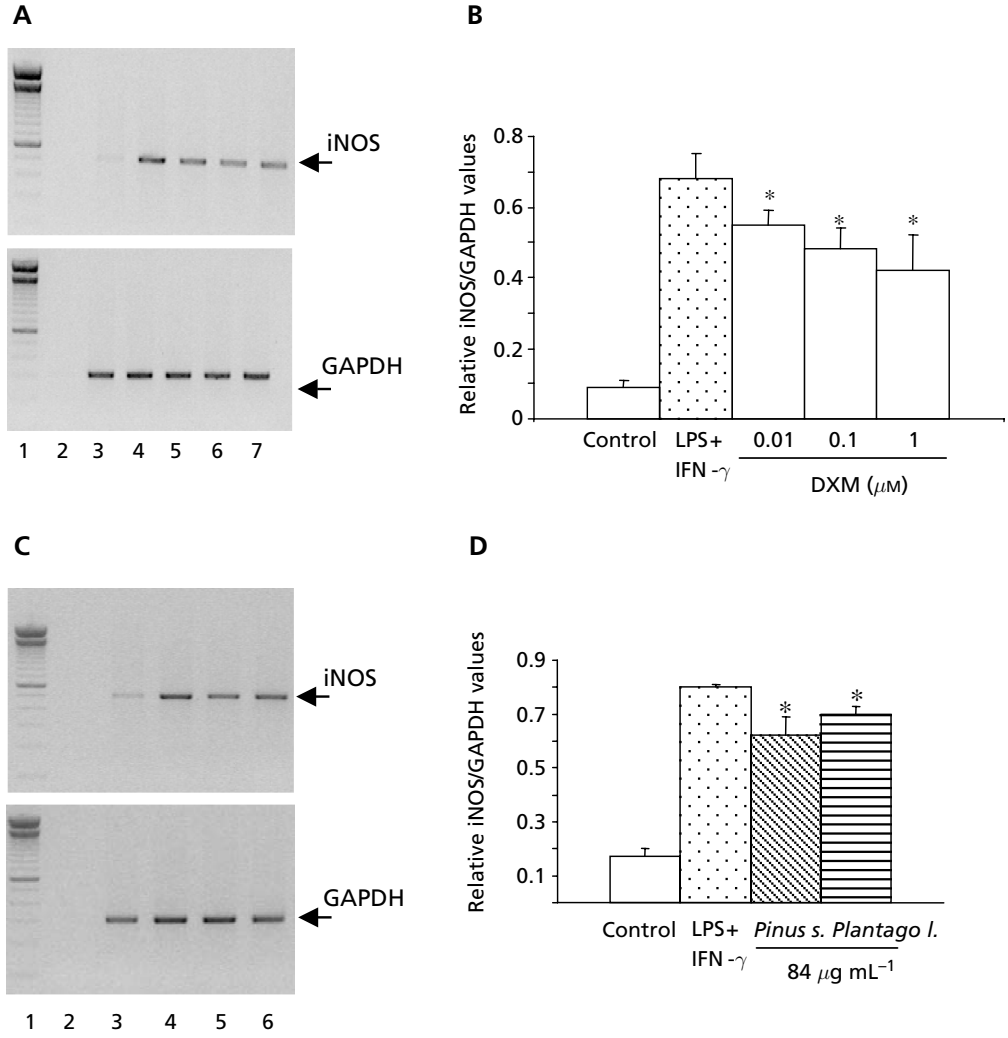

Figure 1 RT-PCR analysis of iNOS mRNA expression and GAPDH mRNA expression in activated J774A.1 macrophages following pre-treatment with *Pinus sylvestris* or *Plantago lanceolata* extract. A. Lane 1, molecular weight markers (1000-bp DNA ladder); lane 2, negative control of PCR; lane 3, non-stimulated cells; lane 4, LPS/IFN-γ-stimulated cells; lanes 5–7, LPS/IFN-γ-stimulated cells pre-treated with 0.01, 0.1 or 1 μM of dexamethasone, respectively. B. Relative iNOS mRNA levels (iNOS/GADPH) in non-treated cells, LPS/IFN-γ-stimulated cells and dexamethasone (DXM)-pre-treated LPS/IFN-γ-stimulated cells. C. Lanes 1–4, as for A; lane 5, LPS/IFN-γ-stimulated cells pre-treated with 84 μg mL^{-1} of *Pinus sylvestris* extract; lane 6, LPS/IFN-γ-stimulated cells pre-treated with 84 μg mL^{-1} of *Plantago lanceolata* extract. D. Relative iNOS mRNA levels (iNOS/GADPH) in non-treated cells, LPS/IFN-γ-stimulated cells and *Pinus sylvestris*- or *Plantago lanceolata*-pretreated LPS/IFN-γ-stimulated cells (*$P < 0.05$ with respect to cells treated with LPS and IFN-γ only).

mRNA levels with respect to cells treated with LPS and IFN-γ only (Figure 1A, B).

Effect of the *Pinus sylvestris* and *Plantago lanceolata* extracts on COX-1 and COX-2 mRNA levels

To investigate whether *Pinus sylvestris* and *Plantago lanceolata* extracts affect COX-1 or COX-2 gene expression, RT-PCR was carried out using specific primers for the COX-1, COX-2 and GAPDH genes. PCR amplification of cDNA from J774A.1 cells yielded a 382-bp product corresponding to mouse COX-1, a 245-bp product corresponding to mouse COX-2 and a 309-bp product corresponding to mouse GAPDH (Figure 2A, C). Non-stimulated J774A.1 cells showed detectable COX-1 mRNA levels (0.55 ± 0.14, relative COX-1/GAPDH value; Figure 2A, lane 1). Pre-treatment of cells with the *Pinus sylvestris* or *Plantago lanceolata* extract (84 μg mL^{-1}) reduced COX-1 mRNA levels with respect to the control cells (0.4 ± 0.11 and 0.41 ± 0.08, respectively; Figure 1A, lanes 2 and 3, and Figure 1B), though these reductions were not statistically significant. Non-stimulated J774A.1 cells showed detectable COX-2 mRNA levels (0.48 ± 0.27, relative COX-2/GADPH value). Cells incubated for 24 h with LPS and IFN-γ showed a significant increase in COX-2 levels

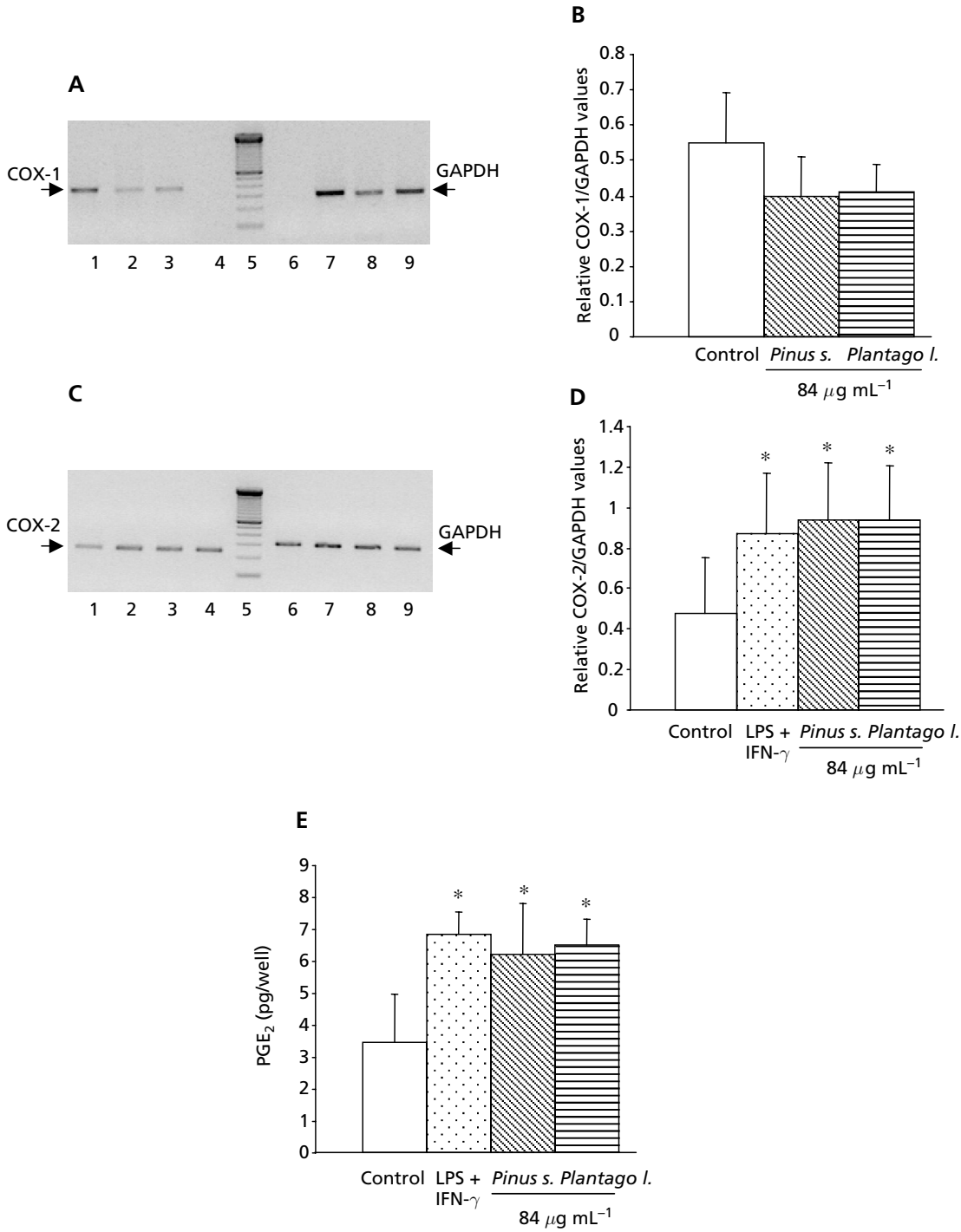

Figure 2 RT-PCR analysis of COX-1 and COX-2 mRNA levels and GAPDH mRNA levels in activated (COX-2) or non-activated (COX-1) J774A.1 macrophages following pre-treatment with *Pinus sylvestris* or *Plantago lanceolata* extract. A. COX-1 RT-PCR: Lane 1, non-stimulated cells; lane 2, cells treated with 84 μg mL^{-1} of *Pinus sylvestris* extract; lane 3, cells treated with 84 μg mL^{-1} of *Plantago lanceolata* extract; lane 4, negative control of PCR; lane 5, molecular weight markers (1000-bp DNA ladder). GAPDH RT-PCR: lanes 6, 7, 8 and 9, as for 4, 1, 2 and 3, respectively. B. Relative COX-1 mRNA levels (COX-1/GADPH) in non-treated cells (control) and cells pre-treated with *Pinus sylvestris* or *Plantago lanceolata* extract. C. COX-2 RT-PCR: lane 1, non-stimulated cells; lane 2, LPS/IFN-γ-stimulated cells; lanes 3 and 4, LPS/IFN-γ-stimulated cells pre-treated with 84 μg mL^{-1} of *Pinus sylvestris* or *Plantago lanceolata* extract. GAPDH RT-PCR: lanes 6, 7, 8 and 9, as for lanes 1, 2, 3 and 4. D. Relative COX-2 mRNA levels (COX-2/GADPH) in non-treated cells (control), in LPS/IFN-γ-stimulated cells and in LPS/IFN-γ-stimulated cells pre-treated with *Pinus sylvestris* or *Plantago lanceolata* extract. E. Effects of the *Pinus sylvestris* or *Plantago lanceolata* extract on LPS/IFN-γ-induced PGE_2 in J774A.1 macrophages (*$P < 0.05$ with respect to control cells).

(0.87 ± 0.3, $P < 0.05$, with respect to non-stimulated cells). Pre-treatment of LPS/IFN-γ-stimulated cells with the *Pinus sylvestris* or *Plantago lanceolata* extract ($84\,\mu g\,mL^{-1}$) significantly increased ($P < 0.05$) COX-2 mRNA levels with respect to the control cells (0.94 ± 0.28 and 0.94 ± 0.27, respectively; Figure 2C, lanes 3 and 4, and Figure 2D), but not with respect to LPS/IFN-γ-stimulated cells.

Effect of the *Pinus sylvestris* and *Plantago lanceolata* extracts on PGE_2 levels

Treatment of J774A.1 macrophages with LPS and IFN-γ caused a significant accumulation of PGE_2 (6.82 ± 0.72 pg/well), compared with the release by unstimulated cells (3.45 ± 1.57 pg/well) (Figure 2E). Pre-treatment of LPS/IFN-γ-stimulated cells with the *Pinus sylvestris* or *Plantago lanceolata* extract ($84\,\mu g\,mL^{-1}$) did not significantly affect PGE_2 production with respect to LPS/IFN-γ-stimulated cells (6.2 ± 1.65 pg/well and 6.5 ± 0.86 pg/well, respectively).

Discussion

In this work, it is demonstrated that the enhanced production of NO induced by LPS and interferon-γ in a murine macrophage cell line, J774A.1, is significantly and dose-dependently inhibited by the previous administration of *Pinus sylvestris* or *Plantago lanceolata* extract. In MTT assays of effects on cell viability, we rather surprisingly found that both the *Pinus sylvestris* and *Plantago lanceolata* extracts reduced cell viability at low and intermediate concentrations but not at higher concentrations; this was confirmed by replicate assays. Regardless of possible explanations for these apparent dual effects, these results indicate that the observed inhibition of NO production by the extracts cannot be attributed – at least at high concentrations – to cytotoxicity. Furthermore, both extracts (at least at high concentrations) showed scavenging of NO radicals released by the NO generator PAPA-NONOate. Our results also indicate that pre-treatment with *Pinus sylvestris* or *Plantago lanceolata* extracts inhibits iNOS mRNA expression without affecting COX-1 mRNA levels. In addition, neither the *Pinus sylvestris* nor the *Plantago lanceolata* extracts decreased COX-2 mRNA expression or PGE_2 levels in LPS/IFN-γ-stimulated macrophages.

The anti-inflammatory steroid drug dexamethasone and the non-steroidal anti-inflammatory drug indometacin, used as reference controls, significantly inhibited NO production by LPS/IFN-γ-stimulated cells, without affecting (at least at the low dose tested) cell viability. These results for dexamethasone and indometacin are in accordance with previously published data (Di Rosa et al 1990; Ogawa et al 2000).

It is well known that some chemical constituents of medicinal plants show biological activity affecting different aspects of the inflammation process. Many flavonoids and phenylethanoids are antioxidants (Rice-Evans et al 1996; Xiong et al 2000) and some of these compounds are also known to show free-radical scavenging activity (Kandaswami & Middleton 1994; Xiong et al 2000). In this study, the *Plantago lanceolata* extract showed NO scavenging activity at all the doses tested, whereas the *Pinus sylvestris* extract only showed NO scavenging activity at the highest concentration. This could be due to the fact that the scavenging activity of phenolic compounds requires a large number of phenol groups per molecule (Wang et al 1996; Xiong et al 1996). For example, *Plantago asiatica*, a member of the same genus family as *Plantago lanceolata*, does not show scavenging activity, but significantly inhibits both NO production and iNOS mRNA expression (Tezuka et al 2001). NO production by macrophages depends on iNOS, which can be activated by various agents, including interferons, tumour necrosis factor-α (TNF-α) and LPS (Moncada et al 1991). The onset of the NO production cascade induced by LPS or cytokines requires a number of steps, including the activation of nuclear factor NF-κB and subsequent iNOS mRNA expression. Some flavonoids decrease iNOS protein levels and activity and NO production by reducing the expression of iNOS mRNA, and this reduction may occur through prevention of the binding of NF-κB to the iNOS promoter, thereby inhibiting the induction of iNOS transcription (Lin & Lin 1997). Thus, we cannot rule out the possibility that the observed inhibition of transcription of the iNOS gene in LPS/IFN-γ-stimulated J774A.1 macrophages following pre-treatment with *Pinus sylvestris* or *Plantago lanceolata* extracts may be mediated by inhibition of, or interference with, NF-κB. Similar findings have been obtained for dexamethasone, which decreases the activity of the iNOS promoter and reduces the formation of cytokine-induced NF-κB complexes that bind to the NF-κB site in the human iNOS promoter (Kleinert et al 1996).

Macrophage activation by LPS leads to a functionally diverse series of responses, including the activation of phospholipase A_2 leading to the production of lipid metabolites of arachidonic acid (such as prostaglandins). COX is a rate-limiting enzyme in the conversion of arachidonic acid to PGH_2, the precursor of a large group of biologically active mediators, such as PGE_2, prostacyclin and thromboxane B_2 (TXB_2). COX-1 is constitutively expressed in many tissues and predominates, for example, in gastric mucosa. Inhibition of COX-1, which reduces the basal production of cytoprotective PGE_2 and PGI_2 in the stomach, may contribute to gastric ulceration. In this study, we did not observe a significant decrease in COX-1 mRNA levels in J774A.1 macrophages pre-treated with *Pinus sylvestris* or *Plantago lanceolata* extracts, suggesting that neither extract inhibits COX-1 expression. Pre-treatment with *Pinus sylvestris* or *Plantago lanceolata* extracts did not decrease the significantly increased COX-2 mRNA expression or PGE_2 levels observed in macrophages after LPS/IFN-γ challenge. It has been reported previously (Liang et al 1999) that different flavonoids affect iNOS and COX-2 expression in different ways: apigenin reduced both iNOS and COX-2 expression, whereas flavonoids, such as quercetin, reduced iNOS expression but enhanced COX-2 expression. These results suggest that the anti-inflammatory properties of *Pinus*

sylvestris and *Plantago lanceolata* extracts are related to an inhibition of NO via reduced iNOS mRNA production or via their NO scavenging activity, not via reduced COX-2 mRNA production. The fact that pre-treatment with these extracts did not decrease COX-1 mRNA levels suggests that these extracts will not cause gastric pathologies.

Conclusions

This study has demonstrated that pre-treatment with *Pinus sylvestris* or *Plantago lanceolata* extracts inhibits NO production and iNOS mRNA expression by LPS/IFN-γ-stimulated murine macrophages of the J774A.1 cell line. Pre-treatment with these extracts did not modify COX-1 mRNA production or decrease the high levels of COX-2 and PGE_2 induced by LPS/IFN-γ challenge, suggesting that the observed anti-inflammatory properties of *Pinus sylvestris* and *Plantago lanceolata* extracts may be related to the inhibition of NO, not to reduced prostaglandin production.

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