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In-vitro anti-inflammatory effect of *Eucalyptus globulus* and *Thymus vulgaris*: nitric oxide inhibition in J774A.1 murine macrophages

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Abstract

It is well known that nitric oxide (NO) plays an important role in the pathogenesis of inflammatory diseases. *Eucalyptus globulus* Labill. and *Thymus vulgaris* L. have been used in traditional medicine in the treatment of bronchitis, asthma and other respiratory diseases. The present study focuses on the effects of these two extracts on NO production induced by lipopolysaccharide (LPS) and interferon-γ (IFN-γ) in the murine macrophage cell line J774A.1. In addition, cell viability, scavenging activity and inducible nitric oxide synthase (iNOS) mRNA expression were evaluated. *E. globulus* and *T. vulgaris* extracts significantly inhibited the enhanced production of NO induced by LPS and IFN-γ in a dose-dependent manner. Treatment with these two extracts did not reduce cell viability at any dose used. Both plant extracts showed significant scavenging of NO radicals released by an NO donor, PAPA-NONOate. Results also show that pre-treatment with *E. globulus* and *T. vulgaris* extracts significantly inhibits iNOS mRNA expression. This study thus suggests that the inhibition of net NO production by these two extracts may be due to their NO scavenging activity and/or their inhibitory effects on iNOS gene expression.

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Introduction

Nitric oxide (NO) is synthesized from L-arginine in numerous mammalian cells and tissues. It has diverse physiological roles and may also contribute to pathological processes. The physiological actions of NO are implemented by NO at low concentrations (about 10^{-12} M) acting as a labile intracellular messenger molecule in, for example, the regulation of vascular tone or in neurotransmission. Physiological NO production is mediated by two enzymes (constitutive nitric oxide synthases, cNOS), which were originally detected in endothelial cells (eNOS) and in neurons (nNOS), and which are generally constitutive. However, when NO is synthesized in large quantities (10^{-9} M) by activated inflammatory cells, it has cytotoxic properties and may be involved in the pathogenesis of acute and chronic inflammatory conditions (Moilanen et al 1999). This NO production is mediated by an enzyme termed inducible nitric oxide synthase (iNOS), which is inducible by several stimuli, including bacterial lipopolysaccharide (LPS) and interferon-γ (IFN-γ). iNOS is present in macrophages and hepatocytes. During inflammation associated with different pathologies, NO production increases significantly (Kharitonov et al 1994) and may become cytotoxic. Moreover, the free radical nature of NO and its high reactivity with oxygen to produce peroxynitrite ($ONOO^-$) makes NO a potent pro-oxidant molecule able to induce oxidative damage and to be potentially harmful towards cellular targets (Epe et al 1996). Thus inhibition of NO production in response to inflammatory stimuli might be a useful therapeutic strategy in inflammatory diseases (Hobbs et al 1999; Sautebin 2000).

Eucalyptus globulus Labill. and *Thymus vulgaris* L. extracts have been used in traditional medicine in the treatment of bronchitis, asthma and other respiratory diseases (Alonso 1998). It has been demonstrated that the antibacterial properties of eucalyptus oil are effective not only against *Streptococcus* D isolated from bronchial aspirates, but also against pathogenic strains of *Proteus mirabilis*, *Klebsiella* sp., *Escherichia coli* and *Staphylococcus aureus* (Benouda et al 1988). In addition, eucalyptus oil increases

respiratory tract fluid in guinea pigs (Boyd & Pearson 1946). Thyme oil also has strong antibacterial action against Gram-positive and Gram-negative bacteria (Essawi & Srour 2000), and has been shown to have bronchospasmolytic properties (Van den Broucke & Lemli 1983). However, rather little is known about the anti-inflammatory properties of these extracts. Here, we report a study of the in-vitro effects of *E. globulus* and *T. vulgaris* extracts on cell viability, iNOS mRNA expression and net NO production by murine macrophages.

Materials and Methods

Materials

Powdered preparations of leaf buds from *E. globulus* and flowering tops of *T. vulgaris* (Bioserum Laboratorios, S.L., Zaragoza, Spain) were dissolved in ethanol:water (1:24). Both extracts were used at a stock concentration of 1.68 $\mu g\,\mu L^{-1}$. Essential oils (1% and 0.2% for *E. globulus* and *T. vulgaris*, respectively) and flavonoids (4% for *E. globulus*) were quantified by gas chromatography and HPLC. 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. Lipopolysaccharide (LPS, *E. coli* 0111:B4), IFN-γ, dexamethasone and PAPA-NONOate were obtained from Sigma, St Louis, MO. 3-(4,5-dimethylthiazo l-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 5 $mg\,mL^{-1}$) was purchased from Roche Diagnostics Corporation, Indianapolis, USA. All reagents used were 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 continuously in DMEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 $U\,mL^{-1}$ penicillin, 100 $mg\,mL^{-1}$ streptomycin, and 130 $mg\,mL^{-1}$ Na 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 of a suspension of the cells in fresh medium. For stimulation with LPS and IFN-γ, cells were seeded into 24-well plastic plates at 1×10^5 cells per well and allowed to adhere for 12 h at 37 °C under 5% CO_2. The medium was replaced with fresh medium without FBS for 5 h, then replaced again with fresh medium containing 10% FBS, 1 $\mu g\,mL^{-1}$ LPS and 15 $ng\,mL^{-1}$ IFN-γ. These doses of LPS and IFN-γ were chosen in view of preliminary findings indicating that they give maximal induction of iNOS in J774A.1 cells. To evaluate the effects of the extracts, cells were first incubated for 5 h with the *E. globulus* or *T. vulgaris* extracts (8.5, 16.8, 50.4 and 84 $\mu g\,mL^{-1}$), 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 and 1 μM).

Measurement of nitrite production

As an indicator of NO production, nitrite concentration in the culture medium was determined by the Griess reaction (Dirsch et al 1998). One hundred microlitres of each culture supernatant, assayed in triplicate, were reacted with an equal volume of the Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediami ne-HCl in 2.5% phosphoric acid) at room temperature for 10 min. Absorbance was then measured at 540 nm, and nitrite concentration was 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 per well) in 96-well plates were incubated with increasing doses of test compound (8.5, 16.8, 50.4 and 84 $\mu g\,mL^{-1}$ of *E. globulus* or *T. vulgaris* extract or 0.01, 0.1 and 1 μM of dexamethasone) 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% SDS 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 test extract (8.5, 16.8, 50.4 and 84 $\mu g\,mL^{-1}$) was 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 mRNA expression: isolation and 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 $\mu g\,mL^{-1}$ for both *E. globulus* and *T. vulgaris*) and 5 h later LPS plus IFN-γ (1 $\mu g\,mL^{-1}$ and 15 $ng\,mL^{-1}$ respectively) was administered. Total RNA was isolated with TRIzol reagent (GibcoBRL, Life Technologies, Grand Island, NY) as previously described (Gil-Puig et al 2002). RNA concentration and purity were determined by spectrophotometry. cDNA synthesis was as follows: 2 μg of total RNA was incubated for 50 min at 37 °C, 15 min at 42 °C and 5 min at 95 °C with 400 units M-MLV reverse transcriptase (GibcoBRL) in buffer (50 mM Tris-HCl, pH 8.3; 50 mM KCl, 10 mM $MgCl_2$; 10 mM DTT and 0.5 mM spermidine) with 2 mM of each deoxynucleotide triphosphate, 20 units RNAse inhibitor (RNasin Rnase inhibitor, Promega, Madison, WI) and 500 ng of random primers (Promega) in a total volume of 40 μL.

Five microlitres of the cDNA generated under these conditions were amplified by PCR using 2 units of Taq polymerase (Promega) in a buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% triton X-100 and 1.5 mM $MgCl_2$, with 0.2 mM of each deoxynucleotide triphosphate and 25 pmol of each of the two oligonucleotide primers (for iNOS or for rat GAPDH as control), to a total volume of 50 μL. The sample was denatured at 94 °C for 1 min, annealed at 60 °C for 1 min and extended at 72 °C for 1 min, for 33 cycles, with an extension step of 10 min at 72 °C in the last cycle. To ensure that PCR was performed in the linear amplification range, samples were taken after 15, 20, 25, 30, 35 and 40 cycles. The reaction was linear over this range for both iNOS ($r = 0.92$) and GAPDH ($r = 0.81$).

To determine the relative amounts of iNOS mRNA in each sample, iNOS mRNA amounts were standardized with respect to GADPH mRNA amounts. Thus, PCR products were separated on 2% agarose gel, stained with ethidium bromide, examined 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′-TCCATGCAGACAACCTT-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 rat GAPDH were 5′-TGATGACATCAAGAAGGTGGTGAAG-3′ (forward), corresponding to residues 758–782 of the rat coding sequence, and 5′-TCCTTGGAGGCCATGTAGGCCAT-3′ (reverse), corresponding to residues 974–997 of the coding sequence. The PCR product obtained was 309 bp in length.

Statistical analysis

Each experiment was performed at least three times. All values are expressed as means ± s.d. Means were compared by one-way analysis of variance with the Tukey–Kramer multiple comparison test for post-hoc comparisons. Statistical significance was indicated by $P < 0.05$.

Results

Effects of dexamethasone, *E. globulus* extract and *T. vulgaris* extract on NO production by LPS/IFN-γ stimulated J774.1 cells

Incubation of the cells with LPS plus IFN-γ resulted in a maximal increase in NO concentrations in culture medium (8.99 ± 1.9 μM) after 24 h incubation vs no detectable nitrite in untreated cultures. This value (8.99 μM) was considered to be 100% nitrite production for the calculation of the percentage decrease in NO production in the presence of *E. globulus* extract, *T. vulgaris* extract or dexamethasone.

Pre-treatment with dexamethasone (5 h before LPS plus IFN-γ bolus) was associated with a significant reduction of NO production, starting from a dexamethasone concentration of 0.01 μM ($P < 0.05$) in the medium. From this concentration, the observed effect was dose-dependent and reached approximately 47% of the positive control value at a concentration of 1 μM ($P < 0.001$) (Table 1).

E. globulus extract (at doses of 8.5, 16.8, 50.4 and 84 μg mL^{-1}) or *T. vulgaris* extract (at the same doses) were administered 5 h before LPS plus IFN-γ stimulation, and nitrite concentration was measured 24 h later. In all cases (Table 1 and Figure 1), a significant

Table 1 Effects of *E. globulus* extract, *T. vulgaris* extract and dexamethasone on NO production, cell viability and plant extract only scavenging of PAPA NONOate-released NO radicals.

	Nitrite (% of control)	Cell viability (% of control)	NO scavenging (% of control)
Control	100	100	100
E. globulus Labill. (μg mL^{-1})			
8.5	66.7 ± 11.3***	119.8 ± 19.7	93.1 ± 3.3
16	62.7 ± 9.6***	132.1 ± 22.5	84.7 ± 6.5**
50.4	54.2 ± 12.4***	136.5 ± 21.6	69.3 ± 7.5***
84	49.1 ± 13.7***	154.5 ± 31.4**	56.7 ± 5.1***
T. vulgaris L. (μg mL^{-1})			
8.5	71.4 ± 7***	129.8 ± 20.9	88.3 ± 7.4
16	67.2 ± 3.9***	128.9 ± 20.3	80.3 ± 15.9*
50.4	50.4 ± 8***	137.3 ± 28.9	74.5 ± 2.1**
84	52.6 ± 9.8***	145 ± 37.6	59.8 ± 9.9***
Dexamethasone (μM)			
0.01	84.4 ± 5.2*	93.3 ± 6.7	
0.1	79.3 ± 7.9***	88.8 ± 6.8	
1	47.7 ± 13.3***	86.5 ± 7.8*	

In all assays J774A.1 macrophages were incubated with the test compound and 5 h later stimulated with LPS plus IFN-γ in 24-well culture plates for 24 h. All results are expressed as mean percentage inhibition with respect to the control ± s.d. (***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$).

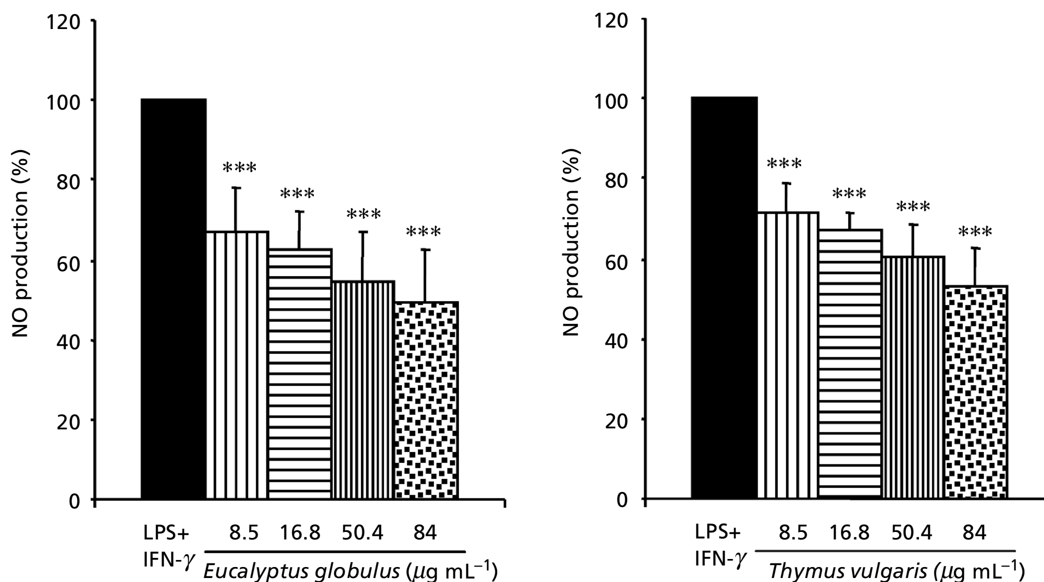

Figure 1 Effects of pretreatment with different doses of *E. globulus* or *T. vulgaris* extract on NO production by LPS/IFN-γ-stimulated macrophages. ***$P < 0.001$ vs LPS plus IFN-γ. Results shown are means $\pm$ s.d. of the value for cells treated with LPS + IFN-γ only, from four to six independent experiments each performed in triplicate.

($P < 0.001$) dose-dependent inhibition of nitrite production was observed.

Effects of dexamethasone, *E. globulus* extract and *T. vulgaris* extract on cell viability

To evaluate the possible cytotoxic activity of *E. globulus* and *T. vulgaris* in the absence of LPS + IFN-γ, an MTT assay was used. Neither *E. globulus* nor *T. vulgaris* had significant effects on cell viability at low to intermediate concentrations (8.5, 16.8 and 50.4 μg). The highest concentration of *E. globulus* (84 $\mu g\,mL^{-1}$) significantly increased cell viability (154.5 $\pm$ 31.4% vs 100% control; $P < 0.01$) (Table 1). Dexamethasone showed significant ($P < 0.05$) cytotoxic activity at 1 μM (Table 1).

Scavenging of NO by the *E. globulus* and *T. vulgaris* extracts

The NO-radical-scavenging activity of the *E. globulus* and *T. vulgaris* extracts was evaluated in experiments using the NO donor PAPA-NONOate. Both extracts showed significant NO-scavenging activity (Table 1 and Figure 2).

Effects of *E. globulus* extract and *T. vulgaris* extract on iNOS mRNA levels

To investigate whether *E. globulus* and *T. vulgaris* extracts affect iNOS gene expression, RT-PCR was carried out using specific primers for iNOS and GAPDH gene. PCR amplification of cDNA prepared from J774A.1 cells gave rise to a 499-bp product corresponding to mouse iNOS and a 309-bp product corresponding to mouse GAPDH (Figure 3). Non-stimulated J774A.1 macrophages had barely detectable iNOS mRNA (0.14 $\pm$ 0.05, relative iNOS/GAPDH value; Figure 3). In contrast, 24-h incubation with LPS plus IFN-γ induced a significant ($P < 0.001$) increase (0.86 $\pm$ 0.06) in iNOS mRNA expression vs control macrophages. Treatment of cells with *E. globulus* or *T. vulgaris* extracts significantly reduced iNOS mRNA expression with respect to the LPS plus IFN-γ treated cells (*E. globulus* 0.67 $\pm$ 0.03, $P < 0.01$; *T. vulgaris* 0.56 $\pm$ 0.05, $P < 0.001$) (Figure 3).

Discussion

In this paper it is demonstrated that *E. globulus* and *T. vulgaris* extracts significantly inhibit the production of NO induced by LPS and IFN-γ in a murine macrophage cell line, J774A.1. The LPS-treated J774A.1 macrophage model is widely used in studies of mechanisms of iNOS induction (Szabo et al 1993; Swierkosz et al 1995). Activation of J774A.1 macrophages with LPS plus IFN-γ resulted in accumulation of nitrite in culture medium, reflecting increased NO production due to induction of iNOS. Treatment with *E. globulus* and *T. vulgaris* extracts did not reduce cell viability (as assessed by the MTT assay) at any dose used; on the contrary, the *E. globulus* extract at the highest dose increased cell viability. Thus, the inhibitory effect of *E. globulus* and *T. vulgaris* on NO production seems not to be due to cell death. Both extracts showed significant scavenging of NO radicals released by an NO donor, PAPA-NONOate, at intermediate-high extract doses. Results also showed that pre-treatment with *E. globulus* and *T. vulgaris* extracts significantly

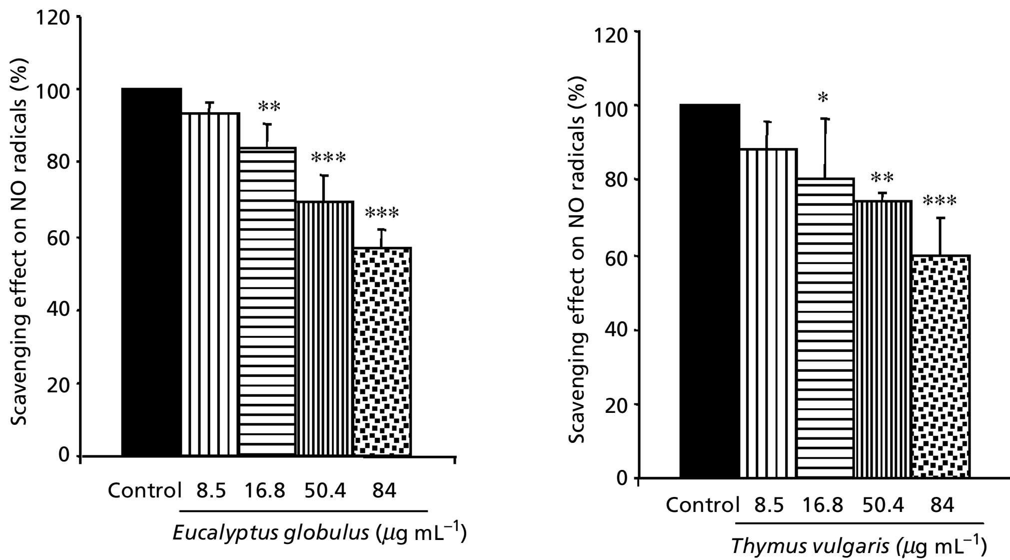

Figure 2 Scavenging of NO by different doses of *E. globulus* or *T. vulgaris* extract. Values are expressed as means ± s.d. vs control (100%). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

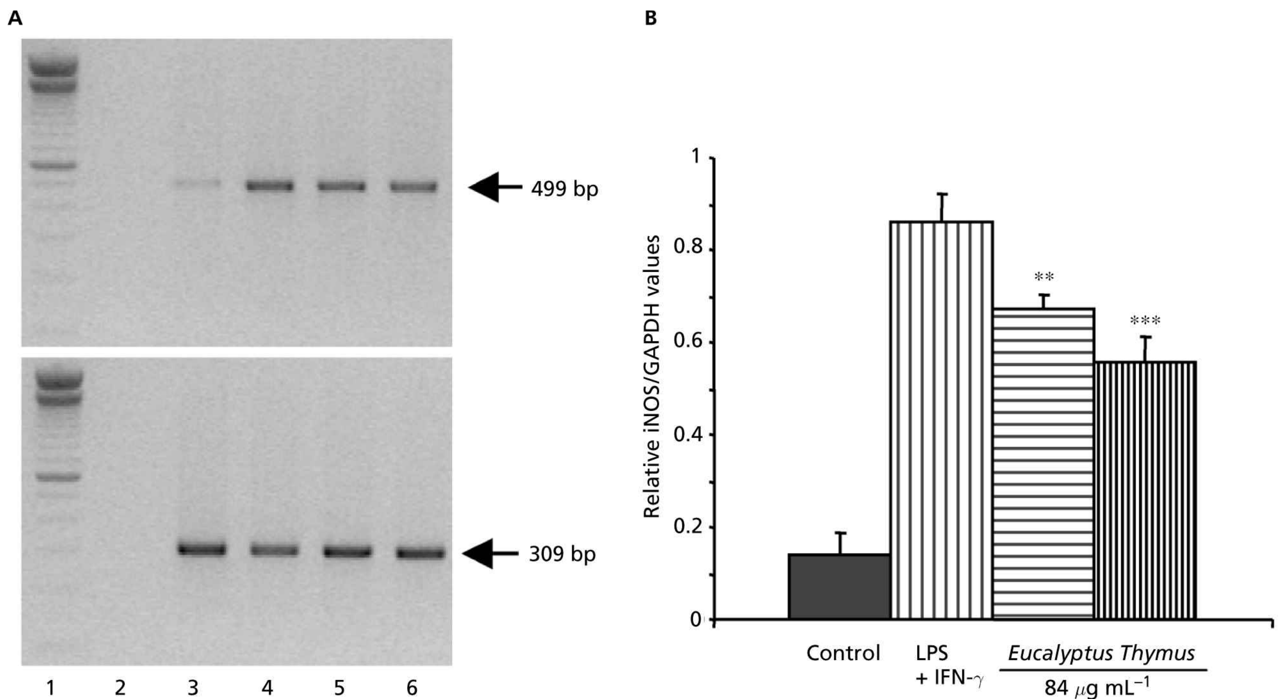

Figure 3 RT-PCR analysis of iNOS and GAPDH mRNA expression in activated J774A.1 macrophages following pretreatment with *E. globulus* or *T. vulgaris* extract. A: upper panel RT-PCR of iNOS mRNA; lower panel RT-PCR of GAPDH mRNA. Lane 1: molecular weight markers (100-bp DNA ladder). Lane 2: negative control of PCR. Lane 3: non-stimulated cells. Lane 4: LPS/IFN-γ-stimulated cells. Lane 5: LPS/IFN-γ-stimulated cells pretreated with 84 μg mL^{-1} of *E. globulus* extract. Lane 6: LPS/IFN-γ-stimulated cells pretreated with 84 μg mL^{-1} of *T. vulgaris* extract. B: relative iNOS mRNA levels (iNOS/GADPH) in non-treated cells, LPS/IFN-γ-stimulated cells, and cells pretreated with *E. globulus* or *T. vulgaris* extract before stimulation with LPS/IFN-γ cells ($**P < 0.01$ and $***P < 0.001$ with respect to cells treated with LPS and IFN-γ only).

inhibited iNOS mRNA expression. The reference control, the steroid anti-inflammatory dexamethasone, significantly inhibited production of NO induced by LPS plus IFN-γ, again without affecting cell viability, at least at low and intermediate doses. These results with dexamethasone are in agreement with previously published data (Di Rosa et al 1990). GADPH mRNA levels remained unchanged, thereby excluding non-specific inhibitory actions of the *E. globulus* and *T. vulgaris* extracts on gene expression.

The onset of the NO production cascade induced by LPS or cytokines in macrophages requires a number of steps, including the activation of nuclear factor (NF)-κB and subsequent iNOS mRNA expression (Moncada et al 1991). Although the cellular mechanisms of suppression of iNOS induction by flavonoids are not clearly understood, it has been demonstrated that some flavonoids decrease iNOS induction and thus inhibit NO production (Cheon et al 2000; Xiong et al 2000). Lin & Lin (1997) have also demonstrated that some polyphenols decrease the iNOS levels and activity by reducing the expression of iNOS mRNA, possibly through prevention of binding of NF-κB to the iNOS promoter, thereby inhibiting the induction of iNOS transcription. The reduced expression of iNOS mRNA levels in IFN-γ/LPS-stimulated J774A.1 macrophages after pre-treatment with *E. globulus* and *T. vulgaris* extracts (both of which contain polyphenols) could also be due to inhibition of transcription of the iNOS gene mediated by inhibition and/or interference with NF-κB. A similar finding has been reported for dexamethasone, which decreased the activity of the iNOS promoter and reduced the formation of cytokine-induced NF-κB complexes that bind to the NF-κB site in the human iNOS promoter, suggesting that the dexamethasone-activated glucocorticoid receptor is likely to interact with the cytokine-activated NF-κB complex, thereby repressing the binding of this complex to the NF-κB response element in the 5′-flanking sequence of the iNOS gene (Kleinert et al 1996).

T. vulgaris and *E. globulus* also showed NO scavenging activity, which could be due to their phenolic components: phenolic components of *T. vulgaris* are known to possess antioxidant activity (Haraguchi et al 1996; Zheng & Wang 2001), protecting biological systems against oxidative stress. This is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa 1994).

In conclusion, these results demonstrate that *T. vulgaris* and *E. globulus* extracts show NO-scavenging activity, and that pretreatment with these extracts decreases iNOS mRNA levels and inhibits NO production in J774A.1 murine macrophages stimulated with LPS and IFN-γ. In accordance with these findings we can speculate that both extracts may modulate net NO production through scavenging activity and/or through inhibition of iNOS gene transcription by interference with some regulatory transcription factor, such as NF-κB. Although these hypotheses cannot be resolved on the basis of the current experimental results, all data obtained indicate that *T. vulgaris* and *E. globulus* extracts have anti-inflammatory properties without affecting cell viability, and therefore may possibly be useful in the treatment of inflammatory respiratory pathologies.

Conclusions

This study has demonstrated that pre-treatment of LPS/IFN-γ stimulated cells with *E. globulus* or *T. vulgaris* extracts leads to a significant reduction in nitric oxide levels in the medium. This effect is mediated (a) by inhibition of inducible nitric oxide synthase (iNOS) mRNA expression and/or (b) by NO scavenging. Neither extract affects cell viability. Thus, these findings indicate that *E. globulus* and *T. vulgaris* extracts may be useful anti-inflammatory drugs in some respiratory pathologies, in line with their traditional use.

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