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In-vivo and in-vitro anti-inflammatory effect of *Echinacea purpurea* and *Hypericum perforatum*

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Abstract

Echinacea purpurea (L.) Moench and *Hypericum perforatum* (L.) were evaluated for their anti-inflammatory activity against carrageenan-induced paw oedema in mice. Each drug was administered orally to mice at 30 and 100 mg kg^{-1}, twice daily. Only the higher dose significantly inhibited, time dependently, the formation of oedema, evaluated as area under the curve (echinacea $P < 0.01$; hypericum $P < 0.05$). Western blot analysis showed that in-vivo treatment with these extracts could modulate lipopolysaccharide (LPS) and interferon-γ induced cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression in peritoneal macrophages. In particular, treatment with 100 mg kg^{-1} hypericum inhibited both iNOS and COX-2 expression, whereas treatment with 100 mg kg^{-1} echinacea down-regulated only COX-2 expression. The present study suggests that the anti-inflammatory effect of these extracts could be in part related to their modulation of COX-2 expression.

Introduction

Natural products represent a source of chemical structures of varying pharmacological interest. *Echinacea purpurea*, one of the three species of Echinacea (*E. purpurea*, *Echinacea angustifolia* and *Echinacea pallida*), is used in phytotherapy for the treatment of the common cold, coughs, bronchitis, and inflammation of the mouth and pharynx (Bauer 1998; Percival 2000; Schulten et al 2001). *E. purpurea* extracts or its purified polysaccharides, used in-vitro or in-vivo, have been shown to activate human and murine phagocytes. These effects include an increase in phagocytosis, chemotaxis, oxidative burst and macrophage cytokine release (Stimpel et al 1984; Wagner et al 1988; Gaisbauer et al 1990). Studies in the 1980s showed an anti-inflammatory effect after topical application of the polysaccharide fraction derived from *E. angustifolia* root (Tubaro et al 1987; Tragni et al 1988). Later, other in-vitro studies demonstrated that the polyunsaturated alkamides from *E. angustifolia* inhibited microsomal cyclo-oxygenase and leukocyte 5-lipoxygenase activity, suggesting an anti-inflammatory effect (Muller-Jakic et al 1994). More recently, Sloley et al (2000) confirmed the antioxidant and the free-radical scavenging activities of Echinacea species. However, these studies did not clarify whether the extract could be effective when taken orally. Many phytochemicals are poorly absorbed when taken orally and thus may have poor bioavailability.

Hypericum perforatum, commonly called St John's wort, is an herbaceous perennial plant belonging to the family Clusiaceae, which is used in popular medicine and phytotherapy for its well documented antiseptic and antidepressant effects (Schwarz & Cupp 2000; Di Carlo et al 2001). Moreover, it has been proposed to have antibacterial and antiviral effects and to exert anti-inflammatory and analgesic activity (Barnes et al 2001; Kumar et al 2001), the former at least partially mediated via inhibition of the transcription factor NF-kB (Bork et al 1999), and to interfere with some serine/threonine kinases of the PKC family (Agostinis et al 1995). Many of the effects of

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hypericum are attributed to hypericin, one of the major active constituents of this plant. In particular, hypericin and pseudohypericin have been reported to inhibit 12-lipoxygenase activity, contributing to the anti-inflammatory effect of hypericum (Bezakova et al 1999).

Macrophages play an important role in the immune and inflammatory response. It is well known that in macrophages and in other cell types, cytokines, such as interferon-γ (IFN-γ), and lipopolysaccharide (LPS) induce the co-expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Di Rosa et al 1996). These isoenzymes are responsible for the production of large amounts of proinflammatory mediators, nitric oxide and prostaglandins, at the inflammatory site (Lee et al 1992; Nussler & Billiar 1993).

In the present study, the anti-inflammatory effect of hypericum and echinacea was evaluated in-vivo against carrageenan-induced paw oedema in mice. We also demonstrated, by Western blot analysis, that in-vivo treatment with these extracts inhibits iNOS and COX-2 enzyme expression in stimulated peritoneal macrophages.

Materials and Methods

Plant material

E. purpurea root dry powder and *H. perforatum* flowering top dried extracts were kindly provided by Arkopharma (Carros, France). Hypericum contained 0.27% of anthraquinone derivatives, calculated as hypericin, and 2.5% of hyperforin; the extract was not characterized for flavonoids. Echinacea contained 1.5% of total polyphenols, calculated as chlorogenic acid. Both drugs were suspended in 1% arabic gum.

Materials

Fetal bovine serum (FBS), tissue culture media, and supplements were purchased from Hy Clone (UK). *Escherichia coli* LPS (serotype 0111:B4) was purchased from Fluka (Milan, Italy). iNOS and COX-2 were detected by Western blot with monoclonal antibodies from Transduction Laboratories (Lexington, KY, USA). The peroxidase conjugated secondary antibody was purchased from Jackson (West Grove, PA, USA). Type-IV lambda carrageenan, antibody against β-actin, bovine serum albumin (BSA) and Bio-Rad protein assay were purchased from Sigma (Milan, Italy).

Animals

Male BALB/c mice, 25–30 g (Harlan, Italy), were housed under controlled temperature conditions ($22 \pm 1^{\circ}C$), with a fixed 12-h light–dark cycle, and received food and water *ad libitum* for the whole period of experimental manipulations. All animal experiments complied with the Italian D.L. no.116 (27 January 1992) and associated guidelines in the European Communities Council Directive (24 November 1986) (86/609/ECC).

Carrageenan-induced paw oedema in mice

Groups of at least eight mice per group received subplantar injections of 50 μL of a 1% w/v carrageenan solution into one hind paw (Calhoun et al 1987; Henriques et al 1987). Paw volume was measured using a water plethysmometer, specially modified for small volumes (Ugo Basile, Milan, Italy), immediately before injection and at set intervals (2, 24, 48 and 72 h) thereafter.

The increase in paw volume was evaluated as the difference between the paw volume at each time point and the basal paw volume. Mice were treated by gavage with echinacea or hypericum (30 and 100 mg kg^{-1}, twice daily) administered 3 days before induction of oedema and up to 72 h after. The control group was treated with vehicle (1% arabic gum, 0.1 mL/10 g bodyweight) at the same time points. A group of animals received indometacin (0.25 mg kg^{-1}, s.c.) as a reference drug, 1 h before the carrageenan injection and every 12 h thereafter.

Haematological parameters

After the paw oedema experiment, seven mice from each group were anaesthetised with enflurane for blood collection by cardiac puncture. Blood (9 vols) was mixed with 3.8% w/v trisodium citrate (1 vol) solution and a sample was used to evaluate leukocyte, platelet and red cell counts, and haematocrit and haemoglobin concentration using a cell counter (Cell Dyn 610; Sequoia-Turner, USA).

Peritoneal macrophage culture and Western blot analysis

In another set of experiments, mice were divided into groups of eight and treated by gavage with echinacea (100 mg kg^{-1}), hypericum (100 mg kg^{-1}) or vehicle twice daily. After 14 days (chosen on the basis of preliminary experiments), control and drug-treated mice were killed and pools of peritoneal macrophages, obtained from two mice in each group, were collected.

The macrophages (2×10^6/dish, cell viability $>95\%$) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 2 mM L-glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 130 μg mL^{-1} Na pyruvate, and 25 mM HEPES at 37°C under 5% CO_2 humidified air. After 3 h, the non-adherent cells were removed and the medium was replaced by complete DMEM with 5% FBS. After 2 h, cells were stimulated with 1 μg mL^{-1} LPS plus 100 U mL^{-1} IFN-γ for 24 h. Cells were then washed twice with ice-cold phosphate-buffered saline (PBS), scraped off, harvested and resuspended in Tris-HCl (20 mM, pH 7.5), 10 mM NaF, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, leupeptin and trypsin inhibitor (10 μg mL^{-1}).

After 1 h, cell lysates were obtained by centrifugation at 100 000 *g* for 15 min at 4°C. Protein concentrations were estimated by the Bio-Rad protein assay using BSA as standard.

Equal amounts of the protein (20 μg) of the cell lysates were dissolved in Laemmli's sample buffer, boiled for 5 min, and then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (8% polyacrylamide). Western blotting was performed by transferring proteins from a slab gel to a sheet of polyvinylidene difluoride membrane at 240 mA for 40 min at room temperature. The filter was blocked with 1 × PBS, 5% non-fat dried milk for 40 min at room temperature and incubated with the primary antibody (iNOS, 1 : 10 000; COX-2, 1 : 500) in 1 × PBS, 5% non-fat dried milk, 0.1% Tween 20, overnight at 4°C. The filter was then incubated with a secondary antibody (anti-mouse IgG–horseradish peroxidase conjugate 1 : 10 000 dilution) for 1 h at room temperature. Subsequently, the blot was developed using enhanced chemiluminescence detection reagents (Amersham) according to the manufacturer's instructions, and exposed to Kodak X-Omat film. To confirm that the blot was loaded with equal amounts of protein lysates, it was also incubated in the presence of the antibody (1 : 10 000) against the β-actin protein. The protein bands of iNOS (~ 130 kDa) and COX-2 (70 kDa) on the X-ray film were scanned and densitometrically analysed with a model GS-700 imaging densitometer.

Data analysis

All data are expressed as mean ± s.d. of n animals in the in-vivo experiments. Statistical analysis was performed by analysis of variance for multiple comparisons followed by Dunnett's test. In-vitro experiments were performed three times and analysed using a non-parametric multiple comparisons test (Kruskal–Wallis test) followed by Dunn's test.

Statistical significance was set at $P < 0.05$.

Results and Discussion

The present experiments were conducted to determine the effects of hypericum and echinacea on an animal model of inflammation. Preliminary experiments showed that the 3-day drug treatment was able to induce an anti-inflammatory effect. Both echinacea and hypericum significantly ($P < 0.01$ and $P < 0.05$, respectively) inhibited the formation of oedema, evaluated as the area under the curve and expressed as surface units (mL oedema h^{-1}), only at 100 mg kg^{-1} (Figure 1). This effect was similar to that of indometacin (0.25 mg kg^{-1}).

In the carrageenan-induced mouse paw oedema, the initial change in paw oedema is unrelated to prostaglandin production, since it is not inhibited by indometacin, however the second phase of oedema is very sensitive to cyclooxygenase inhibitors (Sugishita et al 1981). In mice, the early phase consists of a low intensity oedema, which

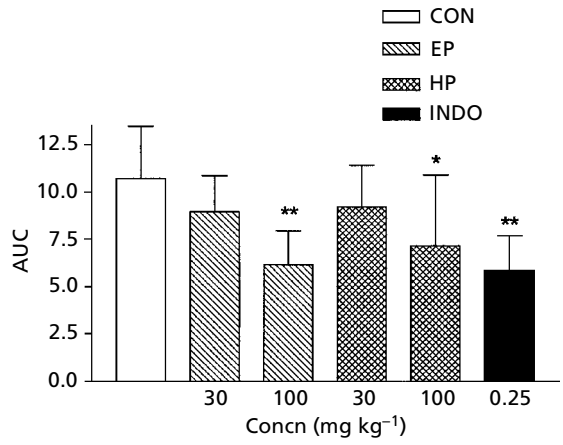

Figure 1 Oral administration of echinacea (EP) or hypericum (HP) (30 or 100 mg kg^{-1}) inhibited carrageenan-induced paw oedema in mice. Indometacin (INDO; 0.25 mg kg^{-1}, s.c.) was used as reference drug. Data are expressed as mean ± s.d. of the area under the curve (AUC) expressed as surface units (mL oedema h^{-1}). $*P < 0.05$; $**P < 0.01$ vs control (CON).

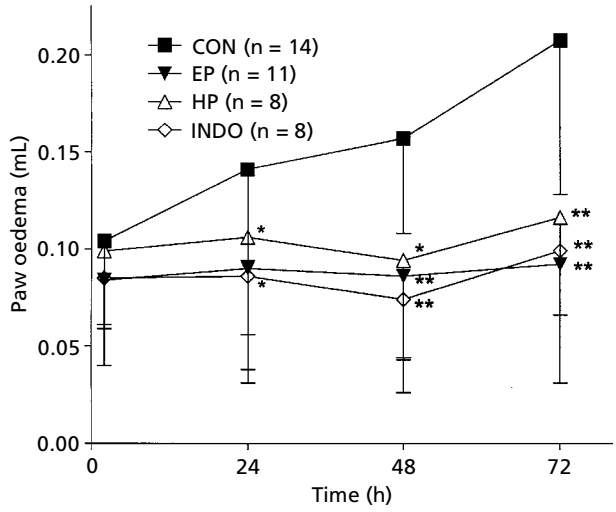

Figure 2 Anti-inflammatory effect of 100 mg kg^{-1} echinacea (EP) and 100 mg kg^{-1} hypericum (HP) against carrageenan-induced paw oedema in mice. Indometacin (INDO; 0.25 mg kg^{-1}) was used as reference drug. Each point is the mean ± s.d. obtained from values related to n animals per group. At time = 0 h, carrageenan was injected into the footpad and at set intervals thereafter (2, 24, 48 and 72 h) the volume of the paw was measured using a plethysmometer. $*P < 0.05$, $**P < 0.01$ vs control (CON).

increases after 24 h, reaching a peak at 72 h after induction of oedema and decreasing thereafter (Henriques et al 1987).

As expected, in our experiments, indometacin significantly inhibited the second phase of carrageenan-induced mouse paw oedema. Hypericum and echinacea also dose-dependently inhibited paw oedema; inhibition was significant at 48 and 72 h (Figure 2).

Treatment with hypericum, echinacea or indometacin did not significantly modify any of the haematological

Table 1 Blood samples obtained from control inflamed mice and mice treated with echinacea (100 mg kg^{-1}, p.o.), hypericum (100 mg kg^{-1}, p.o.) or indometacin (0.25 mg kg^{-1}, s.c.).

Haematological parameter	Control	Echinacea	Hypericum	Indometacin
Total leukocytes (10^3 μL^{-1})	2.2±0.7	4.8±1.5	3.6±0.7	4.0±0.4
Lymphocytes (10^3 μL^{-1})	2.0±0.6	3.7±1.4	3.0±0.6	3.6±0.4
Granulocytes (10^3 μL^{-1})	0.3±0.1	1.1±0.3**	0.6±0.1	0.4±0.1
Platelets (10^3 μL^{-1})	676.2±80.5	856.3±78.2	845.2±54.7	742.3±48.9
Red blood cells (10^6 μL^{-1})	7.3±0.3	7.9±0.9	7.4±0.6	7.0±0.1
Haemoglobin (g dL^{-1})	8.2±0.3	8.9±1.0	8.4±0.4	8.1±0.2
Haematocrit (%)	36.9±1.5	39.7±4.0	37.6±3.1	35.5±1.2
Medium corpuscular volume (fL)	51.6±1.3	50.7±2.0	50.5±1.0	50.7±2.0

Each value is representative of seven animals. $^{**}P < 0.01$ vs control.

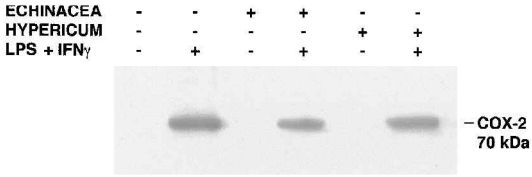

Figure 3 Effect of oral treatment with echinacea or hypericum (100 mg kg^{-1} every 12 h for 14 days) on cyclooxygenase-2 (COX-2) expression by mouse peritoneal macrophages stimulated in-vitro with lipopolysaccharide (LPS; 1 μg mL^{-1}) plus interferon-γ (IFN-γ; 100 U mL^{-1}). Western blot analysis is representative of three separate experiments. All lanes contain 20 μg of total proteins.

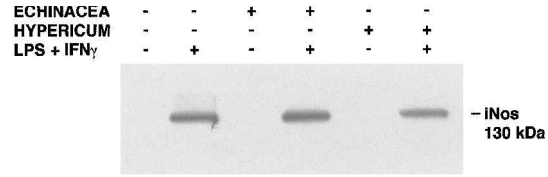

Figure 4 Effect of oral treatment of echinacea or hypericum (100 mg kg^{-1} every 12 h for 14 days) on inducible nitric oxide synthase (iNOS) expression by mouse peritoneal macrophages stimulated in-vitro with lipopolysaccharide (LPS; 1 μg mL^{-1}) plus interferon (IFN-γ; 100 U mL^{-1}). A representative immunoblot of iNOS expression is shown. All lanes contain 20 μg of total proteins.

parameters examined, except that of the granulocyte count, which was significantly increased by echinacea treatment ($P < 0.01$) (Table 1). These data are consistent with previous studies in which polysaccharides isolated from echinacea were reported to induce an increase in leukocyte number in the peripheral blood and multiplication of phagocytes in spleen and bone marrow, contributing to protection against lethal infections with *Listeria monocytogenes* and *Candida albicans* (Roesler et al 1991; Steinmuller et al 1993).

In the present study, we demonstrated that the anti-inflammatory effects of hypericum and echinacea are probably owing to their down-regulation of the inflammatory enzyme COX-2 and/or iNOS expression. This effect was evaluated by Western blot analysis to assess the modulatory effect of in-vivo treatment with echinacea or hypericum on the induction of these enzymes. For this purpose, we used the dose of both drugs that showed a significant anti-inflammatory effect in-vivo. Both echinacea and hypericum exerted an inhibitory effect on LPS- and IFN-γ-induced COX-2 expression in peritoneal macrophages (Figure 3). Densitometric analysis was performed on three different experiments. COX-2 expression was decreased by 57.0±3.0% by treatment with echinacea, and by 29.9±3.7% by treatment with hypericum. When densitometric unit values of control and hypericum or echinacea bands were compared, only the inhibitory effect of echinacea treatment on COX-2 expression was significant ($P < 0.05$).

A different pattern was observed for the effect of echinacea and hypericum on LPS- and IFN-γ-induced iNOS expression (Figure 4). The densitometric analysis of three different experiments demonstrated that hypericum inhibition of iNOS expression was 21.9±3.3% (not significant), while echinacea had no effect. The equal loading of lysates was confirmed by β-actin blotting (data not shown).

The pharmacologically active components responsible for the anti-inflammatory effects of echinacea and hypericum are still unresolved. Further investigations are needed to clarify whether this activity could be attributed to the high concentration of polyphenolic substances found in *H. perforatum*, including quercetin. Recently, this flavonoid was reported to be able to exert a similar anti-inflammatory activity in-vitro (Mattace Raso et al 2001). Besides flavonoids, other substances could also be related to the anti-inflammatory activity of St John's wort (e.g. hypericin). Hypericin was shown to possess a non-antioxidant inhibitory activity on NF-kappa B, a factor involved in immune and inflammatory responses (Bork et al 1999). Regarding echinacea activity, it could be related to its content of polysaccharides. These compounds were previously shown to stimulate macrophages and increase non-specific immunity both in-vitro and in-vivo (Steimuller

et al 1993). Moreover, extracts of roots and leaves of all three echinacea species were found to prevent lipid peroxidation and to scavenge free radicals, phenomena all related to caffeoyl conjugates and flavones (Sloley et al 2000). The active principles of echinacea are not yet clearly defined and the mechanism(s) through which echinacea inhibits the inflammatory process needs to be further characterized.

Several studies have shown that hypericum and echinacea are not toxic in mice, rats or humans (Okpanyi et al 1990; Mengs et al 1991), and so they are widely used in phytotherapy. Conclusions on the possible clinical efficacy of echinacea and hypericum preparations cannot yet be drawn, but our results, demonstrating their effect in-vivo on carrageenan-induced paw oedema and in-vitro on down-regulation of COX-2 and iNOS enzyme expression, strongly support the anti-inflammatory activity of these plants.

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