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Evaluation of anti-inflammatory activity of essential oils from two Asteraceae species

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Received December 9, 2002, accepted January 15, 2003

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Pharmazie 58: 582–586 (2003)

The essential oils from two Asteraceae species, *Porophyllum ruderale* (PR) and *Conyza bonariensis* (CB) were screened for anti-inflammatory activity in the mouse model of pleurisy induced by zymosan (500 μg/cavity) and lipopolysaccharide (LPS) (250 ng/cavity). The main monoterpene constituents of each oil, β-myrcene (in PR) and limonene (in CB), were tested in the LPS-induced pleurisy model and assayed also for immunoregulatory activity by measurement of the inhibition of NO and production of the cytokines, γ-interferon and IL-4. The oils, when administered orally, were able to inhibit the LPS-induced inflammation including cell migration; with a similar effect being observed for pure limonene. Pure β-myrcene and limonene were also effective in inhibiting production of nitric oxide at doses below the cytotoxicity of these monoterpenes. A significant inhibition of γ-interferon and IL-4 production by limonene and β-myrcene was also observed.

1. Introduction

Porophyllum ruderale (Jacq.) Cass. (PR) is a common weed widespread in subtropical America and *Conyza bonariensis* (L.) Cronq. (CB) is known throughout the world as a prolific agricultural weed. Both are herbaceous species of the family Asteraceae used in folk medicine for treating a number of infirmities. Among other traditional uses, *P. ruderale* is reputed to be a remedy for genital inflammation and also to alleviate epilepsy [1], but is mostly used in alcoholic tinctures for treating oedemas. The alcoholic extract also showed activity *in vitro* against the promastigote forms of leishmaniasis [2]. Among reported chemical data, dithienylacetylene derivatives have been isolated from the aerial parts of both species [3]. The essential oil from *Porophyllum tagetoides* was shown to contain predominantly oxygenated monoterpenes (29% citronellal) [4].

Conyza bonariensis finds popular use in the treatment of diarrhoea and hemorrhoids [1]. Species of this genus are used, among other purposes, for treating diabetes, malaria and gastrointestinal inflammation. Some pharmacological properties of species of this genus have been investigated. *C. lobata* has been demonstrated to have anti-microbial activity as well as *in vitro* and *in vivo* activity against *P. berghei* and *T. cruzi* [5]. Alcoholic extracts of *C. canadensis* and *C. floribunda* show anti-inflammatory activity in the formalin or carrageenan-induced paw oedema model in mice [6, 7] and the antispamodic activity of *C. filaginoides* was demonstrated through an activity-guided fractionation [8]. The essential oil of *C. canadensis* L. (syn. *Erigeron canadensis*) is said to be haemostatic and is sometimes employed in medicinal preparations [9]. Acetylenic compounds together with butenolides were isolated from this species [10], as well as polyphenolic compounds [11].

In this study, the anti-inflammatory activity of the essential oils of *P. ruderale* and *C. bonariensis* were assayed by the use of the mouse pleurisy model induced by zymosan (500 μg/cavity) and LPS (250 ng/cavity). The same assays were carried out for myrcene and limonene, two of the main constituents in each plant oils. The *in vitro* immunoregulatory activities of these monoterpenes were also evaluated by assaying the inhibition of NO production.

2. Investigations, results and discussion

2.1. Analysis of essential oils

Monoterpenes predominate in both oils. The oil from *P. ruderale* was rich in trans-β-ocimene (53%), myrcene (16%) and limonene (13%), followed by 1-undecene and α-pinene, whereas oil from *C. bonariensis* showed limonene (46%) and trans-β-ocimene (13%) as the main constituents. The presence of sesquiterpenes was more prominent in *C. bonariensis*, the yield of these being increased by prolonged extraction during 8 additional h (Table 1). An extended extraction of *C. bonariensis*, for a second day, resulted in the almost total predominance of sesquiterpenes in the less volatile fraction (0.03% additional yield). In *P. ruderale*, yields varied from 0.03 to 0.09% for extractions using the whole plant, whereas the

Table 1: Chemical constituents of essential oils from *Porophyllum ruderale* (PR) and *Coniza bonariensis* (CB)

Constituent	Relative abundance (%)		Retention index
	PR	CB	
α-Pinene	5.4	–	929
2-β-Pinene	2.7	1.8	972
Myrcene	16	0.96	986
Limonene	13	45	1024
trans-β-Ocimene	53	13	1044
1-Undecene	10	–	1086
trans-Epoxy-ocimene	*	0.39	–
Terpinen-4-ol	*	–	1169
α-Terpineol	*	–	1183
Terpinen-4-ol	*	–	1169
α-Terpineol	*	–	1183
β-Elemene	–	0.85	1391
Trans-caryophyllene	*	4.5	1411
α-Trans-bergamoptene	–	0.90	1436
α-Humulene	*	1.1	1445
Trans-β-farnesene	–	6.6	1450
γ-Muurulene	–	0.67	1463
Germacrene D	*	6.4	1472
Bicyclogermacrene	–	1.7	1489
δ-Cadinene	*	0.8	1514
Nerolidol	–	1.2	1556
Spathulenol	–	2.2	1571
Caryophyllene oxide	–	2.1	1576
iso-Patulenol	–	1.36	1630
α-Cadinol	–	0.86	1646

* Present in traces. For retention index see reference [20]

use of only leaves increased this value to 0.29%. Very little difference was observed in the chromatographic profile of both oils, which points to the leaves as the oil source in the plant. Over different years, the amount of acyclic terpenes (β-myrcene and *trans*-β-ocimene) varied in the range of 64–74% and the pinenes and limonene varied between 6.8 to 12% and 5.0 to 14%, respectively.

2.2. Anti-inflammatory activity

Injection of zymosan into the mouse pleural cavity induces an acute (4 h) inflammatory reaction characterized by neutrophil migration and protein extravasation [12]. Pre-treatment with PR and CB at the dose used (100 mg/kg p.o.) failed to prevent protein extravasation and did not change the total leukocyte accumulation in the pleural cavity. No inhibition of mononuclear cell accumulation is observed in pre-treated animals when compared with the untreated zymosan-injection group, in the case of PR. The capacity of the oils to prevent a delayed inflammatory reaction (at 24 h) induced by LPS was also verified. In this case, where the reaction is characterized by cell migration with an important eosinophil migration and without protein extravasation [13, 14], the total leukocytes were inhibited to the degree of 37% for PR and 39% for CB, as were mononuclear cells to the extent of 43% and 50% respectively. Neutrophil migration was inhibited 12% and 54%, as well as eosinophil accumulation 63% and 37% by PR and CB, respectively (Fig. 1a–d). For the LPS-induced inflammation, a similar set of results was obtained with limonene, the main constituent in CB, at the doses from 50 to 400 μg/kg. A dose-dependent inhibition of the migration of leucocytes, neutrophils, mononuclear macrophages and eosinophils was verified (Fig. 2a–d). In the same way, β-myrcene inhibited the migration of leucocytes (33% and 22%), neutrophils (36% and 18%), mononuclear macrophages (25% and 18%) and eosinophils (64% and 51%) at the lower (50 mg/kg) or higher dose used (400 mg/kg), respectively (Fig. 3a–d).
In the NO assay, both limonene and β-myrcene were tested at concentrations below the toxic level to the cells.

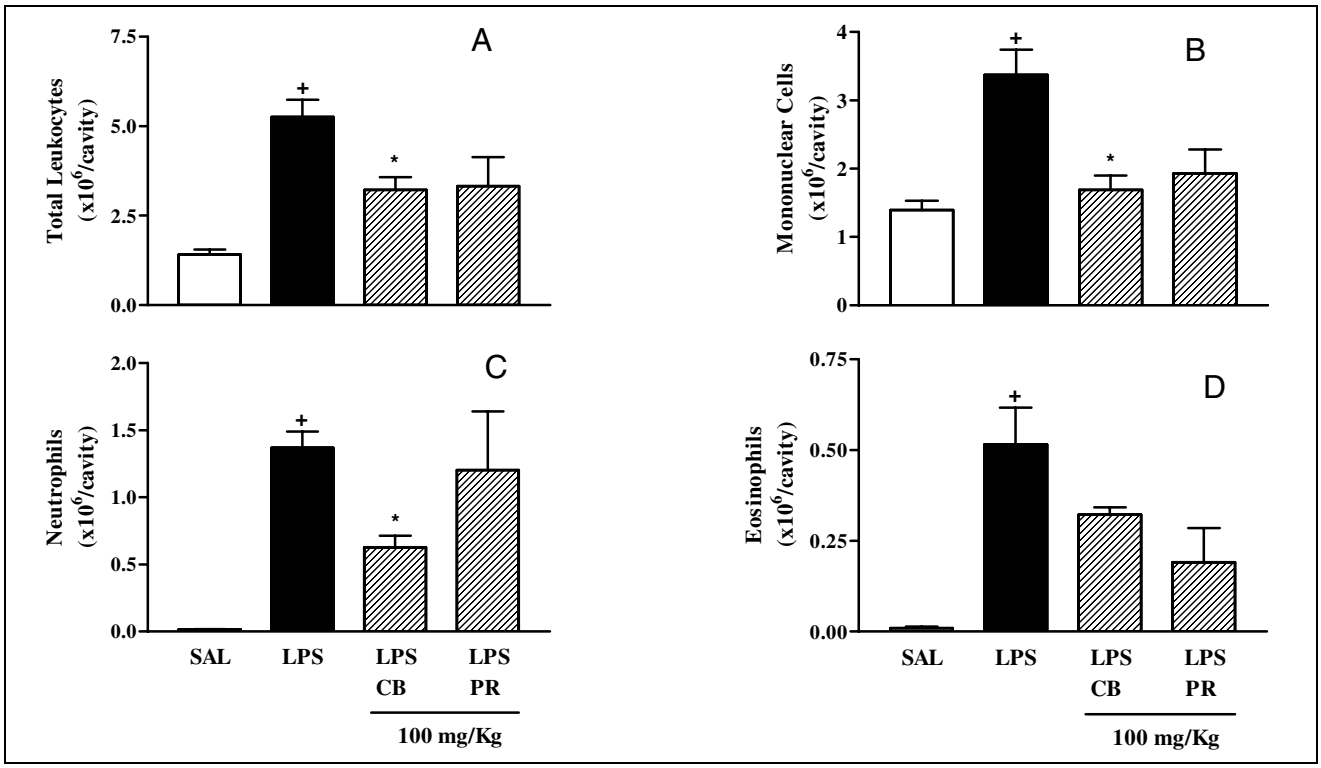

Fig. 1: Effect of essential oil from on LPS-induced leukocyte recruitment. PR and CB (100 mg/kg; hatched columns) or vehicle (open columns) was administered i.p. 1 h prior to LPS (250 ng/cavity) and pleural fluid was collected 24 h later. + and * indicate $P < 0.05$ when compared to control non-stimulated saline injected (open columns) or LPS-stimulated saline-treated group (closed columns), respectively

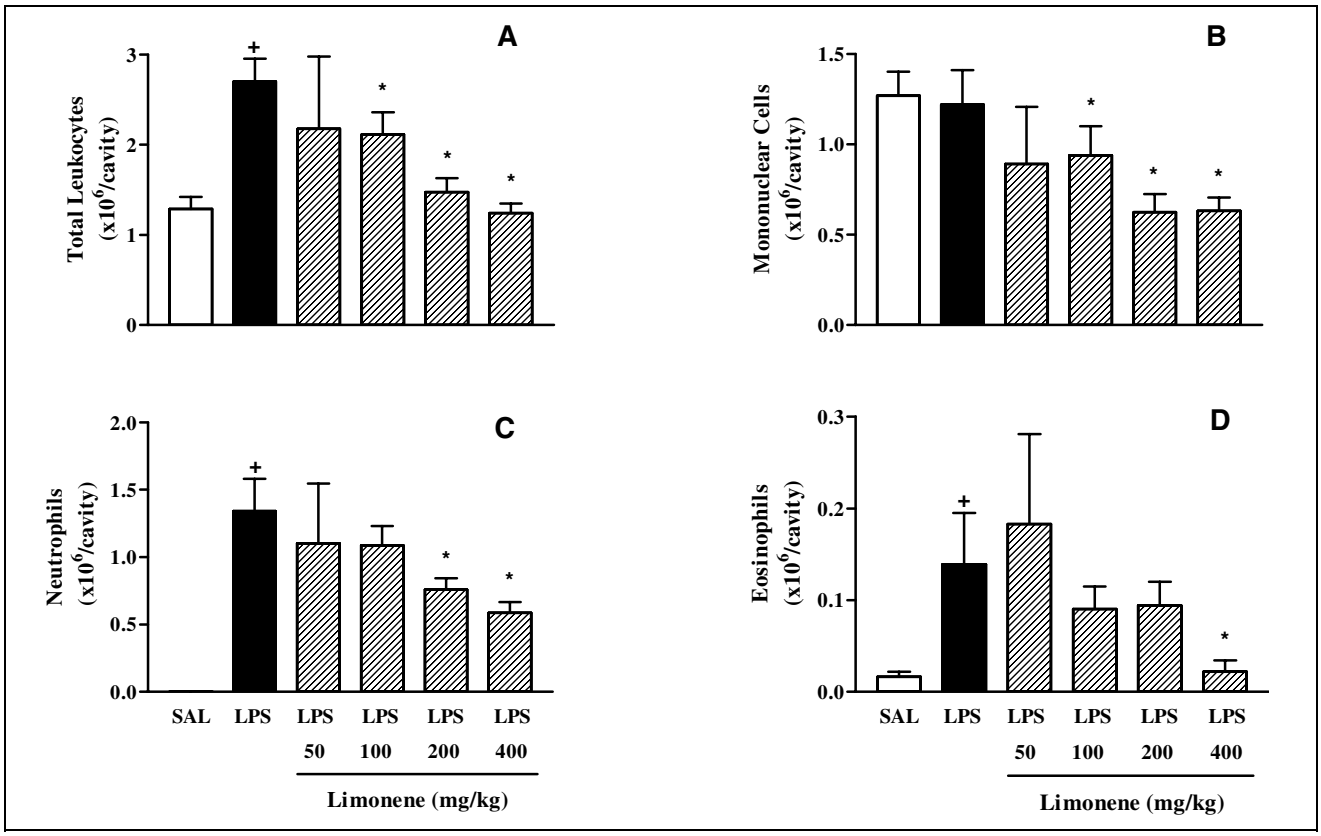

Fig. 2: Effect of limonene on LPS-induced leukocyte recruitment. Limonene (50–400 mg/kg; hatched columns) or vehicle (open columns) was administered i.p. 1 h prior to LPS (250 ng/cavity) and pleural fluid was collected 24 h later. + and * indicate $P < 0.05$ when compared to control non-stimulated saline injected (open columns) or LPS-stimulated saline-treated group (closed columns), respectively

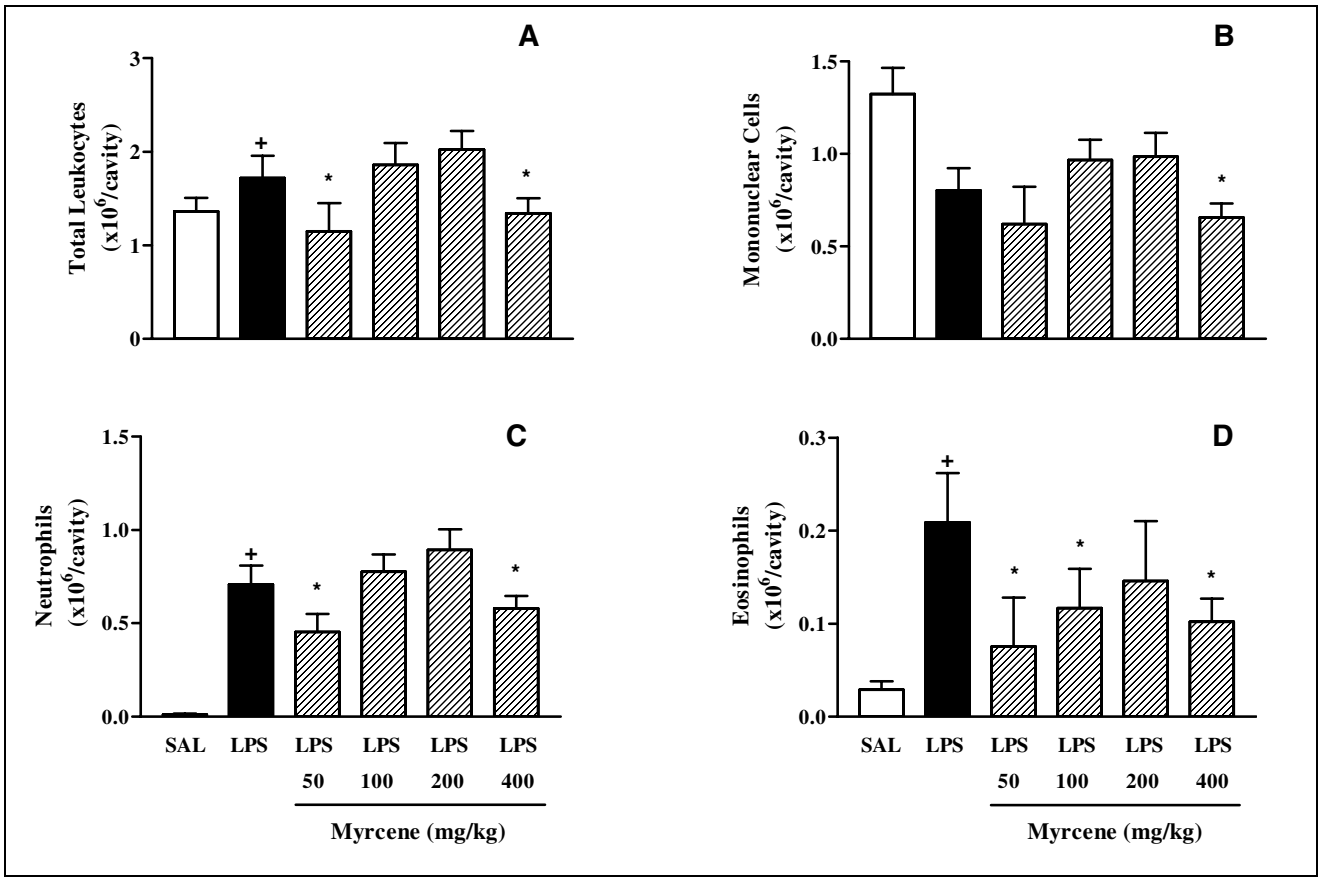

Fig. 3: Effect of myrcene on LPS-induced leukocyte recruitment. Myrcene (50–400 mg/kg; hatched columns) or vehicle (open columns) was administered i.p. 1 h prior to LPS (250 ng/cavity) and pleural fluid was collected 24 h later. + and * indicate $P < 0.05$ when compared to control non-stimulated saline injected (open columns) or LPS-stimulated saline-treated group (closed columns), respectively

Table 2: Effect of the pure monoterpenes on NO and IFN-γ production

	Limonene					Myrcene				
Dose (μg/well)	12.5	25	50	100	200	12.5	25	50	100	200
Cell viability (%)*	100	100	80	27	9	128	107	118	115	51
NO Inhibition (%)*	12	50	–	–	–	14	12	28	35	–
IFN-γ Inhibition (%)*	2	86	–	–	–	94	97	95	98	–

* Values represent the means of triplicate

The former only inhibited NO production by 50% at a dose close to the toxic level (25 μg/well); the latter showed a slightly greater inhibition (28 to 35%) with 50 to 100 μg/well. The production of γ-interferon is inhibited by up to 86% for limonene (25 μg/well) and near to 100% (at several doses) by β-myrcene (Table 2). The same occurred to the cytokine IL-4, where inhibition reached 59 and 91% for limonene and β-myrcene, respectively (Fig. 4a–b).

All these results, taken together, showed that the oils from PR and CB were effective in delayed inflammatory cell migration and this could be helpful in controlling the inflammatory process during some bacterial infections. Eosinophil accumulation is an important component in allergic diseases, parasites and bacterial infections and has been considered one of the causes of lung tissue damage during this process [15].

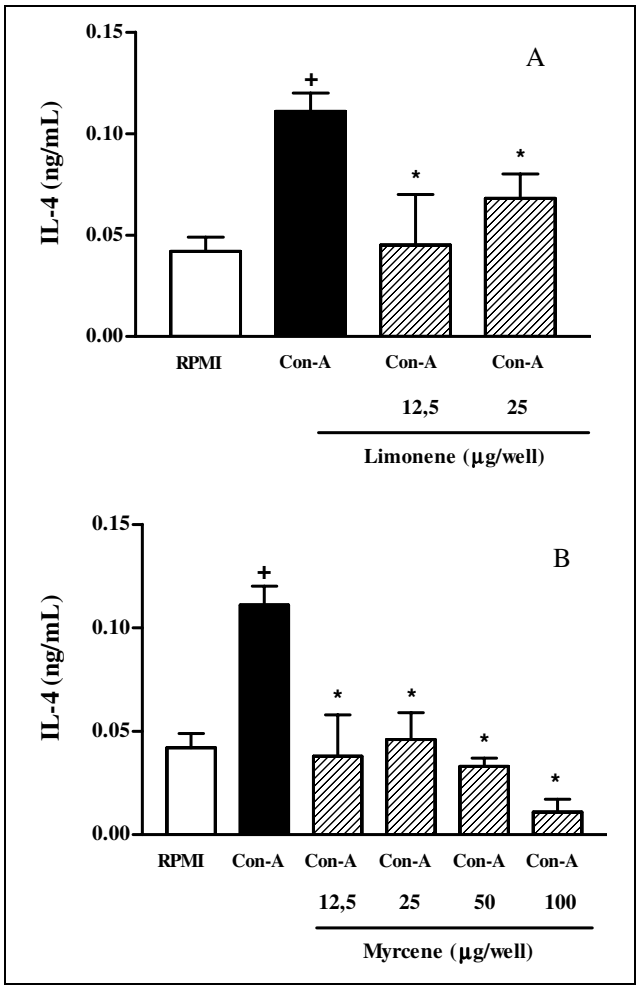

Fig. 4: Effect of limonene (A) and myrcene (B) on IL-4 production. Murine macrophages were incubated with compounds (12.5–100 mg/kg; hatched columns) or medium (open columns) and the IL-4 production was evaluated 24 h later by ELISA assay. + and ∗ indicate $P < 0.05$ when compared to control medium (open columns) or Con-A-stimulated control group (closed columns), respectively

Some atopical diseases, like asthma, are dependent on the production of the cytokines IL-4, IL-5 and IL-13, by allergen-specific Th-2 cells that are generated from a naïve precursor. Mast cells are a source of these cytokines and others mediators that herald the onset of local inflammation with Th-2 cells, eosinophils, monocytes and basophils [16]. Limonene and β-myrcene were able to inhibit both eosinophil accumulation and IL-4 production, suggesting that both of them can modulate the secretion of Th-2 cytokines and, as a consequence, the eosinophil accumulation.

It is well established that T cells, NO and IFN-γ are necessary components of anti-bacterial protection. NO and IFN-γ also modulate the local cellular response by regulating lymphocyte activation and driving T cells into apoptosis, as well as exacerbate inflammation [17]. Both monoterpenes were able to inhibit IFN-γ and partially NO production, indicating that these compounds are not involved in the modulation of bacterial killing by the macrophages.

The monoterpene constitution of the oils is associated with the pharmacological effects observed. There is a noticeable correlation between the diverse usage of *P. ruderale* and its content of ocimene and myrcene. Both are close related isomers, and the latter is established as an analgesic agent [18]. The monoterpene composition may also be thought of as responsible for the use of *P. ruderale* to minimize epileptic seizures, since this group of compounds is known to have anticonvulsant effects [19].

3. Experimental

3.1. Plant material and essential oil extraction

P. ruderale (PR) and *C. bonariensis* (CB) were colleted in the grounds of the Oswaldo Cruz Foundation, city of Rio de Janeiro, Brazil, in May (PR) and December (CB) 1996, and identified by Dr. João Semir, from the Institute of Biology, University of Campinas, Brazil. Voucher specimens have been deposited in the Herbarium of the Botanical Garden of Rio de Janeiro under Nos. # RB 328068 and # RB 328066, respectively. Essential oil of the aerial parts of the fresh plants were obtained by a modified Clevenger-type distillation, during a period of 8 h. The yields were 0.03% (PR, η_D^{25} 1.4712) and 0.16% (CB, η_D^{25} 1.5113).

3.2. Oil analysis

Qualitative data were determined by GC-MS. Analyses of the oils were performed using a Hewlett Packard 6890 GC-MS instrument under the following conditions: injection of 1 μl from 2 mg/ml sample in CH_2Cl_2, HP-5 MS capillary column (30m × 0.32 mm id × 0.25 μm film thickness); carrier gas He, flow 0.5 ml/min; injector temperature 250 °C; column inlet split ratio 1/20; T_i 70 °C, ti 2 min, T_f 280 °C, rate 3°/min; mass spectra: eletronic impact, 70 eV, ion source temperature 250 °C. Individual components were identified by comparison of mass spectra with those in the data system library (Wiley library software 59943B) and their retention index with reference to an n-alkane series in that temperature-programmed run [20].

3.3. Sample preparation

Essential oils (50 mg) were dissolved in chloroform (10 ml) in a round bottom flask. The solvent was removed by low vacuum evaporation to produce a thin film on the interior of the flask. PBS (10 ml) was then added, and the film was dispersed by sonication (50 Hz, 5 min). All the procedures were carried out under sterile conditions. Pure components were dissolved in Tween 80 (1 μL to each oil mg) and than diluted in sterile saline.

3.4. Determination of cytoxicity and toxicity assessment

Balb/c mice received an i.p. injection of thioglycolate 3% and 96 h afterwards were sacrificed and peritoneal cells recovered. The cells were washed RPMI and viability was determined by trypan blue. The cells were incubated in 96 well plates (2.5×10^6 cells/mL) with different concentrations of pure compounds during 20 h when stock MTT (3-(4,5-di-

methylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (5 mg/ml; 22.5 μL/well) was added to all wells, and plates were incubated at 37 °C for 4 h. DMSO (150 μL/well) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. The plate was read on a microplate reader, using a wavelength of 540 nm

3.5. Nitric oxide production

Cells obtained as described above were incubated in 96 well plates (2.5×10^6 cells/mL) and stimulated with or without IFN-γ enriched media plus LPS (30 ng/mL) in the presence of different concentrations of pure compounds. After 24 h the nitrite released into the supernatant was determined by the Greiss method.

3.6. Anti-inflammatory evaluation

Pleurisy was induced by the technique of Spector [12] as modified for mice by Henriques et al. [21]. An adapted needle (13×5 gauge) was inserted carefully 1mm through the right side of the thoracic cavity of mice to enable injection of zymosan (500 μg/cavity) or LPS (250 ng/cavity) in a volume of 100 μl. Control animals received an equal volume of sterile saline. The animals were killed by CO_2 inhalation, 4 or 24 h after the injection. Their thoracic cavities were washed with 1 ml of PBS containing heparin (20 IU ml^{-1}) and the fluid lavage collected for assessment of leukocyte accumulation and Evans blue extravasation.

The pleural lavage fluid collected was diluted 40 times in Turk's solution and total leukocyte counts were made in Neubauer chambers under light microscopy. Differential leukocyte counts were determined in cyto-centrifuged smears stained with May-Grünwald-Giemsa dye using an oil immersion objective (100 ×).

Mice were given intravenous injections of Evans blue (25 mg · kg^{-1}), 24 h before the inflammatory stimulus. The pleural lavage fluid was collected at the same times and as described above, centrifuged (2,500 rpm for 10 min) and the absorbance of the cell-free supernatant was read in a spectrophotometer (Shimadzu, Japan) at 600 nm.

For the *in vivo* experiments, statistical significance ($p < 0.05$) was analyzed by ANOVA followed by Student Newuman Keuls test. Results were expressed as mean ± S.M.

3.7. ELISA assay

Spleens recovered from C57Bl/10 male mice were pooled and cultured for 3 days, as previously described [22]. Mononuclear cell enriched suspension was washed and incubated (3×10^5 cells/well) with or without Con-A (5 μg/ml) and different concentrations of pure compounds. After 72 h, the supernatant was recovered and submitted to ELISA assay.

To measure IFN-γ and IL-4, Maxsorp microplates were coated overnight at 4 °C with either antimouse-IFN-γ or antimouse-IL4 diluted in binding solution (4 μg/ml) and washed twice with PBS/Tween 0.5%. After blocking during 1h with PBS/MILK 3%, plates were washed three times and 100 μL of either standard IFN-γ or standard IL-4 and culture supernatant was added to each well overnight. The plates were washed three times and 100 μL of either biotinylated antimouse-IFN-γ or antimouse-IL-4 were added and the plates were incubated for an additional hour at room temperature. After three washes, 100 μL streptavidin-peroxidase (1 : 800) was added and the mixture was incubated for 30 min at room temperature and washed six times. The peroxidase substrate OPD (5 mg/mL), diluted in perborate buffer, was added and incubated in the dark for 30 min. After stopping the reaction with 3 N HCl, absorbance was measured at 490 nm and cytokine levels were estimated by comparison with a standard curve.

For toxicity assessment, male Swiss mice (20–25 g) received 100 mg/kg of pure compounds (i.p.). An equivalent dose of vehicle was administered to the control group. Both test and control groups (6 animals each) were observed for 24 h under normal environmental conditions, with free access to food and water.

Acknowledgements: The authors thank the CNPq for the fellowships to O. Menezes-de-Lima and A. L. F. Sampaio, and FAPERJ for the fellowship to M. F. S. Ramos.

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