Screening of five essential oils for identification of potential inhibitors of IL-1-induced NF-κB activation and NO production in human chondrocytes: characterization of the inhibitory activity of α-pinene

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Abstract

Nuclear Factor-κB is a key transcription factor activated by pro-inflammatory signals, like interleukin-1β (IL-1), being required for the expression of many inflammatory and catabolic mediators, namely nitric oxide (NO), that play an important role in arthritic diseases. This work aimed at screening and identifying natural inhibitors of IL-induced NF-κB activation and NO production in human articular chondrocytes. Five essential oils obtained from plants of the Iberian flora, as *Mentha x piperita* L. (Lamiaceae), *Origanum virens* L. (Lamiaceae), *Lavandula luiseri* L. (Lamiaceae), and *Juniperus oxycedrus* L. subsp. *oxycedrus* (Cupressaceae), were screened for the ability to prevent IL-1-induced NO production. The oil showing higher inhibitory activity was fractionated, concentrated, analyzed for composition elucidation and prepared for further assays. For this purpose, the human chondrocytic cell line, C-28/I2, was used to evaluate NF-κB activation by determining the cytoplasmic levels of the total and phosphorylated forms of the inhibitory protein, IκB-α, and NF-κB-DNA binding activity. The essential oil from the leaves of *J. oxycedrus* in a concentration of 0.02 % (v/v) achieved the greatest inhibition (80 ± 8 %) of IL-1-induced NO production. Chemical analysis showed that this essential oil is predominantly composed of monoterpene hydrocabons, being α-pinene [2,6,6-trimethyl-bicyclo(3.1.1)hept-3-ene] the major constituent (76 %). As does the whole oil, a fraction containing 93 % α-pinene reduced significantly IL-1-induced IκB-α degradation. Moreover, α-pinene also decreased IκB-α phosphorylation, NF-κB-DNA binding activity and NO production. Another fraction containing oxygenated mono- and sesquiterpenes, was nearly as effective as α-pinene. The ability of the α-pinene-containing fraction to reduce IL-1-induced NF-κB activation and NO production warrants further studies to demonstrate

the usefulness of α-pinene in the treatment of arthritic diseases and other conditions in which NF-κB and NO play pathological roles.

Keywords

Arthritis; α-Pinene; IL-1β; Essential oil; NF-κB; Nitric Oxide.

Abbreviations

Bay 11-7082, Bay; DMSO, dimethylsulfoxide; Fα-p, α-pinene-containing fraction, Fox, oxygenated compounds-containing fraction; IκB-α, NF-κB inhibitor-α; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; NF-κB, Nuclear Factor-κB; NO, nitric oxide; p-IκB-α, phosphorylated IκB-α.

Introduction:

Arthritic diseases include a plethora of conditions characterized by cartilage degradation and variable local inflammation. Loss of the articular cartilage results from decreased synthesis of the cartilage matrix components and increased expression of matrix-degrading enzymes [1-3]. Pro-inflammatory cytokines, among which interleukin-1β (IL-1) is the prototype, contribute to inflammation and cartilage degradation by inducing articular chondrocytes and cells of the synovial membrane to express catabolic and inflammatory genes. Among these, the inducible nitric oxide synthase (iNOS) has a prominent pathophysiologic role [4,5], being upregulated in arthritic joints [6,7]. NO, produced in large amounts by iNOS [8], has been shown to mediate many of the inflammatory and catabolic responses elicited by IL-1 and other pro-inflammatory cytokines in chondrocytes [9-13], contributing to reduce major anabolic processes, while increasing catabolic responses and, therefore, driving the imbalance between matrix synthesis and degradation characteristic of arthritic diseases [2,3].

The transcription factor, Nuclear Factor-κB (NF-κB), plays a crucial role in the pathogenesis of arthritic diseases by mediating the expression of both catabolic and inflammation-related genes, namely that encoding iNOS, in chondrocytes and cells of the synovial membrane [1-3]. The importance of NF-κB in the pathogenesis of arthritis is further highlighted by the findings that several drugs currently used for the treatment of osteoarthritis inhibit NF-κB activity to some extent [14,15] and that specific small inhibitors targeting this transcription factor have anti-inflammatory and anti-degradative effects in animal models of inflammatory arthritis [16].

NF-κB can be activated in many cell types, including chondrocytes, in response to a large number of extracellular stimuli [17]. The major or canonical pathway leading to

NF-κB activation, which is involved in IL-1-induced NF-κB activation in chondrocytes [15,18], requires the phosphorylation of an inhibitory protein, termed IκB-α, that is bound to NF-κB dimmers in the cytoplasm, preventing their translocation to the nucleus. Upon phosphorylation by a specific kinase, IκB-α undergoes ubiquitination followed by proteasome degradation. Thus, the freed NF-κB dimmers translocate to the nucleus where they promote the expression of specific genes, many of which are involved in the immune, inflammatory and stress responses [19,20].

Considering their role in the development and/or progression of arthritis [1-3], the screening for compounds capable of down-regulating NF-κB and NO is a promising strategy for the development of new drugs with potential disease-modifying effects for therapeutic intervention in arthritic diseases. Plant extracts are valuable collections offering a huge diversity of compounds, most of them commercially unavailable and structurally difficult to synthesize. In this context, essential oils, plant extracts prepared by distillation and composed by small hydrophobic molecules with potential to diffuse readily across cell membranes, are particularly valuable for the screen of pharmacologically active compounds. Accordingly, aiming at identifying compounds with inhibitory activity towards IL-1-induced NF-κB activation and NO production in human articular chondrocytes, this study screened a set of essential oils whose compositions are representative of the major chemical families reported for this kind of plant extracts. Five essential oils were selected: *Mentha x piperita* L. (Lamiaceae) oil, mainly composed of oxygen-containing monoterpenes; *Lavandula luiseri* (Rozeira) Riv. Mart. (Lamiaceae) oil, rich in irregular monoterpenes; *Juniperus oxycedrus* L. subsp. *oxycedrus* (Cupressaceae) berries and leaves oils, predominantly composed of monoterpene hydrocabons, and *Origanum virens* Hoffmanns. & Link (Lamiaceae) oil whose major components are phenolic monoterpenes. The essential oil showing the

highest inhibitory activity towards NO production was analyzed, fractionated and its fractions studied in order to identify active compounds.

Material and Methods

Preparation of the essential oils

Plant material was taxonomically identified by J. Paiva, taxonomist of the Herbarium of the Botanical Institute of the University of Coimbra (COI) and vouchers deposited under the references CC1024, CC890, CC1039 and CC1046.

Essential oils from those plants were prepared at laboratory by hydrodistillation for 3h using a Clevenger-type apparatus and the procedure described in the European Pharmacopoeia [21]. The oils were stored at 4°C in the dark, prior to screening assays or chemical analysis.

Oil fractionation

Oil (n x 1.0 g) fractionation was achieved by flash chromatography on silica gel (63–200 µm, Merck, Darmstadt, Germany) using a 2 cm x 40 cm Omnifit (Sigma-Aldrich, St. Louis, MO, USA) glass column. Multiple 20 mL fractions were collected with a automatic fraction collector (Gilson FC 204, Middleton, WI, USA) using *n*-pentane (Panreac Quimica, Barcelona , Spain) as mobile phase (flow rate, 1.5 $mL.min^{-1}$). After complete elution of the hydrocarbons, mobile phase was changed to diethyl oxide (Panreac Quimica, Barcelona, Spain) seeking the elution of the oxygenated compounds. Following solvents evaporation in a vacuum concentrator centrifuge (Univapo 100, UniEquip GmbH, Munich, Germany), all fractions were monitored by gas chromatography-mass spectroscopy and subsequently recombined in eight major fractions, accordingly their compositions.

Chemical analysis of the essential oils and their fractions

Gas chromatography

Analytical GC was carried out in a Hewlett-Packard 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph with a HP GC ChemStation Rev. A.05.04 data handling system, equipped with a single injector and two flame ionization detection (FID) systems. A graphpak divider (Agilent Technologies, part no. 5021-7148) was used for simultaneous sampling to two Supelco (Supelco, Bellefonte, PA, USA) fused silica capillary columns with different stationary phases: SPB-1 (polydimethylsiloxane 30m × 0.20mm i.d., film thickness 0.20μm), and SupelcoWax-10 (polyethyleneglycol 30m × 0.20mm i.d., film thickness 0.20 μm). Oven temperature program: 70–220 ºC (3 ºC.min^{-1}), 220 ºC (15 min); injector temperature: 250 ºC; carrier gas: helium, adjusted to a linear velocity of 30 cm.s^{-1}; splitting ratio 1:40; detectors temperature: 250 ºC.

Gas chromatography–mass spectrometry

Analyses were carried out in a Hewlett-Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane 30m × 0.25mm i.d., film thickness 0.25μm), interfaced with an Hewlett-Packard mass selective detector 5973 (Agilent Technologies) operated by HP Enhanced ChemStation software, version A.03.00. GC parameters as described earlier; interface temperature: 250ºC; MS source temperature: 230 ºC; MS quadrupole temperature: 150ºC; ionization energy: 70 eV; ionization current: 60 μA; scan range: 35–350 units; scans.s^{-1}: 4.51.

Qualitative and quantitative analysis

Components of each essential oil and fraction were identified by their retention indices on both SPB-1 and SupelcoWax-10 columns and from their mass spectra. Retention indices, calculated by linear interpolation relative to retention times of C8–C23 of *n*-

alkanes, were compared with those of authentic samples included in our own laboratory database. Acquired mass spectra were compared with reference spectra from our own library or from the literature [22], [23].

Cartilage samples

Normal human knee cartilage was collected from the distal femoral condyles of multi-organ donors (20-54 years old, mean=38) within 24h *post-mortem*, at the Tissue Bank of the University Hospital of Coimbra (HUC). Cartilage from all donors appeared macroscopically normal without signs of erosion, but the cartilage from the older donors (>45 years old) had a yellow coloration. All the procedures had the approval of the Ethics Committee of HUC.

Isolation and cell culture

Articular chondrocytes were isolated from the cartilage samples by enzymatic digestion and non-proliferating monolayer cultures were established as described previously [7]. Non-proliferating monolayer cultures were subsequently treated with recombinant human IL-1 (Peprotech, Rocky Hill, NJ, USA), in the presence or absence of the essential oils and fractions or of control compounds. Culture supernatants and the adherent cells were used, respectively, to measure NO production and to evaluate cell viability.

The human chondrocytic cell line, C-28/I2, was used to evaluate NF-κB activation.

Dilution of the essential oils and their fractions

Essential oils and their fractions were diluted 1:5 in DMSO and then in culture medium to achieve the final concentrations. The highest concentration of DMSO in the final solution was 0.2% (v/v) and the lowest 0.05% (v/v).

Measurement of nitric oxide production

Nitric oxide production was measured as the amount of nitrite released into the culture supernatant. Nitrite concentration was determined in cell-free culture supernatants using the spectrophotometric method based on the Griess reaction [24].

Preparation of cytoplasmic and nuclear extracts

C28/I2 cells were lysed in 150 μl of buffer 1 [10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0,5% Nonidet P-40, protease inhibitors (Complete Mini, Roche, Indianapolis, IN, USA), pH=7.5] and incubated on ice for 15 minutes. After centrifugation at 2300g/5 min at 4ºC, the supernatants (cytoplasmic extracts) were stored at -20ºC. The pellets were suspended in 25 μl of buffer 2 [20 mM HEPES, 5 mM $MgCl_2$, 0,2 mM EDTA, 1 mM DTT, 300 mM NaCl, 20% glycerol, protease inhibitors, pH=7.5], incubated on ice for 20 minutes and centrifuged at 18000g/20 minutes at 4ºC. Supernatants (nuclear extracts) were stored at -20ºC. Protein concentration was measured using the bicinchoninic acid/copper (II) sulphate protein assay kit (Sigma Chemical Co., St. Louis, MO, USA).

Western blot analysis

Cytoplasmic extracts and molecular weight markers (Precision Plus, Bio-Rad Laboratories Inc., Hercules, CA) were subjected to SDS/PAGE, electroblotted onto PVDF membranes and probed with anti-IκB-α or anti-phospho-IκB-α antibodies (Cell

Signaling Technology, Inc., Danvers, MA, USA) and ~~then~~ with an anti-rabbit alkaline phosphatase-conjugated secondary antibody (Amersham Biosciences, Buckinghamshire, UK). The membranes were reprobed with an anti-actin monoclonal antibody (Chemicon International, Inc., Tremecula, CA) as an internal control. Immune complexes were detected with the Enhanced ChemiFluorescence reagent (Amersham Biosciences) and the bands analyzed using ImageQuant^{Tm} TL (Amersham Biosciences).

Transcription factor assay

A colorimetric ELISA-based assay (NoShift Transcription Factor Assay kit, Novagen, La Jolla, CA) was used to evaluate the presence of active NF-κB dimmers, capable of binding to the cognate consensus oligonucleotide sequence. For this, nuclear extracts from C28/I2 cells were incubated with a biotinylated consensus NF-κB oligonucleotide (NoShift NF-κB Reagents, Novagen) and the assay performed according to the manufacturer's instructions. In parallel and to determine the specificity of the reaction, competition assays were set up by adding a 10-fold molar excess of non-biotinylated wild type or mutant oligonucleotides (NoShift NF-κB Reagents, Novagen) to binding reactions containing nuclear extracts from IL-1-treated cells.

Data analysis

Statistical significance was assessed by one-way ANOVA with Dunnett's post test for comparison of all conditions versus IL-1. In some experiments, the Bonferroni post test was used for simultaneous comparison to IL-1 and the control, untreated condition. Analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Results were considered significant for $P<0.05$.

Results and Discussion

None of the essential oils or fractions tested affected chondrocyte viability under the experimental conditions used (supporting information).

The specific NF-κB inhibitor, Bay 11-7082 (Bay), almost completely abolished IL-1-induced NO production as evaluated by the amount of nitrite accumulated in the supernatants of primary human chondrocyte cultures (Fig. 1). Moreover, four of the five essential oils tested were effective in reducing IL-1-induced NO production. The greatest inhibition (80 ± 8 % relative to IL-1) was achieved with the highest concentration (0.02 %, v/v) of the oil from the leaves of *J. oxycedrus*, closely followed by the same concentration of the essential oil from the berries of the same species (76 ± 19 %). Essential oil from *O. virens* was completely inactive while that from *M. piperita* showed only modest inhibitory activity (32 ± 18 % relative to IL-1), even in the higher concentration. DMSO, used for the initial dilution of the essential oils and fractions, did not affect IL-1-induced NO production either in the lowest or the highest concentrations used (data not shown).

Since the essential oil from the leaves of *J. oxycedrus* showed the highest inhibitory activity, we proceeded with its fractionation and chemical analysis (table 1). As the oils from plants of different geographic origins [25,26], this composition is dominated by monoterpene hydrocarbons (87 %), being α-pinene the major constituent (76.4 %). Oxygen-containing monoterpenes represent only 6.5 % while sesquiterpenic compounds do not exceed 2.6 % of the total composition

Upon fractionation by flash chromatography, seven fractions eluted with *n*-pentane and one eluted with diethyl oxide, were collected. The major fraction is mainly composed of α-pinene (chromatographic purity, 93.2 %). The second relevant fraction was eluted

with diethyl oxide, being composed by a mixture of oxygen-containing mono- and sesquiterpenes. Other six fractions represent only a minor portion of this essential oil and include a huge diversity of compounds at a very low concentration. Since the α-pinene and the oxygenated compounds-containing fractions together constitute 84.3 % of the oil, these two fractions were further screened to evaluate their ability to downregulate IL-1-induced NF-κB activation.

Since human cartilage samples were seldom available and a large number of cells is required, the human chondrocytic cell line, C28/I2, was used to evaluate the ability of that essential oil and its most abundant fractions to inhibit IL-1-induced NF-κB activation. The essential oil was tested in the same concentrations used to evaluate its effect on IL-1-induced NO production. The lowest concentration (0.02 %, v/v) of the α-pinene-containing fraction (Fα-p) roughly corresponds to that present in the highest concentration of the parent essential oil, since this fraction accounts for over 80 % of its composition. Considering its concentration in the parent essential oil, the concentrations of the oxygenated compounds-containing fraction (Fox) used (0.02 % and 0.01 %, v/v) are approximately ten fold higher than those found in the highest and lowest concentrations of the parent essential oil, respectively.

As expected, IκB-α was almost completely absent in the cytoplasmic extracts obtained from cells treated with IL-1 alone (Fig. 2). The amount of IκB-α remaining represented approximately 11 % of that found in the control. This shows that IL-1 induced IκB-α degradation which is an indicator of NF-κB activation. Pre-treatment with the *J. oxycedrus* leaves´ oil in concentrations of 0.02 % or 0.013 % (v/v), decreased IκB-α levels to 38 and 26 %, respectively, of those found in the control, thus representing an inhibition of IL-1-induced IκB-α degradation of 31 and 18 %, respectively. Our previous studies showed that, in chondrocytes, IL-1-induced NO production results

from induction of iNOS expression which requires NF-κB activation subsequent to IκB-α degradation [15-18]. Therefore, the results obtained strongly suggest that the essential oil from the leaves of *J. oxycedrus* inhibits IL-1-induced NO production by preventing, at least in part, IκB-α degradation and NF-κB activation. Nevertheless, inhibition of NO production was much more pronounced than that of NF-κB activation, suggesting that other mechanisms contribute to inhibition of NO production by this essential oil.

In a concentration of 0.02 % (v/v), Fα-p inhibited IL-1-induced IκB-α degradation by 33 %, whereas in a concentration two fold higher the degree of inhibition increased to only 46 %. Similarly, treatment with 0.01 % and 0.02 % (v/v) Fox inhibited IL-1-induced IκB-α degradation by 22 % and 31 %, respectively. Together, Fα-p and Fox constitute almost 85 % of the whole *J. oxycedrus* leaves oil, and both fractions partially decreased IL-1-induced IκB-α degradation in a dose-dependent manner (Fig. 2). Therefore, the inhibitory effect observed with the parent essential oil is likely due to the activity of these two fractions, especially to Fα-p which is considerably more abundant than Fox. Nevertheless, one or more of the components of this fraction are probably, at least, as active as α-pinene, since the two fractions, in identical concentrations (0.02 %, v/v), were similarly effective. Future work will be directed at purifying those oxygenated components and identifying those capable of preventing NF-κB activation induced by pro-inflammatory stimuli, like IL-1, in chondrocytes.

Since Fα-p is the most pure and accounts for most of the *J. oxycedrus* essential oil, additional tests were performed to further assess its ability to inhibit IL-1-induced NF-κB activation. Figure 3A shows that both Fα-p and Bay prevented IL-1-induced IκB-α phosphorylation, a step that precedes and is required for IκB-α degradation. Additionally, Fα-p also reduced NF-κB-DNA binding activity in matching nuclear

extracts (Fig. 3B). Competition assays performed as described in Materials and Methods, confirmed the specificity of the assay. Then, the ability of Fα-p to inhibit IL-1-induced NO production was further tested to confirm whether inhibition of NF-κB activation correlated with reduced production of NO, as demonstrated for Bay. In a concentration of 0.02 % (v/v), Fα-p reduced NO production to 11.3 ± 0.4 % of that obtained with IL-1 alone, representing an inhibition of approximately 89 %. This degree of inhibition is not much different from that obtained with the same concentration of the parent essential oil (~80 %), indicating that Fα-p accounts for most of its activity. However, even in the highest concentration used (0.04 %, v/v), Fα-p reduced IκB-α degradation by only 46 %, whereas in a concentration two-fold lower NO production was inhibited by nearly 90 %. Similarly to the parent essential oil, these results suggest that other mechanisms, besides inhibition of NF-κB activation, may account for the ability of Fα-p to inhibit IL-1-induced NO production. Previous studies [27] showed that α-pinene has anti-inflammatory properties *in vivo*, but no cellular or molecular mechanisms were identified. More recently, α-pinene was reported to dose-dependently decrease IκB-α degradation and the nuclear translocation of NF-κB induced by LPS in THP-1 cells [28]. Results presented in the current study are in agreement with those reported by Zhou and co-workers [28] and further demonstrate that the inhibitory activity of α-pinene on NF-κB activation is not restricted to a specific cell type or to a particular inflammatory stimulus.

In summary, this study shows that the most abundant component in the essential oil from the leaves of *J. oxycedrus* is α-pinene which probably accounts for most of its inhibitory activity, both at the levels of IL-1-induced NF-κB activation and NO production. This suggests that α-pinene may be promising as a small NF-κB and NO

inhibitor and warrants further studies to demonstrate its usefulness in the treatment of arthritic diseases and other conditions in which both play pathological roles.

Acknowledgements

Authors are grateful to Prof. Jorge Paiva for help in plant taxonomy and to Prof. Mary Goldring (Beth Israel Deaconess Medical Center and New England Baptist Bone and Joint Institute, Boston, MA, USA) for the kind gift of the C28/I2 cell line. Rosa S. is supported by a Ph.D. fellowship (SFRH/BD/19763/2004) from the Portuguese Foundation for Science and Technology (FCT). This work was supported by grant PTDC/SAU-OSM/67936/2006 from FCT and by C.E.F. / POCI 2010 /FEDER.

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Legends for Figures

Fig **1** Effect of the essential oils on IL-1-induced NO production. NO was evaluated as the amount of nitrite accumulated in the supernatant of normal human chondrocyte cultures left untreated (Control, C) or treated with IL-1, 30 ng.mL^{-1} (IL-1) for 18 h following pre-treatment for 2 h with or without the indicated concentrations of each essential oil or of the specific NF-κB inhibitor, Bay 11-7082 (Bay). Results are expressed relatively to the concentration of nitrite obtained in cells treated with IL-1 alone (14.3 ± 2.5 μM). Each column represents the mean ± SEM of 3 independent experiments each performed in duplicate. ***P*<0.01; ****P*<0.001 relatively to IL-1-treated cells.

Fig **2** Effect of the essential oil from *J. oxycedrus* leaves and of its fractions containing α-pinene (Fα-p) and oxygenated terpenoids (Fox) on IL-1-induced IκB-α degradation. Intensities of the IκB-α bands normalised to the intensity of the respective actin band and representative images of a membrane showing the IκB-α and the actin bands in C28/I2 cells untreated (Control, C) or treated with IL-1, 30 ng.mL^{-1} (IL-1) for 30 min following pre-treatment for 2 h with or without the indicated concentrations of the essential oil and its fractions. Each column represents the mean ± SEM of 4 independent experiments. §P<0.001 relatively to C; *P<0.05, **P<0.01 and ***P<0.001 relatively to IL-1.

Fig. 3: Effect of the α-pinene-containing fraction (Fα-p) on IL-1-induced IκB-α phosphorylation and NF-κB-DNA binding activity. A) Intensities of the phospho-IκB-α bands normalised to the intensity of the respective actin band and representative images of a membrane showing the phospho-IκB-α and the actin bands in C28/I2 cells untreated (Control, C) or treated with IL-1, 30 ng.mL^{-1} (IL-1) for 5 min following pre-treatment with or without Fα-p (0.04 %, v/v) or Bay (5 μM). Each column represents the mean ± SEM of 4 independent experiments. ***P<0.001 relatively to IL-1. B) Detection of NF-κB-DNA complexes by ELISA. Absorbance intensity is directly proportional to the amount of active NF-κB dimmers present in the nuclear extracts obtained from the control (Ctrl) or from cells treated with IL-1, 30 ng.mL^{-1} for 30 min following pre-treatment with or without 0.04 % (v/v) Fα-p. Each column represents the mean ± SEM of 4 independent experiments. **P<0.01 and ***P<0.001 relatively to IL-1; §P<0.05 relatively to Ctrl.

Table 1

Composition of the essential oil from *Juniperus oxycedrus* leaves.

RI SPB-1	RI SPWax	Compound	Percent in sample
921	1030	α-Thujene	0,1
921	1016	Tricyclene	0,2
933	1030	**α-Pinene**	**76,4**
943	1074	Camphene	0,6
943	1064	α-Fenchene	0,4
946	1131	Verbenene	0,5
960	1447	Oct-1-en-3-ol	0,1
960	n.d.	*m*-Cymene	0,2
964	1124	Sabinene	0,2
970	1115	β-Pinene	1,8
980	1160	Myrcene	1,3
996	1138	δ-2-Carene	0,2
1005	1151	δ-3-Carene	2,7
1011	1271	*p*-Cymene	0,9
1020	1204	Limonene	1,0
1020	1213	β-Phellandrene	0,6
1103	1486	α-Campholenal	0,4
1117	1511	Camphor	0,2
1120	1645	*trans*-Pinocarveol	0,7
1122	1645	*cis*-Verbenol	0,2

Table 1 (continued)

1125	1668	*trans*-Verbenol	1,5
1134	1511	*trans*-Pinocamphone	0,0
1134	1561	Pinocarvone	0,1
1145	1695	Borneol	0,3
1152	1661	Cryptone	0,4
1157	1598	Terpinene-4-ol	0,4
1165	1620	Myrtenal	0,2
1168	1695	α-Terpineol	0,6
1175	1695	Verbenone	0,9
1194	1830	*trans*-Carveol	0,3
1264	1571	Bornyl acetate	0,2
1328	1687	α-Terpinyl acetate	0,2
1375	1514	β-Bourbunene	0,3
1462	1678	γ--Muurolene	0,2
1497	1747	γ-Cadinene	0,4
1542	2037	*E*-Nerolidol	0,2
1557	1965	Caryophyllene oxide	0,5
1581	2021	Humulene epoxide	0,4
1614	2154	*T*-Cadinol	0,3
1624	2215	α-Cadinol	0,2
1644	2024	*(Z)*-6-Pentadecen-2-one	0,7
1967	n.d	Manoyl oxide	0,2
2021	n.d.	Abietatriene	0,2

Table 1 (continued)

Monoterpene hydrocarbons	87,1
Oxygen containing monoterpenes	6,5
Sesquiterpene hydrocarbons	0,9
Oxygen containing sesquiterpenes	1,7
Other compounds	1,3
Total identified	97,5

Compounds listed in order to their elution on the SPB-1 column;

RI SPB-1 - Retention indices on a Supelco polydimethylsiloxane column relative to C8–C23 *n*-alkanes.

RI SPWax - Retention indices on a Supelco polyethylleneglycol column relative to C8 to C23 *n*-alkanes.

n.d. = not determined

Fig. 1

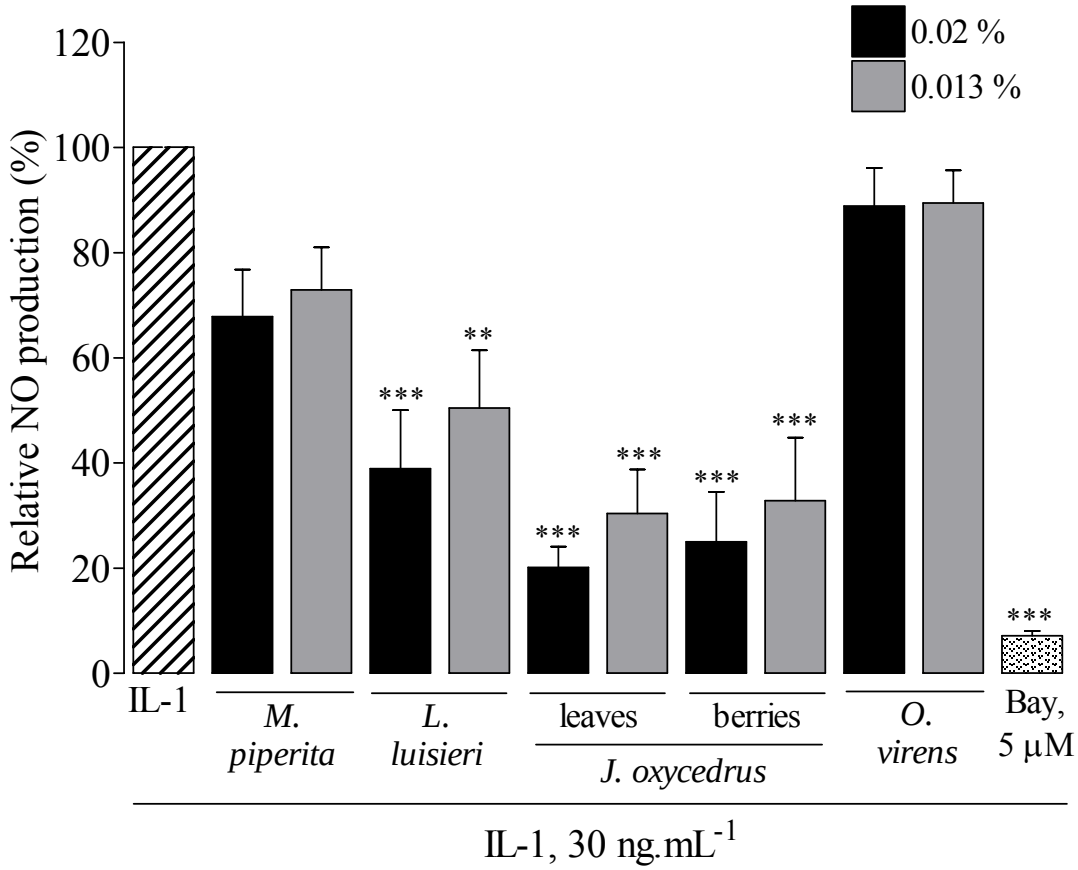
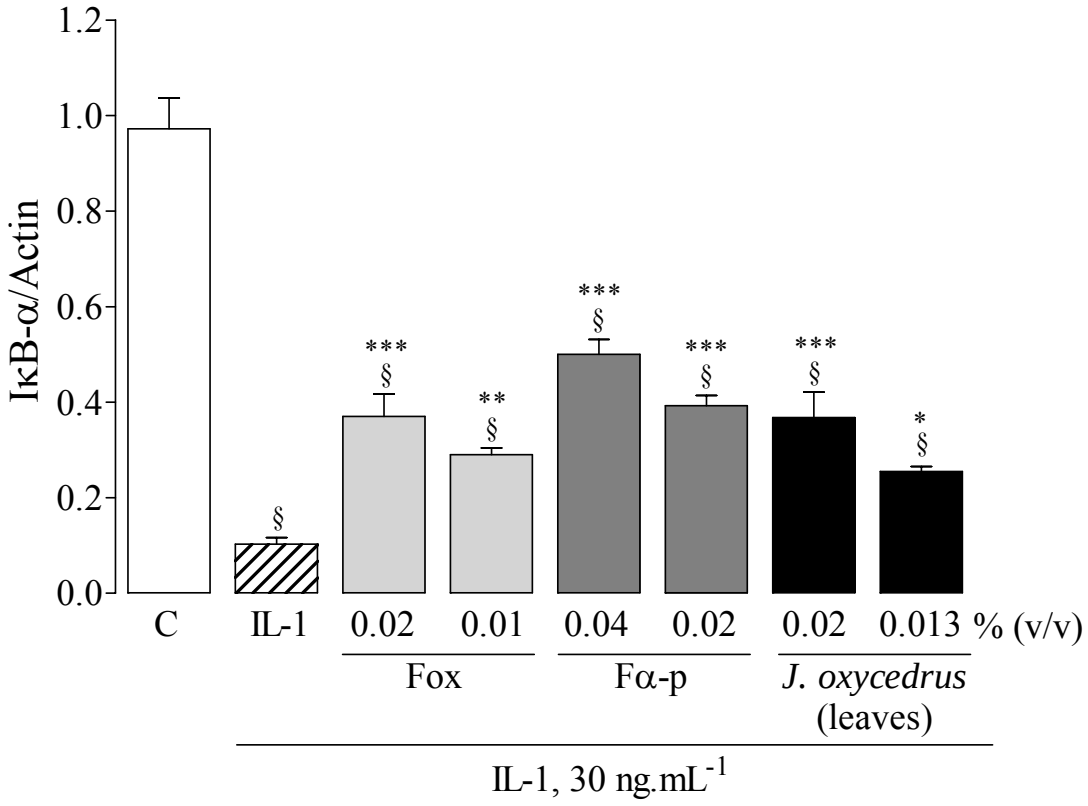

0.02 %
0.013 %
Relative NO production (%)
120
100
80
60
40
20
0
***
**
***
***
***
***
***
IL-1
M. piperita
L. luisieri
leaves
berries
J. oxycedrus
O. virens
Bay, 5 μM
IL-1, 30 ng.mL-1

Fig. 2


IκB-α/Actin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
§
**
§
*
§
C
IL-1
0.02
0.01
0.04
0.02
0.02
0.013
% (v/v)
Fox
Fα-p
J. oxycedrus
(leaves)
IL-1, 30 ng.mL-1

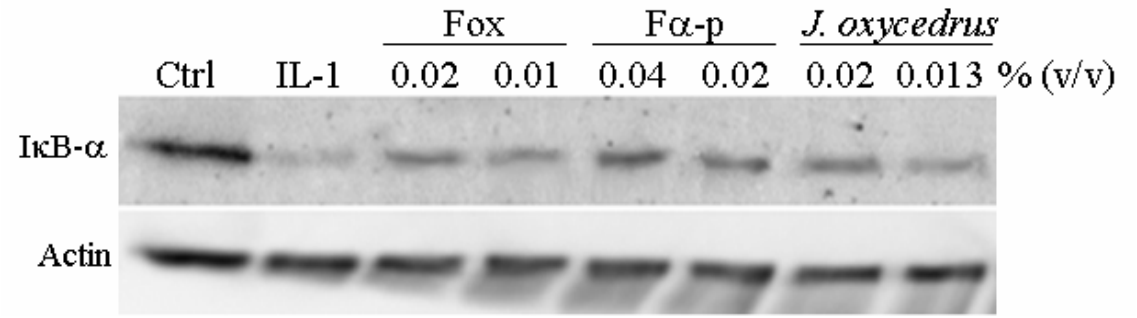
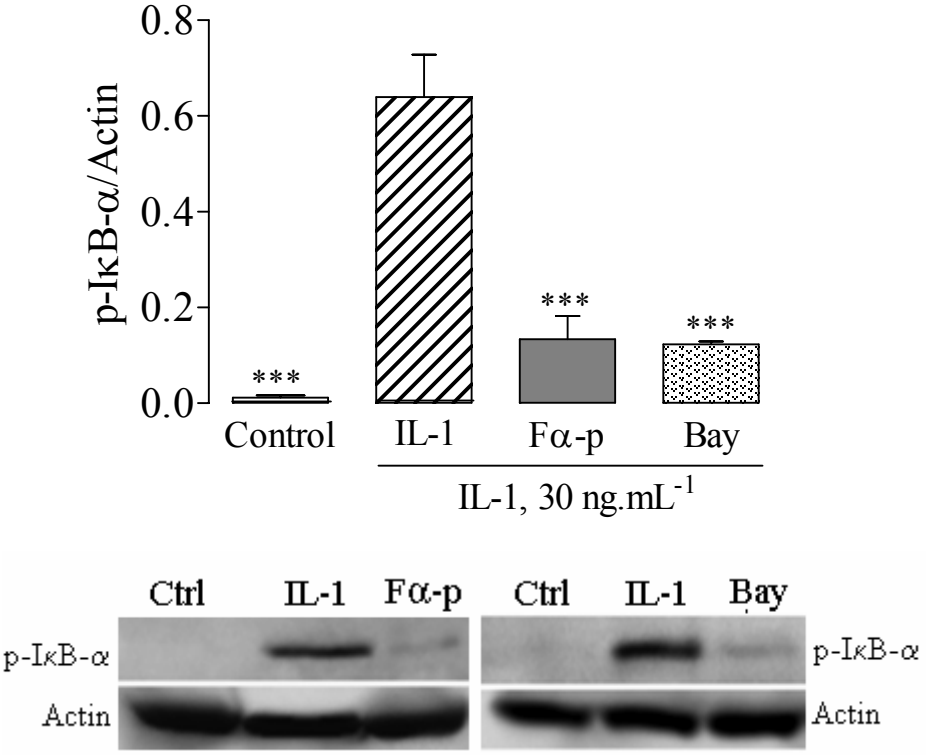
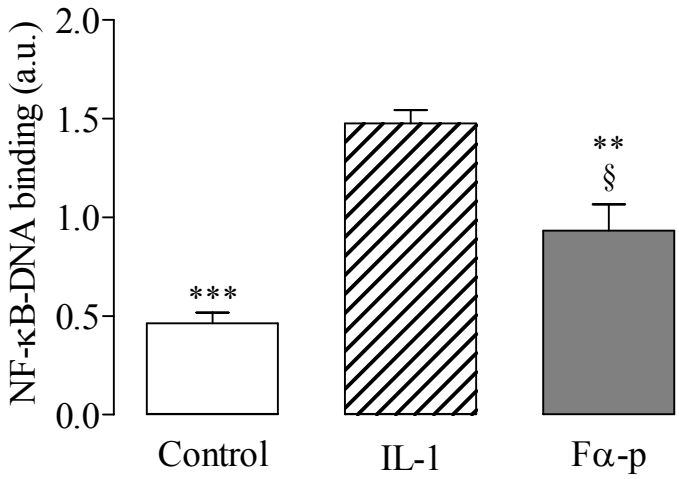

Fox
Fα-p
J. oxycedrus
Ctrl
IL-1
0.02
0.01
0.04
0.02
0.02
0.013
% (v/v)
IκB-α
Actin

Fig. 3

A


p-IκB-α/Actin
0.8
0.6
0.4
0.2
0.0
***
***
***
Control
IL-1
Fα-p
Bay
IL-1, 30 ng.mL-1
Ctrl
IL-1
Fα-p
Ctrl
IL-1
Bay
p-IκB-α
Actin
p-IκB-α
Actin

B


NF-κB-DNA binding (a.u.)
2.0
1.5
1.0
0.5
0.0
***
**
§
Control
IL-1
Fα-p