Composition and anti-inflammatory activity of extracts from three *Paeonia* species

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Abstract

*Ethnopharmacological relevance:* The roots of *Paeonia* species are widely used in Traditional Chinese Medicine for various diseases and, mainly, for their anti-inflammatory activity. *Aim of the study:* This research aimed to investigate the composition of extracts from three peonies, the herbaceous species, *P. lactiflora*, and the tree peonies of the section Moutan, *P. rockii*, and *P. ostii*, and their property to inhibit inflammation in bronchial epithelial and Human Embryonic Kidney (HEK 293/T) cells. *Materials and methods:* *Paeonia* apolar extracts were obtained by maceration from dried roots of three species using chloroform as extraction solvent. Composition and concentrations of the chemotaxonomic markers, benzoic acid and monoterpene derivatives, were established by a HPLC-DAD method. Total polyphenols content was determined by the Folin-Ciocalteau colorimetric assay. Anti-inflammatory activity was studied in bronchial epithelial cells affected by Cystic Fibrosis (CF), CuFi1, and the normal counterpart NuLi1. Cell proliferation and viability were evaluated by BrdU incorporation and MTT assays. The ability of the extracts to modulate cytokines (IL-6) and chemokines (IL-8 and RANTES) secretion was tested by ELISA specific immunoassays. Moreover, the anti-inflammatory activity was confirmed in HEK 293/T cells transfected with a NF-κB reporter plasmid determining NF-κB activity by luciferase assay. *Results:* The total polyphenol content, expressed as benzoic acid equivalents, ranged from 105.2-110.0 (P.rockii and P.ostii) to 347.0 (P. lactiflora) μg/mg. HPLC analysis indicated that the amount of benzoic acid and monoterpenes, paeoniflorigenone and benzoylpaeoniflorin, was almost superimposable (10.4%, 15.1%, and 6.5% w/w) in *P. rockii* and *P. ostii*, whereas *P. lactiflora* was characterized by a very high concentration of benzoic acid (34.5% w/w). All *Paeonia* extracts at sub-toxic concentrations strongly reduced RANTES production in unstimulated as well as TNFa-stimulated CuFi1 cells. Moreover, *P. lactiflora* also reduced IL-8 secretion. In NuLi1 cells *Paeonia* extracts determined only a reduction of RANTES, even though with a lesser extent. This interesting effect on chemokine secretion seems to be correlated to a direct inhibition of NF-κB activity, as revealed in HEK 293/T cells. *Conclusion:* Among the three peonies, *P. lactiflora*, possess anti-inflammatory activity due to inhibition of chemokines (IL-8 and RANTES) release, particularly in CF cells. Results confirmed the traditional anti-inflammatory use of peony and suggested a relevant potential application in the treatment of Cystic Fibrosis.

Keywords: P. rockii, P. ostii, P. lactiflora extracts; Cystic Fibrosis; Inflammation; Chemokines
Introduction

The genus *Paeonia* (Paeoniaceae family) comprises ca. 30 species divided into two groups: the herbaceous kinds, such as *P. lactiflora*, and the tree Peony, such as *P. rockii* and *P. ostii*, belonging to the section Moutan, distributed in Asia and Mediterranean region (Zhao et al., 2008). The dried root of *P. lactiflora* (*Radix Paeoniae*) is one of the most well-known traditional medicinal herb in China, Korea, and Japan for more than 1200 years, used for various biological activities (He et al., 2010; Li et al., 2012; Wu et al., 2010). The anti-inflammatory property of *P. lactiflora* have widely been reported (He and Dai, 2011; Jiang et al., 2011; Ou, 2008; Wu et al., 2010); extracts from peony have been included in health formulations for treating airway (Jiang et al., 2009; Liu and Ma, 2006; Ma et al., 2008; Zhong, 2013), gynecological (Wu and Chai, 2010; Zhao 2011) or skin inflammatory-based diseases (Lee et al., 2010; Liu et al., 2011); *Radix Paeoniae* is found to be effective in the treatment of rheumatoid arthritis (Zhang et al., 2008; Zhang and Dai, 2012).

The anti-inflammatory activity of *P. lactiflora* seem to be correlated to total glucosides (TGP) content of the roots, mainly to paeoniflorin. TGP acts suppressing the increase of intracellular calcium ion concentration and reducing the production of several inflammatory mediators such as prostaglandin E2, leukotriene B4, nitric oxide, reactive oxygen species, pro-inflammatory cytokines and chemokines (He and Dai, 2011; Kim and Ha, 2009; Li et al., 2011; Xu et al., 2007). Whereas *P. lactiflora* is widely studied, only the antioxidant and antimicrobial activity of a polar extract from *P. rockii* (Picerno et al., 2011) as well as cytotoxic and pro-apoptotic effects on human cancer cell lines apolar of an apolar extract were reported (Mencherini et al., 2011). No paper reports on activity of *P. ostii*, but it is considered similar in chemical composition as well as in morphology to *P. rockii* (Guo et al., 2002).

The traditional use of *Paeonia* as anti-inflammatory agent in the treatment of airway diseases (Jiang et al., 2009; Liu and Ma, 2006; Ma et al., 2008; Zhong, 2013), has led us to investigate the effect of *Paeonia* extracts on both normal (NuLit, wild type) and Cystic Fibrosis (CF) affected (CFTR ΔF508/ΔF508 mutant genotype, CuFi1) bronchial epithelial cells. CF is an autosomal recessive disease with high frequency among the white population, caused by mutations in the gene encoding Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein, a cAMP-regulated and ATP-gated chloride channel, regulating epithelial cell surface fluid secretion in respiratory and gastrointestinal tracts (Flume and Van Devanter, 2012). Inheritance of mutant CFTR alleles results in surface liquid depletion, defective mucociliary clearance, infection and inflammation leading to pulmonary failure (Ratjen, 2009). Defects in CFTR also perturb the regulation of many intracellular signaling pathways including the nuclear transcription factor-κB (NF-κB) activation and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK). The consequence seems to be the excessive production of NF-κB-dependent pro-inflammatory mediators such as cytokines IL-1 and IL-6 (Nichols et al., 2008) and chemokines IL-8 and RANTES (Regulated on Activation Normal T cells Expressed and Secreted) in the airways of CF patients (Lyczak et al., 2002; Jacquot et al., 2008). In this respect, herbal remedies may be of great interest in the management of CF inflammation (Borgatti et al., 2011; Nicolis et al., 2008; Prota et al., 2011).

The present research reports on the composition and anti-inflammatory properties of the apolar extracts from the roots of *P. lactiflora*, *P. rockii*, and *P. ostii* in CF and non-CF bronchial epithelial cells. Composition and concentrations of the chemotaxonomic markers, benzoic acid and monoterpenes derivatives, were established by a HPLC-DAD method. Total polyphenols content was determined by the Folin-Ciocalteau colorimetric assay. The effects of each extract on intrinsic as well as TNFa-stimulated inflammation were evaluated by determining IL-8, IL-6 and RANTES production in cell cultures supernatants. Furthermore, to study the direct effect of the extracts on NF-κB activation, NF-κB activity was determined in Human Embryonic Kidney (HEK 293/T) cells transfected with a NF-κB reporter plasmid. Chemokine release under unstimulated or TNFa-stimulated condition was also evaluated in the last cell model system.
Material and methods

Plant material
The roots of P. rockii ssp. rockii, P. ostii and P. lactiflora were purchased from Whitesessence Srl, Viterbo (Italy), in October 2005. Voucher samples (PR101, PO101, and PL101) were deposited at the Herbarium of the Department of Pharmacy, University of Salerno. The authentication of herbal materials was supported as a HPLC trace of the crude plant extracts.

Preparation of the extracts
Powdered, dried roots of P. rockii (2.73 kg), P. ostii (2.00 Kg) and P. lactiflora (2.20 Kg) were defatted with n-hexane and then extracted by exhaustive maceration with chloroform and dried under vacuum, giving 12.80, 9.20, and 17.20 g of dried extracts.

Quantitative HPLC analysis of the extracts
Quantitative HPLC analysis of each extract was conducted with the method validated for the analysis of the chloroform extract from P. rockii (Mencherini et al., 2011). Benzoic acid (from Sigma-Aldrich, Italy), benzoylpaeoniflorin (from 3B Scientific Corporation, USA), and paeoniflorigenone (isolated from the P. rockii and characterized by UV, NMR, and MS data) were used to prepare standard solutions at three concentration levels in the range 6.25-1.50 mg/mL for benzoic acid, 6.00-1.50 mg/mL for paeoniflorigenone, and 2.50-0.25 mg/mL for benzoylpaeoniflorin. The standard curves were analyzed using the linear least-squares regression equation derived from the peak area (regression equation \( y = 1006.3x + 53743 \), \( r = 0.9999 \) for benzoic acid; \( y = 700.64x + 12355 \), \( r = 0.9990 \) for paeoniflorigenone, and \( y = 1907.8x - 252.89 \), \( r = 1.000 \) for benzoylpaeoniflorin, where \( y \) is the peak area and \( x \) the concentration). The peaks associated with the three compounds were identified by retention time. UV and mass spectra were compared with the standards and confirmed by co-injections. Each crude chloroform-soluble extract was dissolved in MeOH and analyzed under the same chromatographic conditions.

Quantitative determination of total phenols
Each extract, dissolved in MeOH, was analyzed for its total phenolic content according to the Folin-Ciocalteau colorimetric method (Mencherini et al., 2011). Total phenols were expressed as benzoic acid equivalent (μg/mg extract).

Cell lines and culture conditions
CuFi1 and NuLi1 cell lines, were grown in human placental collagen type VI coated flasks (Sigma-Aldrich, Milan, Italy) in bronchial epithelial basal medium, BEBM (Clonetics, Lonza, Walkersville, Inc.) supplemented with BPE, Hydrocortisone, hEGF, epinephrine, insulin, triiodothyronine, transferring and retinoic acid (all from Lonza Walkersville, Inc).

Human Embryonic Kidney (HEK 293/T) cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin (50 U/mL) and streptomycin (50 μg/mL). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2.

Proliferation assay
Cell growth was assessed by using a colorimetric bromodeoxyuridine (BrdU) cell proliferation ELISA kit (Roche Diagnostics, Milan, Italy). Briefly, \(10^4\) cells were seeded into 96-well plates and left to adhere to the plate. Cells were then treated with increasing concentrations of P. lactiflora, P. rockii and P. ostii extracts ranging from 0.5 to 10.0 μg/mL for CuFi1 and NuLi1, and from 10.0 to 200.0 μg/mL for HEK 293/T cells for 24 and 48 h.

BrdU was added for the final 16 h (10 μM final concentration). At the end of the whole culture period, the medium was removed and the ELISA BrdU immunoassay was performed as described by the manufacturer.

The colorimetric reaction was stopped by adding \(H_2SO_4\), and the absorbance at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Milan, Italy) as previously described (Prota et al., 2011). Solvent alone (DMSO) did not give any significant result in all biological in vitro assays.

Viability assay
Cell viability was analyzed using the MTT assay. Briefly, cells were seeded in 96-well plate at the density of \(10^4\)/well, left to adhere to the plate and then treated with increasing concentrations of the extracts ranging from 0.5 to 10.0 μg/mL for CuFi1 and NuLi1, and from 10.0 to 200.0 μg/mL for HEK.
293/T cells for 24 h. 3-(4,5-Methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was added (0.5 mg/mL final concentration) to each well and incubated at 37 °C for additional 4 h. Formazan products were solubilized with 10% Triton X-100, 0.1 N HCl in 2-propanol. Absorbance was determined at 595 nm using a microplate reader (Bio-Rad Laboratories srl, MI, Italy) as previously described (Aquino et al., 2012).

**HEK 293/T cell transfection and NF-kB activity evaluation**

Transfection of HEK 293/T was carried out using CaPO₄ method. All transfections included IgkB-Luc reporter plasmid (500 ng), an internal control TK-renilla (20 ng) and a supplemental empty vector to ensure that the total amount of transfected DNA was kept constant in each culture dish. 24 h after transfection, cells were treated with *P. rockii* (30 µg/mL), *P. ostii* (30 µg/mL), *P. lactiflora* (10 µg/mL) extracts or DMSO (30 µg/mL) for 6 h. After the first 2 h of treatment, cells were stimulated with TNFα (10 ng/mL). 4 h later, cells were lysed using a Luciferase Passive Lysis buffer (P/N E1941, from Promega Corporation). Cell lysates were then harvested and assayed using the Dual Glo luciferase reporter assay system (Promega Corporation). Luciferase activity was measured using a multiplate reader (Promega Corporation), and values were normalized to the *Renilla* luciferase activity.

**Interleukin-8 (IL-8), interleukin-6 (IL-6) and RANTES release determinations**

1 x 10⁶ cells were plated. The cells were left to adhere to the plate and then were pretreated with *P. lactiflora*, *P. rockii* and *P. ostii* extracts dissolved in DMSO for 2 h and stimulated with TNFα 20 ng/mL (CuFiI and NuLiI cells) or 10 ng/mL (HEK 293/T cells) for 14 h. The cultured media were then collected, centrifuged for 5 min at 2000 rpm and the release of IL-8, IL-6 and RANTES was determined by Enzyme-linked immunosorbent assay ELISA (R&D Systems) following manufacture’s instructions. The used ELISAs for IL-8, IL-6 and RANTES were sensitive at 3.5 pg/mL, 0.7 pg/mL and 2.0 pg/mL respectively. Cytokine concentration in cell free media was calculated as pg/mL/10⁶ cells and expressed as the percentage of the control in absence of any stimulation or treatment.

**Statistical analysis**

Measurements were performed in triplicate, unless otherwise stated. Values were expressed as means of at least three experiments with three replicates each ± SD. Statistical differences between the treatments and the control were evaluated by the Student’s t-test A (P values less than 0.05 were considered statistically significant).

**Results and discussion**

**Preparation and analysis of the extracts**

Air-dried roots of *P. rockii*, *P. ostii* and *P. lactiflora* were defatted with n-hexane and extracted with chloroform, which was evaporated to dryness to give a chloroform-soluble dried extract. Each extract was characterized using a HPLC-DAD method. The main components were identified as benzoic acid and two monoterpenes, paeniflorigenone and benzoylpaeoniflorin, chemotaxonomic markers reported from a number of *Paonia* species. Their concentrations resulted almost superimposable in *P. rockii* and *P. ostii* extracts, and determined as 10.4%, 15.1%, and 6.5% w/w, respectively. On the contrary, *P. lactiflora* extract showed a higher concentration of benzoic acid (34.5% w/w), a lower concentration of paeniflorigenone (1.7% w/w) and no detectable amount of benzoylpaeoniflorin. This results was confirmed by the total phenolic content, as determined by the Folin-Ciocalteau method and expressed as a benzoic acid equivalent, which was similar in the tree peony, *P. rockii* and *P. ostii* (110.0 and 105.2 µg/mg, respectively) and three fold higher (347.0 µg/mg) in the herbaceous species, *P. lactiflora*.

**Cytotoxic effects of the extracts on CF and non-CF cells**

Preliminarly, BrdU incorporation was used to assess cytotoxicity of *Paonia* extracts in NuLiI cells derived from the bronchial epithelium of a non-CF patient and CuFiI cells from CF-affected patient (Prota et al., 2011). Results show no cytotoxic effects of each *Paonia* extract in the range from 0.5 to 4.0 µg/mL at 24 h treatment (Fig. 1), and a significant reduction of cell proliferation (about 38% at a concentration of 4.0 µg/mL) at 48 h in both CuFiI and NuLiI cells. All the extracts were highly cytotoxic above 8.0 µg/mL. At equivalent concentrations, *P. ostii* and *P. lactiflora* extracts seem the most cytotoxic in CuFiI cells (see Fig.1A at
8.0 and 10.0 µg/mL), while non-CF cells are more susceptible to the effects on cell growth induced by P. ostii and P. rockii (Fig. 1B, 8.0 and 10.0 µg/mL). MTT assay gave similar results in the reduction of cell viability corroborating results from the BrdU incorporation assay (data not shown).

**Anti-inflammatory effects of the extracts on CF and non-CF bronchial epithelial cells**

Since bronchoalveolar fluids (BALS) of CF patients contain increased levels of the chemotactic cytokine IL-8 and other pro-inflammatory cytokines and chemokines responsible for macrophage and neutrophil infiltration (Lyczak et al., 2002; Jacquot et al., 2008), we investigated the effects of the *Paeonia* extracts on the production of IL-8, IL-6 and RANTES. The anti-inflammatory effect of each extract was assessed in both CuFi1 and NuLi1 cells by analyzing cytokine (IL-6) and chemokines (IL-8 and RANTES) release after TNFα stimulation and treatment of cells with two non-toxic concentrations (2.5 and 4.0 µg/mL) at 24 h. Results obtained by treating CuFi1 cells with the different *Paeonia* extracts are shown in Fig. 2. Neither *P. rockii* nor *P. ostii* were able to inhibit the production of IL-6 and IL-8 at tested concentrations, while *P. lactiflora* reduced both interleukins in unstimulated as well as TNFα-stimulated CF cells. The effect was not marked even though statistically significant.

The activity observed for *P. lactiflora* extract in non-stimulated cells suggests that it might be used also in reducing CF-related intrinsic inflammation. Anyway it appears that all *Paeonia* extracts strongly reduced RANTES production in the presence and absence of TNFα stimulation suggesting a specific effect in CF cells.

In Fig. 3 are shown the results obtained by treating normal bronchial epithelial cells (NuLi1) with the same concentrations of each extract in the presence and absence of TNFα-induced inflammatory stimuli. As in CuFi1 cells, there was no effect of *P. rockii* and *P. ostii* extracts on IL-8 and IL-6 release, even though a slight reduction of IL-8 was observed at 4.0 µg/mL for *P. lactiflora*.

Interestingly, *Paeonia* treatments inhibit TNFα-induced stimulation of RANTES, thus suggesting that the extracts are selective in inhibiting RANTES production.

It is noteworthy that the levels of each cytokine is more elevated in CuFi1 cells than in NuLi1 both in unstimulated and stimulated conditions (see y axis in Fig. 2 and 3). All *Paeonia* extracts seem to reduce efficaciously RANTES production, particularly in CF cells characterized by chemokine over-expression. On the other hand, the extracts have no effects in absence of an inflammatory status such as in NuLi1 cells.

All together the data indicate that among the three peonies, *P. lactiflora* is the most efficacious in reducing inflammation; moreover, because of the selectivity of all extracts in inhibiting RANTES, you can suggest the use in other pathological conditions characterized by enhanced RANTES production such as lung cancer (Henriquet et al., 2007). Previously anti-inflammatory activity of *Paeonia* extracts and particularly of *P. lactiflora* Pall. has been reported in vitro and in vivo (He and Dai, 2011; Kim and Ha, 2009). However, this is the first report on peony anti-inflammatory properties in CF cells. The interesting activity observed for *P. lactiflora* extract might be correlate to its high polyphenol (347.0 µg/mg) and benzoic acid (34.5% w/w) content, as well as to characteristic monoterpenes constitutents. In fact, compounds derived from salicylic and benzoic acids are well known as standard anti-inflammatory drugs possessing ciclo-oxygenase inhibitory activity (Singh et al., 1984). Their ability of inhibiting prostaglandin production in mouse macrophages has been reported (Mehler et al., 1987), as well as a potent inhibition on superoxide anion generation and elastase release by human neutrophils (Chen et al., 2008). Also monoterpenes such as paeoniflorin are able to reduce the levels of TNFα, IL-6 and high-mobility group-box 1 protein in LPS-induced RAW264.7 cells (Kim and Ha, 2009); they may inhibit the IkB pathway, modulate NF-κB and have shown anti-inflammatory activity in various experimental animal models (Jiang et al., 2009; Tang et al., 2010).

**Anti-inflammatory activity of the extracts in HEK 293/T cells**

An increased expression of pro-inflammatory cytokines in CF patients, primarily due to iper-activated NF-κB signaling, has been reported (Jacquot et al., 2008; Verhaeghe et al., 2007). NF-κB activation is often assessed by analyzing the increased expression levels of downstream target
gene of transcription such as NFKB1A, encoding IkBα (Prota et al., 2011). Therefore, in order to further evaluate anti-inflammatory properties of Paeonia extracts, their effects on Human Embryonic Kidney (HEK 293/T) cells, transfected with a NF-κB reporter plasmid having five tandem copies of human NF-κB binding site fused to luciferase gene (Gautheron et al., 2010), were determined. Particularly, the experiments were conducted in the presence or not of the potent TNFα pro-inflammatory agent. Preliminary studies were carried out to determine the higher not cytotoxic concentrations of each Paeonia extract. Figure 4 shows cell proliferation curves of HEK 293/T after the treatments with increasing concentrations of each peony extract. Results demonstrate that P. rockii and P. ostii were not cytotoxic until 30 µg/mL in HEK 293/T cells, while P. lactiflora induced its effect already at 20 µg/mL. All the extracts determined a strong reduction of cell growth (90% of inhibition) at concentrations higher than 50 µg/mL. As shown, there is a substantial difference with respect to results obtained in bronchial epithelial CuFi and NuLi cells, since higher concentrations are required to induce any effect on cell growth in HEK 293/T cell line. On the other hands, MTT assay did not give comparable results showing no significant reduction of cell viability up to 100 µg/mL (data not shown) (Mencherini et al., 2011). All the above data indicate that Paeonia extracts reduced cell proliferation without affecting HEK 293/T cells viability suggesting that the extracts might cause a cell cycle arrest rather than cell death. Anyways this needs further investigations. To directly evaluate NF-κB activity, sub-toxic concentrations of each extract were used to treat TNFα stimulated and unstimulated HEK 293/T cells transiently transfected as reported in section 2.8. Results (Fig. 5) indicated that all Paeonia extracts were able to reduce TNFα induced activation of NF-κB, with P. ostii being the most effective in this cell model system. Furthermore, we determined chemokine production in the same cell model possessing a normal expression and activation of NF-κB signaling. A marked decrease in IL-8 and RANTES production following the treatments with Paeonia extracts was found (Fig. 6A and B). The inhibitory trend of the chemokines production was comparable to NF-κB-dependent transcription activation (Fig. 5). Therefore, the results obtained indicate that Paeonia extracts possess clear anti-inflammatory property and this activity might be ascribable to reduced NF-κB activation leading to an inhibition of pro-inflammatory mediators, particularly, the chemokine RANTES.

Conclusion

Among the extracts from three Paeonia species examined in the present study, similar composition and bioactivity is shown by the morphologically and cytologically closely related tree Peonies, P. rockii and P. ostii, belonging to the section Moutan DC. The anti-inflammatory potency of the herbaceous species P. lactiflora appears higher and correlated to a higher polyphenol content. P. lactiflora is confirmed to be an anti-inflammatory remedy able to reduce the expression of pro-inflammatory mediators such as RANTES and IL-8 proteins, over-expressed in inflammation and, particularly, in lung inflammation of CF patients. Interestingly, all three Paeonia extracts possess a selective activity on RANTES release, chemokine which is involved in various severe diseases (Henriquet et al., 2007; Elsner et al., 2004). Therefore, the anti-inflammatory traditional activity reported for Radix Paeoniae (P. lactiflora) in the traditional Chinese medicine seems to be partially shared with other species of the section Moutan.

References


Fig. 1. Cell proliferation analysis in CuFi1 (A) and NuLi1 (B) cells treated with different concentrations of Paeonia extracts. Cell proliferation was determined for 24 (upper panels) and 48 (lower panels) h by using a colorimetric bromodeoxyuridine (BrdU) cell proliferation assay. The histograms report the percentage of growing cells compared to controls (100% proliferation). All data are shown as mean ± SD of three independent experiments each done in duplicate. * P<0.05 and # P<0.01 vs. control.
Fig. 2. Effect of extracts from P. rockii, P. ostii and P. lactiflora on the release of IL-8 (A), IL-6 (B) and RANTES (C) in CuFi1 supernatants. Briefly cells were treated with each extract at indicated concentrations and then stimulated with TNF-α. After additional 14 h of incubation, supernatants were collected and cytokines production was detected by ELISA. Data are expressed as mean ± SD of three independent experiments, each done in duplicate. * P<0.05 and # P<0.01 vs. the corresponding control.

Fig. 3. Effect of extracts from P. rockii, P. ostii and P. lactiflora on the release of IL-8 (A), IL-6 (B) and RANTES (C) in NuLi1 supernatants. Cells were treated with each extract at indicated concentrations and then stimulated with TNFα. After additional 14 h of incubation, supernatants were collected and cytokines production was detected by ELISA. Data are expressed as mean ± SD of three independent experiments, each done in duplicate. * P<0.05 and # P<0.01 vs. the corresponding control.
**Fig. 4.** Effect of *P. rockii*, *P. ostii* and *P. lactiflora* on HEK 293/T proliferation at 24 (A) and 48 (B) h, by using BrdU cell proliferation assay. Results are shown as mean ± SD of three independent experiment each done in duplicate. *P*<0.05 and # *P*<0.01 vs. control.

**Fig. 5.** Effect of extracts from *P. rockii*, *P. ostii* and *P. lactiflora* on NF-xB transcriptional activation in HEK 293/T cells. Cells were treated with each extract at indicated concentrations and then stimulated with TNFα. NF- xB activation was measured using a luciferase assay as described in section 2.8. Fold activation is shown. *P*<0.01 vs. control.
Fig. 6. Effect of extracts from P. rockii, P. ostii and P. lactiflora on IL-8 and RANTES production in HEK 293/T cells. For chemokine determinations, cells were treated with extracts and stimulated with TNFα. Supernatants were collected and analyzed by ELISA. Data are expressed as mean ± SD of three independent experiments, each done in duplicate. * P<0.01 vs. control.