Lichen Metabolites Modulate Hydrogen Peroxide and Nitric Oxide in Mouse Macrophages

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The activities of perlatolic acid (**1**), atranorin (**2**), and lecanoric acid (**3**) and their derivatives, such as orsellinates and *β*-methyl orsellinates obtained by alcoholysis, were assessed for stimulation of the release of hydrogen peroxide and nitric oxide in cultures of peritoneal macrophage cells from mice. The hydrogen peroxide production was estimated by oxidation of phenol red, while the Griess reagent was used to determine the nitric oxide production. **1** and 4-methoxy-ethyl orsellinate (**XVII**) were the compounds that induced the greatest release of H_2O_2, whereas *n*-pentyl orsellinate (**IV**), *iso*-propyl orsellinate (**V**), *sec*-butyl orsellinate (**VI**), and **XVII** induced a small release of NO. These results indicate that lichen products and their derivatives have potential immune-modulating activities.

Key words: Lichens, Hydrogen Peroxide, Nitric Oxide

Introduction

Lichens are symbiotic associations of a fungus, which forms the main structure of the organism (thallus), with the photosynthetic cells of a green alga and/or cyanobacterium (González *et al*., 2005). They are slow-growing organisms and their secondary metabolites are mainly depsides, depsidones, dibenzofurans, xanthones, anthraquinones and terpene derivatives (Müller, 2001).

Lichen-forming fungi are unique organisms, producing biologically active metabolites with a great variety of effects, including antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic activities (Boustie and Grube, 2005; Carli *et al.*, 2009). However, only very limited numbers of lichen substances have been screened for their biological activities and their therapeutic potential in medicine.

Macrophages are the first cells to participate in the immunological response and they can be activated by a variety of stimuli such as bacterial components, cytokines and chemicals. Their principal functions include the phagocytosis of foreign particles, presentation of antigens to other cells, production of cytokines and the release of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) during the "oxidative burst" (Pick and Keisari, 1980; Pick and Mizel, 1981).

Hydrogen peroxide (H_2O_2) plays an important role in the functions of macrophages (Puri *et al*., 1994; Ramasarma, 1990). The products of the oxidative burst are used to kill phagocytosed pathogens and for the extracellular destruction of other cells. In addition to H_2O_2 and O_2^-, two other highly reactive oxygen species have been implicated in the killing process, *viz*. hydroxyl radicals (OH) and singlet oxygen. This coordinated sequence of biochemical reactions is initiated by an increase in oxygen uptake followed by the one-electron reduction of oxygen to the superoxide anion (O_2^-), with NADPH or NADH as the electron donor, catalyzed by an NAD(P)H oxidase. O_2^- is subsequently converted to H_2O_2 (Pick and Mizel, 1981).

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Nitric oxide (NO) is involved in many physiological processes in mammals, including neurotransmission, blood pressure control, inflammation, immune responses and also in the mechanism of defense against invasive organisms and tumours (Costa *et al.,* 2003). It is identified as the major effector molecule in the destruction of tumour cells by activated macrophages. The involvement of NO during nonspecific host defense, macrophage-mediated killing or inhibition (*in vitro* and *in vivo*) of the proliferation of microorganisms and tumour cells has been demonstrated in previous research. Furthermore, several studies have shown that activated macrophages produce NO, and RNI are believed to play a significant role in tumouricidal and microbicidal activities (Chul *et al*., 2001). Peroxynitrite ($ONOO^-$) is produced by spontaneous combination of NO and O_2^-, released, for example, during reperfusion (Ferdinandy, 2006). Of all the ROS and RNI generated during oxidative stress, peroxynitrite is one of the most reactive and toxic ones (Beckman and Koppenol, 1996). In sum, RNI and ROS represent important mediators of the microbicidal and antitumoural activity of macrophages, contributing to the nonspecific response of the host and the inflammatory process (Xie and Fidler, 1998).

As a continuation of our work aimed to discover phenolic compounds isolated from lichens and their cytotoxic effects on cultured macrophages (Santos *et al.*, 2004), the focus of the present study was to determine *in vitro* the cytotoxicity to mouse peritoneal macrophages of perlatolic acid (**1**), atranorin (**2**), and lecanoric acid (**3**) and their derivatives, obtained by alcoholysis, and to investigate the liberation of H_2O_2 and NO by these cells in their presence.

Experimental

Lichens

The lichen *Parmotrema tinctorum* (Nyl.) Hale was collected in the Brazilian state of Mato Grosso do Sul in July 2005; *Cladina confusa* (Sant.). Folmm & Ahti was obtained from a shop of decoration products. Specimens were identified by Dr. Mariana Fleig, Botany Department, Universidade Federal do Rio Grande do Sul (UFRGS), Brazil, and Dr. Marcelo P. Marcelli, São Paulo Institute of Botany, Brazil. A voucher specimen of each species was deposited in our laboratory for future reference.

Extraction and isolation of compounds

The dried lichen *C. confusa* (240.0 g) was extracted with hexane (4 × 0.6 L) and then with acetone (3 × 0.6 L) at room temperature, and. *P. tinctorum* (300.0 g) with chloroform (3 × 0.7 L), followed by acetone (3 × 0.7 L), at 45 ºC in a water bath. The extracts were concentrated *in vacuo*. The concentrated hexane extract from *C. confusa* (2.5 g) and the chloroform extract from *P. tinctorum* (0.5 g) were fractionated on a silica-gel chromatography column and eluted with a hexane/acetone gradient of rising polarity, to give perlatolic acid (**1**) (*C. confusa)* and atranorin (**2**) (*P. tinctorum*). The concentrated acetone extract of *P. tinctorum* was treated by the method described by Ahmann and Mathey (1967), in which lecanoric acid (**3**) was finally precipitated from an aqueous alkaline solution by acidification. The degree of purity of all the lichen compounds isolated was higher than 95%, as determined by TLC and NMR analyses. In the immunological assays the compounds were diluted in 4% DMSO (dimethylsulfoxide) and the lichen materials were used as soon as they were obtained.

Derivatives

The alcoholysis of lecanoric (**3**) and perlatolic (**1**) acids and purification of the products were conducted as described by Gomes *et al.* (2006) and Gianini *et al.* (2008). The 2,4-dihydroxy-6-methylbenzoates **I**–**VII** (orsellinates) and 2-hydroxy-4-methoxy-6-*n*-pentylbenzoates **VIII**–**XV** were obtained from **3** and **1**, respectively.

Methyl-*β*-methyl orsellinate (**XVI**) was obtained by alcoholysis of **2** with methanol. The mixture was fractionated on a silica-gel column with a mobile phase of chloroform/toluene 1:1 (v/v).

4-Methoxy-ethyl orsellinate (**XVII**) was obtained by methylation of ethyl orsellinate, as described by Elix *et al*. (1990). It was separated on a flash silica-gel column with hexane/ethyl acetate 70:30 (v/v).

The structures were confirmed by NMR spectroscopy (^1H and ^{13}C) (Huneck and Yoshimura, 1996). 2D NMR spectra (HMBC and HMQC) were also recorded.

In the immunological assays the derivatives were diluted in 4% DMSO and the lichen derivatives were used as soon as they were obtained.

Animals

Swiss mice (6–8 weeks old, weighting 18–25 g) were maintained in a polycarbonate box at (23 ± 1) ºC, (55 ± 5)% humidity, 10–18 circulations/h and a 12 h light/dark cycle, with water and food available *ad libitum*. At least five animals were used for each experiment. All animals were maintained and handled according to International Ethical Guidelines for the Care of Laboratory Animals (Faculty Ethics Committee # 06/2005).

Peritoneal exudate cells

Thioglycolate-elicited peritoneal exudate cells (PECs) were harvested from Swiss mice in 5.0 mL of sterile phosphate-buffered saline (PBS), pH 7.4. The cells were washed three times by centrifugation at 200 × *g* for 5 min at 4 °C with 3.0 mL PBS. The cells were then resuspended in 1.0 mL RPMI-1640 culture medium (Sigma) containing $2 \cdot 10^{-5}$ M β-mercaptoethanol (Sigma), 100 U/mL penicillin (Sigma), 100 U/mL streptomycin (Sigma), 2 mM L-glutamine (Sigma), and 5% fetal bovine serum (Sigma). This medium was denoted complete RPMI-1640 (RPMI-1640-C) medium and was used for cell counts in a Neubauer chamber (Boeco, Germany). For the proposed tests, the cell suspension was adjusted to a concentration of $5 \cdot 10^6$ cells.

MTT assay for cell viability

PECs ($5 \cdot 10^6$ cells/mL) were resuspended in RPMI-1640 culture medium containing 5% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, 100 μg/mL streptomycin and 50 mM 2-mercaptoethanol. The suspension (100 μL) and the test material (100 μL) were added to each well of a 96-well tissue culture plate, and the plates were incubated for 24 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed and the absorbance at 540 nm determined with an ELISA microplate reader (Multiskan Ascent Labsystems, Helsinki, Finland). Cells and culture medium (RPMI-1640) alone were used as a control representing 100% macrophage viability (Mosmann, 1983).

H_2O_2 measurement

PECs ($2 \cdot 10^6$ cells/mL) were cultivated as described above and suspended in a solution containing 140 mmol NaCl, 10 mmol potassium phosphate buffer, pH 7.0, 5.5 mmol dextrose, 0.56 mmol phenol red, and 0.01 mg/mL type II horseradish peroxidase, called complete buffer. Next, 100 μL of this suspension were added to each of the wells of a 96-well flat-bottom tissue culture plate and exposed to 50 μL of each compound. Phorbol 12-myristate 13-acetate (PMA) solution in phosphate buffer was used as a positive control and complete buffer alone as a negative control. The cells were incubated for 1 h at 37 °C in a mixture of 95% air and 5% CO_2. The reaction was stopped with 10 μL of 4 M NaOH and the absorption of the samples was read at 620 nm in an ELISA microplate reader (Multiskan Ascent Labsystems) against a blank containing phenol red solution and 4 M NaOH. The results, expressed in nmol of H_2O_2 per $2 \cdot 10^5$ PECs, were read from a standard curve established in each test with known molar concentrations of H_2O_2 in complete buffer. At least four experiments were used for the test (Pick and Keisari, 1980; Pick and Mizel, 1981).

NO measurement

PECs ($5 \cdot 10^6$ cells/mL) were resuspended in RPMI-1640-C culture medium, and 100 μL of this suspension were added to each well of a 96-well tissue culture plate, together with various derivatives obtained from lichens. *Escherichia coli* O26:B6 lipopolysaccharide (LPS) was used as a positive control and RPMI-1640 medium alone as a negative control. After incubation for 24 h at 37 ºC in humid atmosphere, 50-μL aliquots of culture supernatant were mixed with 50 μL of Griess reagent (1% w/v sulfanylamide, 0.1% w/v naphthylethylenediamine, and 3% H_3PO_4), incubated at room temperature for 10 min and the colour reaction was determined at 540 nm in an ELISA microplate reader (Multiskan Ascent Labsystems). Supernatants from quadruplicate cultures were assayed in four experiments and reported as µmol NO per $5 \cdot 10^5$ cells, calibrated against solutions of known $NaNO_2$ concentration (Green *et al.*, 1982).

Statistical analysis

Data are expressed as mean ± standard deviation (SD), and Dunn's *t*-test (Microcal Origin 5.0) was used to determine the significance of the differences between the control and experimental compounds.

Results and Discussion

Perlatolic acid (**1**) was isolated from *C. confusa* and atranorin (**2**) and lecanoric acid (**3**) from *P. tinctorum*. The derivatives **I**–**XVII** were obtained by alcoholysis of **1**, **2** and **3** (Fig. 1).

To examine the cytotoxic effects, the viability of PECs was measured by the MTT assay after their treatment with the compounds at various concentrations (Table I). The data presented in this table show the concentration used in the subsequent tests, at which the viability was 50% of the cells or higher (Fig. 2).

In the MTT assay, the yellow tetrazolium salt MTT is taken up into cells and reduced by a mitochondrial dehydrogenase to yield a purple formazan that is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The ability of cells to reduce MTT provides an indication of the mitochondrial integrity and activity which, in turn, may be interpreted as a measure of viability and/or cell number (Mosmann, 1983).

RNI and ROS represent important mediators of the microbicidal and antitumoural activity of the macrophages and contribute to the nonspecific immune response of the host and the inflammatory process (Xie and Fidler, 1998; Carlos *et al.*, 2003; Carli *et al.*, 2009). The macrophages act as a first line of defense of the host and release a great number of factors, including NO and H_2O_2,

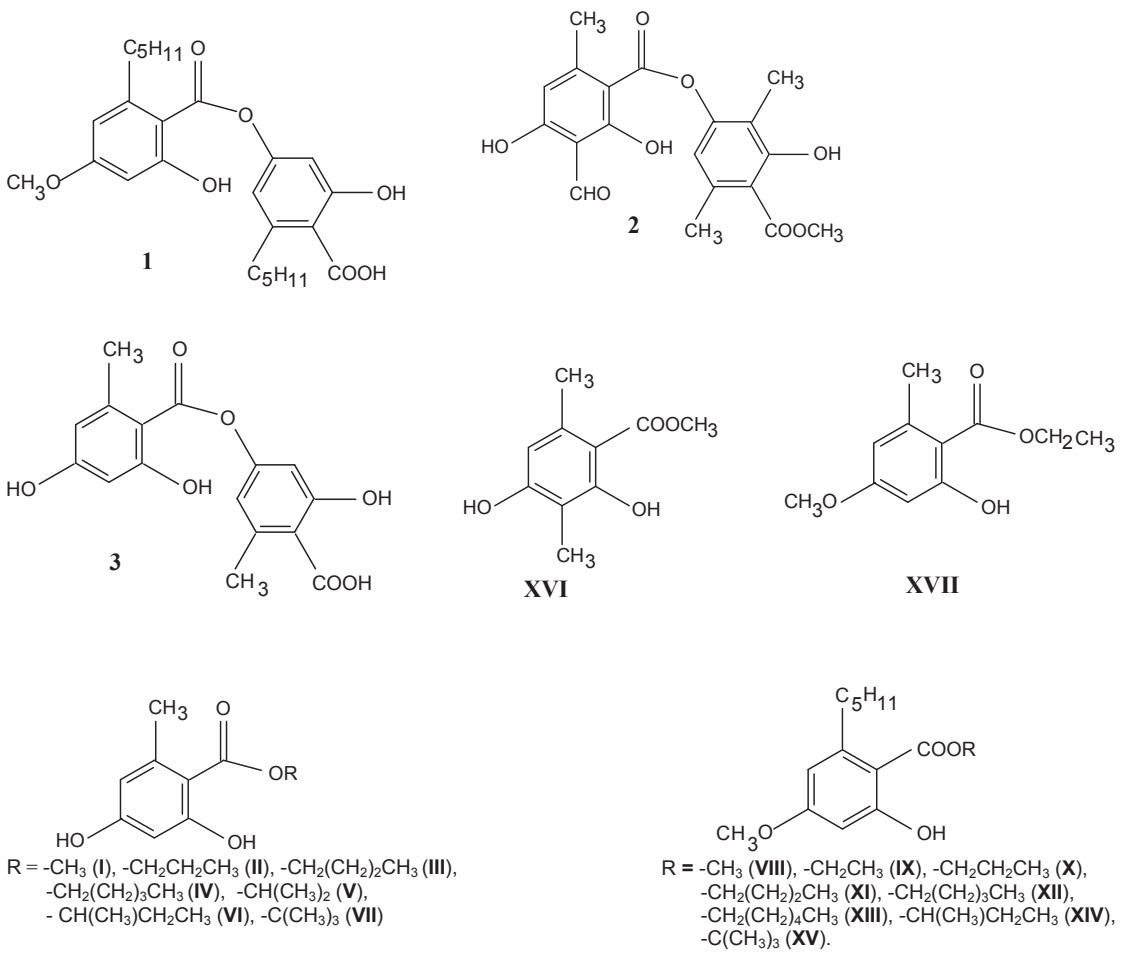

Fig. 1. Chemical structures of the compounds assayed for viability of peritoneal macrophages.

Table I. Compounds and their concentration used in the tests.

Compound	Test concentration [mg/mL]
Perlatolic acid (**1**)	0.031
Methyl orsellinate (**I**)	0.25
n-Propyl orsellinate (**II**)	0.0625
n-Butyl orsellinate (**III**)	0.031
n-Pentyl orsellinate (**IV**)	0.031
iso-Propyl orsellinate (**V**)	0.0625
sec-Butyl orsellinate (**VI**)	0.031
tert-Butyl orsellinate (**VII**)	0.031
Methyl-2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**VIII**)	0.0625
Ethyl-2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**IX**)	0.125
n-Propyl-2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**X**)	0.0156
n-Butyl-2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**XI**)	0.0625
n-Pentyl-2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**XII**)	0.031
n-Hexyl-2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**XIII**)	0.031
sec-Butyl-2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**XIV**)	0.031
tert-Butyl-2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**XV**)	0.25
Methyl-*β*-methyl orsellinate (**XVI**)	0.0625
4-Methoxy-ethyl orsellinate (**XVII**)	0.5

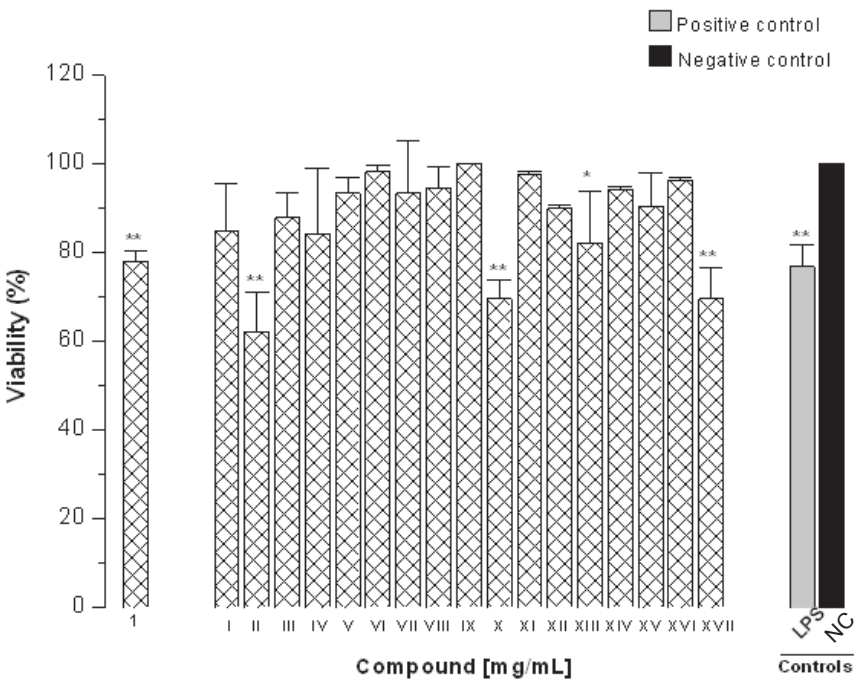

Fig. 2. Viability of peritoneal macrophages activated by compounds isolated from the lichens. PECs ($5 \cdot 10^6$ cells/mL) were resuspended in RPMI-1640-C medium and the suspension (100 μL) was incubated with the test compounds (100 μL) for 24 h. LPS solution was the positive control and the medium (RPMI-1640) alone was the negative control (NC, 100% viability). MTT solution was added (100 μL) to the cells and the plate was incubated for 3 h. Each bar represents the mean ± SD of five experiments. *$p < 0.5$ and **$p < 0.01$, significantly different from the negative control.

powerful mediators of the immune and inflammatory responses (Laskin and Pendino, 1995).

H_2O_2 plays a variety of roles in the body, for example as signaling molecule, intermediate in metabolism, cytotoxic agent in host defense and cytotoxic agent in pathology (Halliwell *et al.*, 2000; Samali *et al.*, 1999). H_2O_2 has important functions in the intracellular and intercellular reactions of various cells. Under normal physiological conditions, it is generated in small quantities and is rapidly used or degraded, but longer exposures to high concentrations of this molecule can destroy biological structures and lead to irreversible cell damage. H_2O_2 is toxic to cells and is indeed responsible for killing internalized bacteria in phagocytosis (Ramasarma, 1990).

The release of H_2O_2 was measured as the amount of substance (in nmol) produced by $2 \cdot 10^5$ cells after 1 h of incubation with the compounds. Among the tested compounds, perlatolic acid (**1**) stimulated the release of (10.48 ± 0.96) nmol H_2O_2, and 4-methoxy-ethyl orsellinate (**XVII)** led to the release of (13.34 ± 1.17) nmol H_2O_2. It was observed that the compounds *n*-pentyl orsellinate (**IV**), *tert*-butyl orsellinate (**VII**) and methyl-*β*-methyl orsellinate (**XVI**) stimulated a more modest release of this mediator, while PMA (positive control), a powerful immunostimulator, was able to release (26.73 ± 1.23) nmol H_2O_2. The quantity of H_2O_2 released in response to compounds **VIII**–**XV**, synthesized by alcoholysis of perlatolic acid (**1**), was very low**,** though even a small amount more than the negative control is an important result (see Fig. 3), as it may modulate the immune response without causing cytotoxicity. The relationships between the chemical structures of the orsellinates **IV**, **VII** and **XVI** obtained by alcoholysis of lecanoric acid (**3**) are shown in Fig. 1. The aromatic ring of **IV** and **VII** bears hydroxy groups at positions 2 and 4 and a methyl group at position 6. The difference among them lies in the ester chain. Results show that chain elongation of the series of 2,4-dihydroxy-6-*n*-methyl benzoates (methyl to pentyl orsellinates, **II**–**IV**) and compounds with branched chains *(iso*-propyl, *sec*-butyl and *tert*-butyl orsellinates, **V**–**VII**) lead to higher lipophilicity and induce a greater release of H_2O_2. However, compounds **VIII** to

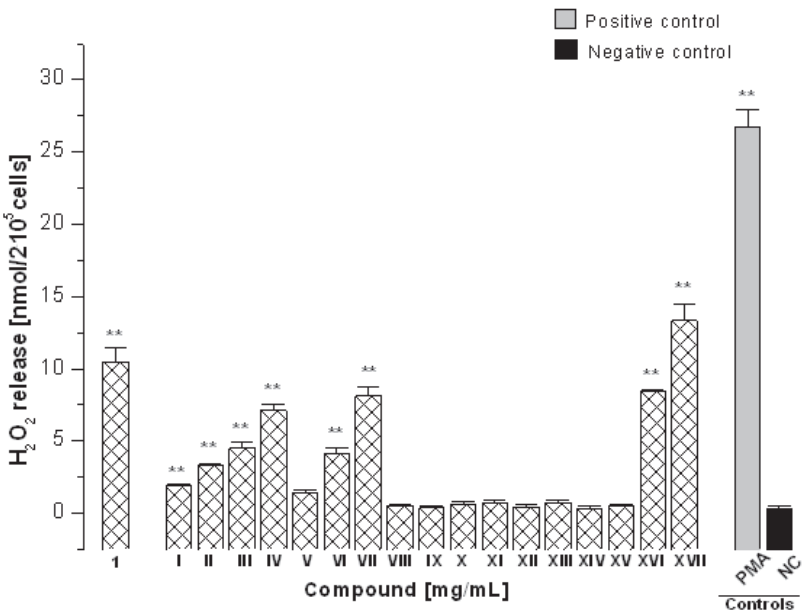

Fig. 3. Hydrogen peroxide production by PECs in the presence of compounds isolated from the lichens. Adherent cells (100 μL PECs suspended at $2 \cdot 10^6$ cells/mL) were incubated for 1 h with 50 μL of test solution. PMA was the positive control and the medium (RPMI-1640) alone was the negative control (NC). The reaction was stopped with 10 μL of 4 M NaOH. Each bar represents the mean ± SD of five experiments. ** $p < 0.01$, significantly different from the negative control.

XV, obtained by alcoholysis of perlatolic acid (**1**), were weakly active. These compounds differ from orsellinates in having a methoxy group at C-4 and an *n*-pentyl chain at C-6. Thus, the esters of 2-hydroxy-4-methoxy-6-*n*-pentylbenzoates are more lipophilic than the orsellinates and the lower activity may be related to the *n*-pentyl group at C-6 in the aromatic ring of these compounds. The good activity shown by methyl-*β*-methyl orsellinate (**XVI**) suggests that the methyl group at C-3 also causes an increase in the release of H_2O_2. The greatest activity was shown by 4-methoxy-ethyl orsellinate (**XVII**) and this may be explained by the lower polarity of this compound. These results suggest that the activity of these compounds depend on an equilibrium between the hydrophilic and lipophilic properties.

Reactive oxygen metabolites (like H_2O_2) have been suggested as potentially important signaling molecules in both intra- and intercellular reactions in a number of different cell types. It has been well documented that reactive species such as H_2O_2 are produced by activated inflammatory cells (Rochele *et al.*, 1998).

NO produced by activated macrophages plays a role in both inflammatory and anti-inflammatory processes. It is involved in the killing or inhibition of the proliferation of microorganisms, destruction of tumour cells by activated macrophages, and nonspecific host defense (Stuehr and Nathan, 1989; Hibbs *et al.*, 1987). At the site of an acute inflammatory reaction, all the conditions are met for the generation of NO and for a role of this compound as an inflammatory mediator (Moncada, 1999). NO is a messenger molecule involved in the regulation of diverse physiological processes and the cytotoxic actions of immunological cells. When found in high concentrations, this mediator can become involved in numerous pathological processes (Hibbs *et al.*, 1987; Maia *et al.*, 2006).

The compounds *n*-pentyl orsellinate (**IV**), *iso*-propyl orsellinate (**V**), *sec*-butyl orsellinate (**VI**) and 4-methoxy-ethyl orsellinate (**XVII**) stimulated a small release of NO (Fig. 4) compared to the positive control LPS. However, the quantities are significant, showing that these compounds are able to stimulate the immune system without

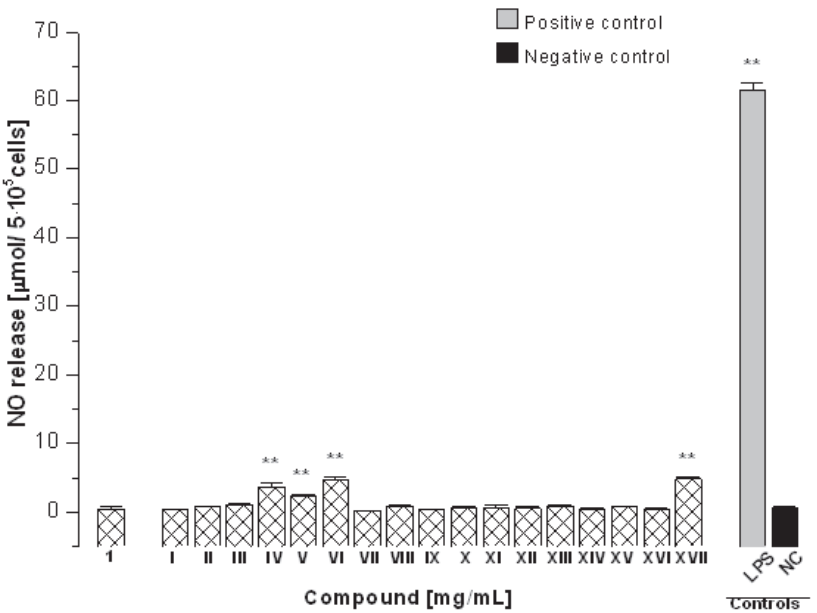

Fig. 4. Nitric oxide production by PECs in the presence of compounds isolated from the lichens. Adherent cells (100 μL PECs suspended at $5 \cdot 10^6$ cells/mL) were incubated for 24 h with 100 μL of test solution. Cell-free supernatant was drawn off and mixed with Griess reagent. LPS was the positive control and the medium (RPMI-1640) alone was the negative control (NC). Each bar represents the mean ± SD of five experiments. ** $p < 0.01$, significantly different from the negative control.

causing cell damage due to the cytotoxic effect of NO at high concentrations (Park, 2006).

The methyl (**I**), *n*-propyl (**II**), *n*-butyl (**III**) and *n*-pentyl orsellinates (**IV**) showed an increasing release of NO with increasing lipophilicity, although the amount of NO produced was little compared to the positive control. The esters of 2-hydroxy-4-methoxy-6-*n*-pentylbenzoates **VIII**–**XV** and methyl-*β*-methyl orsellinate (**XVI**) were less active, all of them showing practically the same activity.

In conclusion, the best activities for the H_2O_2 release were observed with compounds **IV**, **VII**, **XVI** and **XVII**, while for NO release the best activities were observed with compounds **IV**, **V**, **VI** and **XVII**, in comparison with PMA and LPS as positive controls. These results indicate that certain compounds isolated from lichens and their derivatives have potential immune-modulating activities.

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