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Anti-infective Activities of *Pelargonium sidoides* (EPS® 7630): Effects of Induced NO Production on *Leishmania major* in Infected Macrophages and Antiviral Effects as Assessed in a Fibroblast-Virus Protection Assay

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Abstract

EPs® 7630 is an aqueous-ethanolic extract of the roots of *Pelargonium sidoides*, employed in the treatment of upper respiratory tract infections. Its anti-infective activity is supposed to be associated with the activation of the nonspecific immune system. Using *Leishmania major* GFP-infected murine BMMΦ, the NO production of EPs® 7630-activated macrophages was correlated with the reduction of the GFP signal measured at single cell levels using flow cytometry. The anti-infectious effect of EPs® 7630 (3–10 µg/mL) on its own (NO production: 4–13 µM; signal reduction: 25–73%) was less prominent than that in combination with IFN-γ (100 U/mL) (NO production: 20–27 µM; signal reduction: 35–78%). Furthermore, supernatants of EPs® 7630-stimulated BMMΦ (10 µg/mL) significantly reduced the cytopathic effect of EMCV on L929 fibroblasts (antiviral activity 80 U/mL) when compared with an IFN-γ standard (100 U/mL). Direct addition of EPs® 7630 to L929 did not mediate cytoprotective effects. The antiviral components induced in BMMΦ by EPs® 7630 remain to be identified. Detection of any IFNs by ELISA was unsuccessful, which may be due to their very low concentrations in cell supernatants. The current data provide convincing support for the induction of anti-infectious responses by EPs® 7630.

Abbreviations

BMMΦ: bone marrow-derived macrophages

rINF: recombinant interferon

EMCV: encephalomyocarditis virus

L-NMMA: NG-monomethyl-L-arginine

CPE: cytopathic effect

GFP: green fluorescent protein

PI: propidium iodide

Introduction

EPs® 7630 is an aqueous-ethanolic extract from the roots of *Pelargonium sidoides* (Geraniaceae), exclusively contained in Umckaloabo®, an herbal medicinal product marketed by Spitzner Arzneimittel, Ettlingen, Germany. Although a number of clinical studies documented the effectiveness and safety of this herbal remedy for the treatment of upper respiratory tract infections [1–6], its mode of action is still insufficiently understood. We recently reported that murine BMMΦ incubated with 30 µg/mL of EPs® 7630 for 6 h showed a significant increase in the production of proinflammatory mediators such as nitric oxide (NO), IL-1α, TNF-α and IL-12. In addition, this effect was significantly enhanced in BMMΦ infected with *Listeria monocytogenes* and exposed to EPs® 7630 [7].

The present paper is a continuation of our work and provides further insight into the anti-infectious principles of EPs® 7630. BMMΦ treated with EPs® 7630 (10 µg/mL) were effectively activated to kill intracellular GFP-transfected *Leishmania major* parasites (*L. major* GFP) as assessed by a recently established FACS-based method [8]. Taking into account previous studies [9], this finding suggests that EPs® 7630 exerts its anti-infectious activity against a broad spectrum of pathogens. Furthermore,

significant inhibition of the cytopathic effect of EMCV on a sensitive murine fibroblast L929 cell line gives credence to an improved antiviral protection in afflicted patients treated with this herbal medicine.

Material and Methods

Plant material

EPs® 7630 (batch Ch.-No. 007) is an aqueous ethanolic (11% m/m) extract of *Pelargonium sidoides* DC roots with a yield of 9–11% [10] and was kindly provided by Dr. Willmar Schwabe GmbH & Co. The dried extract was dissolved in DMSO (Merck) at a concentration of 20 mg/mL for use as stock solution.

Mice

Six- to 12-week-old female C57BL/6 mice were bred at and supplied by Zentrale Versuchstierzucht, Federal Institute for Risk Assessment (BfR), Berlin, Germany. Animal care and the experimental procedures were in accordance with the institutional guidelines and German law. The animal experiments were approved by the Landesamt für Gesundheit und Soziales, Berlin on April 27, 2004 under the number 01707/04.

Pathogens and cell lines

Green fluorescent protein transfected *L. major*, strain LT 52, clone CC-1pXG-GFP (*L. major* GFP; [11]), stable under the influence of geneticin, were kindly provided by S.M. Beverley (Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO, USA). *L. major* GFP were cultured in *Leishmania* growth medium consisting of RPMI 1640 medium (Gibco-Invitrogen), 5% heat-inactivated fetal calf serum (FCS), penicillin (100 U/mL; PAA Laboratories), streptomycin (100 µg/mL; PAA), Na-pyruvate (10 mM; Sigma-Aldrich Chemie), HEPES (25 mM; Gibco), Geneticin (25 µg/mL; G418, Gibco; only with *L. major* GFP), and hemin (0.25% v/v solution B of Hoesmem II medium; [12]) at 25°C in a humidified atmosphere with 5% CO₂. Murine EMC-virus (single stranded RNA poliovirus, Picornaviridae) and the murine L929 fibroblast cell line, selected for IFN sensitivity, were a kind gift of the late Dr. M.L. Lohmann-Matthes (Fraunhofer Institut für Toxikologie, Hannover, Germany).

Bone marrow derived macrophages (BMMΦ)

Mice were killed by cervical dislocation and bone marrow was obtained by flushing the femura and tibiae with cold Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS). BMMΦ were produced as described previously [13] with minor modifications. Briefly, the pooled bone marrow precursor cells were matured in polystyrene petri dishes (Sarstedt) for 6–8 days in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Bio Whittaker), 5% heat-inactivated horse serum (Gibco), 15% fibroblast (GM-CSF transfected L929 cells) conditioned medium, penicillin (100 U/mL), streptomycin (100 µg/mL), D-glucose (20 mM, Sigma), Na-pyruvate (10 mM), and HEPES (25 mM) at 37°C in a humidified atmosphere with 5% CO₂. For harvesting, the medium was replaced twice by cold PBS, thereby removing most nonadherent cells and debris. BMMΦ were chilled to 4°C for 1 h, rinsed off the plastic with a 5-mL pipette and washed by centrifugation (250 × g, 10 min, 4°C). The viable cells, as assessed by trypan-blue exclusion, were counted and kept at 4°C until use.

Fibroblast-virus protection assay (IFN activity)

BMMΦ (1.0 × 10⁶ cells/mL) were incubated for 24 h with EPs® 7630 (10, 5, 1 µg/mL, respectively), in R5 medium consisting of RPMI 1640 (Gibco-Invitrogen) plus 5% heat-inactivated fetal calf serum (FCS), HEPES (25 mM, Gibco), Na-pyruvate (10 mM, Sigma-Aldrich). Untreated cells served as the negative control, LPS (1 ng/mL; obtained from *Escherichia coli*; Sigma-Aldrich) treated BMMΦ as the positive control, and DMSO (0.5%) as the vehicle control. Experimental culture supernatants were collected and stored at -70°C until use. Dead cells were detected by flow cytometry after staining with PI [14] (PI-positive cells were below 10% in all experiments). Interferon activity was assessed by observing the protection of murine L929 fibroblasts against the cytopathic effect induced by infection with EMCV. For this, a monolayer of EMCV- and IFN sensitive murine L929 fibroblasts (1.0 × 10⁴ cells/well) was incubated overnight in R5 medium alone (negative control) or with serial 1:2 dilutions of the experimental culture supernatants described above. Serial 1:2 dilutions of murine rIFN-γ (100 U/mL; Genentech) served as a reference for cytoprotection. Then 100 µL of a pretested EMCV suspension (3680 PFU/mL in R 2.5 medium consisting of RPMI 1640 plus 2.5% heat-inactivated FCS, 25mM HEPES, 10mM Na-pyruvate, 100 µg/mL streptomycin and 100 U/mL penicillin [both PAA

Laboratories]) was added to each well and cell destruction was monitored microscopically in the control samples. At this point, when non-protected cells had disintegrated due to the cytopathic effect of the virus, the relative number of viable cells per well was assessed colorimetrically by the crystal violet method. For this, cells were fixed and stained for 30 min using crystal violet solution (0.1% crystal violet, 2% ethanol, 10% formaldehyde in PBS). Excess of the dye was washed off with water. After drying, the remaining dye crystals were dissolved in acetic acid (33% acetic acid in PBS) and the preparations subjected to spectral photometric analysis. Relative optical density, correlating with the relative amount of viable cells/well was measured at 595 nm using an ELISA reader (SpectraFluor) and Magellan V5.03 software (both Tecan). The cytoprotective effect was compared to that of recombinant murine IFN- γ . Antiviral activity was expressed in U/mL, defined as reciprocal value of the supernatant dilution that would inhibit 50% of the cytopathic effect. These values were correlated with the IFN standard to account for any fluctuation in assay sensitivity. This functional assay does not discriminate between IFN- α , IFN- β , and IFN- γ [15]. In a modified procedure, L929 fibroblasts were directly incubated with EPs@ 7630 samples overnight.

Interferon ELISA

Production of murine IFN- α (α A α 1 α 4 α 5 α 6, and α 9 according to the manufacturer) and IFN- β (PBL Biomedical Laboratories) was determined by ELISA according to the manufacturer's protocol, with detection limits of ca. 16 pg/mL. For IFN- γ (clone R4-6A2 for coating; biotinylated clone AN-18 for detection), high protein binding 96-well polystyrene microtiter plates (Immunomodule F16 Maxisorb; Nunc) were coated overnight at 4°C with antibody and washed four times with PBS containing 0.05% (v/v) Tween-20. Protein binding capacity was saturated using PBS containing 10% FCS (v/v) for 1 h at room temperature. Serial dilutions of recombinant standard cytokine and test supernatants, respectively, were loaded and incubated overnight at 4°C, followed by the addition of a biotinylated detecting antibody. After 1 h at room temperature, streptavidin-coupled horseradish peroxidase was added, and cells incubated for one more hour. Then microtiter plates were washed repeatedly to minimize background and a 3,3',5,5'-tetramethylbenzidine (0.1 mg/mL; Sigma-Aldrich) substrate solution was added. After 15 min, the conversion into a light blue color by the horseradish peroxidase was stopped by adding 2 N H₂SO₄ (100 μ L/well). The absorbance was read immediately at 450 nm using an ELISA reader (SpectraFluor) and Magellan V5.03 software (Tecan).

In vitro infection of BMM Φ with Leishmania GFP parasites

In vitro infection of BMM Φ with L. major GFP and activation of macrophages with IFN- γ (100 U/mL) and IFN- γ (100 U/mL) plus LPS (10 ng/mL) have been fully described elsewhere [8]. Noninfected and non-treated as well as infected non-treated BMM Φ were used as negative controls and amphotericin B (Sigma Aldrich), a standard antileishmanial drug, was used as the positive control of parasite killing. For treatment of cells, R10 medium was completely replaced by 2 mL R10 medium containing different concentrations (0.1– 10 μ g/mL) of EPs@ 7630. After 48 h of incubation, BMM Φ were pelleted (200 \times g, 10 min, 4°C), the experimental culture supernatants were collected and stored at – 70°C until use.

Flow cytometric measurements of Leishmania GFP-infected BMM Φ

After treatment, cells were resuspended in 300 μ L of PBS and stored on ice to facilitate detachment of the remaining adherent cells. To discriminate dead from living cells, propidium iodide (PI; Sigma) solution was added ca. 30 s before FACS measurement, giving a final concentration of 0.33 μ g PI/mL. Information on the rate of infection was obtained by comparing the GFP-signal with that of noninfected cells. A total of 30000 events were counted using a FACS Calibur cytometer and CellQuest Pro software (both BD Biosciences).

Griess-assay for NO-production by activated BMM Φ

After incubating, the supernatants of EPs@ 7630 treated BMM Φ cultures collected as a source of secreted NO were subjected to the Griess assay for determining the total nitric oxides produced by activated BMM Φ [16]. For this, 100 μ L of the supernatant were mixed with equal volumes of freshly prepared Griess reagents [1 :1 v/v mixture of 1% sulphanilamide, 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 3% H₃PO₄ (Sigma Aldrich)]. After 5 min at room temperature, the absorbance was measured at 550 nm using an ELISA reader (SpectraFluor; Tecan), and the relative NO concentrations were calculated from a standard curve generated with NaNO₂.

Statistical analysis

Data are expressed as the mean \pm standard deviation (SD) of at least two independent experiments. Statistical analysis was performed by the "test of two means" using OpenStat program (<http://www.statpages.org/miller/openstat/>). P values < 0.05 were considered to be statistically significant.

Results and Discussion

Experimental infection of macrophages constitutes a particularly versatile model for assessing the immunoregulation that occurs during a cell-mediated response to an intracellular pathogen. Using obligate intracellular *Leishmania* parasites, the potential of EPs® 7630 for activating effective cytotoxic host defense mechanisms was evaluated. The elimination of the pathogen in macrophages was determined applying a flow cytometric (FACS) assay with transgenic *L. major* GFP. This elegant method allows to measure simultaneously antiparasitic effects, host cell viability, and immunological response markers at the single cell level [8]. *Leishmania* parasites exist as extracellular promastigotes within an insect vector and as intracellular amastigotes in phagocytes of vertebrate hosts. The latter form is of clinical and pharmacological importance. Due to the individual biological cycle, initial experiments were aimed at evaluating possible effects of the extract against promastigotes.

Extracellular *L. major* GFP were incubated with 10 µg/mL of EPs® 7630 or 1% DMSO (vehicle control), respectively. After 48 h, the GFP signal as a measure of the viability of promastigotes was analyzed by FACS, while PI-positive events indicated dead parasites in a complementary manner. EPs® 7630 did not show any detectable effect on the viability of *L. major* GFP promastigotes (Fig. 1). On the other hand, exposure to the antileishmanial drug amphotericin B (1 µM) significantly reduced the GFP signal and increased, in parallel, the number of PI-positive (dead) cells. This finding demonstrates that EPs® 7630 does not directly affect the viability of *L. major* GFP promastigotes. Next, EPs® 7630 was tested for inducing microbicidal mechanisms in infected macrophages with an emphasis to reduce the parasitic burden. Activated murine BMMΦ are known to produce reactive oxygen and nitrogen species that exert potent antimicrobial activity. For example, there is ample evidence that NO produced by inducible NO synthase (iNOS) plays a decisive role as a microbicidal effector molecule in macrophage cytotoxicity towards a number of microorganisms including the *Leishmania* species [17–20]. That EPs® 7630 induces significant NO production in parasitized BMMΦ has already been demonstrated in a recent study but without any information on the rate of intracellular killing [7].

In a first set of experiments, the effect of the known macrophage stimulating agents IFN-γ and LPS on the parasites' viability and the host cells was tested and compared with that under EPs® 7630 treatment (Fig. 2). For this, BMMΦ experimentally infected with *L. major* GFP promastigotes were rested for 24 h at 37°C to allow internalized parasites to transform into amastigotes. Although LPS (10 ng/mL) alone is known to activate BMMΦ for intracellular leishmanicidal activity, the combination IFN-γ + LPS proved to be highly effective and was used in comparative experiments. When *L. major* GFP-infected BMMΦ were exposed to rIFN-γ (100 U/mL) + LPS (10 ng/mL) or to rIFN-γ (100 U/mL) + EPs® 7630 (10 µg/mL) for an additional 48 h, the resulting GFP signals were considerably reduced (ca. 6% and 9% GFP events, respectively) compared to the non-treated population (ca. 49% GFP events). In contrast, rIFN-γ alone was less effective in reducing the GFP signal (ca. 26% GFP events). Although priming of macrophages with IFN-γ alone does usually not result in any NO production, there was a clear stimulation of NO synthesis under our experimental condition (Fig. 3). This apparent discrepancy may be explained by the presence of a very low endotoxin contamination [21] in the rIFN-γ standard used in the current experiments, which was produced in gene-transfected *E. coli*. This conjecture finds support by previous studies using rIFN-γ of different batches with no detection of any NO production and missing effects on the survival rates of *Leishmania* parasites [8].

This finding shows that EPs® 7630 significantly potentiates the killing of intracellular *L. major* GFP parasites in combination with rIFN-γ similar to that of LPS. Upon treatment of infected BMMΦ with amphotericin B (1 µM), a known antileishmanial agent [22], the GFP signal vanished rapidly without a concomitant stimulation of NO synthesis, indicating that the parasites had been killed directly (Figs. 2 and 3). It should be noted that intracellular *L. major* GFP amastigotes show a characteristic GFP signal that correlates well with the relative number of BMMΦ still infected with viable parasites [8]. Importantly, host cell toxicity was less than 10% in all incubations as assessed by PI staining (Fig. 2). DMSO (0.5%) did not affect the survival rate of parasites and the NO production discussed below (data not shown).

In cultures activated with rIFN-γ + LPS, a strong inverse correlation between NO levels and the intracellular survival rates of *Leishmania* parasites was noted [23]. Having in mind that EPs® 7630 also showed an NO-inducing potential [7], attention was subsequently given to the question whether the enhanced parasite killing observed for the combination of rIFN-γ + EPs® 7630 similarly correlated with the NO production. For this reason, aliquots of the supernatants were collected for measuring NO

concentrations (Griess assay), then the GFP signal of infected cells was determined by FACS analysis. As shown in Fig. 3, noninfected and infected BMM Φ produced relatively little detectable NO (0.6 μ M). On the other hand, the combination rIFN- γ (100 U/ mL) + LPS (10 ng/mL) strongly induced NO synthesis (ca. 28 μ M NO) compared with infected and rIFN- γ treated infected cells (ca. 7 μ M NO). Interestingly, the combination rIFN- γ (100 U/mL) + EPS $^{\text{®}}$ 7630 (10 ng/mL) was similarly effective with respect to NO-production (ca. 22 μ M) and intracellular parasite kill (vide supra). Accordingly, the anti-infective principle of EPS $^{\text{®}}$ 7630 appears to be strongly related to BMM Φ activation.

In order to assess the role of iNOS, experiments were carried out in parallel with the known inhibitor L-NMMA (Sigma Aldrich) [24]. The stereoisomer D-NMMA (Sigma Aldrich) is known to have no inhibitory effect on the enzyme and served as a negative control. As is evident from Fig. 4, inhibition of iNOS caused significantly lower NO levels and concomitantly increased the GFP signals, providing strong evidence for the crucial role of NO as a toxic effector molecule in the host defense to microbial infections and the pronounced NO-inducing capabilities of EPS $^{\text{®}}$ 7630.

More detailed information about the induced NO release in killing amastigotes in EPS $^{\text{®}}$ 7630-activated BMM Φ was obtained from exposure to different concentrations of the extract (0.1– 10 μ g/mL) in combination with rIFN- γ (100 U/mL). A strong inverse relationship between NO release and the intracellular survival of *L. major* GFP was evident (Fig. 5). Notably, treatment of infected BMM Φ with IFN- γ (100 U/mL) + EPS $^{\text{®}}$ 7630 (10 and 5 μ g/ mL) was almost as effective as IFN- γ (100 U/mL) + LPS (10 ng/mL). Even at a concentration of 3 μ g/mL EPS $^{\text{®}}$ 7630, a remarkable NO release (ca. 20 μ M) was induced and the resulting GFP signal was reduced compared with untreated controls although this effect was not statistically significant.

In a modified procedure, cell cultures were similarly incubated with EPS $^{\text{®}}$ 7630 in the absence of rIFN- γ . As is obvious from Fig. 6, the NO-inducing effect was clearly less pronounced than in cultures exposed to the combination IFN- γ + EPS $^{\text{®}}$ 7630 but was still statistically significant at concentrations between 3 and 10 μ g/ml when compared to vehicle treated controls. Similarly, events related to living (GFP-positive) parasites were reduced in a concentration-dependent manner in cultures containing EPS $^{\text{®}}$ 7630.

One important function of IFN- γ , independent of the time of incubation [25], is its potential to sensitize macrophages to react more effectively when needed. The signal transduction is initiated via Toll-like receptors after pathogen recognition, for example, LPS, causing a stimulation of innate immune responses [25, 26]. The present data suggest that EPS $^{\text{®}}$ 7630 may similarly synergize with IFN- γ to induce an infectious-resistant state in neighboring cells. Comparable results have been reported for taxol which was shown to mimic LPS by triggering signal pathways via TLR-4 interaction [27, 28]. It may be speculated that EPS $^{\text{®}}$ 7630 also mediates anti-infective activities by TLR signalling. This is to be studied in the near future.

EPS $^{\text{®}}$ 7630 has been shown to be an efficient herbal medicine for the treatment of upper respiratory tract infections, e.g. bronchitis. The causative pathogens are predominantly viruses including the respiratory syncytial virus, Coxsackie, influenza, para-influenza, and ECHO viruses, although bacterial infections may play a role. The destabilized defense mechanisms resulting from a viral infection can clear the way for a secondary bacterial infection. Induction of nonspecific host defense mechanisms against a number of pathogens including viruses is intimately related to the IFN-system. Both type I and type II IFNs can protect cells from viral infections, while IFN- γ functions as the major macrophage activating factor. In light of the documented efficacy of EPS $^{\text{®}}$ 7630 in the treatment of respiratory diseases with primary viral infections, we investigated this special extract for its direct antiviral potential and for its capacity to induce the production of antiviral components in BMM Φ using a fibroblast-virus protection assay.

After 24 h of incubation, supernatants of EPS $^{\text{®}}$ 7630-activated BMM Φ were analyzed for their capacity to protect L929 fibroblasts from the CPE of EMCV using crystal violet as a staining reagent for protected cells. LPS (1 ng/mL) served as a positive control [29, 30], while untreated BMM Φ and DMSO (0.5%) were used as negative controls (Fig. 7). In parallel, NO production was measured as an indicator for cell activation and as a possible contributing factor to the overall antiviral activity [31]. Dead BMM Φ (< 10 in all experiments) were quantified by FACS using PI-staining [14]. The cytoprotective effect was compared to that of rIFN- γ (100 U/mL). Antiviral protection was expressed in U/mL, defined as the reciprocal value of the supernatant dilution that would inhibit 50% of the CPE induced by EMCV in L929 cells. These values were correlated with the IFN standard to account for any fluctuation in assay sensitivity.

Prominent cytoprotective effects were observed for EPs® 7630 in a concentration-dependent manner (1–10 µg/mL) (Fig. 7). Conspicuously, when BMMΦ were incubated with 10 µg/mL of EPs® 7630, the resulting antiviral activity (ca. 80 U/mL) was similar to that of the LPS response. However, the induced NO production was remarkably enhanced in EPs® 7630-stimulated cell cultures, but dropped noticeably close to detection limits when exposed to a lower sample concentration (5 µg/mL). On the other hand, EPs® 7630 at this concentration still exerted fairly high cytoprotective effects (ca. 35 U/mL) in the virus protection assay, suggesting that different cellular defense mechanisms are activated independently. The NO production induced by EPs® 7630 does not seem to represent a major contributing factor to the overall antiviral activity. EPs® 7630 on its own did not induce any cytoprotective effects when directly added to L929 fibroblasts (Fig. 8).

Known antiviral factors are members of the IFN family classified in type 1 and type 2 interferons according to receptor specificity and sequence homology [20]. The functional virus protection assay used in this study does not discriminate between IFN-α, IFN-β, and IFN-γ. In order to gain insight into the underlying antiviral principle, we tried to measure distinct IFN proteins by using commercially available ELISA kits. Surprisingly, measurement of IFN proteins proved to be invariably unsuccessful in a series of experiments. The failure to detect any IFN proteins by applying ELISAs may be rationalized by the production of IFN quantities below the detection limits of the assays (IFN-α: 16 pg/mL, IFN-β: 16 pg/mL, and IFN-γ: 40 pg/mL, respectively). On the other hand, the virus protection assay represents a very sensitive functional method. Only very few IFN molecules may readily interact with receptors, triggering signal transduction pathways for antiviral responses [32]. Future studies are required to support this conjecture by using highly sensitive methods such as quantitative PCR analysis. Another possible explanation may be that IFN molecules of different types may act synergistically in the protection of L929 cells. Besides, other antiviral factors such as type III IFN [33] or eicosanoids [34, 35] may play a role in the control of viral infection. Very recent work showed that EPs® 7630 suppressed rather than stimulated the synthesis of eicosanoids in calcium ionophore stimulated human granulocytes [36], rendering their implication less likely.

Using BMMΦ experimentally infected with *L. major* GFP, incubation with EPs® 7630 induced release of NO in a concentration-dependent manner and significantly reduced the parasitic burden at relatively low concentrations. The assessment of the killing of the pathogen carried out at single cell levels reaffirmed the anti-infectious potential of this herbal medicine, previously concluded from diverse functional assays. The mode of action of activated cytotoxic defense mechanisms, e.g., NO production, on intracellular pathogens remained unknown. The present data provide convincing evidence of the effectiveness of the immune response induced in infected cells.

Since respiratory tract infections are frequently caused by viruses, the modulatory potential of EPs® 7630 as assessed in a fibroblast/EMCV protection assay deserves to be explicitly mentioned. Supernatants of EPs® 7630-activated BMMΦ showed prominent cytoprotective effects. Although it appears reasonable to relate the antiviral activity to the IFN system, detection of IFN molecules using ELISA kits were unsuccessful, possibly due to very low amounts. Conspicuously, when L929 cells were directly incubated with the extract, inhibition of the cytopathic effect of the virus on fibroblasts was not evident and in contrast with similar incubations using an IFN-γ standard.

Further studies are needed to identify the signalling pathway and the antiviral components. A closer look into the composition of EPs® 7630 remains a challenge towards a better understanding of the responsible biologically active substances.

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Figures

Figure 1. EPs® 7630 per se did not kill *L. major* GFP promastigotes. Propidium iodide-positive (dead) vs. GFP-positive (living) parasites: **A** Untreated *Leishmania* parasites, **B** promastigotes (1.0×10^6) treated with 10 Mg/mL of EPs® 7630, **C** DMSO (1 %) for 48 h, and **D** with amphotericin B (1 mM/10 mM) for 24 h, respectively. One of two examples with similar results is shown.

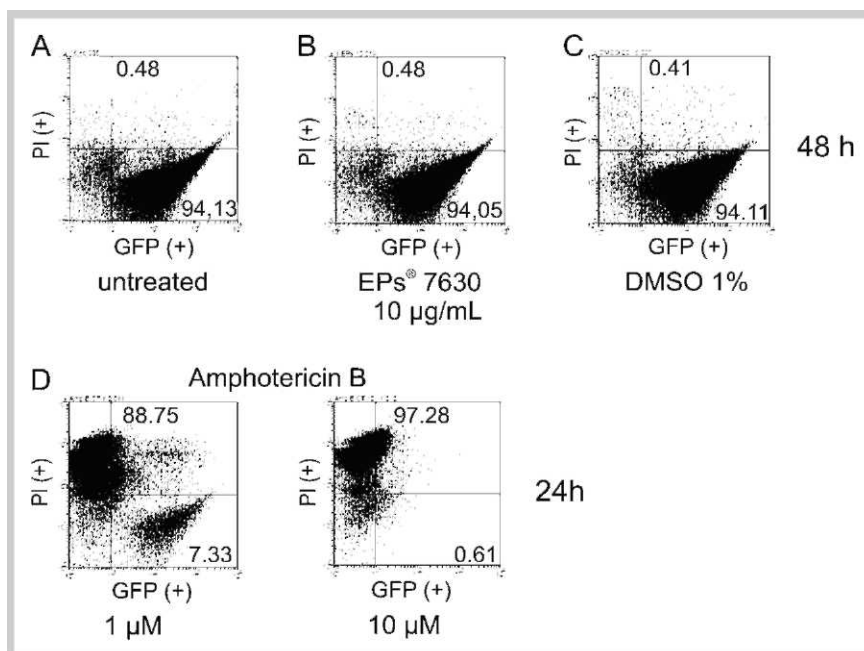


Figure 2. Effect of EPs® 7630 in the presence of IFN- γ on the viability of *L. major* GFP amastigotes in infected BMM<t> as assessed by FACS analysis after 48 h incubation. **A** noninfected, **B** infected cells, **C** infected cells + IFN- γ (100U/mL), **D** infected cells + IFN- γ (100 U/mL)/LPS (10 ng/mL), **E** infected cells + IFN- γ (100 U/ml)/EPs® 7630 (10ug/mL), **F** infected cells + amphotericin B (1 μ M). The depicted experiment is one out of six independent experiments with similar results.

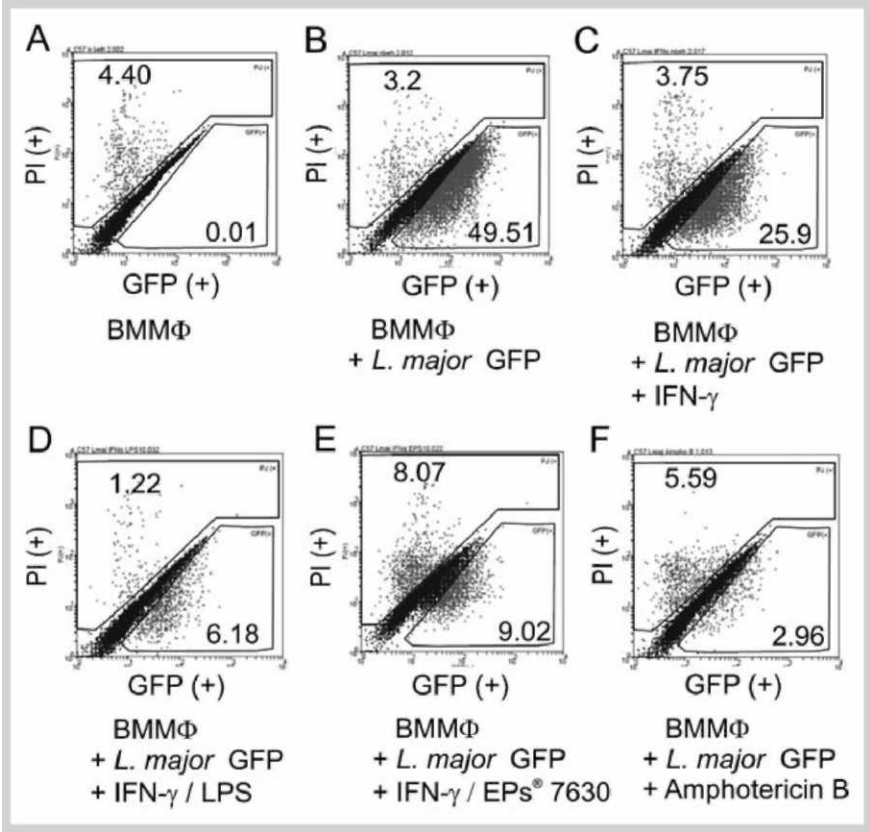


Figure 3. Relative survival rates of intracellular *L. major* GFP parasites (GFPpositive events) and concomitant NO production as assessed by FACS analysis and in the Griess assay, respectively. A non-infected, B infected cells, C infected cells + IFN- γ (100 U/mL), D infected cells + IFN- γ (100 U/mL)/LPS (10 ng/mL), E infected cells + IFN- γ (100 U/mL)/EPs® 7630 (10 μ g/mL), F infected cells + amphotericin B. The values (mean \pm SD) are derived from six independent experiments [$p < 0.05$; GFP-signal (*), NO production (*)].

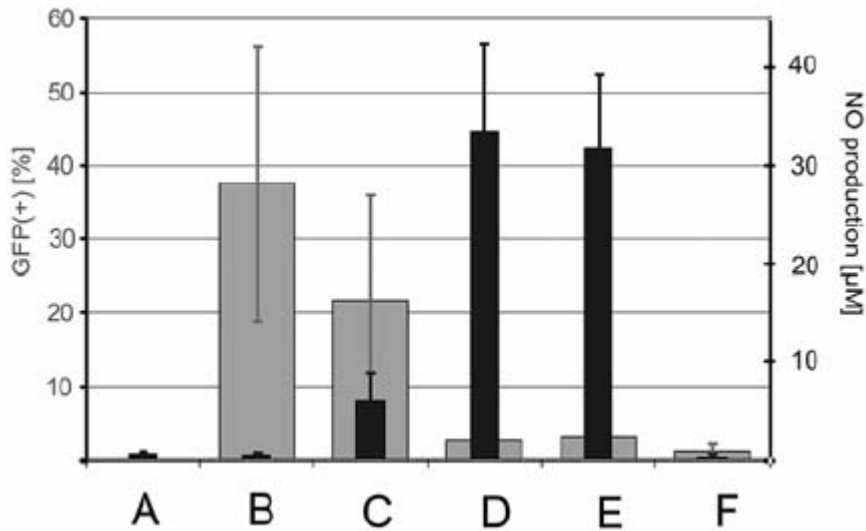


Figure 4. Relative survival rates of intracellular *L. major* GFP parasites (GFPpositive events; FACS analysis) and concomitant NO production (Griess assay) in infected BMM Φ stimulated in the presence and absence of L-NMMA and D-NMMA (each at 500 μ M), respectively. The depicted experiment is one of two independent experiments with similar results.

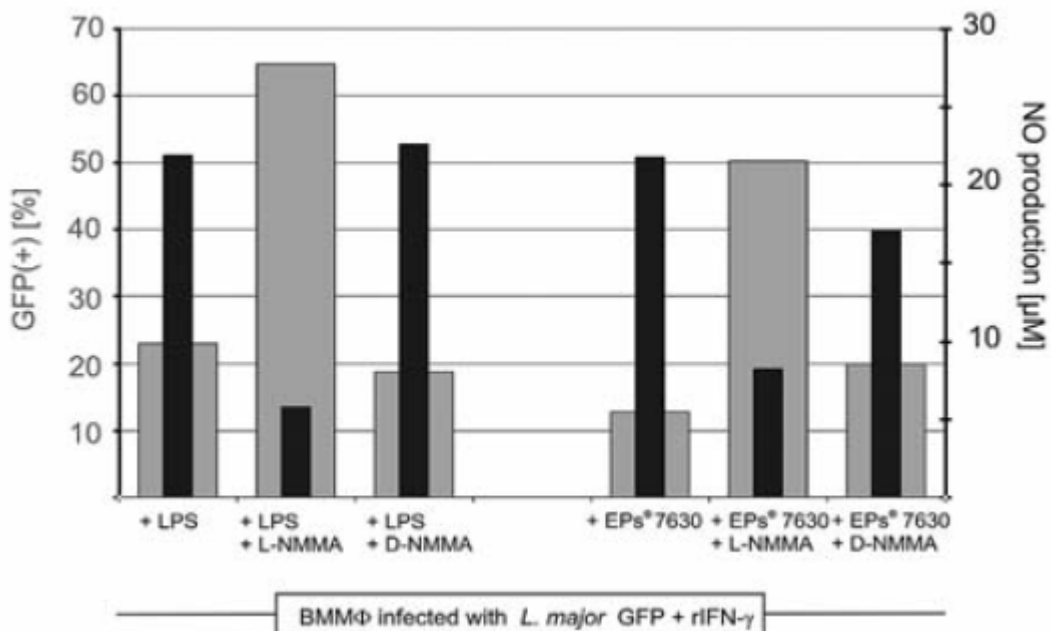


Figure 5. Relative survival rates of intracellular *L. major* GFP parasites (GFPpositive events; FACS analysis) and concomitant NO production (Griess assay) in infected BMMΦ treated with EPs® 7630 (10–0.1 µg/mL) + IFN-γ (100 U/mL). Control experiments included the exposure of cells to IFN-γ (100 U/mL) and IFN-γ (100 U/mL)+ LPS (10 ng/mL), respectively. The values (mean ± SD) are derived from six independent experiments [$p < 0.05$; GFPsignal (*), NO production (*)].

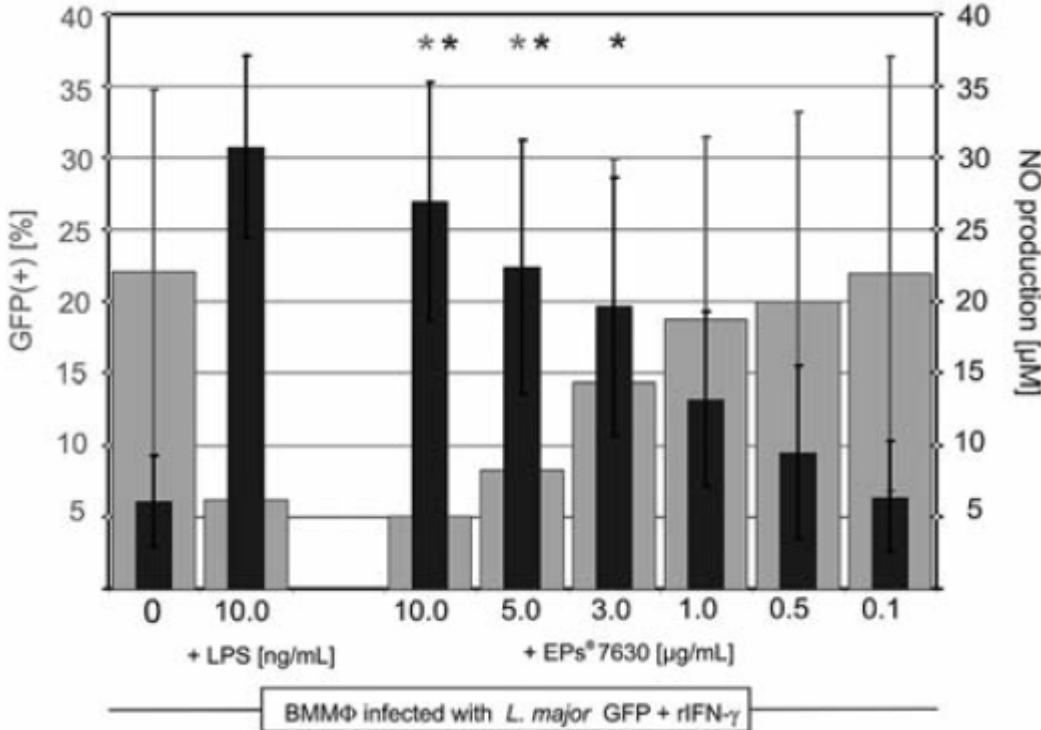


Figure 6. Relative survival rates of intracellular *L. major* GFP parasites (GFPpositive events; FACS analysis) and concomitant NO production (Griess assay) in infected BMMΦ treated with EPs® 7630 (10–0.1 µg/mL). IFN-γ (100 U/mL) + LPS (10 ng/mL) stimulated cells served as positive and untreated cells as negative controls. The values (mean ± SD) are derived from six independent experiments ($p < 0.05$; NO production [*]).

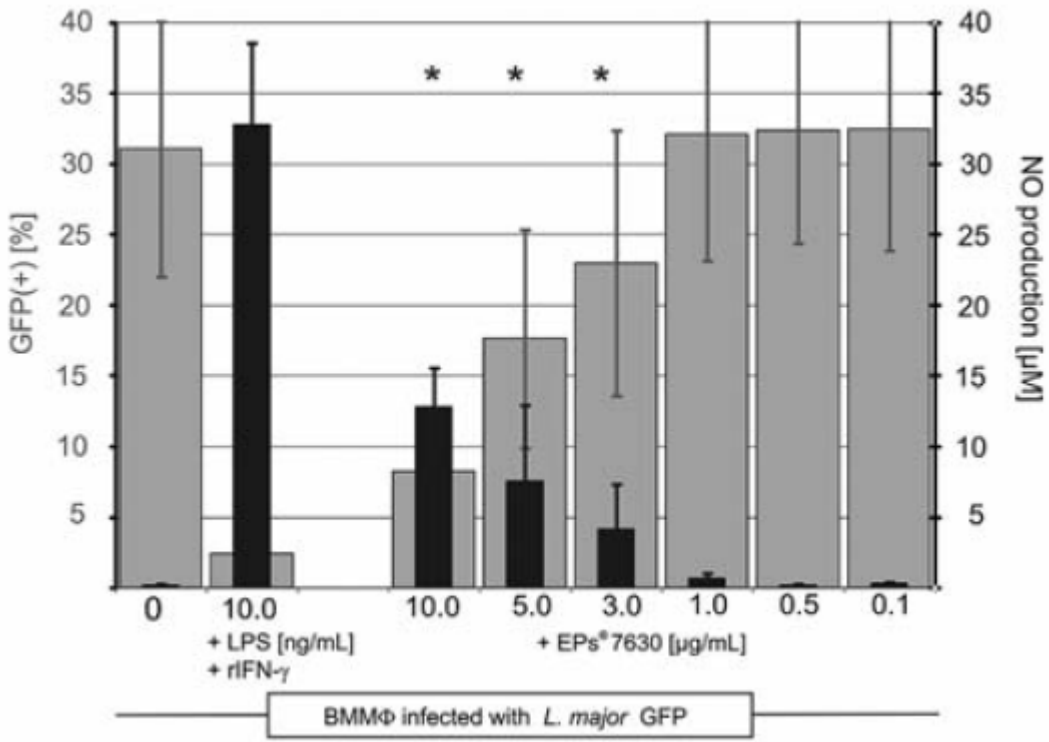


Figure 7. Fibroblast-virus protection assay. A Antiviral effects of an IFN- γ standard (100 U/mL) and supernatants from BMM Φ treated with EPs[®] 7630 (10 μ g/mL) shown by serial dilutions in microtiter plates and crystal violet staining. B Cytoprotective effects of EPs[®] 7630 as assessed in the fibroblast/EMCV protection assay and simultaneous measurement of NO production (Griess assay). The values (mean \pm SD) are derived from three independent experiments [$p < 0.05$; NO production (*), antiviral activity (*)].

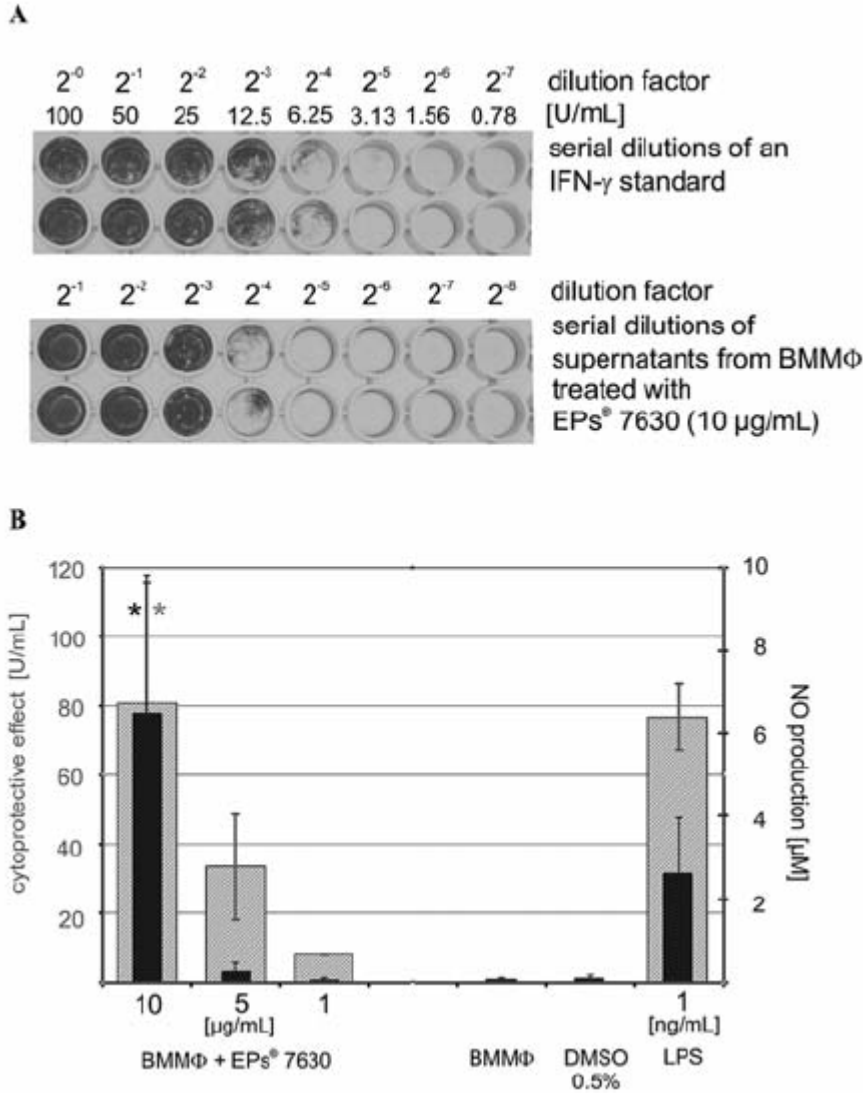


Figure 8. Antiviral effects of an IFN- γ standard (100 U/mL) and EPs® 7630 (10 μ g/mL) on L929 cells shown by serial dilutions in microtiter plates and crystal violet staining following direct incubations.

