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Polysaccharides from the fungus *Scleroderma nitidum* with anti-inflammatory potential modulate cytokine levels and the expression of Nuclear Factor kB

Marília S. Nascimento,[1] Joedyson E. M. Magalhães,[1] Thuane S. Pinheiro,[1] Thayse Azevedo da Silva,[2] Leonam Gomes Coutinho,[2] Iuri G. Baseia,[3] Lucymara F. Agnez Lima,[3] Edda Lisboa Leite*[*,1]

[1]Laboratório de Glicobiologia, Departamento de Bioquimica, Universidade Federal do Rio Grande do Norte, Brazil,
[2]Laboratório de Biologia e Genômica Molecular, Departamento de Genética e Biologia Celular, Universidade Federal do Rio Grande do Norte, Brazil,
[3]Laboratório de Micologia, Departamento de Botânica, Ecologia e Zoologia, Universidade Federal do Rio Grande do Norte, Brazil.

Abstract: Several pharmacological properties are attributed to polysaccharides and glucans derived from fungi such as tumor, anti-inflammatory, and immunomodulatory activity. In this work, the anti-inflammatory potential of polysaccharides from the fungus *Scleroderma nitidum* and their possible action mechanism were studied. The effect of these polymers on the inflammatory process was tested using the carrageenan and histamine-induced paw edema model and the sodium thioglycolate and zymosan-induced model. The polysaccharides from *S. nitidum* were effective in reducing edema (73% at 50 mg/kg) and cell infiltrate (37% at 10 mg/kg) in both inflammation models tested. Nitric oxide, a mediator in the inflammatory process, showed a reduction of around 26% at 10 mg/kg of body weight. Analysis of pro- and anti-inflammatory cytokines showed that in the groups treated with polysaccharides from *S. nitidum* there was an increase in cytokines such as IL-1ra, IL-10, and MIP-1β concomitant with the decrease in INF-γ (75%) and IL-2 (22%). We observed the influence of polysaccharides on the modulation of the expression of nuclear factor κB. This compound reduced the expression of NF-κB by up to 64%. The results obtained suggest that NF-κB modulation an mechanisms that explain the anti-inflammatory effect of polysaccharides from the fungus *S. nitidum*.

Introduction

Inflammation is a complex event that consists of recognizing the lesion stimulus and the consequent attempt at restoring the damaged tissue (Nathan, 2002). This involves a series of cellular and vascular events (Rocha & Silva, 1978). In inflammatory reactions, nitric oxide derived from cells stimulated by the action of cytokines promotes changes in the vascular permeability of the inflamed tissue (Barnes & Karin, 1997). The Nuclear κB (NF-κB) factor, an important transcription factor in the progression of inflammation, is related to gene activation in many of the mediators involved in inflammatory response, such as TNF-α, IL-1β and iNOS (Barnes & Karin, 1997; Ben-Neriah & Schmidt-Supprian, 2007). The biodiversity found in Brazil accounts for 15 to 20% of total biodiversity worldwide and is considered a source of biologically active compounds with enormous potential as a source of new drugs (Barreiro & Fraga, 1999).

A number of mushroom-based compounds with pharmacological potential can be mentioned, such as triterpenes (Liu et al., 2006), lectins (Koyama et al., 2002), and polysaccharides. Among the various polysaccharides isolated from basidiomycetes are glucans, heterogalactans, and heteromanans (Schepetkin & Quinn, 2006). The β-glucans, for example, exert potent effects on the immunological system through the binding to macrophage receptors and other white cells activating them (Brown & Gordon, 2001). Recently, polysaccharides derived from mushrooms have been considered an important class of bioactive compounds and their anti-

inflammatory potential has been widely studied (Dore et al., 2007; Carbonero et al., 2008; Smiderle et al., 2008). Several *in vitro* and *in vivo* mechanisms have been related to the anti-inflammatory activity of polysaccharides and extracts rich in fungal polysaccharides. Among these mechanisms are cyclooxygenase inhibition (Hong et al., 2004), inhibition, or decrease in cytokine levels (Kim et al., 2003) and inhibition of inflammatory cell recruitment (Dore et al., 2007).

Scleroderma genus Pers.: Fr (Gasteromycetes, Sclerodermatales) has included species commonly known as "earth balls", worldwide distributed (Kirk et al., 2001). Although this genus poisonous mushroom contain, species have been investigated their anti-inflammatory and haemostatic porperties (Guzmán & Ovrebo, 2000; Liu et al., 1984). Thus, the aim of this study was to verify the anti-inflammatory potential of polysaccharides from the fungus *Scleroderma nitidum*, as well as investigate the possible mechanism involved in this process.

Material and Methods

Fungus

Fresh fruiting bodies of the fungus *Scleroderma nitidum,* Phylum Basidiomycota, Class Agaricomycetes, Order Boletales, Family Sclerodermataceae, were collected in the Dunes State Park in Natal, Brazil, and identified by Iuri Goulart Baseia from the Department of Botany, Ecology, and Zoology of UFRN. The species were deposited in the Herbarium at UFRN (UFRN-fungos 688).

Reagents

Sodium thioglycolate, zymosan, carrageenan, histamine, and *N*-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma Aldrich, EUA (St Louis, MO, USA). Diclofenac sodium was purchased from Novartis. Other reagents and their respective sources include the following: polyvinylidene difluoride (PVDF) membrane (Qbiogene); primary monoclonal antibodies against NF-kappa B (Santa Cruz biotechnology, INC); secondary antibodies conjugated to peroxidase (Santa Cruz biotechnology, INC); DAB (0.5%) (Across Organics, R&D systems and β-actin antibody (SIGMA).

Animals

The studies were conducted with *Wistar* rats (150-200 g) and Swiss mice (25-30 g) kept in the vivarium of the Department of Biochemistry-UFRN. All the animals used in the experimental phase were submitted to water and diet *ad libitum* under controlled illumination (12 h light/dark cycle) and constant temperature at 23±2 ºC. The animals were acclimatized in the laboratory for at least 4 h before the experiments and were used only once. The assays conducted were approved (nº012/2009) by the Ethics Committee (of the Federal University of Rio Grande do Norte (UFRN), and followed established norms.

Polysaccharide extraction

The polysaccharides from fruiting bodies of the fungus *Scleroderma nitidum* were washed and dissected at 40 ºC and then pulverized. For the extraction of polysaccharides, 50 g of pulverized fungal tissue was used. Two volumes of 80% acetone were added and the mixture was kept at 25 °C for 24 h and then filtered. Extraction with chloroform-methanol (2:1, v/v) was performed for 2 h at 60 ºC, under reflux, followed by centrifugation at 8000 x *g* for 10 min. The supernatant was discarded and 500 mL of distilled water was added to the supernatant, and the mixture remained in a double boiler at 100 ºC for 3 h. The precipitate was treated with 4 volumes of ethanol, resulting in a precipitate that was separated from the supernatant by centrifugation (8000 x *g* for 20 min at 4 ºC). Next, the precipitate obtained was dried and pulverized.

Chemical analyses

The proteins present in the polysaccharide fraction of the mushroom were determined using the Bradford method (Bradford, 1976). Total sugars were determined by the method proposed by Dubois (1956). Total sulphate content was determined by turbidimetry using the gelatin-barium method (Dodgson & Price, 1962) after previous acid hydrolysis with HCl 6 N, for 6 h at 100 ºC.

Monosaccharide composition

The polymers (25 mg) were hydrolyzed (2M HCl, 100 °C, 2 h) and monosaccharide composition was determined by HPLC with a refractive index detector using a column LiChroCART® (250-4 mm). The following sugars were used as standard for analysis: glucose, galactose, arabinose, fucose, mannose, rhamnose and xylose.

Carrageenan and histamine-induced paw edema

The carrageenan and histamine-induced paw edema model was performed with modifications to the method proposed by Winter et al., (1962). The rats were anesthetized with ethyl ether and the groups were intraperitoneally (*i.p.*) administered polysaccharides from *S. nitidum* at doses of 10, 30, and 50 mg/kg of

animal weight or L-NAME. In the negative and positive controls, sterile saline solution was injected. After 30 min carrageenan (0.1 mL at 1%) or histamine (0.1 mL at 0.1%) was injected subcutaneously into the right paw of the rats, except the negative control, which received saline solution. Immediately after this procedure a new measurement of right paw thickness was taken in all the groups and this was repeated after 1, 2, 3, and 4 h.

Histological examinations

Paw tissue samples were removed after the second induction of inflammatory reaction in all the groups of histamine-induced paw edema. The tissue was fixed in formaldehyde, embedded in paraffin and sectioned. The sections were stained in hematoxylin and eosin (HE).

Sodium thioglycolate and zymosan-induced peritonitis

The animals were treated subcutaneously with polysaccharides at different doses. The positive (zymosan) and negative (saline) control groups were submitted to the same procedure, but received 500 µL of PBS buffer. After 30 min the animals received an intraperitoneal injection containing 1 mL of 3% sodium thioglycolate or zymosan, according to methodology proposed by Xie et al., (2000). The negative control group received an injection of 1 mL of PBS buffer. Three hours after the application of 3% sodium thioglycolate (1 mL) or zymosan (1 mg/600 µL), we anesthetized and sacrificed the animals and washed the peritoneal cavity with 5 mL of a PBS buffer solution. The peritoneal liquid of each animal was collected and placed into hemolysis tubes containing EDTA. Next, the leukocytes present in the liquid collected in the peritoneal cavity of the animals were stained with Türck solution and counted in a Neubauer counter.

Drug potentiation

The animals were subcutaneously treated with the concomitant application of sodium diclofenac and polysaccharides, both at 10 mg/kg, and L-NAME and polysaccharides also at 10 mg/kg each.

Nitric oxide

The nitrite-nitrate concentration was measured using the Griess reaction and the addition of 100 µL of peritoneal wash obtained in item 2.8. Absorbance at 540 nm was measured using the ELISA reader.

Ex vivo *determination of cell viability*

Cell viability was determined using the trypan blue exclusion method described by Phillips, (1973). Different doses of polysaccharides were inoculated and after 4 and 24 h the animals were sacrificed. The control group received only PBS (pH 7.4). After peritoneal wash collection, as previously described, an aliquot of 20 µL of the cell suspension was removed and diluted in 20 µL of trypan blue. The percentage of viable cells was determined after differential counting in Neubauer chamber, with the help of a light microscope. The viable cells, which excluded this stain, were translucent and the dead cells were purplish.

Cytokine analysis

Concentrations of IL-1ra, IFN-γ, IL-2, MIP-1β, and IL-10 in the peritoneal wash were measured in duplicate by LINCOplex assay (Linco Research, Inc. St Charles Missouri, USA). This assay is based on the use of polystyrene beads marked with fluorescent stains, in which each group of spheres has a distinct color and can be distinguished using a Luminex100 system analyzer (Luminex Corp, Austin, TX). Each bead was coated with a specific antibody for the cytokine of interest. Each specific cytokine-antibody pair in this multiplex assay did not react with the other analyses on the panel.

Dot-blot assay for NF-kappa B

Proteins were obtained with 5 µL aliquots of the peritoneal wash from the mice; these aliquots were centrifuged at 15000 x *g*, at 4 °C, for 5 min. The samples were transferred to an activated membrane of PolyVinylidene DiFluoride (PVDF) and the non-specific ligand was blocked by incubation with blocking buffer (5% powdered milk (non-fat) diluted in TBS) for 1 h. The membranes were then incubated with primary monoclonal antibodies against NF-kappaB. After washing, the membrane was incubated with peroxidase-conjugated secondary antibodies. The protein-antibody reaction was detected by the reaction with diaminobenzidine (0.5%), amonium acetate at 50mM, pH 5.0 and H_2O_2 (0.06%). β-actin antibody was used as control of the endogenous expression. To quantify the expression levels, the membranes were photographed digitally and the images were analyzed using ImageJ software. The NF-kappaB levels were determined from the division of the respective optical density obtained by the optical density of the β-actin membrane.

Statistical analysis

Values were presented as mean±SEM. Analyses of variance (ANOVA) and Tukey-Kramer were used, considering $p<0.05$ as statistically significant.

Results

Extraction

Aqueous extraction is one of the most widely used techniques in scientific studies (Barbisan et al., 2002; Bellini et al., 2006; Giacomini & Eira, 2003; Lavi et al., 2006). Its efficiency in obtaining polysaccharides and the low cost may be the main reasons for its wide use.

Chemical analyses

The chemical analyses conducted after obtaining polysaccharides from the fungus *S. nitidum* showed high carbohydrate content (94.71%), low protein content (5.29%), and no sulphate (Table 1).

Table 1. Percentual chemical components after fractionation of bodies fructification from *Scleroderma nitidum.*

Components	(%)
Total sugar	94,71
Proteins	5,29
Sulfate	0
Phenolic compounds	0

Monosaccharide composition

The polysaccharide on acid hydrolysis by 2M HCl showed the presence of glucose, galactose, mannose and xylose that were detected by HPLC analysis and found to be present in a molar ratio of 1 :0.4:0.6 :0.2 respectively.

Carrageenan and histamine-induced paw edema

To determine the effect of polysaccharides on the inflammatory process, we performed carrageenan and histamine-induced paw edema assays in *Wistar* rats. Glucans from the fungus *S. nitidum* reduced carrageenan-induced paw edema by up to 41±7% at the inflammatory peak (2rd h) and by approximately 73% at the end of the 4th h at a dose of 50 mg/kg (Figure 1A). In the histamine-induced paw edema, all the doses of polysaccharides (10, 30 and 50 mg/kg) were efficient in reducing the edema. The greatest reduction observed occurred in the second h, where the reduction obtained using a dose of 50 mg/kg of polysaccharides exceeded the effect obtained with L-NAME by 26.5%. At the end of the fourth h, all the groups treated with the different doses of polysaccharides exhibited no edema (Figure 1B).

Histological examinations

Histological examinations demonstrated that the animals sensitized only with histamine (positive control) exhibited intense cell infiltrate, characteristic of the inflammatory reaction (Figure 2A). However, those that received only saline solution (negative control) showed an absence of inflammatory reaction (Figure 2B). It was also observed that in the animals treated with polysaccharides from the fungus *S. nitidum* (10, 30, and 50 mg/kg, respectively) showed a significant decrease in PMN cell recruitment at the inflammation site (Figure 2D,E,F). These results were confirmed by histological analysis of the paw treated with L-NAME (Figure 2C).

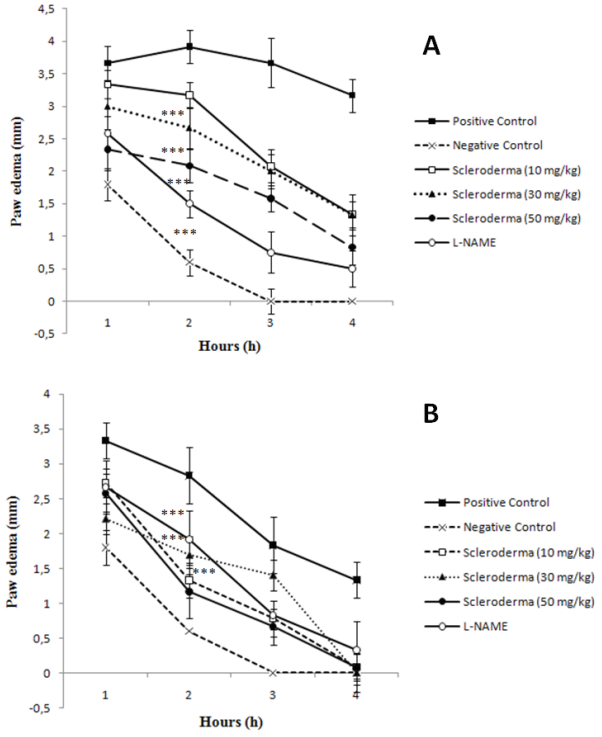

Figure 1. Anti-inflammatory effect of polysaccharides from the fungus *Scleroderma nitidum* in the carrageenan and histamine-induced paw edema model. A. Effect of different doses of polysaccharides and L-NAME on the carrageenan-induced plantar edema model; B. Effect of different doses of polysaccharides and L-NAME on the histamine-induced plantar edema model.

Sodium thioglycolate and zymosan-induced peritonitis

The anti-inflammatory action of polysaccharides from the fungus *Scleroderma nitidum* was observed in sodium thioglycolate and zymosan-induced peritonitis assays, where leukocyte migration to the injury site in both models tested was reduced. According to the results obtained in sodium thioglycolate-induced peritonitis, a reduction of around 38±3% in the overall number of leukocytes was observed when a 10 mg/kg dose of

polysaccharides from *S. nitidum* was administered. The other polysaccharide concentrations tested (30 and 50 mg/kg) reduced the inflammatory process by 31±1% and 15±3%, respectively (Figure 3A). In the zymosan-induced peritonitis model, a reduction of around 66±3% was observed in overall leukocytes with 10 mg/kg and 30 mg/kg of polysaccharides from *S. nitidum*. At polysaccharide concentrations of 50 and 100 mg/kg, the reduction observed in the inflammatory process was 60±10% and 52±10%, respectively (Figure 3B). Thus, the polysaccharides showed no dose-dependent effect. In this inflammation model, the 10 mg/kg dose had a better anti-inflammatory effect.

Drug potentiation

To check if the polysaccharides were capable of potentiating drug action, two drugs were used for this assay: L-NAME and diclofenac. Diclofenac is a COX 1 and 2 inhibitor, whereas L-NAME is a competitive inhibitor of arginine that promotes a decrease in the enzymatic activity of the iNOS (Bogle et al., 1992). Figure 3C shows that the dose of the polysaccharides tested (10 mg/kg), applied jointly with diclofenac, exceeded the action of this drug by around 10±1%. However, the polysaccharides applied in conjunction with the L-NAME showed no significant differences. The data demonstrate that these fungal polysaccharides may act synergically with other inflammatory drugs, potentiating their effect.

Nitric oxide

Nitric oxide in most body fluids is rapidly metabolized to stable products such as nitrite and nitrate. According to the results obtained (Figure 3D) in the sodium thioglycolate-induced model, the polysaccharides from the fungus *Scleroderma nitidum* showed a reduction of NO2/NO3 content of around 32.4±10%. However, in the zymosan-induced peritonitis model, no significant reduction in NO_2/NO_3 levels were observed at any of the doses tested (Figure 3E).

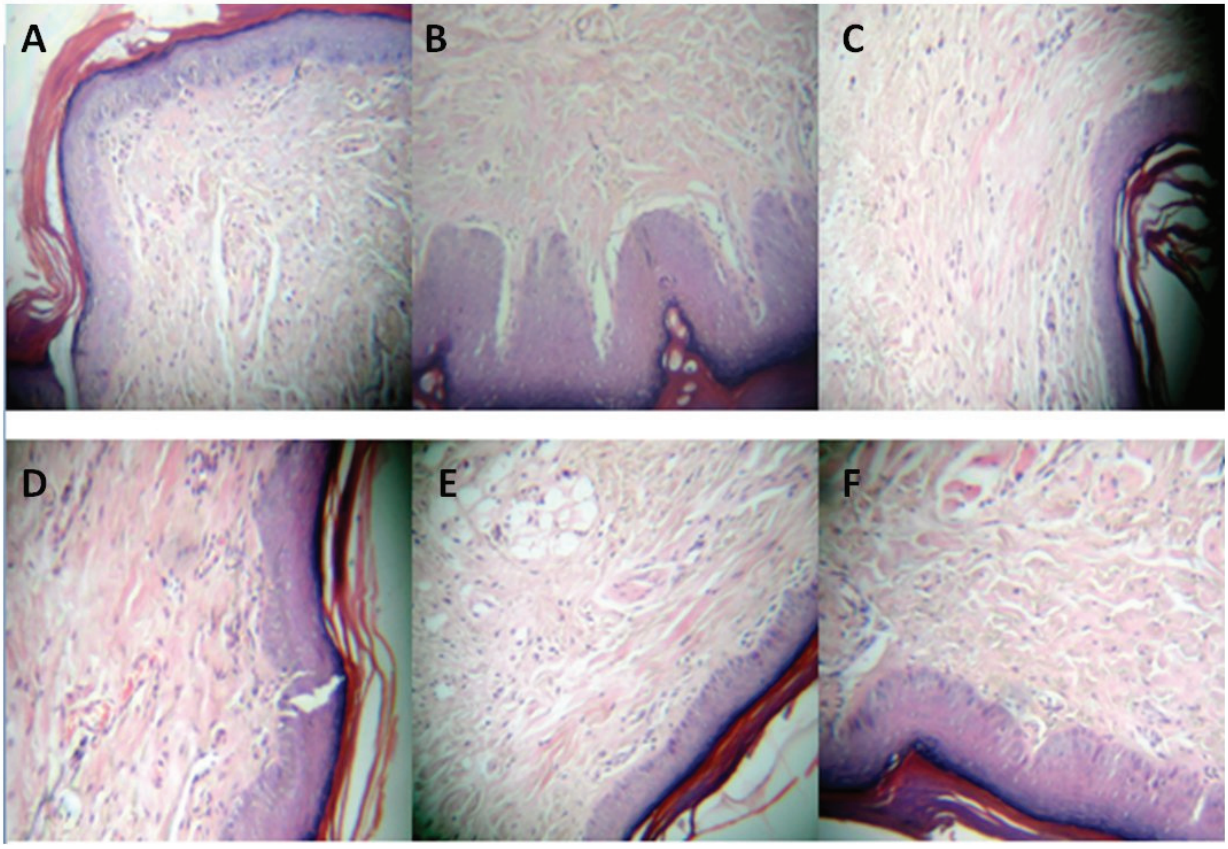

Figure 2. Histological analyses (HEX200) of paws from different groups belonging to the histamine-induced paw edema model. A. Positive control (Histamine, without treatment); B. negative control (saline); C: L-NAME (50 mg/kg); D: polysaccharides from *S. nitidum* at 10 mg/kg; E. polysaccharides from *S. nitidum* at 30 mg/kg; F. polysaccharides from *S. nitidum* at 50 mg/kg.

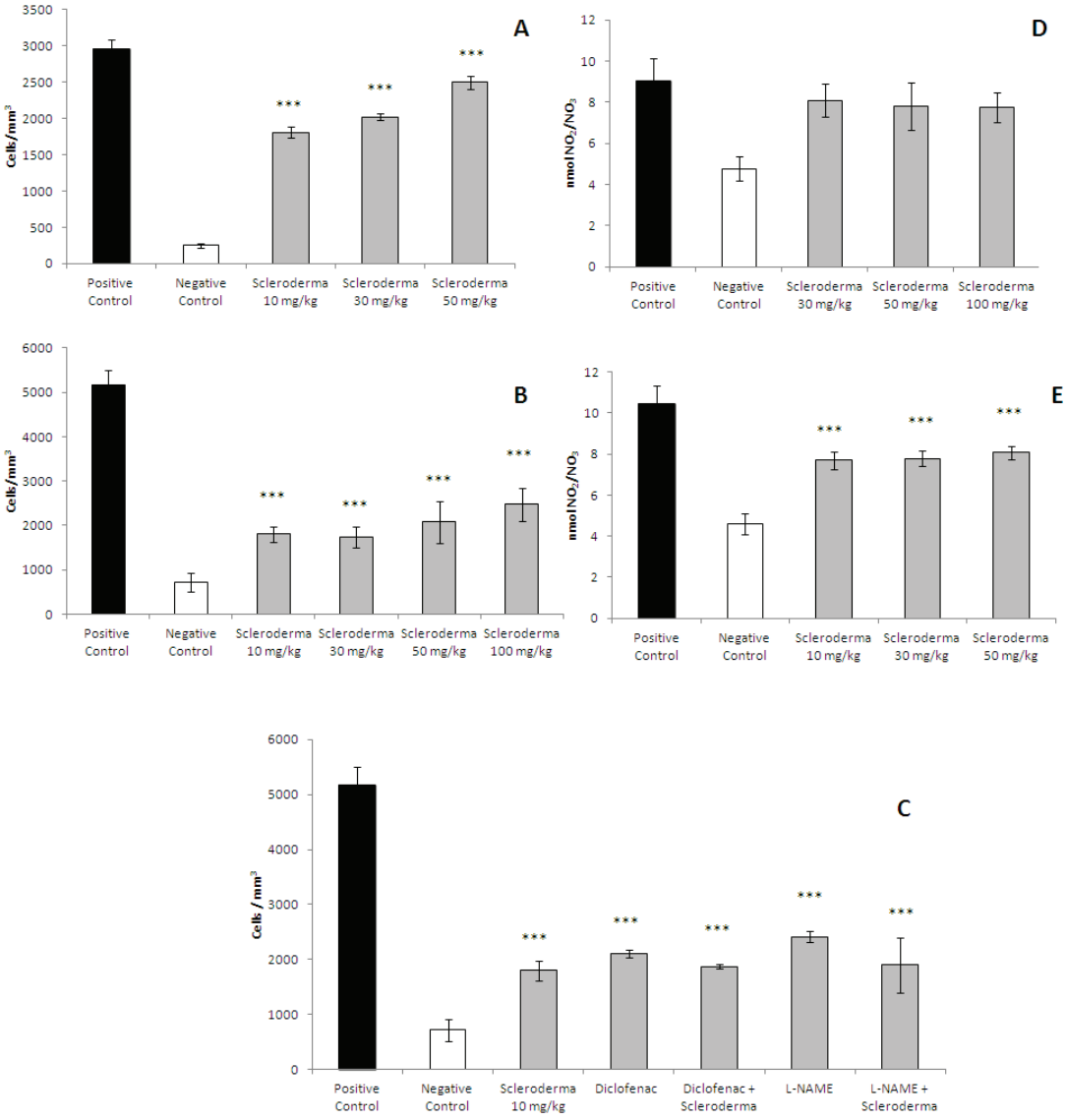

Figure 3. Anti-inflammatory activity of polysaccharides from *Scleroderma nitidum* in the sodium thioglycolate and zymosan-induced peritonitis model. A. on the sodium thioglycolate-induced peritonitis model; B. on the zymosan-induced peritonitis model. C. Anti-inflammatory effect of polysaccharides from *Scleroderma nitidum* (10 mg/kg) on drug potentiation in the zymosan-induced peritonitis model. The factors were expressed as mean±SD. A value of $p<0.001$(***) was considered statistically significant. D. Effect of different doses of polysaccharides from *S. nitidum* in NO (NO_2/NO_3) content on the sodium thioglycolate-induced peritonitis model; E. Effect of different doses of polysaccharides in NO content on the zymosan-induced peritonitis model. The values were expressed as mean±SD. A value of $p<0.001$(***) was considered statistically significant.

Ex vivo *cell viability*

These results show that the polysaccharides had no toxic effect at the doses tested. The maximum percentage of non-viable cells was 10% at a dose of 75 mg/kg 24 h after administration. Thus, the polysaccharides did not reach IC50 at any of the doses tested (Figure 4A).

Cytokine analysis

The glucans from *S. nitidum* showed an effect on cytokine expression. We observed a two-fold and three-fold increase in the release of anti-inflammatory cytokines IL-1ra and IL-10 respectively, in the groups treated with polysaccharides when compared with the positive control. A significant decrease (75%) was also

observed in IFN-γ levels as well as a slight reduction in IL-2 and MIP-1β levels of 22% and 29% respectively, in the groups treated with the different polysaccharide doses. In addition, the effect of polysaccharides was not dose-dependent (Figure 4B).

Dot-blot assay for NF-κB

Nuclear factor kB activation is a critical event in inflammation. NF-kB activation is related to gene activation for many cytokines, chemiokines, and adhesion molecules. This experiment was conducted to determine whether the polysaccharides from the fungus *Scleroderma nitidum* are capable of modulating NF-κB expression. According to the results obtained, the dose that had the best effect was 30 mg/kg, reducing NF-κB expression by 63±8%, whereas doses of 50 and 100 mg/kg reduced it by only 30±1%. Thus, it can be observed that all the fungal polysaccharide doses tested significantly reduced nuclear factor NF-kB expression (Figure 4C).

Discussion

Acute inflammation is essentially mediated by exudate formation and leukocyte migration. Innumerable studies have proven the potential of polysaccharides extracted from fungi in reducing leukocyte migration to the inflammation sites. To confirm the anti-inflammatory potential of fungal polysaccharides, the object of this study, we tested their effect in models of plantar edema induced by carrageenan and histamine and of peritonitis induced by sodium thioglycolate and zymosan.

During the inflammatory process, monocytes are recruited for parenchyma tissue, where they are activated to become cells with a phagocytic function, that is, macrophages. These cells can release inflammatory cytokines, free radicals, and nitric oxide, which can mediate the tissue lesion related to inflammatory response (Almeida at al., 1980; Gordon, 2001; Hermann et al., 2001). Studies on mushrooms with medicinal potential have been conducted to discover compounds that can

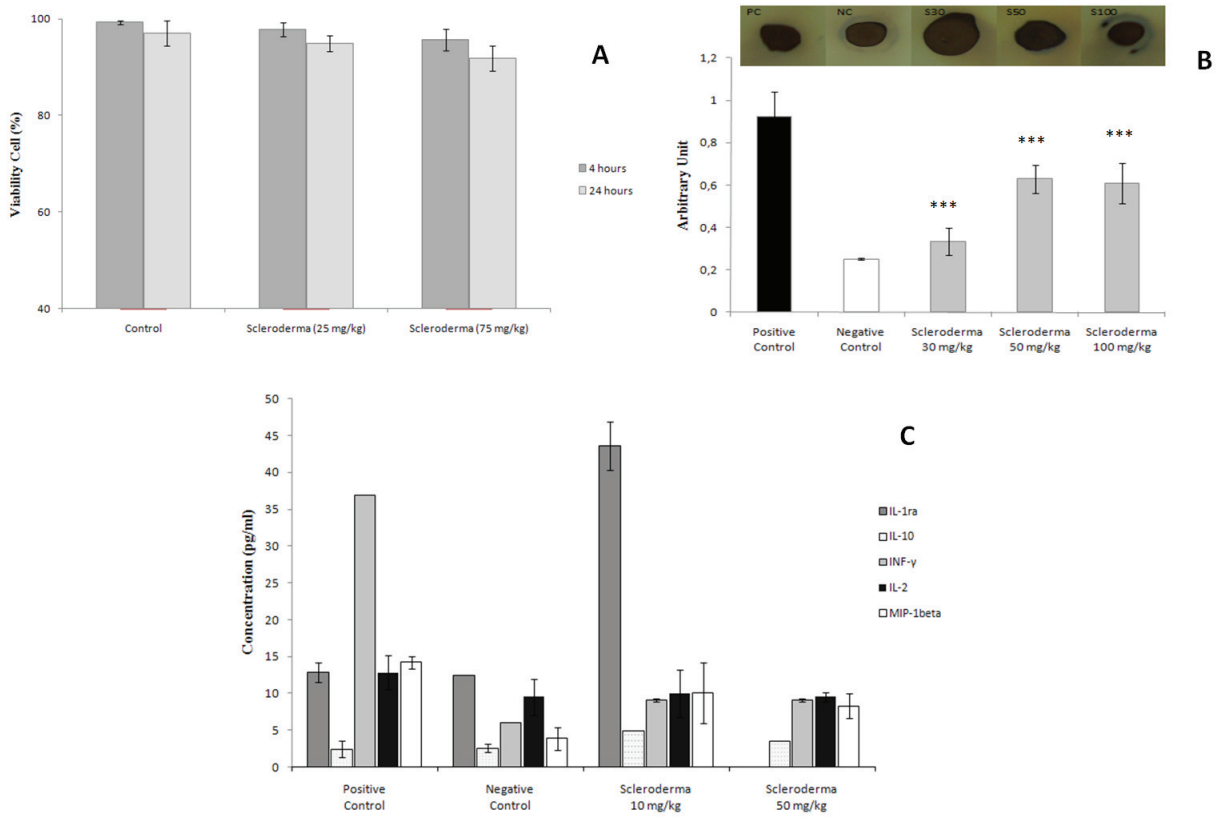

Figure 4. A. Assessment of the cytotoxicity of polysaccharides from *S. nitidum* (25 and 75 mg/kg) in the *ex vivo* cell viability model. Polysaccharides were intraperitoneally administered in mice, and cells were collected and counted after 4h and 24h. Cell viability was analyzed using the trypan blue method. B. Effect of polysaccharides from *S. nitidum* (10 and 50 mg/kg) on the expression of the cytokines IL-1ra, IL-10, IFN-γ, IL-2, and MIP-1β in the peritoneal wash of mice with zymosan-induced peritonitis. *a: Values of IL-1ra that were out of range (OOR). C. Assessment of the modulation of NF-κB expression in the peritoneal wash of mice with zymosan-induced peritonitis and previously treated with different doses of polysaccharides from *S. nitidum* (30, 50 and 100 mg/kg). The values were expressed as mean±SD. A value of $p<0.001$(***) was considered statistically significant.

positively or negatively modulate the immunological system. The significant reduction of edema observed in the rats treated with polysaccharides from the fungus *Scleroderma nitidum* can be observed in two plantar edema models, suggesting their anti-inflammatory effect.

Macrophage activation via the classic pathway leads to the secretion of nitric oxide and pro-inflammatory cytokines, whereas activation via the alternative pathway leads to the release of anti-inflammatory cytokines (Gordon, 2003).The polysaccharides from the fungus (glucans) *Scleroderma nitidum* did reduce INF-γ levels in the peritoneal wash of the mice. Futhermore, we observed a reduction in the levels of IL-2, a cytokine that stimulates T cell proliferation (Malek & Bayer, 2004).

The macrophage inflammatory protein 1 beta (MIP-1β) is a chemiokine that induces chemiotaxis and T cell adhesion (Tanaka et al., 1993). The MIP-1β levels were found to be reduced after treatment with polysaccharides from *Scleroderma nitidum*. IL-1ra can inhibit the pro-inflammatory effects of IL-1 (Dinarello, 1992; 1994). Polysaccharides from the fungus *Scleroderma nitidum* caused a three-fold increase in IL-1ra levels in the peritoneal wash of mice with zymosan-induced peritonitis. In the present study a substantial increase in IL-10 levels was also found.

The increase in anti-inflammatory cytokine levels concomitant with the decrease in pro-inflammatory cytokine release is a plausible explanation for the anti-inflammatory activity of polysaccharides extracted from the fungus *Scleroderma nitidum.* Given that polysaccharides from the fungus *Scleroderma nitidum* were capable of decreasing nitric oxide levels and modulating cytokine expression, we decided to assess a possible action mechanism to explain their biological effects. Thus, an assay was conducted to determine the expression of transcription factor NF-kB. In the groups treated with polysaccharides from the fungus *Scleroderma nitidum,* a significant reduction in NF-κB expression was observed, showing that the polysaccharides could modulate nitric oxide and cytokine levels through the regulation of NF-κB expression. This fact may explain the anti-inflammatory effect of these polysaccharides.

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***Correspondence**

Edda Lisboa Leite
Department of Biochemistry, Bioscience Center, UFRN
Av Sen Salgado Filho, 3000, Natal-RN, Brazil
eddaleite@cb.ufrn.br
Tel.: +55 84 3215 3216