Inhibition of Nitric Oxide and Tumour Necrosis Factor-α Production in Peritoneal Macrophages by *Aspergillus nidulans* Melanin

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The naturally occurring pigment, melanin is found in organisms of all phylogenetic kingdoms, including fungi, and exhibits a wide range of biological activities. Our objective was to investigate the effects of melanin extracted from the fungus *Aspergillus nidulans* on the production of the pro-inflammatory mediators nitric oxide (NO) and tumour necrosis factor-α (TNF-α) in peritoneal macrophages and on the viability of McCoy mouse fibroblasts. The results showed that *A. nidulans* melanin did not stimulate NO production in macrophages, but it inhibited the NO production in lipopolysaccharide (LPS)-stimulated macrophages by approximately 82%. Similarly, *A. nidulans* melanin inhibited LPS-stimulated TNF-α production by 52% and showed a slight stimulatory effect on TNF-α production in macrophages. In addition, the toxicity of *A. nidulans* melanin to McCoy cells was much lesser (IC_{50}=373.5±2.4 μg/mL) than that of known agents such as cisplatin (IC_{50}=41.2 μg/mL). The viability of peritoneal macrophages was greater than 90% at the highest melanin concentration tested (100 μg/mL). Thus, the combination of low cytotoxicity and marked inhibition of TNF-α and NO production suggests that *A. nidulans* melanin has potential as an anti-inflammatory agent and may be used in the future for development of new drugs with therapeutic utility.

Key words *Aspergillus nidulans*; melanin; nitric oxide; tumour necrosis factor-α; fungus

Recently, the discovery of natural compounds that may be involved in the modulation of the immune system has attracted great attention because the inflammatory processes can induce oxidative stress and reduce antioxidant capacity.[1)] Several studies have shown that overproduction of free radicals can be a predisposing factor for cancer and age-related disorders such as Alzheimer's disease, Parkinson's disease, diabetes, asthma, and heart disease.[2–4)]

Inflammation is a complex process initiated by several factors ranging from bacterial infection and chemical agents to environmental pollution that result in cellular injury or tissue destruction. This trauma causes the release of inflammatory mediators, including cytokines such as tumour necrosis factor-α (TNF-α) and reactive nitrogen intermediates, such as nitric oxide (NO), which are produced by immune effector cells, including macrophages. Many studies have shown that overproduction of TNF-α and NO contributes to excessive inflammatory reactions and the subsequent deleterious effects on the human body.[5–8)]

TNF-α is an important pro-inflammatory cytokine with pleiotropic effects on biological and immunological processes. It is involved in the stimulation of the secretion of other inflammatory mediators and plays a pivotal role in the host defence to bacterial, viral, and parasitic infections. TNF-α is synthesized by various activated phagocytic and non-phagocytic cells, including macrophages, neutrophils, lymphocytes, endothelial cells, smooth and cardiac muscle cells, and osteoclasts. According to the literature, the excessive production of this cytokine contributes to many diseases, including septic shock, rheumatoid arthritis, and multiple sclerosis.[9–11)]

NO is involved in the regulation of various physiological processes, it acts as a biological mediator similar to neurotransmitters in the nervous system, it regulates the blood vessel tone in vascular systems, and it is an important host defence effector in the immune system. On the other hand, NO is a nitrogen free radical and can act as a cytotoxic agent in pathological processes, particularly in inflammatory disorders.[12–14)]

NO is mainly produced by microglia and macrophages from L-arginine by a family of enzymes known as NO synthases (NOS). There are three distinct isoforms of NOS, and their distribution is largely related to their respective functions.[15)] Neuronal NOS (nNOS), is constitutively expressed in the neuronal cells and is thought to play a role in neurotransmission. The endothelial isoform (eNOS) is also constitutively expressed and regulates the vascular tone and platelet aggregation. The third isoform of NOS is inducible NOS (iNOS) found in macrophages, which produce NO during bacterial infection or tumour cell cytolysis. The constitutive NOS isoforms produce small quantities of NO that are required for normal physiological functions, while iNOS is expressed under pathological conditions such as in response to bacterial lipopolysaccharide (LPS) or pro-inflammatory cytokines such as TNF-α, interleukin 1β (IL-1β), and interferon α (IFN-α), and it could be involved in the multistage process of carcinogenesis.[16,17)]

A recent review showed that many natural products used to treat human diseases originated from microbial species.[18)] Among these, fungi represent an economically significant source of bioactive compounds because they are capable of producing high yields of substances with greatly diverse chemical structures, which exhibit a wide range of activi-

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ties.[19–23] However, only a small fraction of the estimated fungal biodiversity has been investigated for these compounds and there is great possibility to discover species that produce new bioactive metabolites.[22,23]

The pigment melanin is found in organisms from all phylogenetic kingdoms, including fungi, and melanin exhibits thermoregulatory, photoprotective, antimicrobial, antiviral, antioxidant, cytotoxic, and anti-inflammatory activities.[24,25] This pigment, formed by oxidative polymerization of phenolic or indolic compounds, is not essential for fungal growth or development but has been reported to act as 'fungal armour' because of its ability to protect the microorganisms from harmful environmental conditions such as ultraviolet (UV) radiation, extreme temperatures, hydrolytic enzymes, heavy metal toxicity, and antimicrobial drugs.[25–27]

Previously, we showed that melanin isolated from the MEL1 mutant of the fungus *Aspergillus nidulans*, which produces high levels of this pigment, is a potential hypochlorous acid scavenger and may be a promising component for cosmetic formulations that protect the skin against oxidative damage.[28] Recently, we characterized this pigment as 3,4-dihydroxyphenylalanine (DOPA)-melanin according to its physicochemical properties and tests with melanin biosynthesis inhibitors.[29]

Some naturally occurring bioactive substances with antioxidant properties, such as phenols, vitamins, carotenoids, and terpernoids, also exhibit anti-inflammatory activity and could be used in prevention of diseases and stimulation of the immune system.[30]

The purpose of the present study was to evaluate the immunomodulatory properties of the melanin extracted from the fungus *A. nidulans*. We examined the effect of this pigment on the production of NO and TNF-α in peritoneal macrophages and evaluated its cytotoxic effect on macrophages and fibroblasts in order to identify its biological activities with an aim of developing therapeutic applications in the future.

MATERIALS AND METHODS

Materials McCoy cell line (ATCC CRL-1696b) and Eagle's medium were obtained from Adolfo Lutz (São Paulo, Brazil). Foetal bovine serum (FBS) from Cultilab (Campinas, Brazil). Neutral red (NR) from Riedel-de-Haën AG (Seelze, Hannover). RPMI 1640, LPS (*Escherichia coli* O111:B4), dimethyl sulfoxide (DMSO), penicillin, streptomycin, L-glutamine, actinomycin D and 2-mercaptoethanol were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals not mentioned were obtained from Sigma Chemical Co.

Strains and Growth Conditions The present study was performed with MEL1, a highly melanised mutant, isolated from *A. nidulans* fungus by Pombeiro.[31] The mutant was grown in Erlenmeyer flasks containing 100 mL of liquid minimal medium, as described in Cove,[32] supplemented with 55 mmol/L glucose as the carbon source, 70 mmol/L sodium nitrate as the nitrogen source, and inositol (20 μg/mL) as nutritional requirement. The medium was inoculated with 10^6 conidia/mL and incubated at 37°C in a rotary shaker at 220 rpm for 72 h. The mycelium was then collected by vacuum filtration and the melanin present in the culture medium was extracted.

Melanin Extraction and Purification Melanin in the culture medium was extracted using the method described by Paim *et al.*[33] The culture medium was acidified to pH 1.5 with 6 mol/L hydrochloric acid (HCl) and allowed to stand overnight. The precipitated pigment was then recovered by centrifugation at 4500×***g*** for 15 min.

Melanin purification was performed according to the procedure of Sava *et al.*,[34] with minor modifications. The precipitate obtained after centrifugation was washed with distilled water and subjected to acid hydrolysis with 7 mol/L HCl and 5% phenol in a nitrogen atmosphere for 7 days to remove carbohydrate and protein. The sample was then treated with an organic solvent (chloroform, ethyl acetate, and ethanol) to wash away lipids. The residue obtained was then dried at room temperature, re-dissolved in 2 mol/L NaOH and centrifuged at 4000×***g*** for 15 min. The supernatant was precipitated by adding 1 mol/L HCl, washed with distilled water and lyophilized. The amount of pigment produced was approximately 175 μg/mL of the culture medium.

Cytotoxicity Assay The mouse fibroblast McCoy cell line (ATCC CRL-1696b) was maintained in Eagle's medium with 7.5% FBS. The cells were harvested from culture by trypsinization and aliquots of 200 μL medium (10^4 cells/mL) were seeded into 96-well tissue-culture plates and incubated at 37°C in a 5% CO_2 atmosphere for 24 h. The culture supernatants were removed and replaced with the medium alone (controls) or with medium containing various concentrations of melanin and cells were incubated for a further 24 h under the same conditions. The medium was removed and the cells were subjected to the Neutral Red (NR) viability assay, as described in Borenfreund and Puerner.[35] The optical density of each well was measured on a microplate reader (Multiskan, Labsystem) at 540 nm, with 620 nm as a reference wavelength. All experiments were performed at least 3 times, with triplicate wells for each condition. Linear regression analysis with 95% confidence limit was used to construct a dose–response curve and to estimate the concentration that reduced the absorbance by 50% (inhibitory concentration, IC_{50}).

Animals Swiss mice (6 to 8 weeks of age, weighing 18 to 25 g) were supplied by Faculty of Pharmaceutical Science of Araraquara-UNESP. Animals were housed in polycarbonate boxes and maintained on a 12-h light/dark cycle at 23± 1°C and 55±5% humidity, with 10–18 air exchanges per hour, with water and food available *ad libitum*. The UNESP Institutional Animal Care and Use Committee approved all of the employed protocols (06/2005).

Peritoneal Macrophages Thioglycollate-elicited peritoneal exudate cells (PEC) were harvested from Swiss mice by using 5.0 mL of sterile phosphate-buffered saline (PBS) pH 7.4. The cells were washed twice by centrifugation at 200×***g*** for 5 min at 4°C and resuspended in the appropriate medium for each test.

Cell Viability To determine PEC viability in the presence of melanin, the cells were resuspended at a concentration of 5×10^6/mL in RPMI 1640 (Gibco) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, and 0.05 mol/L 2-mercaptoethanol. Samples of cell suspension (5×10^5 cells/100 μL per well) were placed in a 96-well tissue-culture plate and mixed with 100 μL of medium containing 0, 25, 50 or 100 μg melanin/mL in 0.1% DMSO. The cells were incubated for 24 h at 37°C in a 7.5% CO_2 humidified atmosphere. The medium was then removed

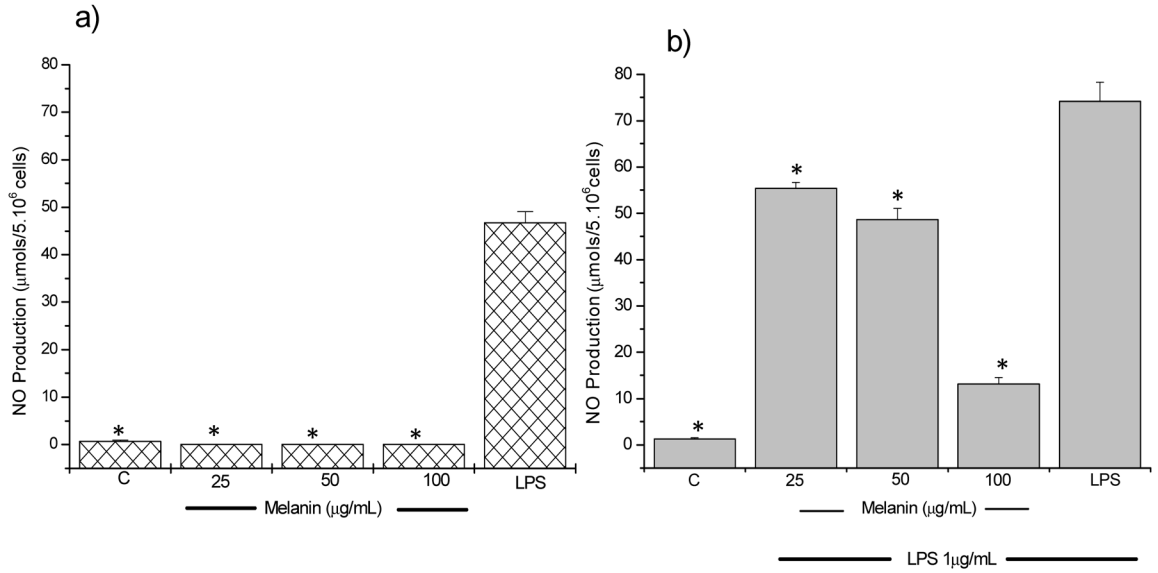

Fig. 1. Effects of Melanin from *A. nidulans* on Nitric Oxide (NO) Production in Peritoneal Macrophages

Adherent cells (5×10^6) were incubated for 24 h with melanin alone (a) or with melanin plus lipopolysaccharide (LPS) (b). Cell-free supernatants were collected and NO production was quantified with Griess reagent. Cells incubated with LPS alone or medium alone served as positive or negative controls, respectively. Data were analyzed by one-way analysis of variance (ANOVA). *$p<0.05$ *vs.* LPS.

and the cells were subjected to the NR assay as described above. All experiments were performed at least 4 times, with triplicate wells for each condition. The optical density of the cells incubated with culture medium alone was taken as 100% viability. The maximum concentration of DMSO used in the experiments had no effect on cell viability and on the NO and TNF-α production.

Measurement of NO Production NO production was determined by measuring the nitrite concentrations in culture supernatants by using Griess reagent (sulphanilamide 0.1%, phosphoric acid 3%, naphthylethylenediamine 0.1%). Adherent PEC at 5×10^6 cells/mL were incubated for 24 h at 37°C in a 7.5% CO_2 atmosphere with 25 μL of 1 μg/mL LPS (*Escherichia coli* O111:B4), either alone or with 25 μL of 25, 50, and 100 μg melanin/mL in 0.1% DMSO. After incubation, 100 μL of cell-free supernatant was mixed with 50 μL of Griess reagent and incubated at room temperature for 10 min. The absorbance was then measured at 540 nm. Nitrite concentrations were determined against a standard curve constructed from dilutions of sodium nitrite in culture medium.[36]

Measurement of TNF-α Production TNF-α production was determined by a bioassay using L929 cells. This mouse fibrosarcoma cell line undergoes cell death in the presence of TNF-α. PEC cells were resuspended in supplemented RPMI 1640 medium at 5×10^6 cells/mL and aliquots of 1 mL were added to 24-well plates. Cells were incubated for 24 h at 37°C in a 7.5% CO_2 atmosphere with 100 μL of LPS (1 μg/mL), either alone or in the presence of 100 μL of 25, 50, or 100 μg melanin/mL in 0.1% DMSO. The culture supernatants were then removed and assayed for the presence of TNF-α. Briefly, L929 cells were resuspended in supplemented RPMI 1640 medium and added to 96-well plates at 4×10^5 cells/well. The cells were mixed with 100 μL actinomycin D (final concentration, 2 μg/mL) together with control or test culture supernatants and incubated at 37°C in a 7.5% CO_2 atmosphere. After 24 h, the viability of L929 cells was assessed by the NR assay as described above. TNF-α was quantified using a standard curve constructed with recombinant TNF-α. One unit of TNF-α was defined as the amount that induced death of 50% L929 cells, and is equivalent to approximately 1 pg of recombinant TNF-α.[37]

Statistical Analysis The results of the NO, TNF-α and NR assays were expressed as mean±standard error (S.E.). Differences between groups were determined by analysis of variance (ANOVA) and were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

During an inflammatory response, cells of the innate and acquired immune systems, including mast cells, neutrophils, and macrophages, release a variety of mediators that enhance specific aspects of the inflammatory response.[8,38] Among these mediators, NO, TNF-α, and reactive oxygen species produced in excess are implicated in the pathogenesis of a number of inflammatory diseases.[39,40] Identification of new drugs that can interfere with the production of TNF-α and/or NO and can be used for the treatment of chronic inflammatory diseases, autoimmune diseases, septic shock, ulcerative colitis, AIDS, and cancer has recently attracted a lot of attention.[41,42]

To evaluate whether *A. nidulans* melanin induces NO or TNF-α production in macrophages, the cells were incubated with various concentrations of melanin and the culture supernatants were collected. The results indicate that macrophages incubated with melanin for 1 h did not produce NO (Fig. 1a). Melanin-treated cells produced significantly lesser amounts of the pro-inflammatory cytokine TNF-α (Fig. 2a) than the LPS control, and this difference was confirmed by statistical analysis.

The possible anti-inflammatory activity of *A. nidulans* melanin was tested in an *in vitro* model of inflammation. Macrophages were treated with LPS, which stimulates the production of pro-inflammatory mediators, in the presence of various concentrations of melanin and then NO and TNF-α levels were measured. The results showed that this pigment inhibited

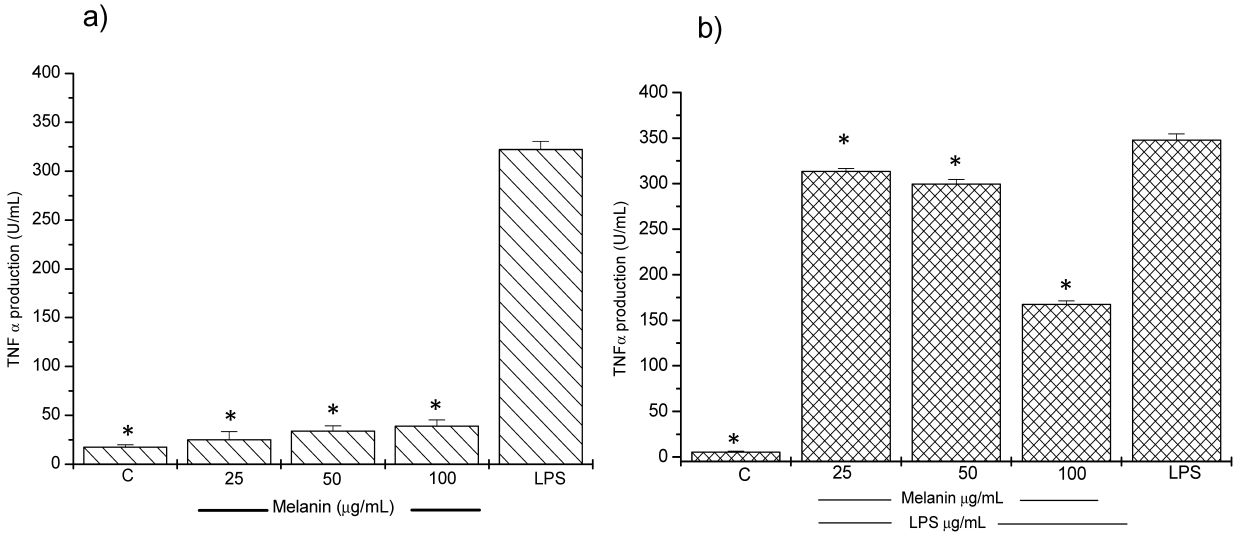

Fig. 2. Effects of Melanin from *A. nidulans* on Tumour Necrosis Factor-α (TNF-α) Production in Peritoneal Macrophages

Adherent cells (5×10^6) were incubated for 24 h with melanin alone (a) or with melanin plus lipopolysaccharide (LPS) (b). Cells incubated with LPS alone or medium alone served as positive or negative controls, respectively. Data were analyzed by one-way ANOVA. *$p<0.05$ *vs.* LPS.

Table 1. Inhibition of NO and TNF-α Production in LPS-Stimulated Macrophages by *Aspergillus nidulans* Melanin

		NO production		TNF-α production	
		μmol/5×10^6 cells	% inhibition	U/mL	% inhibition
Melanin concentrations	25 μg/mL	55.28±1.32	25.44±1.23	313.53±3.01	9.83±0.98
	50 μg/mL	48.66±2.36	34.37±0.98	299.53±5.01	13.85±1.02
	100 μg/mL	13.13±1.32	82.29±2.51	167.36±4.01	51.86±1.14
Positive control (LPS)		74.15±4.12	—	347.70±7.01	—
Negative control		1.27±0.20	—	5.14±1.01	—

NO production in a dose-dependent manner (Fig. 1b), and the percentage inhibition varied from 25.44 to 82.29% (Table 1).

The controlled production of NO plays an important role in immune defence. NO affects virtually every step in the progression of inflammation, mainly because of to its reaction with superoxide to form a strong oxidant, peroxynitrite.[43] Our data showed that melanin has immunomodulatory properties because it strongly inhibited NO production in LPS-stimulated macrophages, with a maximum response of 82% inhibition at a melanin concentration of 100 μg/mL. Two mechanisms can be proposed to explain the inhibition of NO production in LPS-stimulated macrophages by fungal melanin. Melanin may inhibit the induction of iNOS at the transcriptional level, because NO production was inhibited in a concentration-dependent manner.[44] Further, melanin may act by directly neutralising NO, because although NO is relatively stable, it is a nitrogen free radical and fungal melanin can act as a free radical scavenger.

Melanin from *A. nidulans* also showed a dose-dependent inhibitory effect on LPS-stimulated TNF-α production (Fig. 2b), and the percentage of inhibition ranged from 9.83 to 51.86% (Table 1). This is an important result because TNF-α induces a number of pro-inflammatory changes in endothelial cells, including cytokine production, expression of adhesion molecules, release of pro-coagulatory substances, induction of iNOS and subsequent enhancement of NO production.[39,45] In this context, it is likely that the melanin-induced inhibition of TNF-α production contributed to the inhibition of NO production after treatment with this pigment (Table 1).

Our results are consistent with those reported in other studies. Mohagheghpour *et al.*[46] showed that synthetic melanin, produced by tyrosinase oxidation of tyrosine, diminished the production of some cytokines, including IL-1, IL-6, and TNF-α, and showed anti-inflammatory properties. Wang and Mazza[47] showed that various polyphenolics, compounds structurally similar to melanin, diminished the production of NO in macrophages stimulated with IFN-γ and LPS. Bocca *et al.*[48] also found that treatment of IFN-γ and LPS-stimulated macrophages with melanin, obtained from the fungus *Fonsecaea pedrosoi*, inhibited NO production. The study performed by Zhang *et al.*[49]on the pigmented cells of the *Fonsecaea monophora* fungus showed that iNOS expression in activated macrophages decreased after treatment with melanin in a dose-dependent manner, and these results were similar to our results. In the same study, lower levels of cytokines such as IL-12 and TNF-α, were observed, which showed that the pigment causes inhibition of the Th1-mediated response.

Kunwar *et al.*[50] showed that melanin isolated from the fungus *Gliocephalotrichum simplex* reduced the radiation-induced overproduction of pro-inflammatory cytokines (IL-6 and TNF-α), which might help in the recovery from radiation injury by preventing the aggravation of inflammation and oxidative stress. Additionally, this melanin was shown to be a very effective radioprotector in a mouse model. The probable mechanism involved is the modulation of signal transduction pathways since pre-treatment with this pigment reversed the

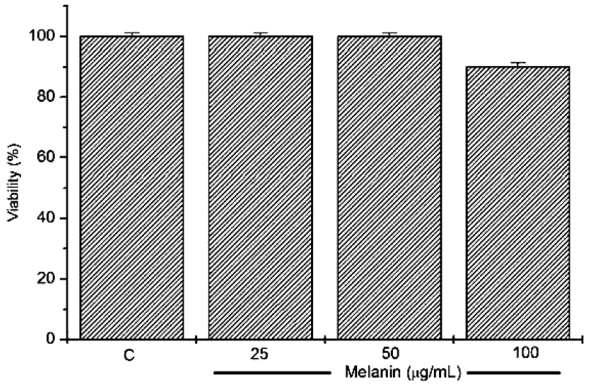

Fig. 3. Effects of Melanin from *A. nidulans* on the Viability of Peritoneal Macrophages

Adherent cells (5×10^6) were incubated with or without melanin for 24 h. Cell viability was determined by the NR assay. Cells in culture medium alone (control) correspond to 100% of viability. Data were analyzed by one-way ANOVA.

radiation-induced decrease in extracellular signal-regulated kinase (ERK) phosphorylation.

A wide variety of natural products are claimed to possess immunomodulatory activity, but it is often difficult to differentiate this activity away from their associated cytotoxicity.[51] In view of a potential practical application of the melanin from *A. nidulans* as anti-inflammatory agent, it was important to evaluate the toxicity of this pigment.

To exclude the possibility that any inhibitory effects of melanin on NO or TNF-α production were due to cytotoxicity, we examined the viability of mouse macrophages cultured in the presence of melanin. The results of these experiments showed that the viability of thioglycolate-elicited peritoneal macrophages remained greater than 90% when these cells were treated with the highest melanin concentration tested (100 μg/mL, Fig. 3). The results also showed that the concentration of melanin that led to a 50% decrease in the viability (IC_{50}) of McCoy cells was 373.5±2.4 μg/mL (Fig. 3), as measured by the neutral red (NR) cell viability assay. This result indicated that melanin has a lower cytotoxicity than known cytotoxic agents such as cisplatin or epirubicin, for which the IC_{50} values are 41.2 μg/mL and 15.9 μg/mL, respectively, against the same cell line.[50] Thus, we showed that *A. nidulans* melanin has a much lower cytotoxic effect under the experimental conditions used to evaluate the immunomodulatory activity of this pigment.

The results obtained in this study suggest that melanin extracted from the MEL1 mutant of the *A. nidulans* fungus has potential as an anti-inflammatory agent because it exhibited marked inhibition of NO and TNF-α production and low cytotoxicity. Our findings may contribute to a better understanding of the biological activities of fungal melanin and might also be useful in the future for the development of new therapeutic agents.

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