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Antifungal-demelanizing properties and RAW264.7 macrophages stimulation of glucan sulfate from the mycelium of the mushroom *Ganoderma lucidum*

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Abstract Underutilized mycelium of *Ganoderma lucidum* BCCM 31549 has been a significant source of a glucan sulfate (GS) possessing therapeutic activities. GS have been evaluated for their antifungal-demelanizing properties and nitrite oxide production from stimulated RAW264.7 macrophages. GS exhibited antifungal activity against *Aspergillus niger* A60 with a minimum inhibitory concentration of 60 mg/mL and a minimum fungicidal concentration of 100 mg/mL. At 60 mg/mL (sublethal) and 30 mg/mL (subinhibitory) doses of GS, the mycelium of *A. niger* A60 was successfully demelanized with a conidiophore head and black pigment reduction. Additionally, GS successfully stimulated RAW264.7 macrophage cells at a concentration of 500 μg/mL to produce 0.45 μM of nitric oxide. The GS-stimulated RAW264.7 macrophages were morphologically similar to those treated with lipopolysaccharide. The results highlight a novel bifunctional property of mycelial GS from *G. lucidum*.

Keywords: antifungal activity, demelanization, *Ganoderma lucidum*, glucan sulfate, nitric oxide production

Introduction

For many years, mushrooms have been used for treating various health conditions or diseases due to their therapeutic properties. The practice of using mushrooms is common in traditional medicine but has also recently attracted increased attention in conventional medicine. With the rise of mushroom-based drugs in the pharmaceutical industry, their therapeutic potential is well established (1,2). Extracted compounds from mushrooms of the genus *Ganoderma* exhibit potential medicinal effects in the treatment of various human diseases (3), particularly cytotoxicity (4), antimicrobial (4), antioxidant (5), immunomodulatory (5), and antifungal (6) properties, demelanization (7), and nitric oxide (NO) production (8).

Ganoderma lucidum is a well-known species of mushroom, and its use in traditional medicine has prompted scientists worldwide to undertake mass cultivation and production of the bioactive metabolites of this fungus (9-11). It produces several functional metabolites with biological activity, such as polysaccharides (4), proteins (6), and ganoderic acids (12), which might explain some of the observed medicinal properties. The polysaccharide fractions appear to be the primary source of the biological activity of *G. lucidum* (13). The solubility of these polysaccharides has been improved to widen their application and ease of experimental use (14), resulting in highly bioactive sulfated polysaccharides (4).

Sulfated polysaccharides or glucans are commonly found in marine organisms (15) and have recently been reported as less prevalent in mushrooms (4). However, the sulfated glucan with a proposed structure of β-1,3-D-glucan, derived from the mycelium of the mushroom *G. lucidum*, has exhibited antimicrobial activity against normal pathogenic bacteria. It has also been shown to be noncytotoxic to normal human cells. This glucan sulfate (GS) is soluble and its therapeutic activities is significantly impactive compared with nonsulfated glucans (4). Based on this properties, *G. lucidum* glucan compounds (16) could be utilized for their prospective bifunctional effects (17).

G. lucidum has been shown to possess various biological activities including a demelanizing effect and antifungal activity (6,18). A great deal of interest has also focused on the immunomodulatory ability of

this species pertaining to macrophages (white blood cells). Macrophages act as the major host barrier against cancer growth and bacterial infection (19,20), and consequently, play an imperative function as an initiator for adaptive immune responses caused by NO.

A highly-reactive free radical compound, NO is produced by NO synthase from L-arginine (21). It causes cytolysis in tumors and is therefore implicated in the antitumor immune response (19). Activated macrophages release NO (8), which provides a defense mechanism against pathogens by modulating inflammation. However, most of the reported positive bioactivities mentioned above were demonstrated in compounds obtained from fruiting bodies and not from their mycelial cultures in bioreactors. Such cultures represent a faster way to produce bioactive compounds from *G. lucidum* compared with extraction from mushrooms, as discussed in a previous study (9). The current study examines the biological activity of β-glucan produced from mycelial cultures.

To date, NO production activity and the antifungal-demelanizing properties of *G. lucidum* mycelial extracts, mostly GS, have not been evaluated. In this study, GS showed significant antifungal-demelanizing activity in addition to activating RAW264.7 macrophage cells, and hence could potentially be utilized as a food additive. In doing so, its presence would inhibit both pathogenic and spoilage fungi while conveying the important health benefits described in this study.

Materials and Methods

Materials The RAW264.7 mouse macrophage cell line was obtained from the European Collection of Authenticated Cell Cultures (ECACC), in partnership with Sigma-Aldrich (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin (Sigma-Aldrich) was used to culture the cell line in a 5% CO_2 atmosphere at 37°C. RPMI-Bio Whittaker® (lacking L-glutamine) was obtained from Lonza (Vergiers, Belgium). Hank's balanced salt solution (HBBS) was obtained from Sigma-Aldrich. For cell culture, 96-well plates (TPP 92096) were obtained from TPP (Trasadingen, Switzerland). A standard Wallac Victor2™ H20 (PerkinElmer, Waltham, MA, USA) multilabel counter with infrared, high-density time-resolved (TR)-fluorometry, robot loading and stacker was used for cell culture spectroscopy analysis. All chemicals and solvents used for analysis were of analytical grade. *Aspergillus niger* A60 was obtained from the Fermentation Centre, Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS), University of Strathclyde, Glasgow, UK and used for the screening study. Potato dextrose agar (PDA) was used for culture maintenance at 4°C. Only cultures from the standardized stock culture stored in a −80°C freezer were used.

Source of sulfated glucan *G. lucidum* BCCM 31549 was obtained from the Belgian Coordinated Collections of Microorganisms (BCCM/MUCL), (Agro) Industrial Fungi and Yeast Collection (Leuven, Belgium) and subcultured onto PDA (Oxoid Limited, Basingstoke, UK). Plates were inoculated and incubated at 30°C for 7 days prior to storage at 4°C. Strain preservation was performed on PDA slants. The liquid fermentation strategy was implemented in a 2.5 L stirred-tank bioreactor (Bioflow 3000 Fermentor; New Brunswick, Ramsey, MN, USA) as described previously (9), and the mycelium was extracted to produce glucan. The sulfated (1,3)-β-D-glucan (4) was obtained from the sulfation process of the native glucan and used in the screening study.

Bioassay for antifungal and demelanizing activity on *A. niger* A60
A modified microdilution technique from Heleno *et al*. (7) was used to examine the antifungal activity of GS in terms of measuring the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). GS was dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) at (mg/mL): 200, 125, 100, 60, 30, 20, 15, 10, 2, and 1 and added to potato-dextrose medium with inoculum. A commercial fungicide, bifonazole (Sigma-Aldrich), was used as a positive control (2 mg/mL) with DMSO as a negative control.

The demelanizing activity of GS on *A. niger* A60 was tested in 96-well microtiter plates in order to determine the minimum demelanizing concentration (MDC) (7). Examination of the treated mycelium after fixation with lactophenol was accomplished using a light microscope (Nikon, Tokyo, Japan). The untreated control plate samples were also examined under the same conditions. DMSO (5%) solution was used as a negative control. The MDC was defined as the lowest concentration that caused the demelanization of conidia and fungal hyphae.

Stimulation of macrophage cells and measurement of NO
RAW264.7 macrophages were cultivated according to the technique of Chiong *et al*. (20), with some modifications. Following centrifugation at 112x*g* and 4°C, cell density was adjusted to 2.5–3×10^5 cells/mL. Cell viability was maintained at above 80%. Seventy-five μL of cells were dispensed into each well of the 96-well plates to obtain 5×10^4 cells/well. Plates were incubated for 6 h at 37°C in a 5% CO_2 atmosphere to attach the cells. Floating cells were discarded gently after 2 h of incubation while attached cells were removed mechanically (Corning® cell scraper, Sigma-Aldrich) to collect the desired cell population. The concentrations of glucan, GS, and 0.1 M NaOH (solvent and negative control) used to stimulate the attached cells were (μg/mL) 500, 200, and 50, in a final volume of 200 μL/well. As a positive control, lipopolysaccharide (LPS) extracted from *Escherichia coli* 0111: B4 (Sigma-Aldrich) was used. Incubation for 24 h at 37°C in a humidified 5% CO_2 atmosphere was standard for all cell cultures.

The level of NO in cell-free culture supernatants, which reflects intracellular NO synthase activity, was evaluated by the Griess reaction using a colorimetric nitrate/nitrite assay kit (23479; Sigma-Aldrich). Assay sample preparation was performed by centrifuging

the reacted medium at 150x*g* for 15 min, and supernatant was used as a sample solution. The determination of nitrite was performed in 96-well plates. Sample solutions (80 μL) were added to each well, followed by 20 μL of buffer solution. Next, 80 μL of Griess Reagent A was added and mixed. After 5 min, 50 μL of Griess Reagent B was added and mixed. Plate incubation was performed at room temperature and absorbance was measured at 570 nm in a microplate reader. For the calculation of NO levels, the blank solution (well A) absorbance was subtracted from the absorbance of each well. Nitrite concentration in the sample solution was determined from a predetermined nitrite standard calibration curve.

Statistical analysis Analyses were carried out in triplicate, and the respective mean±SD was calculated using GraphPad Prism 5 software (Version 5.01). Error bars were shown accordingly, and if not visible, were less than the size of symbols used. Statistical differences were determined using Student's *t*-test, one-way analysis of variance (ANOVA), and Bonferroni's post-test.

Results and Discussion

Antifungal activity of GS on *A. niger* A60 The antifungal activity of GS against *A. niger* A60 is shown in Table 1 with the growth morphology (elongated fungal mycelial filaments) shown in Fig. 1. GS exhibited antifungal activity against the test fungi with an MIC of 60 mg/mL and MFC of 100 mg/mL. In this study, antifungal screening was performed on *A. niger* A60 as previous work on an extract from *G. lucidum* fruiting bodies showed that it had the highest antifungal activity against this fungus (7). Our data showed that the GS derived from *G. lucidum* mycelia inhibited the growth of *A. niger*. However, only nonsulfated compounds present in *Ganoderma* species have been reported as antifungal, including three triterpenes (applanoxidic acids A, C, and F) produced by *G. annulare* sourced from decayed wood (22), the fruiting bodies extract of *G. lucidum* (7), and Ganodermin sourced from *G. lucidum* fruiting bodies (6). Few studies have been reported on the antifungal activity of nonsynthetic polysaccharides containing sulfate residues that are mostly in synthetic forms (23). To the best of our knowledge, our study is the first report of the antifungal activity of any possible sulfated polysaccharides derived from *G. lucidum* mycelium via extended batch cultures (4,9).

Table 1. Antifungal activity, minimum inhibitory concentration (MIC), and minimum inhibitory concentration (MFC) of glucan sulfate (GS) (mg/mL) derived from extended batch cultures of *Ganoderma lucidum* BCCM 31549 mycelium

Fungus	GS level (mg/mL)		Bifonazole (2 mg/mL)	DMSO
	MIC	MFC		
Aspergillus niger A60	60	100	(+) control	(-) control

Demelanizing effect of GS on *A. niger* A60 The demelanizing activity of GS was investigated in *A. niger* A60. The results were expressed in terms of the MDC, which was defined as the subinhibitory and sublethal concentrations that caused demelanization in the fungus during a 72 h. The subinhibitory level was 30 mg/mL, whereas the sublethal concentration was 60 mg/mL of GS (Fig. 2, images A-F).

The morphological changes that occur during the melanization of *A. niger* can be observed in Fig. 2 and represent depigmentation.

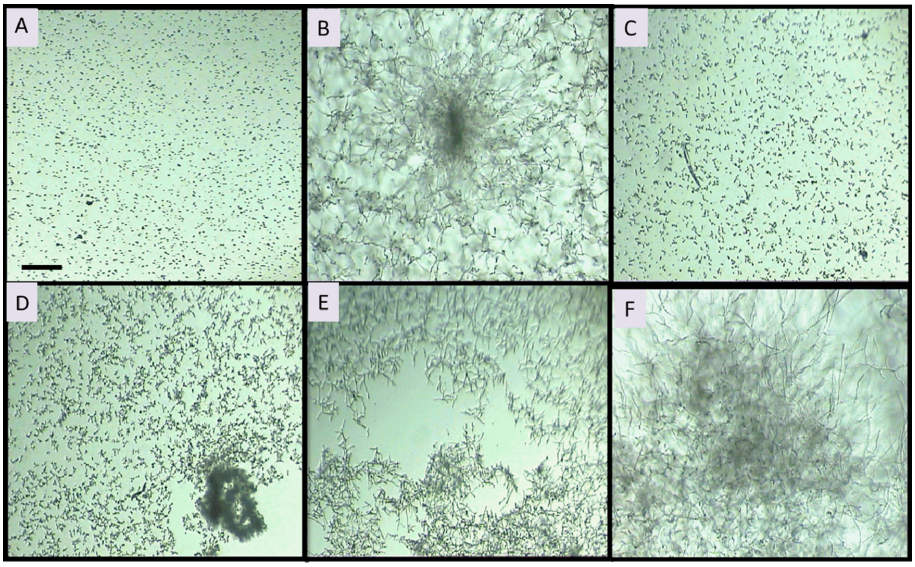

Fig. 1. Growth morphology of *A. niger* A60. (A) Positive control reaction with bifonazole at 2 mg/mL; (B) Negative control reaction with 5% DMSO solution; (C-F) Positive antifungal treatment reactions with glucan sulfate derived from extended batch cultures of *G. lucidum* BCCM 31549 mycelium at (C) 200, (D) 100, (E) 60, and (F) 30 mg/mL. Images were taken at 4-fold magnification using a compound microscope. Bar=150 μm. *Heavier filament growth indicates lower antifungal activity.

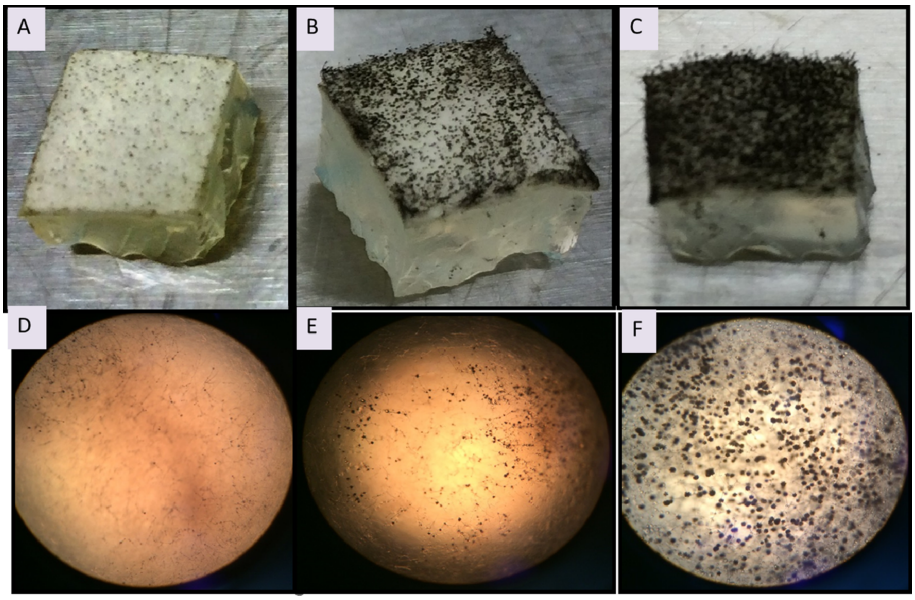

Fig. 2. Effect of glucan sulfate (GS) on antifungal-demelanizing properties of *A. niger* A60. (A) Demelanized mycelium of *A. niger* A60 stimulated with GS at 60 mg/mL; (B) Demelanized mycelium of *A. niger* A60 stimulated with GS at 32.5 mg/mL; (C) Normal mycelium of *A. niger* A60 without stimulation; (D) Culture of *A. niger* A60 with fewer heads, stimulated with GS at 65 mg/mL; (E) Culture of *A. niger* A60 with fewer heads, stimulated with GS at 30 mg/mL; and (F) Typical culture of *A. niger* A60 with numerous heads. (D-F) Images were obtained under light microscopy. GS was derived from extended batch cultures of *G. lucidum* BCCM 31549 mycelium. *Black conidiophores indicate weaker demelanizing reactions.

Upon observing the morphological variations of the conidiophores, it was noted that demelanized cultures of the tested fungi displayed a markedly reduced number of heads (Fig. 2A and 2B) in comparison with those in the untreated culture (Fig. 2C). The reduction of heads and demelanization of *A. niger* A60 spores were also observed under a light microscope (Fig. 2D and 2E) and compared with an untreated control (Fig. 2F). Melanin plays a vital role in fungal virulence and has been described as "fungal armour" due to its ability to shield against a broad range of toxins (24). It may also play a role in the survival of fungi in extreme environments (25). Therefore, disrupting the fungal armour (demelanization) would suppress the growth of pathogenic fungi such as *A. niger.*

Melanin has also been shown to protect the fungus against immune effector cells as it scavenges reactive oxygen species produced by these cells (26). The GS of *G. lucidum* showed a subinhibitory and sublethal MDC as mentioned in the text; thus we believe that the GS is directly involved in the modification or inhibition of the demelanization mechanism. Demelanizing activity outcomes are vital for the present work, since MDC is sublethal to fungus in contrast to the inhibitory and fungicidal doses.

Comparison with antifungal reactions of compounds extracted from *G. lucidum* The use of GS as an antifungal compound has not been reported previously (Table 2); therefore, the comparison of antifungal reactions is limited to available research that utilized any *G. lucidum* constituents extracted from mycelium or fruiting bodies. When compared with previous *A. niger* antifungal work by Heleno (7), the current values for MIC, MFC, and MDC (Table 1 and Fig. 2) were higher with the same pigmentation, shape, and size when viewed under the microscope. However, Fagade and Oyelade (27) tested *G. lucidum* extracts on several microorganisms and found that the MIC of *Candida albicans* was 750 mg/mL, which is higher than the MIC (60 mg/mL) we observed on *A. niger* A60. Higher MIC means that the extract is less potent, so this may indicate that the current extracted compound is more suitable for treating Aspergillosis (caused by *A. niger*) than Candidiasis (caused by *C. albicans*). Further studies are needed to confirm this, however.

Nevertheless, when compared with an earlier medicinal

Table 2. Antifungal activity of active constituents from *Ganoderma lucidum*

Source	Compound	Tested fungus	MIC[2)] (mg/mL)	Reference
Mycelium	Glucan sulfate	*Aspergillus niger* A60	60	This study
Fruiting bodies	Methanol extract	*A. niger*	1.5	(7)
NA[1)]	Ganozhi toothphase, DXN Industries (M) Sdn. Bhd. Malaysia	*C. albicans*	2	(18)
Fruiting bodies	Ethanolic extract	*C. albicans*	750	(27)
Fruiting bodies	Ganodermin (peptides)	*Botrytis cinerea*	NA	(6)

[1)]NA, Not available. The comparison is based on the available literature for *G. lucidum.*
[2)]MIC=minimum inhibitory concentration.

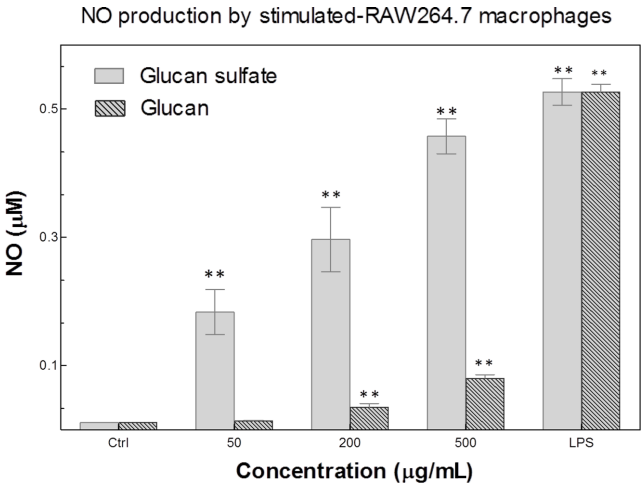

Fig. 3. Effect of glucan (G) and glucan sulfate (GS) on NO production by RAW264.7 macrophages. Cells were treated with different concentrations of compounds or LPS (2 μg/mL) for 48 h and culture supernatants were analyzed for NO production. Each value is the mean±SD of two independent experiments, performed in triplicate. ***p*<0.05, significant difference from the control group.

application of antifungal toothpaste containing *G. lucidum* against *C. albicans* at sensitivity concentrations of (mg/mL): 500, 250, 125, 62.5, 31.25, 16, 8, 4, and 2 (18), the current tested concentrations (mg/mL: 200, 125, 100, 60, 30, 20, 15, 10, 2, and 1) are approximately similar, thus indicating the suitability of *G. lucidum* extracts for future use in the bioproducts industry. To the best of our knowledge, the current *G. lucidum* BCCM 31549 mycelium extract derived from repeated-batch cultures (4,9,10) is the latest compound shown to possess antifungal-demelanizing activity against *A. niger* A60. Further modifications of this compound, including purification and fractionation, and further testing of its applicability as a food additive and in clinical and animal studies would be beneficial.

Effect of GS on NO production in macrophages As shown in Fig. 3, our study shows that NO production from RAW264.7 cells stimulated with GS (500, 200, and 50 μg/mL) for 48 h increased significantly compared with untreated controls. GS was more effective than the native glucan in initiating the release of NO from the macrophages, with 0.45, 0.29, and 0.18 μM of NO released and 0.08, 0.035, and 0.013 μM of NO released, respectively, at concentrations of 500, 200 and 50 μg/mL of GS and glucan. In human health systems, sustained production of NO confers macrophages with cytotoxicity against fungi, protozoa, viruses, bacteria and tumor cells (28). GS may therefore contribute to the high-output NO pathway in order to protect the host from infection.

Furthermore, no macrophage reactions were observed with 0.1 M NaOH, the solvent used for both glucan and GS (no pseudopodia were observed), indicating that the NO increase originated solely from to the compounds themselves (Fig. 4). Meanwhile, the stimulated-macrophage morphological reactions showed that the GS-stimulated macrophage was morphologically similar to 2 μg/mL of LPS at concentrations of 500 and 200 μg/mL. Darker green macrophages were associated with a change in macrophage morphology, causing them to take on a fibroblast-like appearance (29) or pseudopodia formation (21). Meanwhile, glucan-stimulated macrophages showed minimal morphological responses while the solvent proved to be unreactive towards the macrophage cells.

A polysaccharide isolated from *G. lucidum* fruiting body has already been shown to stimulate the release of NO (19). However,

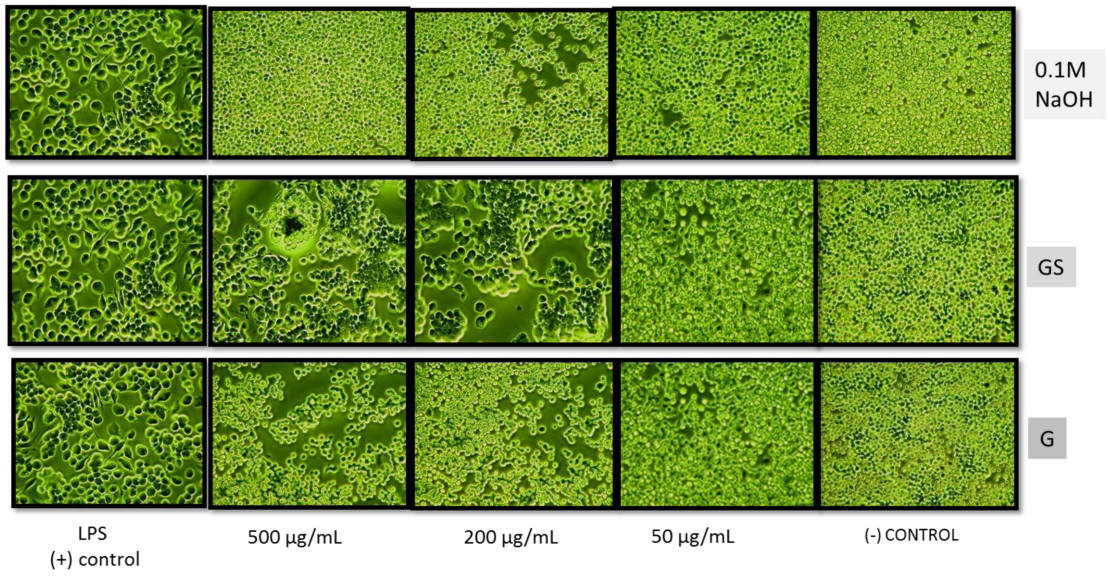

Fig. 4. Reaction diagram of NO production by RAW264.7 macrophages stimulated with glucan (G) and glucan sulfate (GS). NaOH (0.1M) was used as the solvent (indicating a negative reaction) for both compounds indicating a positive reaction independently from G and GS. All images were at 10x magnification. *Darker green indicates greater NO production.

the effect of liquid-mycelial GS has not yet been reported. The current study is the first to utilized a sulfated polysaccharide obtained from *G. lucidum* mycelium that possesses immunomodulatory reactions. The level of NO secreted from RAW264.7 cells in the presence of the glucan was observed to be very low (<0.1 μM), indicating its weak ability to trigger macrophages compared with GS. GS was less potent than the LPS positive control; however, the results are similar to those previously achieved using the aforementioned polysaccharide purified from *G. lucidum* fruiting bodies (19) and has been described as a safe compound in human cell lines (4), suggesting that GS has potential as an immunomodulator.

The GS results are comparable with those of non-sulfated polysaccharides from the mycelia of *G. lucidum* (30). That study reported stimulated growth of macrophage cells and higher levels of NO (16–17 μM at 40 μg/mL of polysaccharides) than the compounds evaluated in previous studies. It is possible that this observation was due to differences in the structure and composition of the polysaccharides when the fungus was grown in different media. The nonsulfated polysaccharides of *G. lucidum* were obtained via solid-state fermentation using soybean curd residue (31), whereas glucan was obtained through a newly implemented repeated-batch strategy with subsequent sulfation to yield GS (4,9). Further experiments comparing the polysaccharides produced through different culture methods should be performed to determine the optimum monosaccharide composition and, subsequently, the optimum culture method for the production of macrophage-stimulating polysaccharides.

In summary, GS extracted from *G. lucidum* BCCM 31549 mycelium not only has strong antimicrobial activity and considerable cytotoxicity against cancer cells (4), but also exhibits substantial antifungal-demelanizing and immunomodulatory activity. It therefore shows potential as a solution to the current research interest in producing drugs with antibacterial, anticancer, or antifungal activity as well as immunomodulating properties (32).

Relationship between antimicrobial, cytotoxicity, antifungal properties and immunomodulation by GS Despite recent advances in the treatment of infections, the issue of drug resistance persists (33). Each year, approximately 15 million people die from contagious infections (34). With the improvement of microorganism survival rate, more research must be dedicated to the development of host immunomodulatory and antimicrobial-synergistic drugs to effectively battle new types of infections. Various drugs are already available on the market that can be prescribed in combination with antifungal or antibacterial agents.

We are now in the third generation of the anti-infective plan (17), which centers on the chemistry between antibiotics and host immunomodulation. Our previous (4) and current results have indicated the potential of the aforesaid anti-infective plan, starting with antibacterial, antifungal, immunomodulatory, and antiproliferative compounds, thus introducing a novel "quad-functional" approach to the nutraceutical potential of the mushroom species, mainly through the *G. lucidum* mycelia-sourced polysaccharides. To date, the closest comparison to the current *G. lucidum* reaction is found in the hot-water mycelial extract of the oyster mushroom, exhibiting "bifunctional" properties (antimicrobial-immunomodulatory) (17).

To date, approximately 83% of all edible-medicinal mushroom products are obtained from fruiting bodies, while 15% are obtained from the mycelial extracts (35). This research indicates that *G. lucidum* mycelia may also represent an easily accessible and renewable resource compared with fruiting bodies for producing anti-infective agents.

The sulfated glucan extracted from the mycelium of *G. lucidum* BCCM 31549 showed antifungal-demelanizing activity in *A. niger* A60 and successfully stimulated RAW264.7 macrophage cells to produce an important mediator, NO. Our findings therefore demonstrate the possible applications of GS as a pharmacological, medicinal, and functional food ingredient with multifunctional benefits (antifungal and immune-stimulatory). Further studies are required, however, to elucidate the relationship between its molecular structure and biological activities.

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Disclosure The authors declare no conflict of interest.

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