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RESEARCH ARTICLE

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A new insight into purinergic pharmacology: Three fungal species as natural P2X7R antagonists

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P2X7 is a purinergic receptor involved in important physiological functions and pathological processes, such as inflammation, neurodegeneration, and pain. Despite its relevance, there is no selective antagonist useful in the treatment of diseases related to this receptor. In this context, research for a selective, safe, and potent antagonist compound that can be used in clinical therapy has been growing. In this work, we evaluated the potential antagonistic activity of three fungal extracts, namely, *Vishniacozyma victoriae*, *Metschnikowia australis*, and *Ascomycota* sp., which were discovered in a high-throughput screening campaign to search for new antagonists for P2X7R from natural products. First, the IC_{50} values of these fungal extracts were determined in J774.G8 (murine macrophage cell line) and U937 (human monocyte cell line) cells through dye uptake assays. The IC_{50} values of *V. victoriae* were 2.6 and 0.92 μg/mL, *M. australis* has IC_{50} values of 3.8 and 1.5 μg/mL, and *Ascomycota* sp. showed values of 2.1 and 0.67 μg/mL in J774.G8 and U937 cells, respectively. These extracts also significantly inhibited propidium iodide and Lucifer yellow uptake via P2X7R pore, P2X7R currents in electrophysiology, IL-1β release, and the production of oxide nitric and reactive oxygen species. The extracts did not cause cytotoxicity within a period of 24 h. The results showed the promising antagonistic activity of these extracts toward P2X7R, thereby indicating that they can be future candidates for phytomedicines with potential clinical applicability.

KEYWORDS

antagonist, drug discovery, fungi, natural products, P2X7R

The

1 | INTRODUCTION

P2X receptors are a kind of purinergic P2 ionotropic receptors cloned in the 1990s. Currently, there are seven subtypes of P2X receptors cloned in mammalian cells (P2X1-7) (Burnstock, 2009). The P2X7 receptor (P2X7R) is expressed in many physiological systems, including the endocrine, cardiovascular, immune, nervous, respiratory, reproductive, and digestive systems. In the immune system, many cell types express P2X7R, including macrophages, monocytes, dendritic cells, lymphocytes, and mast cells (Burnstock, 2009).

When activated, P2X receptors allow the passage of ions according to the electrochemical gradient. P2X7R also has this characteristic, but it can shift from a low-conductance channel (~15 ps) to a nonselective pore, an entity which has been an enigma until now. This pore allows the passage of molecules up to 900 Da, such as the fluorodyes YO-PRO-1, propidium iodide (PI), Lucifer yellow (LY), and ethidium bromide (EB) (Coddou, Stojilkovic, & Huidobro-Toro, 2011).

P2X7R has roles in many physiological processes, such as activation of immune and inflammatory responses and cell death (Coddou, Yan, Obsil, Huidobro-Toro, & Stojilkovic, 2011). Moreover, it was suggested that P2X7R participates in several pathologies, including neurodegenerative, inflammatory and infectious diseases, neuropathic pain, rheumatoid arthritis, and cancer (Broom et al., 2008; Corrêa, Marques da Silva, de Abreu Moreira-Souza, Vommaro, & Coutinho-Silva, 2010; Diaz-Hernandez et al., 2012; Lee et al., 2012; Li, Liang, & Chen, 2008; Lister et al., 2007; Mesuret et al., 2014; Raffaghello, Chiozzi, Falzoni, Di Virgilio, & Pistoia, 2006; Teixeira, de Oliveira-Fusaro, Parada, & Tambeli, 2014; Zhao et al., 2013). Despite its relevance as a therapeutic target, P2X7R antagonists are still not applied in clinical treatment. AZD9056 and CE-224,535 are the unique P2X7R antagonists that arrived in clinical trials to study their efficacy in the treatment of rheumatoid arthritis, but their use did not bring any benefit to patients (Keystone, Wang, Layton, Hollis, & McInnes, 2012; Stock et al., 2012). Thus, the search for new selective, safe, and potent compounds with antagonistic activity toward P2X7R that could be used in clinical therapy is necessary.

High-throughput screening (HTS) campaigns using synthetic chemical libraries have been performed to search new antagonists for P2X7R, especially by pharmaceutical industries. However, natural products are also valuable sources due to their biological richness and chemical structure diversity (Hong, 2011; Koehn & Carter, 2005). Moreover, there are some compounds from natural products described in the literature that have antagonistic activity toward P2X7R, such as amentoflavone from *Rheedia longifolia* Planch & Triana and emodin from *Rhein officinale* Baill (Liu, Zou, Liu, Jiang, & Li, 2010; Santos et al., 2011).

Because P2X7R activation by extracellular adenosine 5′-triphosphate (ATP) in concentrations above 100 μM induces the opening of a nonselective pore that allows the passage of dyes, some HTS methodologies are based on this biophysical property (Namovic, Jarvis, & Donnelly-Roberts, 2012). Thus, after performing an HTS campaign using a methodology previously described by our group (Soares-Bezerra et al., 2015), 1,800 extracts were tested, and three of them significantly inhibited dye uptake. In this study, we described the potential antagonistic activity of these fungal extracts on the physiological roles of P2X7R *in vitro*.

2 | MATERIAL AND METHODS

2.1 | Reagents

ATP, oxidized ATP, Brilliant Blue G (BBG), PI, LY, Triton X-100, HEPES, NaCl, KCl, $MgCl_2$, $CaCl_2$, phosphate-buffered saline, modified Griess reagent, Lipopolysaccharide (LPS) from *Escherichia coli*, dihydroethidium (DHE), and RPMI 1640 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum was obtained from Gibco (Massachusetts, MA,USA). The ELISA Kit for detection of human and murine IL-1β was purchased from R&D Systems (Minneapolis, MN, USA). A kit for the detection of lactate dehydrogenase (LDH) was purchased from Doles (Goiania, GO, Brazil).

2.2 | Extracts

The crude extracts tested in this work are extracts deposited in the Bioprospecting Platform of the Laboratory of Chemistry of Bioactive Natural Products, René Rachou Chemical Research Center, Oswaldo Cruz Foundation, Minas Gerais, Brazil. All extracts used in the screening campaign originated from plant and fungus species of Brazilian and Antarctic biomes and were collected with the authorization of the Brazilian Ministry of Environment and the Brazilian Navy. The extracts tested were randomly chosen, and the screening was carried out in a single-blind manner, because the professional that performed it did not know the names of the originating biological species (Salkind, 2010).

The active extracts were obtained from the Antarctic fungi *Vishniacozyma victoriae* and *Metschnikowia australis*, collected at the Antarctic Specially Managed Area in Admiralty Bay, King George Island, and Port Foster Bay at Deception Island, Antarctica (Vaz et al., 2011), and from the *Ascomycota* sp. endophytic fungus isolated from the macroalga *Padina gymnospora*, collected at Ilha do Mel, Paraná State, along the coast of Brazil (Loque, 2009). To produce the fungal crude extracts, the species were cultivated using solid-state fermentation. Briefly, 5 mm diameter plugs from each filamentous fungus were inoculated into the centers of Petri dishes (60 mm diameter, with 20 mL of Marine Agar). The plates were incubated at 10 ± 2°C (for *V. victoriae* and *M. australis*) and 25 ± 2°C (for *Ascomycota* sp.) for 15 days. The cultured materials from each Petri dish were then cut and transferred into 50-mL vials containing 35 mL of ethanol. After 48 h at room temperature, the organic phase was decanted, and the solvent was removed under a vacuum centrifuge at 35°C (Santiago et al., 2012). An aliquot of each dried extract was dissolved in dimethyl sulfoxide (Merck, USA) to prepare a 100 mg/mL stock solution, which was stored at −20°C. The *Ascomycota* sp., *V. victoriae*, and *M. australis* extracts were deposited under voucher numbers EX8067, EX8549, and EX8568.

2.3 | Liquid chromatography–mass mass spectrometry (LC-MS/MS) data crude extracts

The extracts were dissolved in acetonitrile. LC-MS/MS analyses were performed on a Nexera UHPLC-system (Shimadzu) connected to a maXis ETD high-resolution ESI-QTOF mass spectrometer (Bruker) and controlled by the Compass 1.5 software package (Bruker). Fractions of 20 µL were injected into a Shimadzu Shim-Pack XR-ODS III (C18, 2.2 µm, 2.2 × 200 mm) at 40°C under a flow rate of 200 µL/min. The mobile Phases A and B (0.1% formic acid in water and acetonitrile, respectively) formed an eluent gradient of an initial 0.5 min of 5% B, a linear gradient to 100% B in 12.5 min, and a hold at 100% B for 1 min. The mass spectra were acquired in positive mode at a spectral rate of 2. The ion-source parameters were set to a 500 V end-plate offset, a 4,500 V capillary voltage, a 2.0 bar nebulizer pressure, and an 8.0 L/min and 200°C dry gas flow and temperature, respectively. Data-dependent precursor fragmentation was performed at a collision energy of 40 eV. The ion cooler settings were optimized within a 40–1,000 *m/z* range using a solution of 10 mM sodium formate in 50% 2-propanol as the calibrant. Mass calibration was achieved by initial ion-source infusion of 20 µL calibrant solution and postacquisition recalibration of the raw data. Compound detection was performed by chromatographic peak dissection with subsequent formula determination according to the exact mass and isotope pattern (MS1) and database comparison of the compound fragment spectra (MS2). The sources of the reference ESI fragment spectra were an in-house database of standard compounds as well as the public spectra database MassBank (Horai et al., 2010).

2.4 | Cell culture

The J774.G8 macrophage murine cell line and the U937 human monocyte cell line were donated by Dr. Vinícius Cotta from the Laboratory on Thymus Research, Oswaldo Cruz Institute, Rio de Janeiro, RJ, Brazil. Cells were routinely maintained in culture with RPMI 1640 medium supplemented with 10% FBS at 37°C in a 5% CO_2 atmosphere.

2.5 | Cells treatment

J774.G8 or U937 cells were plated at a density of 4×10^5 cells per well in a 96-well opaque plate (Falcon). After 24 h, the cells were treated with extracts (100 µg/mL) or the P2X7R competitive antagonist BBG (100 nM) for 15 min. With the exception of the dye uptake assays, cells were treated with extracts at their IC_{50} concentration, that is, *V. victoriae* extract (2.6 and 0.92 µg/mL), *M. australis* extract (3.8 and 1.5 µg/mL), and *Ascomycota* sp. (2.1 and 0.67 µg/mL), for the J774.G8 and U937 cell lines, respectively. Then, the cells were treated with the P2X7R agonist, ATP (5 mM), for a further 10 min.

2.6 | Dye uptake assay

To identify extracts that inhibit the activation of P2X7R at a single established concentration (100 µg/mL), we applied the dye uptake methodology in the J774.G8 macrophage cell line. This methodology, previously described by our group, is based on the uptake of PI via the P2X7R pore when this receptor is activated by ATP at concentrations above 1 mM (Soares-Bezerra et al., 2015). Using this method, we tested 1,800 plant and fungus extracts from several Brazilian and Antarctic biomes. Among them, only three extracts significantly inhibited PI uptake via the P2X7R pore: *V. victoriae*, *M. australis*, and *Ascomycota* sp.

The experimental approach consisted on the measurement of PI or LY uptake by J774.G8 or U937 cells through the P2X7R-associated pore. In the beginning of the experiments, the cellular medium was exchanged to an extracellular saline solution (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, and 10 HEPES (pH 7.4). After cell treatment, PI (375 nM) or LY (3 mM; final concentration per well) was added to the cells, and after 5 min, the plate was read in a spectrophotometer (SpectraMax M5: excitation 488 nm and emission 590 nm for PI; excitation 485 nm and emission 528 nm for LY). In the experiments with LY, the saline solution of each well was replaced by phosphate-buffered saline containing 10 mM $MgCl_2$ before plate reading. The maximum permeabilization control was obtained using Triton X-100 (0.1%) directly over the cells. Negative controls were obtained from cells without treatment, but in the presence of PI or LY. The extracts with positive inhibition activity were tested again to determine the IC_{50} value using a concentration–response curve.

2.7 | Electrophysiological measurements

Whole-cell patch clamp experiments were performed at 37°C using an Axopatch-1D amplifier (Axon Instruments). Cells were transferred to a chamber mounted into a microscope stage. Patch pipettes (with a 1.2 mm outer diameter) were pulled from IBBL borosilicate glass capillaries (World Precision Instruments). Whole-cell configuration was performed as previously described (Faria, Farias, & Alves, 2005).

The series resistance was 5–11 MΩ for all experiments in standard saline (Solution A), and no compensation was applied for currents less than 1,500 pA. Above this level, currents were compensated by 88%. Measurements were discarded when the series resistance increased substantially. The mouse peritoneal macrophage (mean–standard deviation, 34.98–11.03 pF; n = 31) cell capacitance was measured by applying a 20 mV hyperpolarizing pulse from a holding potential of 20 mV. The capacitive transient was then integrated and divided by the amplitude of the voltage step (20 mV).

The currents were filtered with a corner frequency of 5 kHz (eight-pole Bessel filter), digitized at 20–50 kHz using a BNC-2110 interface (National Instruments), and acquired on a personal computer using Axoscope software and pCLAMP 9.0 (Axon Instruments). Recordings were then filtered using a low-pass digital filter with a cut-off frequency of 500 Hz in the software (WinEDR V2.6.6.; University of Strathclyde).

2.8 | Saline solutions for electrophysiology

Different saline solutions were used in the pipette or in the bath, depending on the protocol. The bath solution (in mM) consisted of

the following: 150 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, and 10 HEPES (pH 7.4). The pipette solution (in mM) consisted of the following: 150 KCl, 5 NaCl, 1 $MgCl_2$ 10 HEPES, and 0.1 EGTA (pH 7.4).

2.9 | Drug application in electrophysiology

Patch clamp experiments were carried out under perfusion (RC-24 chamber, Warner Instrument Corp.) to confirm the data obtained by micropipette application. All drugs were dissolved in saline solution immediately before use. Ion currents were studied by pulse application of ATP (from 1 to 30 s).

2.10 | LDH detection assay

J774.G8 and U937 cells (4×10^5 cells per well) were previously incubated with extracts at concentrations of four times their respective IC_{50} values. After 24 h, the cells were treated with ATP (5 mM) for a further 1 h. Then, the supernatants were collected and the LDH detection assay was performed following the manufacturer's instructions.

2.11 | ELISA assay for IL-1β detection

J774.G8 and U937 cells (4×10^5 cells per well) were treated with extracts at concentrations of their respective IC_{50} or with BBG (100 nM) for 1 h. Afterward, the cells were treated with ATP (5 mM) for 15 min, and then the supernatants were collected. The experiments were performed following the manufacturer's instructions.

2.12 | Nitrite measurement

Nitrite, a stable oxidation product of nitric oxide, was measured spectrophotometrically through the Griess reaction. J774.G8 and U937 cells were treated as described previously, and the supernatants were collected. Then, 30 μL of the cell culture supernatant was transferred to another plate and reacted with the same volume of Griess reagent. After 40 min at room temperature, the absorbance of the chromophore was measured at 540 nm (Spectramax M5).

2.13 | ROS measurement

J774.G8 and U937 cells were treated following the protocol previously described. Reactive oxygen species (ROS), an indicator of oxidative stress, was measured using DHE dye (10 nM) for 20 min. Then, the fluorescence was determined using a spectrophotometer (Spectramax M5) at excitation and emission wavelengths of 490 and 570 nm, respectively.

2.14 | Data analysis

All experiments were performed in triplicate on at least three independent days. All data presented are the mean ± SDM, with the exception of the concentration-response curves, which are presented as the mean ± SEM. To test whether the samples followed a Gaussian distribution, the D'Agostino and Pearson normality test was used. If data followed a Gaussian distribution, the one-way analysis of variance (ANOVA) parametric test was used, and if not, the Kruskal-Wallis nonparametric test was applied. ANOVA is a parametric test that compares means between three or more groups. Kruskal-Wallis is a nonparametric test equivalent to ANOVA (Knapp, 2018). The tests used are described in figure legends with the appropriate post hoc tests. p values of .05 or less were considered significant.

3 | RESULTS

3.1 | Inhibition of dye uptake associated with the P2X7R pore

V. victoria, *M. australis*, and *Ascomycota* sp. were the extracts that demonstrated the best inhibition performance in the screening campaign, which was based on dye uptake assays. As shown in Figure 1 a, these three fungal extracts inhibited more than half the PI uptake induced by a millimolar ATP concentration. This high extracellular concentration is able to activate P2X7R and its membrane pore, allowing the passage of molecules up to 900 Da. Because the origin of the P2X7R pore still remains undetermined, some authors have observed

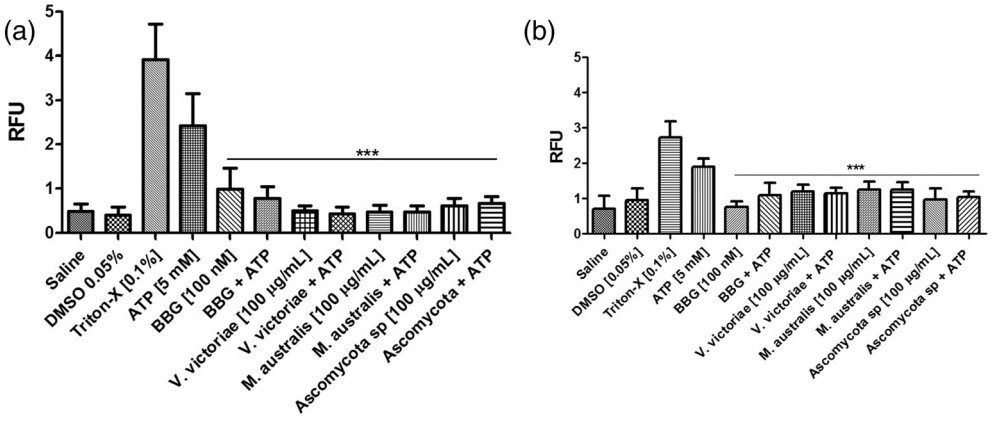

FIGURE 1 Effect of candidate extracts on dye uptake. J774.G8 cells were pretreated with extracts (100 μg/mL) or BBG (100 nM) for 15 min. Then, the cells were stimulated with ATP (5 mM) for a further 10 min. (a) Cationic (PI [375 nM]) or (b) anionic (LY [3 mM]) fluorescent dyes were added to the wells, and the fluorescence of the cells was measured using a spectrophotometer. Data are presented as the mean ± SDM of three independent experiments performed in triplicate. The results were analyzed by ANOVA and Tukey's post hoc test, $p < .05$. Asterisks indicate a significant difference in relation to ATP. RFU means relative fluorescence units

that different cell lines have distinct dye uptake properties, including the permeabilization of cationic (such as PI) or anionic (such as LY) fluorescent dyes (Alves et al., 2014). Because we already demonstrated that the extracts inhibited the uptake of a cationic dye, we also tested their ability to inhibit the uptake of an anionic probe. As demonstrated in Figure 1b, previous treatment of J774.G8 cells with these three extracts significantly inhibited LY uptake. It is noteworthy that these three fungal extracts showed a pattern of antagonism similar to that of BBG for both cationic and anionic uptake.

Subsequently, we treated J774.G8 cells with crescent concentrations of the three selected extracts to obtain the IC_{50} values. IC_{50} values of 2.6, 3.8, and 2.1 μg/mL were identified for the *V. victoriae*, *M. australis*, and *Ascomycota* sp. extracts, respectively (Figure 2a–c). We also identified IC_{50} values in a human cell line expressing P2X7R. The U937 human monocyte cell line was treated with the same extract concentrations used with J774.G8 cells. As a result, the IC_{50} values obtained from U937 were 0.92, 1.5, and 0.67 μg/mL for the *V. victoriae*, *M. australis*, and *Ascomycota* sp. extracts, respectively (Figure 2d–f). The IC_{50} values in human cells (i.e., U937) were lower than those in murine cells (i.e., J774.G8), and this could demonstrate a possible higher sensitivity of the human cells to these fungal extracts. This could also suggest that future treatments in humans will require only low dosages of these compounds.

3.2 | Inhibition of P2X7R ionic currents

Thereafter, we investigated whether these three fungal extracts could inhibit P2X7R ionic currents. For this purpose, we pretreated J774.G8 cells with increasing concentrations of extracts (according to their IC_{50}) and then stimulated the ionic currents with ATP. As we can observe in Figure 3a–c, the *V. victoriae*, *M. australis*, and *Ascomycota* sp. extracts inhibited the relative currents in a concentration-dependent manner. *V. victoriae* and *Ascomycota* sp. extracts inhibited the currents at the nanograms per milliliter range with statistical significance, suggesting that they could act at lower concentrations (Figure 3a,c). In Figure 3d, we observe a representative original recording of whole-cell analysis, with J774.G8 cells treated with extracts at their IC_{50} concentrations. As demonstrated, the peak amplitude and current duration were lower in the presence of extracts, suggesting that they also modulate the ion channel, in addition to the membrane pore.

3.3 | Cytotoxicity assays

The next step was to verify whether these fungal extracts could cause toxicity in cells. The results demonstrate that the treatment with extracts did not cause significant cytotoxicity in J774.G8 (Figure 4a) or U937 (Figure 4b) cells compared with the negative control (cells without treatment). Thus, these data indicate that the extracts alone did not induce cell death, even after prolonged treatment, that is, 24 h. Interestingly, they also prevented P2X7-induced cell death from treatment with ATP for 1 h. These findings, once again, reinforce the role of these extracts in the inhibition of P2X7R.

3.4 | Inhibition of P2X7R physiological functions

It is already described in the literature that P2X7R stimulates several cellular responses, including inflammasome, caspase, phospholipase and mitogen-activated protein kinase (MAPK) activation, and the transcription of proinflammatory genes such as COX-2 and iNOS. Its

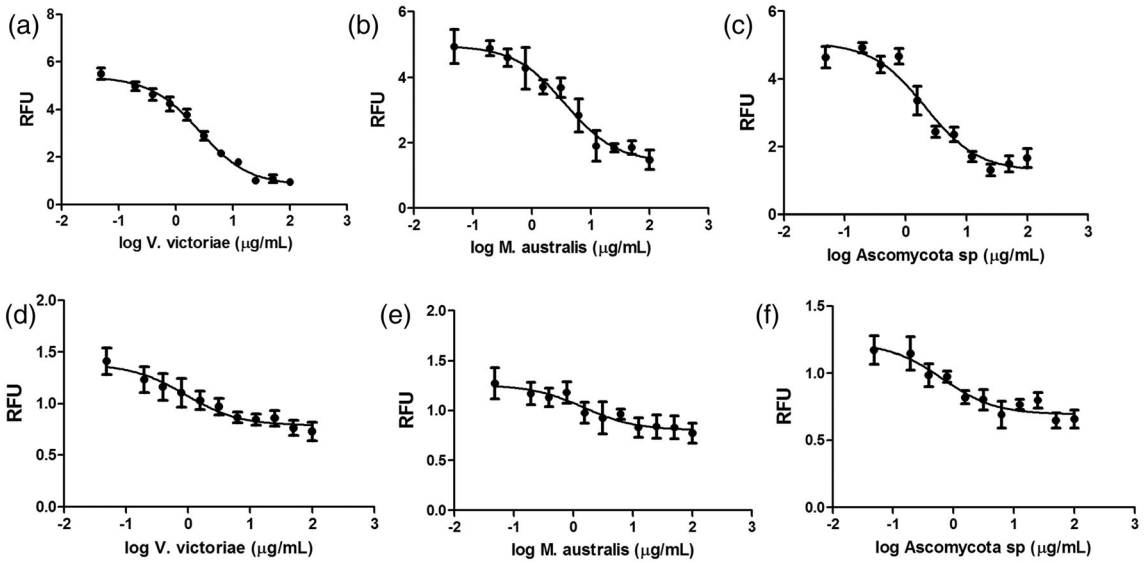

FIGURE 2 Concentration-response curves of the extracts in murine and human cell lines. J774.G8 and U937 cells were pretreated with increasing concentrations of the extracts (2 to 100 μg/mL) for 15 min and then were stimulated with ATP (5 mM). PI was added to the wells, and after 5 min, the cell fluorescence was measured using a spectrophotometer. (a) Concentration-response curve of *V. victoriae* extract in the J774.G8 cell line. (b) Concentration-response curve of *M. australis* extract in the J774.G8 cell line. (c) Concentration-response curve of *Ascomycota* sp. extract in the J774.G8 cell line. (d) Concentration-response curve of *V. victoriae* extract in the U937 cell line. (e) Concentration-response curve of *M. australis* extract in the U937 cell line. (f) Concentration-response curve of *Ascomycota* sp. extract in the U937 cell line. Data are presented as the mean ± SEM of three independent experiments performed in triplicate

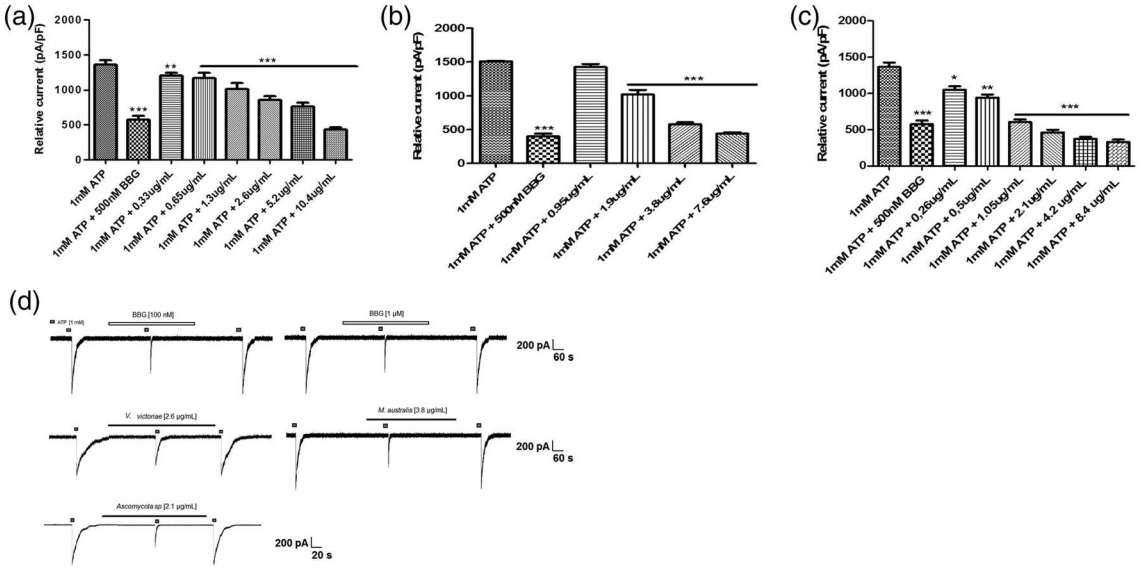

FIGURE 3 Effect of the candidate extracts on P2X7R ionic currents. J774.G8 cells were treated with increasing concentrations of each fungal extract (according to their IC_{50}) or BBG (100 nM, 500 nM or 1 µM) for 15 min and then were stimulated with ATP (1 mM) to evaluate the P2X7R ionic currents. The relative currents of (a) *V. victoriae*, (b) *M. australis*, and (c) *Ascomycota* sp. extracts were quantified, and the data are presented as the mean ± SDM of three independent experiments performed in triplicate. The results were analyzed by ANOVA and Tukey's post hoc test, $p < .05$. Asterisks indicate a significant difference in relation to ATP currents. (d) Representative electrophysiology measurements of fungal extracts at their IC_{50} concentration and of BBG as an antagonist control of P2X7R

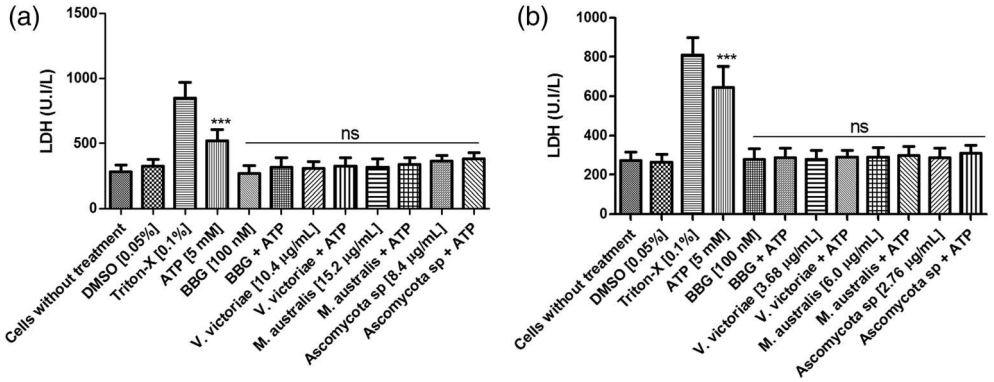

FIGURE 4 Cytotoxicity assay of the candidate extracts in murine and human cells. (a) J774.G8 and (b) U937 cells were treated with the fungal extracts at four times their IC_{50} or with BBG (100 nM) for 24 h and then were incubated for a further 1 h in the presence or absence of ATP (5 mM). Cell supernatants were collected to perform the LDH assay. The concentrations of extracts used were *V. victoriae* (10.4 and 3.68 µg/mL), *M. australis* (15.2 and 6.0 µg/mL), and *Ascomycota* sp. (8.4 and 2.76 µg/mL) for J774.G8 and U937 cells, respectively. Data are presented as the mean ± SDM of three independent experiments performed in triplicate. The results were analyzed by ANOVA and Tukey's post hoc test, $p < .05$. Asterisks indicate a significant difference in relation to the control cells (without treatment). ns means "not significant," when compared with the control cells

activation also promotes cytokines release such as IL-1β, IL-18, and TNF-α, generation of ROS and cell death (Bartlett, Stokes, & Sluyter, 2014; Friedle, Curet, & Watters, 2010; Latz, Xiao, & Stutz, 2013; Lister et al., 2007; Riteau et al., 2012). With the aim of investigating the role of extracts inhibition on the physiological functions associated with P2X7R, first, we performed an IL-1β release assay. As shown in Figure 5a,b, the three fungal extracts significantly inhibited the release of IL-1β in both cell types compared with the control, ATP.

Then, we evaluated the activity of these three fungal extracts on nitrite production. Nitrite is a stable oxidation product of nitric oxide, which is produced when macrophages are activated. As expected, treatment with the extracts significantly inhibited nitrite production (Figure 5c,d).

Finally, we investigated the role of the extracts in ROS production using DHE dye in J774.G8 and U937 cells treated with the extracts and stimulated with ATP. As demonstrated in Figure 5e,f, these fungal extracts significantly inhibited ROS production, confirming their potential anti-inflammatory activity through P2X7R antagonism. Thus, the data suggest that these extracts antagonize P2X7R and its associated physiological functions.

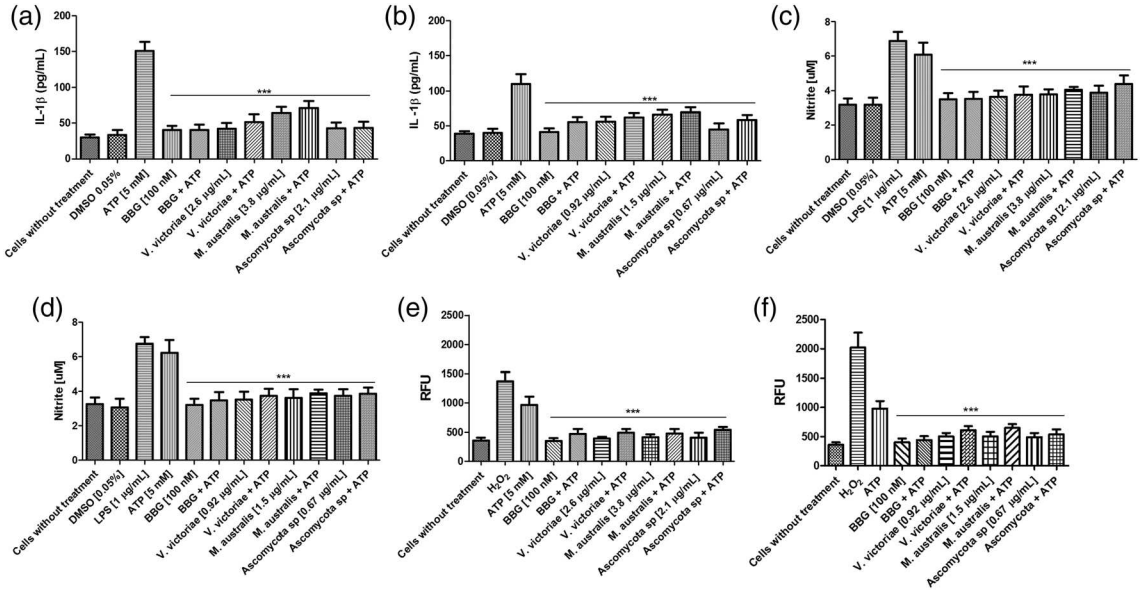

FIGURE 5 Effects of the candidate extracts on the physiological functions of P2X7R. (a) J774.G8 and (b) U937 cells were treated with extracts at their IC_{50} concentrations or with BBG (100 nM) for 1 h and then were stimulated with ATP (5 mM) for a further 15 min. Cell supernatants were collected to perform an ELISA assay for IL-1β detection. (c) J774.G8 and (d) U937 cells were treated with extracts at their IC_{50} concentrations or with BBG (100 nM) for 15 min and then were stimulated with ATP (5 mM) for a further 10 min. Supernatants were collected to perform a Griess reaction to measure nitrite production. (e) J774.G8 and (f) U937 cells were treated with extracts at their IC_{50} concentrations or with BBG (100 nM) for 15 min and then were stimulated with ATP (5 mM) for a further 10 min. DHE dye (10 nM) was added to the wells of plates containing cells to indicate ROS production. Data are presented as the mean ± SDM of three independent experiments performed in triplicate. The results were analyzed by ANOVA and Tukey's post hoc test, $p < .05$. Asterisks indicate a significant difference in relation to ATP

3.5 | Chemical profile of extracts

In this work, we characterized the antagonistic activity of three fungal extracts: *V. victoria* and *M. australis* from the Antarctic, and *Ascomycota* sp. from the Brazilian coast. Because we were working with crude extracts, it was important to obtain information about their molecular content. To obtain this information, we performed tests using the mass chromatography technique. As we can see in Tables 1–3, we were able to identify nine, six, and three compounds from *V. victoria*, *M. australis*, and *Ascomycota* sp., respectively. These data were important in providing a molecular weight profile of the compounds, which are all below 500 Da, meeting one of the criteria of the Lipinski rule of 5. Although it has not been possible to purify or perform structural elucidation of the compounds, these preliminary features suggest that such compounds may be related to the bioactivity under study.

TABLE 1 Mass spectrometry profile of *Vishniacozyma victoriae* extract

Vishniacozyma victoriae				
RT (min)	*m/z* Parent	M + H calc	*m/z* Fragment	M + H calc
13.9	227,1388	$C_{11}H_{19}N_2O_3$	155,0239	NF
14.6	261,1232	NF	170,0984	$C_{12}H_9O$
15.1	243,1337	$C_{11}H_{19}N_2O_4$	NF	NF
15.1	261,1230	$C_{14}H_{17}N_2O_3$	170,0681	NF
16.2	277,1179	NF	NF	NF
17.3	245,1280	NF	154,0736	$C_7H_8N_2O_2$
17.5	206,0810	NF	130,0651	C_9H_8N
18.7	164,1068	$C_{10}H_{14}NO$	105,0658	$C_{10}H_{14}NO$
22.4	274,2738	NF	185,0048	$C_4H_4N_2O_5$
22.6	318,3005	NF	256,2639	$C_{16}H_{34}NO$

Abbreviations: *m/z* fragment, mass divided by the charge number of the dissociated ion; *m/z* parent, mass divided by the charge number of the parent ion; M + H (calculated), protonated molecule; NF, not found; RT, retention time.

4 | DISCUSSION

In this study, we identified three fungal extracts that demonstrated potential antagonistic activity toward P2X7R. They inhibited the macroscopic current associated with the P2X7 pore, membrane pore formation, and the production of proinflammatory mediators, which are all related to P2X7R activation, without toxicity to the cells.

The first fungal specie evaluated was *V. victoriae*. This biological specie was identified by Godinho et al. (2013) during Antarctic expedition XXIV in the Austral Summer of 2009–2010. *V. victoriae* is a basidiomycetous anamorph fungus found in Antarctica but is also described in Portuguese seawater and Germany. This specie is psychrophilic, that is, it has the ability to grow and reproduce at extremely low temperatures. On the Antarctic continent, it was collected with high frequency on different substrates such as rhizospheres, sediment, soil, freshwater, and excrement from penguins (Godinho et al., 2013).

TABLE 2 Mass spectrometry profile of *Metschnikowia australis* extract

Metschnikowia australis				
RT (min)	*m/z* Parent	M + H calc	*m/z* Fragment	M + H calc
13.3	197,1282	NF	153,0658	$C_7H_9N_2O_2$
14.0	227,1385	$C_{11}H_{19}N_2O_3$	170,0677	NF
15.1	243,1333	NF	157,0109	NF
15.1	261,1230	$C_{14}H_{17}N_2O_3$	170,0681	NF
16.4	211,1436	$C_{11}H_{19}N_2O_2$	154,0732	NF
17.3	245,1277	$C_{14}H_{17}N_2O_2$	154,0731	NF
19.4	162,0908	NF	143,0728	NF
22.3	274,2733	NF	256,2618	NF
22.5	318,2987	NF	256,263	$C_{16}H_{34}NO$

Abbreviations: *m/z* fragment, mass divided by the charge number of the dissociated ion; *m/z* parent, mass divided by the charge number of the parent ion; M + H (calculated), protonated molecule; NF, not found; RT, retention time.

TABLE 3 Mass spectrometry profile of *Ascomycota* sp. extract

Ascomycota sp.			
RT (min)	*m/z*	MS/MS	M + H calc
22.3	274,2730	256,2630	$C_{16}H_{36}NO_2$
22.4	288,2874	256,2622	$C_{17}H_{38}NO_2$
23.1	288,2885	185,9645	
23.6	288,2884	270,2772	
24.9	302,3033	284,2919	$C_{16}H_{38}N_4O$

Abbreviations: MS/MS, mass spectrometry/mass spectrometry; *m/z*, mass divided by the charge number; M + H (calculated), protonated molecule; RT, retention time.

The second fungal specie evaluated was *M. australis*. This specie is endemic to Antarctica. *M. australis* is an ascomycetous fungus naturally found in Antarctic seawater. It has a widespread distribution and was isolated from samples of marine sediment and seawater. However, it is abundantly found associated with the thalli of seaweed species (Godinho et al., 2013).

The third fungus belongs to the Ascomycota phylum, and it is a nonidentified biological specie. This fungus is an agricultural yeast, that is, it survives associated with marine macroalgae and has an important role in the cycling of organic matter in the oceans. The specie used in this work is associated with the macroalga *P. gymnospora*. *P. gymnospora* belongs to the Ochrophyta phylum and is found in the Brazilian coast. *P. gymnospora* has the ability to accumulate heavy metals such as zinc and is used as a biological indicator (Loque, 2009).

These extracts were found after a HTS campaign, in which approximately 1,800 extracts were tested. The first results of these extracts are related to their ability to inhibit both cationic and anionic dye uptake through the P2X7R-associated pore. These experiments were performed using the J774.G8 cell line, a murine macrophage cell line that classically expresses P2X7R (Coutinho-Silva et al., 2005). All three extracts similarly inhibited propidium iodide uptake (cationic dye); however, when anionic dye (Lucifer yellow) was used, *Ascomycota* sp. seemed to demonstrate the better inhibition activity. Additionally, the three extracts showed similar inhibition profiles to that of BBG, a P2X7R antagonist used as a control in the experiments.

A human cell line that also expresses P2X7R was used to compare the extract responses between different species. As can be observed in Figure 2, the IC_{50} values of the extracts were lower in the human cell line (U937) compared with those in murine cells (J774.G8). However, it is already described in the literature that P2X7R agonists and antagonists present slight differences in EC_{50} and IC_{50} values between mammalian species such as mice, rats, and humans (Donnelly-Roberts, Namovic, Han, & Jarvis, 2009). These differences could be related to P2X7R selectivity. Despite this finding, treatments with the extracts for 24 h did not cause toxicity for either cell types.

To evaluate the putative pharmacological inhibitory effect of these extracts on P2X7R, electrophysiological measurements were performed. These experiments revealed significant inhibition of the P2X7R ionic currents by the extracts. Among the three extracts, *Ascomycota* sp. seemed to demonstrate the best performance, especially at higher concentrations.

Finally, experiments to understand the role of the extracts on physiological functions related to P2X7R activation were carried out. The first function evaluated was IL-1β release, because P2X7R activation is associated with inflammasome formation in macrophages and LPS-stimulated monocytes (Di Virgilio, 2007). The results showed that all extracts inhibited the release of this cytokine in both macrophage and monocyte cell lines. Subsequently, other proinflammatory mediators were analyzed. P2X7R leads to MAPK activation, which induces nitrogen intermediary and ROS generation (Clark et al., 2010; Miller et al., 2011). The extracts significantly inhibited nitrite production and ROS formation in macrophages and monocytes after ATP stimulation.

In this context, our results have strong relevance, because this is the first study to determine the potential antagonistic activity of Antarctic fungal extracts on P2X7R. However, studies with natural products have some challenges, such as identification of the active principle and discovery of its chemical structure, because the observed activity can be related to a pool of different molecules.

Despite the similarity among the fungal extracts in chromatographic profiles, currently, we have only obtained the molecular formula of some compounds present in the crude extracts. The molecular formulas of these compounds were calculated based on their isotope patterns after deconvolution. Magnetic nuclear resonance data acquired from pure compounds are undoubtedly the best methodology for the chemical structure identification of active compounds. However, due to sample scarcity, it was not possible to carry out previously, but this work is currently in process.

Chemical structure elucidation will also help identify extract potencies and compare them with other P2X7R antagonists, because the extract concentration information was based on micrograms per milliliters. Once identified, the active principle could present activity at micromolar or lower ranges.

P2X7R antagonists from natural products are already described in the literature. Santos et al. (2011) found amentoflavone, a compound present in the leaf extract *of R. longifolia* Planch & Triana, to have antagonistic activity toward P2X7R. The results demonstrated that the methanol extract inhibited P2X7R ionic currents and dye uptake with an IC_{50} value of 2 μg/mL (Santos et al., 2011). Liu et al. (2010) studied the effect of emodin, an anthraquinone from rhubarb *Rheum officinale* Baill, which also presented antagonistic activity toward P2X7R. Emodin inhibited peritoneal macrophage cell death with an IC_{50} of 0.2 μM and dye uptake and the increase of intracellular calcium concentrations with an IC_{50} of 0.5 μM. It also inhibited P2X7R currents with an IC_{50} of 3.4 μM in P2X7-HEK293 cells (Liu et al., 2010).

Currently, research to discover new antagonists of P2X7R has been growing and has gained high financial support from the pharmaceutical industry (Gunosewoyo & Kassiou, 2010). P2X7R is implicated in many pathological processes, including neuropathic pain, inflammatory, and neurodegenerative diseases and cancer (Broom et al., 2008; Corrêa, Marques da Silva, Abreu Moreira-Souza, Vommaro, & Coutinho-Silva, 2010; Diaz-Hernandez et al., 2012; Lee et al., 2012; Li et al., 2008; Lister et al., 2007; Mesuret et al., 2014; Raffaghello et al., 2006; Teixeira et al., 2014; Zhao et al., 2013). The development of new antagonists with efficacy and safety that could be applied in clinical treatments is one of the major challenges of purinergic therapy.

5 | CONCLUSIONS

Collectively, our results demonstrated that the three extracts from the respective fungal species (*V. victoriae*, *M. australis*, and *Ascomycota* sp.) significantly inhibited P2X7R physiological roles in vitro., which necessitates future investigations about these promissory sources for the discovery of new molecules with potential antagonist activity. The elaboration of a new phytopharmaceutical compound could be useful in the clinical treatment of diseases associated with P2X7R.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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