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Extraction optimization and antinociceptive activity of (1⟶3)-β-D-glucan from *Rhodotorula mucilaginosa*

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ABSTRACT

β-D-glucans are polymers of D-glucose monomers found in the cell walls of many bacteria, plants, fungi and yeasts. A variety of β-D-glucans differing in structures have been isolated from various sources and their biological activity to be regulated by various structural factors, such as the primary structure, molecular weight, solubility, and conformation. This study investigated the effect of extraction time and temperature on the yield of β-D-glucan produced by *Rhodotorula mucilaginosa*. A statistical Doehlert design was applied to determine the important effects and interactions of these independent variables on the yield of β-D-glucan, the dependent variable. Significant models were obtained. The best yield was of 25% obtained after 128 min of extraction in a temperature of 72 °C. The polysaccharides were characterized as (1⟶3)-β-D-glucan by methods spectroscopic (FT-IR, ^{1}HNMR and ^{13}CNMR). In addition, the antinociceptive effect was evaluated using different experimental tests (acetic acid-induced writhing test, formalin test and tail immersion test). The (1⟶3)-β-D-glucan showed a potent peripheral antinociceptive effect, possibly by the inhibition of inflammatory mediators.

1. Introduction

Polysaccharide is general category of macromolecules that are made up of monosaccharides through glycosidic bonds and exists ubiquitously in the bodies of plants and microorganisms. Besides, it also possesses many important biological functions, such as skeleton supporting, signal transmission and body defense (Liao, Guo, & Lin, 2011; Han et al., 2012).

The polysaccharides have been the subject of intense research, taking into view of its high potential for application in different sectors. The biopolymers of microbial origin in addition to having properties similar or superior to traditional (vegetable source and seaweed) are often able to form solutions viscous. Some of them also form gels in aqueous media, even at low concentrations and, in some cases, these polymers are used as substrates for development of new products (McIntosh et al., 2005; Majumder et al., 2009).

The fungal polysaccharides and polysaccharide-protein complexes, both belonging to cell wall as the extracellular, have been investigated by presenting a variety of biological response, such as antitumor, antinociceptive, anti-inflammatory, anti-oxidative and immune stimulatory activities (Novak & Vetvicka, 2008; Carbonero et al., 2008; Smiderle et al., 2008; Baggio et al., 2010; Ruthes et al., 2013). The therapeutic application seems to depend on chemical structure and spatial conformation of each macromolecule and small structural differences of each polymer features result in peculiar for new biotechnological applications (Ahmad, 2010).

β-D-glucans are polymers of D-glucose monomers linked by β-glycosidic bonds (with or without (1⟶6)-β-D-glucose side chains) found in the cell walls of many bacteria, plants, fungi and yeasts (Novak & Vetvicka, 2008; Luo, Xu, Yu, Yang, & Zheng, 2008; Hunter et al., 2002). A variety of β-D-glucans differing in structures have been isolated from various sources and their biological activity to be regulated by various structural factors, such as the primary structure, molecular weight, solubility, and conformation (Tada et al., 2007).

β-D-glucan extraction from fungus and yeast cells can consists of two stages: yeast cell lysis and separate insoluble cell wall from cytoplasm. There are several groups of yeast lyse methods – physical, chemical and enzymatic. Physically, yeasts can be disrupted using sonication, homogenizers with high pressure, among other; chemically, yeast cells can be lysed by inorganic acids (e.g., HCl), organic acids (e.g., acetic acid, citric acid); alkali (e.g., NaOH; KOH) or using other chemically aggressive solutions (Hunter et al., 2002). In most cases chemical lysis is held at high temperatures; and

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enzymatic cell lysis using natural enzymes (Javmen et al., 2012; Ahmad, 2010).

We report a sequential optimization strategy for β-D-glucan production by *Rhodotorula mucilaginosa* through statistically designed experiments. Doehlert experimental design was used to describe the nature of the response surface in the experimental region, to search optimal medium composition for maximizing polysaccharides yield. The (1⟶3)-β-D-glucan was characterized by FT-IR, ^{1}H and ^{13}C NMR spectroscopic techniques; and antinociceptive action was examined in experimental protocols on mice.

2. Materials and methods

2.1. Microorganism

Rhodotorula mucilaginosa (33d1) was obtained from the Collection of Cultures of Microorganisms of Bahia (CCMB, Feira de Santana, Brazil). The microorganism was maintained on YM agar at 28 °C in an incubator (IGO 150 Cell Life – Jouan).

2.2. Culture conditions of Rhodotorula mucilaginosa

The strain of *R. mucilaginosa* was incubated in a MMS culture medium for activation of 4 days at 30 °C. Cultivation in liquid media was carried out in 250 mL Erlenmeyer flasks containing 100 mL of (g/L): glucose, 15; KH_2 (PO_4) $3H_2O$, 2.0; $MgSO_4$ $7H_2O$, 0.5; $(NH_4)_2SO_4$.7 H_2O 2.0; $CaCl_2$. $2H_2O$, 0.1, pH 5.0. Flasks were inoculated with ten milliliters of cell suspension contend 10^9 UFC of *R. mucilaginosa*. The incubation was performed at 120 rpm for 96 h in a rotary shaker. After the fermentation, the liquid culture medium, on which *R. mucilaginosa* was grown, was filtered and centrifuged (Centrifuge 5804R –Eppendorf, São Paulo, Brazil) at 8000 g for 15 min at 4 °C, and the precipitate, containing the biomass from *R. mucilaginosa,* was used to extract the biopolymers (Dong et al., 2009).

2.3. Extraction of yeast polysaccharides

The powder of dry biomass (10 g) was extracted several times with 20 volumes of NaOH 0.1 M. The extractions were conducted at different temperatures (50, 70 and 90 °C) with various extraction times (60, 90, 120, 150 and 180 min.). The suspension was centrifuged at 10,000 × g for 10 min. It was added to the supernatant three times of the volume of absolute ethanol, stirred vigorously and left overnight at 4 °C. The precipitated polysaccharides were centrifuged at 10,000 × g for 30 min and the supernatant discarded. The precipitate of crude polysaccharides was dried at 55 °C to a constant weight and the polymer was weighted by a scale (Sartorious ALC-110.4, Germany). The carbohydrate content was determined by phenol–sulfuric acid method (DuBois et al., 1956). The product yield was measured at the (w/w)% of polysaccharides per unit mass of biomass yeast.

2.4. Experimental design

The temperature (°C) and extraction time (minutes) were monitored using Doehlert experimental design to β-D-glucan extraction. Three repetitions of the central point (c) to a total of nine experiments (Table 1) were performed to estimate the possible pure error, and the experimental treatments were varied randomly to detect the presence of possible systematic errors. The temperature was studied at five levels, ranging from 50 to 90 °C, and the time was studied at three time points, ranging from 60 to 180 min (Table 1). All the calculations and graphics in this work were performed using the software Statistica® version 7.0, and including the analysis of variance (ANOVA) for the responses of this study.

Table 1
Doehlert matrix used for the optimization of the (1⟶3)-β-D-glucan extraction from *Rhodotorula mucilaginosa.*

Assay	Temperature (°C)	Time (min)	β-D-Glucan (%)
1	90 (+0.866)	90 (−0.5)	20
2	90 (+0.866)	150 (+0.5)	23
3	70 (0)	60 (−1)	17.9
4c	70 (0)	120 (0)	25.9
5c	70 (0)	120 (0)	24.9
6c	70 (0)	120 (0)	25
7	70 (0)	180 (1)	20.8
8	50 (−0.866)	90 (−0.5)	20.3
9	50 (−0.866)	150 (+0.5)	21

(c): central point; coded values are presented in the parentheses.

2.5. Fractionation

A sample of β-D-glucan (15 mg/mL) was applied to a column (48 × 1.2 cm) of Sephacryl S-200 (Amersham Bioscience, USA) pre-equilibrated and developed with sodium phosphate buffer (0.05 mol L^{-1}, pH 7.0). Fractions of 2.5 mL were collected and assayed for polysaccharides totals by phenol–sulfuric acid method (DuBois et al., 1956).

2.6. The number-average molecular weight (MWn) and degree of polymerization (DPn)

The MWn and DPn of the β-D-glucan were determined by the measurement of the reducing value using 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959) and the measurement of total carbohydrates using the phenol–sulfuric acid method (DuBois et al., 1956) DPn = [(total carbohydrates in μg of D-glucose)/(reducing value in μg of maltose)] × 1.9; MWn = [(DPn) × 162] + 18 (Vettori et al., 2012).

2.7. Determination of the protein concentration

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

2.8. Characterization of β-D-glucan by spectroscopic analysis

The polysaccharide was characterized using a Fourier Transform Infrared Spectrometer (PE IR Spectrum AsciiPeds 1.60, Spectrum One FT-IR Spectrometer). The dried polymer was grinded with potassium bromide (KBr) powder and pressed into pellets for FT-IR spectral measurement in the frequency range of 4000–500 cm^{-1}, with 20 scans. In NMR spectroscopic analysis, all spectra were recorded at 300 K on a Varian Inova 500 spectrometer operating at 11.7 T, observing ^{1}H at 500 MHz and ^{13}C at 125 MHz, and using 5 mm direct detection probe. The samples was dissolved in D_2O/DMSO (6:1) and transferred to NMR tube. TMS was used as a chemical shift reference (δ 0.0). D_2O (99.9%) and DMSO (99.9%) were from Cambridge Isotope Laboratories, Inc. (USA).

2.9. Pharmacological studies

2.9.1. Animals

Experiments were performed using male Swiss mice (23–30 g), housed in a temperature-controlled rooms (22 ± 2 °C), under 12:12 h light-dark cycle, with access to water and food *Ad libitum* until use. All behavioral tests were performed between 8:00 AM and 5:00 PM, and the animals were only used once. Animal care

and handling procedures were performed after protocol approval by the Animal Ethics Committee of University of Feira de Santana and were carried out in accordance with the guidelines of International Association for Study of Pain (IASP) on the use of animals in pain research (Zimmermann, 1983). All efforts were made to minimize the number of animals used and their discomfort.

2.9.2. Writhing test

Mice were treated with (1⟶3)-β-D-glucan (3, 10, and 30 mg/kg), indomethacin (10 mg/kg; reference drug) or saline (control group) by intraperitoneal (i.p.) injection 30 min before acetic acid administration (0.8% v/v, 10 ml/kg). The acetic acid was injected into the peritoneal cavities of mice, which were placed in a large glass cylinder and the intensity of nociceptive behavior was quantified by counting the total number of writhes occurring between 0 and 30 min after the stimulus injection (Collier et al., 1968). The writing response consists of a contraction of abdominal muscle together with stretching of the hind limbs. The antinociceptive activity was expressed as the writing scores over 30 min.

2.9.3. Formalin test

Mice were placed in the observation chamber for 10 min before formalin injection in order to allow acclimatization to the new environment. Mice were gently restrained while the dorsum of the hind paw was subcutaneously administered with 20 μl of formalin 2.5% (0.92% formaldehyde) made up in saline. Following injection, the mice was returned to the observation chamber. Mice were observed from 0 to 5 min (early phase) and from 15 to 30 min (late phase). The nociception score was determined by counting the time that the animal spent licking, biting and shaking the injected limb during the observation time (Dubuisson & Dennis, 1977). Animals were treated with (1⟶3)-β-D-glucan (3, 10, and 30 mg/kg, i.p.), indomethacin (10 mg/kg, i.p.; reference drug) or saline (control group) 30 min before formalin injection.

2.9.4. Tail immersion test

The tail immersion test in mice was conducted as described previously (Sewell & Spencer, 1976), with minor modifications. Before the day of the experiment, each animal was habituated to the restraint cylinder for 4 consecutive days (20 min per day). On the experimental day, mice were placed in the restraint cylinder and the tail tip (2 cm) was immersed in a water bath at 48 °C ± 0.5 °C. The latency for the tail withdrawal reflex was measured. Each mouse was first tested for baseline latency by immersing its tail in the water and recording the time to response. Mice not responding within 5 s were excluded from further testing. Mice were then randomly selected to perform in one of experimental groups: control (saline, i.p.), (1⟶3)-β-D-glucan (30 and 90 mg/kg, i.p.) and morphine (5 mg/kg, s.c.; reference drug) and tested for antinociception at 0.5, 1, 3 and 5 h following the administration of the drugs. To prevent tissue damage of the tail, the cut-off time was 10 s. Antinociception of each mouse was calculated according to the following formula:

Antinociception index = [(test latency − baseline latency)/(10 − baseline latency)] × 100

2.9.5. Drugs and dilutions

The drugs were purchased from Sigma® Chemical Co., St. Louis, MO, USA (Indomethacin), Merck® (Formaldehyde and Acetic acid) and Cristália® (Morphine). Indomethacin was dissolved in Tris-HCl 0.1 M pH 8.0 plus physiological saline. Remaining drugs were dissolved in physiological saline.

2.9.6. Statistical analysis of animal experiments

The data are presented as means ± standard error of the mean (SEM) of measurements made on 7–8 animals in each group. Comparisons between groups were evaluated by one-way analysis of variance (one-way ANOVA) followed by the Tukey's test, repeated measures of two-way ANOVA with Bonferroni's post-hoc test, as appropriate. Statistical differences were considered to be significant at $P < 0.05$.

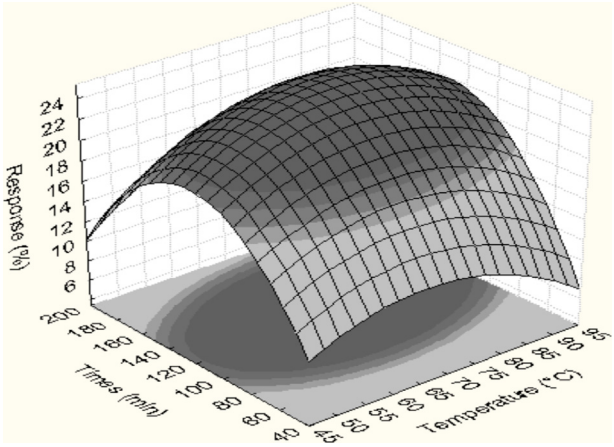

Fig. 1. Response surface of the (1⟶3)-β-D-glucan yield for temperature (°C) versus extraction time (min).

3. Results

3.1. Extraction of polysaccharides

A Doehlert matrix for two variables was applied to find the optimum conditions for the extraction of yeast polysaccharides from *Rhodotorula mucilaginosa*. The experimental results are showed in the Table 1.

The Eq. (1) illustrates the relationship between these two variables and the response R (polysaccharides in %); C is the temperature and T is the extraction time:

$$R = -28.2323 + 0.855625C - 0.00678C^2 + 0.3538T - 0.00164T^2 + 0.00096C \times T \quad (1)$$

The corresponding surface response and level curves graphics for this equation are shown in Fig. 1. A test based on the Fisher distribution (F-test) indicated that the fitted equation is statistically significant ($F = 49.60 > 9.01$). A lack-of-fit sum of squares ($F = 0.35 < 10.12$) indicates that there is good agreement between the model's predicted response and the experimental values studied for each variable. Lagrange criterion applied to this equation indicates that the critical point is characterized as a maximum.

The higher yield extraction (25%) was obtained at 72 °C at extraction time of 128 min (Fig. 1). Temperature between 55 and 90 °C and extraction time between 100 and 160 min showed highest mass of β-D-glucan. However, extraction time higher than 160 min and shorter than 100 min caused a reduction in the amount of β-D-glucan extracted of *Rhodotorula mucilaginosa*. The β-D-glucan showed average molecular weight of 78.2 kDa and DPn of 480. The protein in the sample partially purified was detected by technique described (Bradford, 1976).

3.2. Fractionation

A sample of β-D-glucan extracted was fractionated by means of Sephacryl S-200 leading to four symmetrical peak attribute to four type of β-D-glucan in fractions 15, 20, 25 and 30 (Fig. 2).

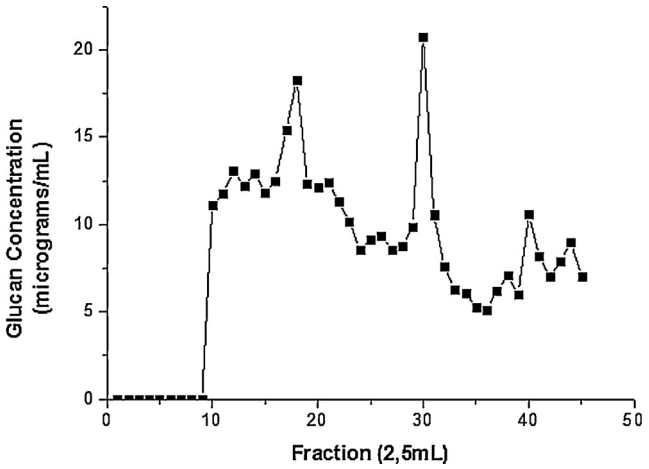

Fig. 2. Elution diagram of (1⟶3)-β-D-glucan from Sephacryl S-200 (50 mM sodium phosphate buffer, pH 7.0).

3.3. Characterization of polysaccharide by spectroscopic analysis

The analysis of the infrared data of biomass obtained was performed to identify the main functional groups. The FT-IR spectrum showed peaks at 3267 cm^{-1} assigned to the hydroxyl stretching vibration of the polysaccharide (Fig. 3). The signal at 1062 cm^{-1} which is due to the stretching of anomeric C_1H group vibration as was observed by Gonzaga et al., 2005 suggested for β-D-glucan gum pellets.

Absorption at 1237 cm^{-1} may be related to the vibration of the axial deformation of the C—O—C group present in a ring of six carbon atoms, characteristic of β-D-glucans (Luo et al., 2008; Yu et al., 2002). It is also important to note that the spectrum showed absorption peak at 883 cm^{-1} that is an indicative of β-linked glycosidic bonds, and no absorption near 830 cm^{-1} showed the absence of α-linked glycosidic bonds. The intense absorption in the region between1500 and 1700 cm^{-1} is related to the presence of functional groups corresponding to proteins such as amines, amides and aromatic compounds (Ahmad, 2010). The presence of proteins was confirmed by the technique described by Bradford (1976).

The ^{13}C NMR spectrum of sample (Fig. 4) reveals signals characteristic of polysaccharide. However, some impurities such as free glucose are present. The six signals at a δC 105, 74.0, 86.1, 72.0, 76.4 and 61.5 were characteristic of a (1⟶3)-β-D-glucan and attributed

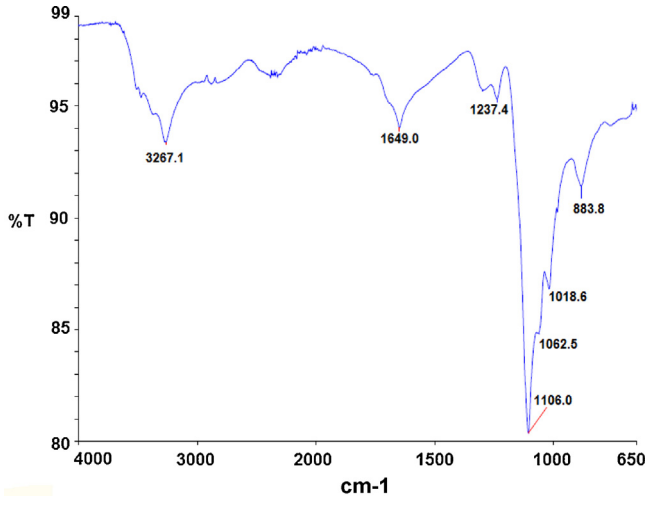

Fig. 3. FT-IR spectrum of (1⟶3)-β-D-glucan produced from *Rhodotorula mucilaginosa*.

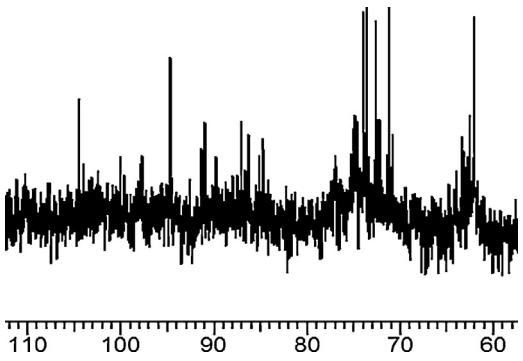

Fig. 4. ^{13}C NMR spectrum (expanded) of (1⟶3)-β-D-glucan from *Rhodotorula mucilaginosa*.

to C-1, C-2, C-3, C-4, C-5, and C-6, respectively, by comparison with data from the literature (Wu et al., 2005; Majumder et al., 2009; Synytsya & Novák, 2013).

3.4. Pharmacological studies

3.4.1. Effect of β-D-glucan on acetic acid-induced writhing response

In order to evaluate the (1⟶3)-β-D-glucan from *Rhodotorula mucilaginosa* for its therapeutic properties, in the first series of experiments, it was addressed whether the (1⟶3)-β-D-glucan inhibits writhing response induced by acetic acid. The dose of 3 and 10 mg/kg of (1⟶3)-β-D-glucan did not inhibit significantly the writhing response. On the other hand, the dose of 30 mg/kg inhibited the writhing response significantly ($P < 0.05$). Indomethacin (10 mg/kg), a standard NSAID used as reference drug, also produced a significant inhibition of acetic acid-induced writhing response ($P < 0{,}005$)(Fig. 5).

3.5. Effect of β-D-glucan on formalin-inducednociception

The antinociceptive property of (1⟶3)-β-D-glucan from *Rhodotorula mucilaginosa* was confirmed in the formalin test. Injection of formalin in control animals induced a biphasic flinching response, with the early phase ranging from 0 to 5 min (Fig. 6A) and the late phase from 15 to 30 min (Fig. 6B) after the injection.

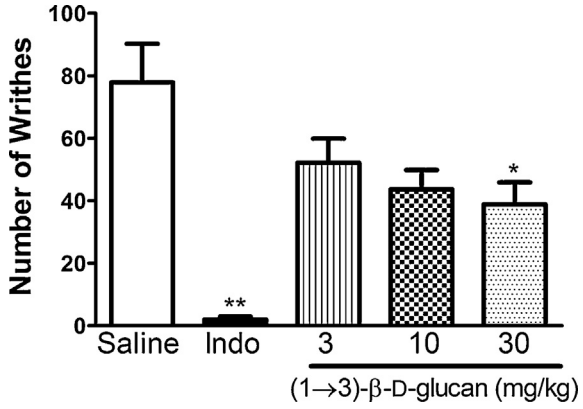

Fig. 5. Effects of intraperitoneal administration of (1⟶3)-β-D-glucan from *Rhodotorula mucilaginosa* on acetic acid-induced writhing in mice. Mice were treated with β-D-glucan (3, 10 and 30 mg/kg) or saline (control group) by intraperitoneal route 30 min before acetic acid 0.8% (injected at time zero). Indomethacin (Indo; 10 mg/kg i.p.) was the reference drug. Data are expressed as means ± S.E.M.; $n = 7$ mice per group. *Significantly different from control group ($P < 0.05$), **significantly different from control group ($P < 0.005$), as determined by ANOVA followed by Tukey's test.

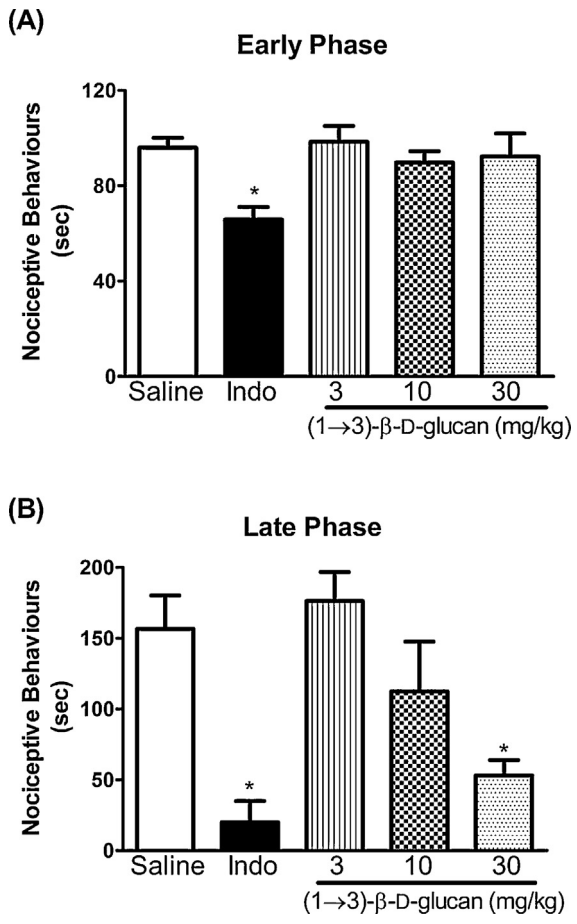

Fig. 6. Effects of the (1⟶3)-β-D-glucan from *Rhodotorula mucilaginosa* treatment on the formalin-induced nociception. Panels (A) and (B) represent effects of β-glucan β-D-glucan on the early and late phases of formalin-induced nociception in mice, respectively. Mice were treated with β-D-glucan (3, 10 and 30 mg/kg) or saline (control group) by intraperitoneal route 30 min before formalin (injected at time zero). Indomethacin (Indo; 10 mg/kg, i.p.) was the reference drug. Data are expressed as means ± S.E.M.; $n = 7–8$ mice per group. *Significantly different from control group ($P < 0.005$) as determined by ANOVA followed by Tukey's test.

Treatment with (1⟶3)-β-D-glucan (30 mg/kg) 30 min before the formalin caused antinociceptive effect ($P < 0.005$) in the late phase, but not early phase, of the formalin test. Indomethacin (10 mg/kg) caused antinociceptive effects in both the early and late phases of the formalin test ($P < 0.005$).

3.6. Effect of β-D-glucan tail immersion test

The tail immersion test was used to evaluate the central antinociceptive effects of (1⟶3)-β-D-glucan from *Rhodotorula mucilaginosa*. The treatment with (1⟶3)-β-D-glucan (30 and 90 mg/kg) did not show significant increase in tail immersion latency time compared to control group ($P > 0.05$). The administration of morphine (5 mg/kg), the reference drug, 30 min before testing caused a significant increase in the latency response just 1 h after administration ($P < 0.001$) (Fig. 7).

4. Discussion

The response surface methodology (RSM) is a collection of mathematical and statistical techniques for designing experiments, building models, evaluating the effects of factors and searching optimum condition of factors for desirable responses (Box & Behnken, 1978). The optimization process of this methodology

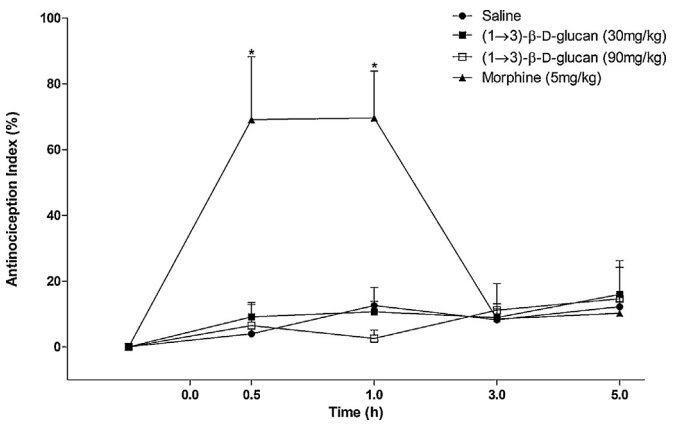

Fig. 7. Effects of the (1⟶3)-β-D-glucan from *Rhodotorula mucilaginosa* treatment on tail immersion test. The figure show data of tail withdrawal reflex latencies represented as antinociception index. To evaluate the time-course of the antinociceptive effect, thermal nociceptive threshold was evaluated before and up to 5 h following oral administration of β-D-glucan (30 and 90 mg/kg, i.p.) or saline (control group). Morphine (5 mg/kg s.c.), was the reference drug. Data are expressed as means ± S.E.M.; $n = 6$ mice per group. *Significantly different from control group ($P < 0.001$) Two-way ANOVA followed by the Bonferroni's test.

involves studying the response of the statistical designed combinations, estimating the coefficients by fitting it in a mathematical model that fits best the experimental conditions, predicting the response of the fitted model and checking the adequacy of the model.

Dohlert design applied in this study described so well-adjusted behavior of the variables applied in extraction of β-D-glucan. The ANOVA of quadratic regression model demonstrates that the model is highly significant, as is evident from the Fisher's F-test with a very low probability value [(Pmodel > F) = 0.0001] (Table 1 and 2). The efficiency of the model was checked by the determination coefficient R^2 (0.9918) to biomass production and R^2 (0.988) to extraction of the β-D-glucan.

Several extraction techniques have been proposed in order to achieve more effectively the β-D-glucan from the cell wall of fungi. But mostly, it uses an alkaline thermal treatment followed by precipitation with ethanol (Yu et al., 2002). Works describe the extraction β-D-glucan of fungi using time ranging from 30 min to 6 h, and temperatures ranging from 60 to 100 °C. In this case, the optimization of these parameters can ensure better performance and generate few costs (Blaschek et al., 1992; Bohn & BeMiller, 1995; Matsuzaki et al., 1986; Luo et al., 2008; Wu et al., 2005).

Crude β-D-glucan content in the medicinal mushroom Chaga (*Inonotus obliquus*) was measured by different extraction (alkali extraction and enzymatic digestion) and followed by a gravimetric analysis was employed to determine the crude β-D-glucan content. The amount obtained was similar (Rhee et al., 2008). However, enzymatic extraction process appeared more efficient in removing impurities during extraction (Javmen et al., 2012). This may explain the signals in the infrared spectrum (1500–1700 cm^{-1}) and the positive resulted to method of Bradford (1976), indicating the presence of protein to β-D-glucan obtained.

Some extraction methodologies of β-D-glucan revealed that temperature and pH have a significant effect on extraction and functional properties of β-D-glucan (Bhatty, 1993; Bhatty, 1995; Temelli, 1997). Compared with other extraction of polysaccharides, the yield of β-D-glucan from *Rhodotorula mucilaginosa* (25%) was higher that β-D-glucan extract of *C. sinensis* found by Dong et al. (2009), in conditions of 89 °C for 110 min; with yield of 16% β-D-glucan extract. This difference is attributed to various forces of interaction between β-D-glucan *Rhodotorula mucilaginosa*. The purification curve shows four peaks, which can be assigned to

different types of β-D-glucan. This difference may be the connection types, the size of the molecule or in association with proteins which may differ in pharmacological activities (Sugawara et al., 2004; Majumder et al., 2009).

The methods spectrometric of infrared-spectroscopy and nuclear resonance magnet were used to structural characterization of β-D-glucan extracted from *R. mucilaginosa*. The profile of the graphics and sings presented were characteristic of (1⟶3)-β-D-glucan, very similar to the profile of spectra obtained from analysis of β-D-glucan extracted from other fungi (Dong et al., 2002; Gorin, 1981; Mizuno et al., 1990; Ohno et al., 2001; Saito et al., 1976; York, 1995).

Several research papers have been published that revealed the monomeric compound within isolated β-D-glucan to be glucose (Lee et al., 2001; Nguyen et al., 1998). In this study, the glucose peak was the only peak found on the HPLC chromatogram, which indicated that the crude β-D-glucan residues were comprised of glucose with β-glycosidic linkages.

Several studies showed that β-D-glucan can have remarkable biological activities. However, some researches shows that the activity of β-D-glucan depends on their structure, molecular weight, solubility, degree of branching (Ruthes et al., 2013), primary structure and conformation (Tada et al., 2007). Then, we started the investigation of the biological activity of (1⟶3)-β-D-glucan from *Rhodotorula mucilaginosa* using acetic acid-induced writhing test in mice, a screening tool for the assessment of the analgesic or anti-inflammatory properties of new agents (Collier et al., 1968). The intraperitoneal administration of (1⟶3)-β-D-glucan produced dose-related suppression of writhing response. This method has good sensitivity, but poor specificity because the abdominal writhing response may be suppressed by muscle relaxants, neuroleptics and other drugs (Le Bars et al., 2001). Thus, we confirmed the antinociceptive effect of (1⟶3)-β-D-glucan using the formalin test, which affords a more specific response compared with acetic acid-induced writhing test. The intraplantar injection formalin promotes two distinct phases of pain sensitivity that cannot only to assess antinociceptive substance but also for elucidating of the mechanisms of antinociception (Dubuisson & Dennis, 1977; Hunskaar & Hole, 1987). The early phase (neurogenic pain), which was not affected by treatment with the (1⟶3)-β-D-glucan, occurs through direct chemical stimulation promoted by formalin on nociceptors, while the late phase (inflammatory pain) is caused by inflammation local and associated with the release of chemical mediator such as histamine, serotonin, bradykinin, prostaglandins and excitatory aminoacids (Hunskaar & Hole, 1987).

In the present study, (1⟶3)-β-D-glucan administration induced antinociceptive activity only in the late phase of formalin; it is possible that its antinociceptive effect is due, at least in part, to an anti-inflammatory activity. Recently, Ruthes et al. (2013) demonstrated that structural differences alter activity of (1⟶3)-β-D-glucan on formalin-induced nociception. This idea is reinforced by our date since (1⟶3)-β-D-glucan from *Rhodotorula mucilaginosa* did not induce antinociception on early phase of intraplantar injection of formalin differently of 1⟶3)-β-D-glucan from *Lactarius rufus* (Ruthes et al., 2013).

The tail immersion test was used to check for a possible central antinociceptive effect. The fact that (1⟶3)-β-D-glucan did not induce antinociceptive effect in the tail immersion test suggest that (1⟶3)-β-D-glucan from *Rhodotorula mucilaginosa* does not block the neural transmission of pain like morphine does, and may induce a peripheral antinociception.

It can be concluded that the method of obtaining was effective and (1⟶3)-β-D-glucan from *Rhodotorula mucilaginosa* demonstrates peripheral antinociceptive activity, which are probably through inhibition of inflammatory mediators. Further studies currently in progress will enable us to understand the precise action mechanisms.

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