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Review

Chemopreventive effect and lack of genotoxicity and mutagenicity of the exopolysaccharide botryosphaeran on human lymphocytes

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ABSTRACT

Carbohydrate biopolymers of fungal-origin are an important natural resource in the search for new bioagents with therapeutic and nutraceutical potential. In this study the mutagenic, genotoxic, antigenotoxic and antioxidant properties of the fungal exopolysaccharide botryosphaeran, a (1 → 3)(1 → 6)-β-D-glucan, from *Botryosphaeria rhodina* MAMB-05, was evaluated. The mutagenicity was assessed at five concentrations in *Salmonella typhimurium* by the Ames test. Normal and tumor (Jurkat cells) human T lymphocyte cultures were used to evaluate the genotoxicity and antigenotoxicity (Comet assay) of botryosphaeran alone and in combination with the mutagen methyl methanesulfonate (MMS). The ability of botryosphaeran to reduce the production of reactive oxygen and nitrogen species (RONS) generated by hydrogen peroxide was assessed using the CM-H_2DCFDA probe in lymphocyte cultures under different treatment times. None of the evaluated botryosphaeran concentrations were mutagenic in bacteria, nor induced genotoxicity in normal and tumor lymphocytes. Botryosphaeran protected lymphocyte DNA against damage caused by MMS under *simultaneous treatment* and *post-treatment* conditions. However, botryosphaeran was not able to reduce the RONS generated by H_2O_2. Besides the absence of genotoxicity, botryosphaeran exerted a protective effect on human lymphocytes against genotoxic damage caused by MMS. These results are important in the validation of botryosphaeran as a therapeutic agent targeting health promotion.

Contents

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1. Introduction

Carbohydrate biopolymers (polysaccharides) of fungal-origin are an important natural resource in the search for new agents with therapeutic potential. Among them are the β-glucans, notably the (1 → 3)(1 → 6)-β-D-glucans, that are known to modify biological responses (Bohn and BeMiller, 1995), and play roles in chemoprevention, and can manifest immunomodulatory and antitumor activities, among others (Kagimura et al., 2015; Kirkland et al., 2011). β-D-Glucans of the (1 → 3)-type are not digested by enzymes such as the β-(1 → 3)-glucanases, which are absent from the gastrointestinal tract of mammals. Thus, they survive longer in the tissues of mammals, and can therefore be a candidate bioagent in treating cancers (Miura et al., 1996). Their ability to non-specifically activate the cellular and humoral components of the host immune system is well documented in the literature, see for example, (Williams, 2009). Accordingly, this activation leads to the increased functional activity of immune cells such as macrophages, mononuclear cells and neutrophils (Liang et al., 1998). Despite the studies that show the absence of toxicity of these compounds and their derivatives (Kagimura et al., 2015; Miranda-Nantes et al., 2011; Miranda et al., 2008; Roupas et al., 2012; Weng et al., 2011), the evidence of the absence of risk to health and the mechanism by which the (1 → 3)-β-D-glucans exert their effects on mammalian cells are key to their validation as bioactive agents.

The exopolysaccharide (EPS) botryosphaeran is of the (1 → 3)(1 → 6)-β-D-glucan type and is secreted by the fungus, *Botryosphaeria rhodina* MAMB-05. Botryosphaeran consists of a linear backbone chain of D-glucose residues linked by (1 → 3)-β-glucosidic bonds, with side-branches comprising glucose and gentiobiose linked through (1 → 6)-β-glucosidic bonds (Barbosa et al., 2003). It is soluble in water and forms gels and viscous solutions that may favor its commercial applications in different sectors such as health, biotechnology, food, pharmaceutical and cosmetology (Kagimura et al., 2015).

Studies from our research groups have evaluated the biological effects of botryosphaeran. This (1 → 3)(1 → 6)-β-D-glucan (i) did not present mutagenic activity (micronucleus test) in mice, and exhibited strong antimutagenic activity (Miranda et al., 2008); (ii) promoted hypoglycemic activity reducing the levels of plasma glucose in diabetes-induced rats by 52%, and reduced LDL-cholesterol levels in hyperlipidaemic-conditioned rats by 18% (Miranda-Nantes et al., 2011); (iii) exerted an antiproliferative effect in breast cancer MCF-7 cells that was associated with apoptosis, necrosis and oxidative stress (Queiroz et al., 2015); (iv) in combination with the antineoplastic agent, doxorubicin, botryosphaeran was selective for leukemic T lymphocyte cells (Malini et al., 2015). The molecular mechanisms of action of botryosphaeran appear to be involved in the repression of genes related to the G1 phase of the cell cycle (Malini et al., 2015). When derivatized by sulfonylation, the sulfonated biopolymer exhibited anticoagulant and antithrombotic activities (Brandi et al., 2011). In addition, botryosphaeran was found to possess free radical scavenging properties and antioxidant activity (Giese et al., 2015). In another study with a different *B. rhodina* strain (RCYU 30101), botryosphaeran demonstrated the ability to activate the lymphoblastogenesis process (Weng et al., 2011).

Understanding the mechanisms by which botryosphaeran exerts its chemoprotective effect is important in validating this β-glucan as a therapeutic agent. Studies on botryosphaeran's ability to inhibit the genotoxic effect caused by several mutagens are key to understanding its mechanism of action. The antimutagenic activity consists of the peculiar ability of a substance to reduce the frequency of spontaneous or induced mutations regardless of the mechanism involved (Von Borstel et al., 1996). Słoczyńska et al. (2014) recently proposed that various classes of compounds can be distinguished, such as compounds with antioxidant activity; compounds that inhibit the activation of mutagens; blocking agents; as well as compounds characterized with multiple mechanisms of action.

It is necessary and important to use *in vitro* tests employing normal and tumor cells in order to rapidly assess the selectivity of botryosphaeran as a therapeutic agent, given its biological effects, and the potential applications of this EPS described in the literature by our research group.

In the present study the objectives were to investigate: (i) the effects of the treatment with botryosphaeran alone, or in combination with the mutagen, methyl methanesulfonate (MMS), on normal and tumor (Jurkat cells) human T lymphocytes in order to evaluate its genotoxic and chemoprotective effects on these cell types; (ii) the possible mutagenicity of botryosphaeran assessed by the Ames test on different *Salmonella typhimurium* strains; and (iii) the antioxidant effect against H_2O_2-induced production of RONS (reactive oxygen and nitrogen species) on normal and tumor lymphocytes.

2. Material and methods

2.1. Microorganism and culture conditions

Botryosphaeria rhodina (MAMB-05 isolate) was grown by submerged fermentation (SmF) on sucrose as sole carbon source for 72 h at 28 °C as described by Steluti et al. (2004).

2.2. Production of botryosphaeran

Following the growth of the fungus, the mycelium was removed by centrifugation (1250g/15 min) and the supernatant recovered to which was then added three volumes of isopropanol, and left to stand overnight at 4 °C. The precipitate was recovered by centrifugation, resolubilized in water with gentle heating, and then dialyzed exhaustively against water for 48 h at 4 °C. Thereafter, the dialyzed solution

containing botryosphaeran was lyophilized, and the dried powder stored at −20 °C until used.

A stock solution of botryosphaeran was prepared by solubilizing 3.0 g of botryosphaeran in 1 L of sterile water, and the solution sterilized by autoclaving (121 °C/20 min) and stored at 4 °C.

2.3. Source of lymphocytes

The biological effects of botryosphaeran were assessed in human lymphocyte cultures (normal and leukaemic - Jurkat cells). Normal lymphocytes were obtained by collecting 10 mL of peripheral blood from three healthy male individuals aged 27, 32 and 34 years. Each person was interviewed for possible confounding factors such as smoking, alcohol drinking, medicine intake and exposure to radiation and/or chemical mutagens. All volunteers signed the free and informed consent form approved by the Ethics Committee on Human Research of FCFRP - University of São Paulo, Brazil (CEP/FCFRP n. 314).

Jurkat cells, a lymphoblastoid cell line, were kindly provided by Hemocentro do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, (Brazil), and were kept under liquid nitrogen (−196 °C) until required.

2.3.1. Lymphocyte culture conditions and treatments

Normal lymphocytes were isolated from peripheral blood using *Ficoll Histopaque®* 1077 (Sigma-Aldrich, St. Louis, MO, USA) immediately after being collected from volunteers in all of the experiments described in the present study. Jurkat cells were thawed and used between the 3rd and 9th passages. Both cell types were cultured in 15 mL sterile conical tubes (Corning, Lowell, MA, USA) during the cytogenetic assays, and in the biochemical assays were cultured in 96-well Microplates (Corning). The cells were cultured for 24 h in RPMI 1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20% fetal bovine serum (Gibco, Carlsbad, CA, USA); 0.01 mg/mL streptomycin (Sigma-Aldrich); 0.005 mg/mL penicillin (Sigma-Aldrich); and 2.38 mg/mL HEPES (Sigma-Aldrich) in all experiments. The tubes and plates were kept in an incubator at 37 °C with 5% CO_2 atmosphere and placed on 45° slopes, and lightly agitated periodically.

Three non-cytotoxic concentrations of botryosphaeran (7.5, 30 and 100 μg/mL) were used to evaluate genotoxic and antigenotoxic activities in normal and tumor (Jurkat) human T lymphocytes. These concentrations were selected based upon a study (unpublished from our research group) on MTT assays (Mosmann, 1983) conducted on Chinese hamster lung fibroblast (V79) and in hepatocarcinoma of *Rattus norvegicus* (HTC) cell lines. This study showed that botryosphaeran was not cytotoxic towards each of these cell lines over the 10 concentrations (ranging from 7.5 to 600 μg/mL) examined. From the MTT results obtained in that study, three concentrations of botryosphaeran (7.5, 30.0 and 100.0 μg/mL) were chosen to perform all the tests as reported here.

Approximately 1×10^5 cells were exposed to botryosphaeran alone, or in combination with the mutagenic agent, MMS (10 μM). The duration of treatment with botryosphaeran was for 20 h, and started 28 h after the cells had been cultured. Cell cultures were harvested and the cells fixed 48 h after onset.

In the *post-treatment* protocol, both types of lymphocytes were cultured for 24 h, and then treated with MMS (10 μM) for 4 h. The cells were then washed with PBS, and fresh culture medium and botryosphaeran solution were added, and the cells were left for a further 20 h before the experiment was terminated at the 48th hour. In the *simultaneous treatment* experiment, after 44 h of the beginning of cell culture, the lymphocytes were treated with botryosphaeran and MMS for 4 h; then fresh medium was added to the cells and they were cultured for a further 4 h, with the cells fixed at the 48th h. An aliquot of the cell suspension was used at the end of the treatment procedures to analyze for cell viability by the Trypan Blue exclusion method (Strober, 2001). Only cultures showing cell viability of ≥80% were considered to be valid.

2.4. Comet assay

The comet assay was performed according to Tice et al. (2000) with modifications. Briefly, 1×10^5 of both lymphocyte cell types were mixed with Low Melting Point agarose (Sigma-Aldrich) and transferred onto pre-coated slides with a basic layer of Normal Melting Point agarose (Sigma-Aldrich) 1.5% (w/v) in PBS (pH 7.4) and then covered with glass coverslips and cooled at 4 °C for 20 min. Next the coverslips were removed and the slides immersed in cold alkaline lysis solution (2.5 M NaCl, 10 mM Tris-HCl buffer (pH 10.0), 100 mM EDTA, 10% dimethyl sulfoxide, 1% Triton X-100, pH 10.0) for one hour at 4 °C in the dark. At the end of lysing period, the slides were transferred to an electrophoresis chamber containing a high pH (13.0) electrophoresis solution (300 mM NaOH, 1 mM EDTA) and incubated at 4 °C for 20 min to allow the DNA to unwind. A current of 25 V: 300 mA (1.25 V/cm) was applied for 20 min, after which, the slides were submerged in a neutralization buffer (400 mM Tris-HCl, pH 7.5) for 15 min, followed by drying at room temperature, and then fixed in 100% ethanol for 5 min.

The slides were then stained with 50 μL GelRed® solution (Biotium, Hayward, CA, USA) (15 μL of GelRed® 10,000× in water, 5 mM of 1 M NaCl, and 45 mL distilled water) and examined by fluorescent microscopy (Nikon-Brazil) using 488 nm (excitation) and 515 nm (emission) barrier filters, and a 40× objective lens. One hundred (100) nucleoids were analyzed per treatment, and DNA damage was quantified visually and classified into four classes (0, 1, 2 and 3) according to the size of the tails (Kobayashi et al., 1995). The cytological analyses were always performed by the same investigator under blind conditions. The score (Manoharan and Banerjee, 1985), the frequency of damaged cells (DC) and the percentage reduction (% R) of damage (Waters et al., 1990), were calculated according to the formulae presented below, wherein N is the number of analyzed cells in each class, and PC and NC correspond, respectively, to the positive and the negative control groups.

$$\text{Score} = \frac{(0\ \text{X}\ N_0) + (1\ \text{X}\ N_1) + (2\ \text{X}\ N_2) + (3\ \text{X}\ N_3)}{100}$$

$$\text{DC} = \left(\frac{(N_1 + N_2 + N_3)}{100}\right) \text{X}\ 100$$

$$\%\text{R} = \frac{[\text{PC score mean}] - [\text{Treatment score mean}]}{[\text{PC score mean} - \text{NC score mean}]} \text{X}\ 100$$

2.5. Reactive oxygen and nitrogen species (RONS)

Intracellular reactive oxygen and nitrogen species were evaluated using the fluorescent probe CM-H_2DCFDA (Sigma-Aldrich). CM-H_2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate) passively diffuses into the cells in which it is easily oxidized by RONS and converted into highly fluorescent compounds (Lu and Finkel, 2008). The fluorescence intensity is directly proportional to the intracellular RONS generated. Hydrogen peroxide (H_2O_2) was used to induce RONS and its concentration was previously established by means of a preliminary assay in which the Jurkat cells were exposed to increasing concentrations ranging from 25 to 200 μM.

The oxidation assay was conducted on both normal and Jurkat human lymphocytes assessing five concentrations of botryosphaeran (7.5, 15, 30, 60 and 100 μg/mL). Concentrations of 15 and 60 μg/mL were included because they were intermediate to those evaluated in the comet assay. Approximately 1×10^4 lymphocyte cells were incubated with botryosphaeran for 3, 9 and 24 h in 96-well microplates. Freshly made H_2O_2 (Sigma-Aldrich; 100 μM) solution in water was added to

each well at the end of each treatment time. Exposure of the lymphocytes to H_2O_2 was for 1 h only. The positive control group consisted of H_2O_2-treated cells (100 μM), and the negative control group consisted of PBS-treated cells (pH 7.4). Subsequently, the cells in the microplate wells were centrifuged (300 × *g*/5 min), the supernatant was discarded, and 100 μL of CM-H_2DCFDA solution (5 mM) was added to each well and incubated for 30 min. Fluorescence analysis was performed in a fluorimeter (Biotek, Synergy™ H1 Multi-Mode Reader, Winooski, VT, EUA), at an excitation wavelength of 485 nm and emission at 528 nm. All treatments were performed in quadruplicate. The results were expressed based upon the values obtained in cells treated with H_2O_2 alone.

2.6. Ames test for mutagenicity

The mutagenicity of botryosphaeran was evaluated by the Ames test (*Salmonella*/liver microsome) using the preincubation methodology according to Maron and Ames (1983). The incubation was conducted in the presence (+S9) and absence (—S9) of the S9 microsomal fraction, which consists of a homogenate derived from rat liver (Maron and Ames, 1983). The present study used *Salmonella typhimurium* strains: TA98, TA97a, TA100 and TA102, and were kindly provided by Dr. Bruce Ames, University of California, Berkeley, CA, USA.

The *S. typhimurium* strains were maintained frozen in Oxoid nutrient broth n. 2 (Oxoid Ltd., Basingstoke, UK; 2.25%) and DMSO 10% and inocula prepared from this, grown for 14 h at 37 °C in Oxoid nutrient broth n. 2 to obtain a cell density of 1–2 × 10^9 colony forming cells/mL. The metabolic activation mixture (S9) was purchased from Molecular Toxicology (Boone, NC, USA), and consisted of a lyophilized preparation comprizing 4% S9 fraction; 1% 0.4 M $MgCl_2$, 1% 1.65 M KCl; 0.5% 1 M glucose-6-phosphate disodium salt, 4% 0.1 M NADP, and 50% 0.2 M sodium phosphate buffer (pH 7.4), and was dissolved in 39.5% sterile distilled water and prepared fresh before each assay.

In the mutagenicity tests, botryosphaeran solutions of final concentrations 75, 150, 300, 450, 600 μg/plate was each separately added to 0.5 mL of 0.2 M phosphate buffer (pH 7.4) for the −S9 assays, followed by the addition of 0.1 mL of the bacterial culture, and the mixtures incubated for 30 min at 37 °C. In the metabolic activation tests (+S9), 0.5 mL of the S9 reagent replaced the phosphate buffer. After the incubation period, 2.0 mL of surface agar (top agar – 0.6% agar and 0.6% NaCl) containing traces of L-histidine (0.05 mM) and D-biotin (0.05 mM) was added to the bacterial culture-botryosphaeran mixture, and then poured onto a Petri-plate containing glucose minimal agar medium (GM agar). The plates were further incubated at 37 °C for 48 h, and His + revertant bacterial colonies scored manually. The compounds used as positive controls (C+) in the assays without metabolic activation (—S9) were 4-nitro-*o*-phenylenediamine (Sigma-Aldrich; NPD, 10 μg/plate) for *S. typhimurium* strains TA97 and TA98, sodium azide (2.5 μg/plate) for strain TA100, and mitomycin C (Sigma-Aldrich; 0.5 μg/plate) for the TA102 strain. Compounds used in the assays with metabolic activation (+S9) included: 2-antramine (Sigma-Aldrich; 2-ANTR, 1.25 μg/plate) for the TA97, TA98 and TA100 strains, and 2-aminofluorene (10 μg/plate) for the TA102 strain. Negative controls were performed using the samples' solvent, *i.e.*, sterile distilled water.

The mutagenicity index (MI) was calculated for each analyzed sample based on the results obtained according to the following formula (Maron and Ames, 1983).

$$MI = \frac{\text{Number of revertants in the plate with test compound}}{\text{Number of revertants in the plate with negative control}}$$

2.7. Statistical analysis

All results were expressed as the mean ± standard deviation. All the means from the tests with Jurkat cells were obtained from three independent experiments. Means derived from tests with normal lymphocytes were obtained from four different individuals donating the peripheral blood. Variance analysis (ANOVA) and Tukey test at 5% significance level were used to analyze genotoxicity, antigenotoxicity and the reactive oxygen and nitrogen species data. These statistical analyses were performed using GraphPad INSTAT software, version 3.02 (http://www.graphpad.com/scientific-software/instat/). Mutagenicity data were analyzed by variance analysis (ANOVA) followed by the linear regression test at the 5% significance level, and using Salanal statistical software (Environmental Protection Agency of the United States, Monitoring Systems Laboratory, Las Vegas, NV, version 1.0, Research Triangle Institute, RTP, NC, USA), according to the model by Bernstein et al. (Bernstein et al., 1982).

3. Results

3.1. Genotoxicity and antigenotoxicity

The results from normal and tumor lymphocytes treated with botryosphaeran, MMS, and a combination of both, are shown, respectively, in Tables 1 and 2. The mutagenic agent MMS (10 μM) was effective at causing damage to the DNA molecule. On the other hand, botryosphaeran showed no evidence of a genotoxic effect at any of the three concentrations tested in the different lymphocyte types under the conditions evaluated in the study.

The protective effect antigenotoxicity of botryosphaeran was observed in both normal and tumor lymphocytes (Tables 1 and 2). As for the normal lymphocytes, this protective effect was observed only in botryosphaeran in the *post-treatment* conditions in comparison to MMS. It was possible to verify that the two higher evaluated concentrations of botryosphaeran (30 and 100 μg/mL) led to protective effects of 72.41% and 91.09%, respectively.

As for the tumor cells, this protective effect was observed in both associated protocols. In the *simultaneous treatment*, botryosphaeran at concentrations of 30 and 100 μg/mL showed 60.71% and 63.39% reduction of DNA damage, respectively, while the *post-treatment* condition using 30 and 100 μg botryosphaeran/mL resulted in a reduction of damage to DNA of 64.52% and 61.29%, respectively (Table 2).

3.2. Reactive oxygen and nitrogen species

Jurkat cells were exposed to increasing hydrogen peroxide concentrations (from 25 to 200 μM) in order to assess intracellular production of RONS and to set the limits of concentration that are able to significantly promote the release of RONS within these cells. Fig. 1 shows that all concentrations of H_2O_2 were equally efficient in generating RONS. Thus, in the experiments of the present study, a H_2O_2 concentration of 100 μM was used, as was also reported by Serpeloni et al. (2012).

Results obtained for normal and tumor lymphocyte cell cultures treated with different concentrations of botryosphaeran in combination with 100 μM H_2O_2 are shown in Fig. 2. Fig. 2A, B and C shows the results from the RONS generated in the Jurkat cells subjected to treatment with botryosphaeran at concentrations of 7.5, 15, 30, 60 and 100 μg/mL for 3, 9 and 24 h in combination with 100 μM H_2O_2. Data analyses showed that all of the tested concentrations did not decrease the formation of H_2O_2-induced RONS for the different times of treatment.

Reactive oxygen and nitrogen species generation in normal human lymphocytes subjected to the same treatments showed similar results to those found for the Jurkat cells. There was significant H_2O_2-induced RONS production by the normal lymphocytes with time, and botryosphaeran did not offer protection to these cells under the conditions evaluated (Fig. 2D, E and F).

Table 1
Frequency of nucleoids observed for each comet class, score and average of nucleoids with damage to normal human lymphocytes after being exposed to botryosphaeran alone or in combination with methyl methanesulfonate (MMS) in *simultaneous treatment* (SIM) and in *post-treatment* (POST).

Treatments (μg/mL)		Comet classes (100 cells/experiment)						% Reduction
		0	1	2	3	Score (X ± SD)	DNA damage (X ± SD)	
PBS		85.00 ± 7.00	6.67 ± 2.89	6.67 ± 2.08	2.33 ± 3.21	21.0 ± 16.64[b]	0.13 ± 0.08[b]	–
EPS	7.5	79.33 ± 8.50	12.67 ± 5.51	5.00 ± 0.00	1.67 ± 1.53	27.67 ± 10.07	0.19 ± 0.07	–
	30	73.33 ± 15.28	12.00 ± 10.54	7.67.3 ± 5.51	2.00 ± 1.73	33.33 ± 22.30	0.22 ± 0.16	–
	100	79.00 ± 13.89	13.00 ± 13.86	4.33 ± 0.58	3.00 ± 1.73	30.67 ± 13.20	0.20 ± 0.14	–
MMS[1]	10 μM	2.33 ± 1.53	10.33 ± 7.57	23.00 ± 8.89	65.00 ± 2.65	251.33 ± 5.51[a]	0.98 ± 0.01[a]	–
SIM	MMS + 7.5	7.00 ± 4.58	22.00 ± 5.20	20.00 ± 1.73	54.67 ± 8.7	226.00 ± 24.06[a]	0.97 ± 0.05[a]	–
	MMS + 30	3.33 ± 4.93	24.33 ± 4.16	30.67 ± 4.04	42.00 ± 6.56	211.67 ± 14.57[a]	0.97 ± 0.05[a]	–
	MMS + 100	14.00 ± 12.77	21.67 ± 4.51	22.00 ± 4.36	41.00 ± 12.53	188.67 ± 44.12[a]	0.85 ± 0.16[a]	–
MMS[2]	10 μM	36.67 ± 13.01	15.0 ± 11.53	11.00 ± 8.66	33.33 ± 8.50	137.00 ± 27.40[a]	0.59 ± 0.14[a]	–
POST	MMS + 7.5	38.33 ± 5.51	28.67 ± 2.52	11.33 ± 1.53	21.67 ± 4.51	116.33 ± 15.63[a]	0.62 ± 0.06[a]	–
	MMS + 30	70.67 ± 16.26	13.33 ± 10.41	8.33 ± 3.21	7.67 ± 3.21	53.00 ± 25.06[b]	0.29 ± 0.16[b]	72.41[b]
	MMS + 100	85.67 ± 2.08	4.00 ± 3.00	3.67 ± 1.53	6.67 ± 1.15	31.33 ± 4.04[b]	0.14 ± 0.02[b]	91.09[b]

X ± SD: mean ± standard deviation; PBS: phosphate buffer - negative control; EPS, botryosphaeran; MMS[1]: methyl methanesulfonate - positive control for SIM protocol (RPMI 1640 - 20 h; MMS - 4 h - inducing damage at the 4 final hours of the cell cycle). SIM: *Simultaneous treatment* with botryosphaeran and MMS[1]; MMS[2]: methyl methanesulfonate - positive control for POST protocol (RPMI 1640 - 20 h; MMS-4 h-inducing damage at the 4 initial hours of the cell cycle); POST: cells treated with botryosphaeran after MMS[2].
[a] Statistically different values of the negative control (PBS) at 5% by ANOVA followed by the Tukey test.
[b] Statistically different values of the positive control (MMS[1] or MMS[2]) at 5% by ANOVA followed by the Tukey test.

3.3. Ames test

Table 3 shows the mean number of bacterial revertants per plate, the standard deviation (SD) and mutagenicity index (MI) observed after the treatment of four different *S. typhimurium* strains (TA98, TA100, TA102 and TA97) with five different concentrations of botryosphaeran (75, 150, 300, 450, and 600 μg/mL) in the presence (+S9) and absence (—S9) of metabolic activation. The mutagenicity tests demonstrated that botryosphaeran did not induce any significant increase in the number of revertant colonies in any of the treatments evaluated in the different bacterial strains used in the Ames test. Furthermore, there were no significant differences in the results with and without metabolic activation.

4. Discussion

β-D-Glucans of the (1 → 3)-, (1 → 3)(1 → 6)- and (1 → 6)-linked types such as produced by certain bacterial, yeast and fungal species and strains have been the subject of intense investigation because of their biological response modifying (BRM) activities in the host, and this attribute favors their application in medical therapy (Chen and Seviour, 2007; Williams, 2009). Identifying and studying the biological effects of macromolecules that include polysaccharides and polysaccharide–protein complexes, have helped in introducing effective cancer therapeutic agents onto the pharmaceutical market (Zong et al., 2012). Although much of the literature associated with these biopolymers is of a protective nature, and usually bears little or no toxic activity, there are literature reports involving the mutagenic potential of molecules found in fungal extracts that may contain biopolymers (Sugimura, 2000), *e.g.*, agaritine from *Agaricus bisporus* (Pool-Zobel et al., 1990), and the carcinogenic, mutagenic and teratogenic activities of 11 compounds derived from 22 mushrooms species (Toth, 1991).

The guidelines of the Organization for Economic Cooperation and Development (OECD) used to identify and characterise potential hazards of chemicals including mutagens recommends two *in vitro* tests that should be conducted to assess chromosomal damage: the reverse mutation (Ames) test using bacteria to assess point mutation (OECD, 1997), and the micronucleus test on mammalian cells (ICH guidance S2(R1) OECD, 2014). In addition, because of its sensitive nature, the Comet test (Di Sotto et al., 2014) should also be considered as this evaluates genotoxicity of chemical compounds revealing very early damage to DNA (Collins, 2014; Kimura et al., 2013) and its universal applicability to all kinds of cells (Cortés-Gutiérrez et al., 2011). Once the absence of

Table 2
Frequency of nucleoids observed for each comet class, score and average of nucleoids with damage to leukaemic human lymphocytes (Jurkat cells) after being exposed to botryosphaeran alone, or in combination with methyl methanesulfonate (MMS) in *simultaneous treatment* (SIM) and in *post-treatment* (POST).

Treatments (μg/mL)		Comet classes (100 cells/experiment)						
		0	1	2	3	Score (X ± SD)	DNA damage (X ± SD)	% Reduction
PBS		96.00 ± 3.61	4.00 ± 3.61	0.00 ± 0.00	0.00 ± 0.00	4.00 ± 3.61[b]	0.04 ± 0.04[b]	–
EPS	7.5	97.00 ± 2.00	2.33 ± 1.53	0.67 ± 0.58	0.00 ± 0.00	3.67 ± 2.52	0.03 ± 0.02	–
	30	95.67 ± 2.08	3.00 ± 1.00	1.33 ± 1.15	0.00 ± 0.00	5.67 ± 3.21	0.04 ± 0.02	–
	100	98.33 ± 2.89	1.33 ± 2.31	0.33 ± 0.58	0.00 ± 0.00	2.00 ± 3.46	0.02 ± 0.03	–
MMS[1]	10 μM	4.33 ± 2.08	55.33 ± 12.50	32.00 ± 17.78	11.33 ± 4.04	153.33 ± 15.18[a]	0.99 ± 0.06[a]	–
SIM	MMS + 7.5	0.00 ± 0.00	88.00 ± 5.57	11.67 ± 4.04	0.67 ± 1.15	113.33 ± 5.86[a,b]	1.00 ± 0.01[a]	–
	MMS + 30	38.00 ± 3.00	55.33 ± 6.66	3.67 ± 2.89	0.00 ± 0.00	62.67 ± 6.66[a,b]	0.59 ± 0.06[a,b]	60.71[b]
	MMS + 100	43.00 ± 4.00	56.00 ± 2.65	1.33 ± 1.15	0.00 ± 0.00	58.67 ± 4.51[a,b]	0.57 ± 0.04[a,b]	63.39[b]
MMS[2]	10 μM	85.67 ± 1.53	8.33 ± 1.53	1.67 ± 1.53	4.33 ± 2.31	24.67 ± 3.51[a]	0.14 ± 0.02[a]	–
POST	MMS + 7.5	88.33 ± 3.06	7.33 ± 3.51	2.67 ± 1.53	1.67 ± 1.53	17.67 ± 1.53[a]	0.12 ± 0.03[a]	–
	MMS + 30	89.67 ± 2.52	9.33 ± 3.06	1.00 ± 1.00	0.00 ± 0.00	11.33 ± 2.31[a,b]	0.10 ± 0.03	64.52[b]
	MMS + 100	92.00 ± 2.00	7.00 ± 2.65	0.00 ± 0.00	1.67 ± 1.53	12.00 ± 2.00[a,b]	0.09 ± 0.01	61.29[b]

X ± SD: mean ± standard deviation; PBS: phosphate buffer - negative control; EPS, botryosphaeran; MMS[1]: methyl methanesulfonate - positive control for SIM protocol (RPMI 1640 - 20 h; MMS - 4 h - inducing damage at the 4 final hours of the cell cycle). SIM: *Simultaneous treatment* with EPS and MMS[1]; MMS[2]: methyl methanesulfonate - positive control for POST protocol (RPMI 1640 - 20 h; MMS-4 h-inducing damage at the 4 initial hours of the cell cycle); POST: cells treatment with EPS after MMS[2].
[a] Statistically different values of the negative control (PBS) at 5% level by ANOVA followed by Tukey test.
[b] Statistically different values of the positive control (MMS[1] or MMS[2]) at 5% level by ANOVA followed by Tukey test.

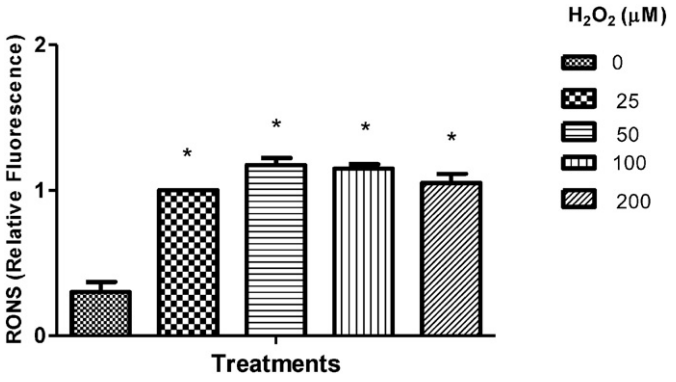

Fig. 1. Reactive oxygen and nitrogen species (RONS) production (in relative fluorescence units) in Jurkat cells exposed to different concentrations of H_2O_2 and their respective control. *Different values from the negative control (0 μM) at 5% level by ANOVA analysis followed by Tukey test.

genotoxicity in a chemical agent has been verified, it can be evaluated for its protective potential against DNA damage.

The investigation reported herein on the *in vitro* genotoxic and mutagenic studies on normal and tumor human T lymphocyte cultures, and on *S. typhimurium* strains, by the exopolysaccharide botryosphaeran, a (1 → 3)(1 → 6)-β-D-glucan, was expanded to include the Ames test and Comet assay, and further advances the knowledge acquired from our earlier studies on the *in vivo* genotoxic approach reported by Miranda et al. (2008). These tests are important in validating the biosafety of botryosphaeran.

Bacterial mutagenicity tests are used worldwide as part of the selection strategy in investigating the safety of chemical compounds by the pharmaceutical industry during drug testing. The mutagenicity index of the five botryosphaeran concentrations evaluated in the Ames test was less than twice of that observed in the negative control group. From this result, we infer that botryosphaeran does not pose a mutagenic hazard in the presence or absence of the S9 fraction, under the conditions evaluated in the Ames test experiments.

The negative data regarding botryosphaeran genotoxicity and mutagenicity obtained in the present study corroborates the results obtained by our research group from the *in vivo* micronucleus tests conducted on mice (Miranda et al., 2008), as well as other still unpublished data (*in vitro* micronucleus test and comet assay with the mammalian cell lines, V79 and HTC). The association between the reverse mutation test and the micronucleus test appeared to be effective to evaluate the genotoxic potential of new therapeutic agents. Thus, when the results from both tests are negative, additional genotoxic tests are not necessary (Kirkland et al., 2011). Therefore, it is possible to consider botryosphaeran as a low mutagenic and genotoxic biomacromolecule; a fact that will assist the validation process as a therapeutic agent.

Given the potential of non-cellulosic β-glucans modifying host biological responses, antigenotoxic tests were performed on normal and tumoral human T lymphocyte cultures to confirm the protective action of botryosphaeran against the damage induced by the alkylating agent, methyl methanesulfonate. Our results indicate that treatment with botryosphaeran was able to exert a protective action on the normal and tumor lymphocyte cells evaluated, and this was more efficient on normal lymphocytes under the *post-treatment* condition assessed. Moreover, the 72.4% and 91.1% reduction in MMS-induced damage in normal human lymphocytes treated, respectively, with 30 and 100 μg/mL of botryosphaeran under both the *simultaneous treatment* and *post-treatment*, suggests the potential of this compound as a bioagent that possibly acts on the repair mechanism of DNA leading to decreasing mutations. This activity was reported by Kada and Shimoi (1987) as bioantimutagenic activity. This kind of cell signal modulation mechanism is possibly triggered by botryosphaeran binding to specific cell membrane receptors, such as CR3-1 and dectin-1 receptors. These receptors have been described as being able to interact with β-glucans (Williams, 2009). Nevertheless, data recently published by our research group (Malini et al., 2015), on gene expression analysis of some β-glucan pattern recognition cell receptors, indicated no interference by botryosphaeran in the expression of genes encoding CR3 and dectin-1

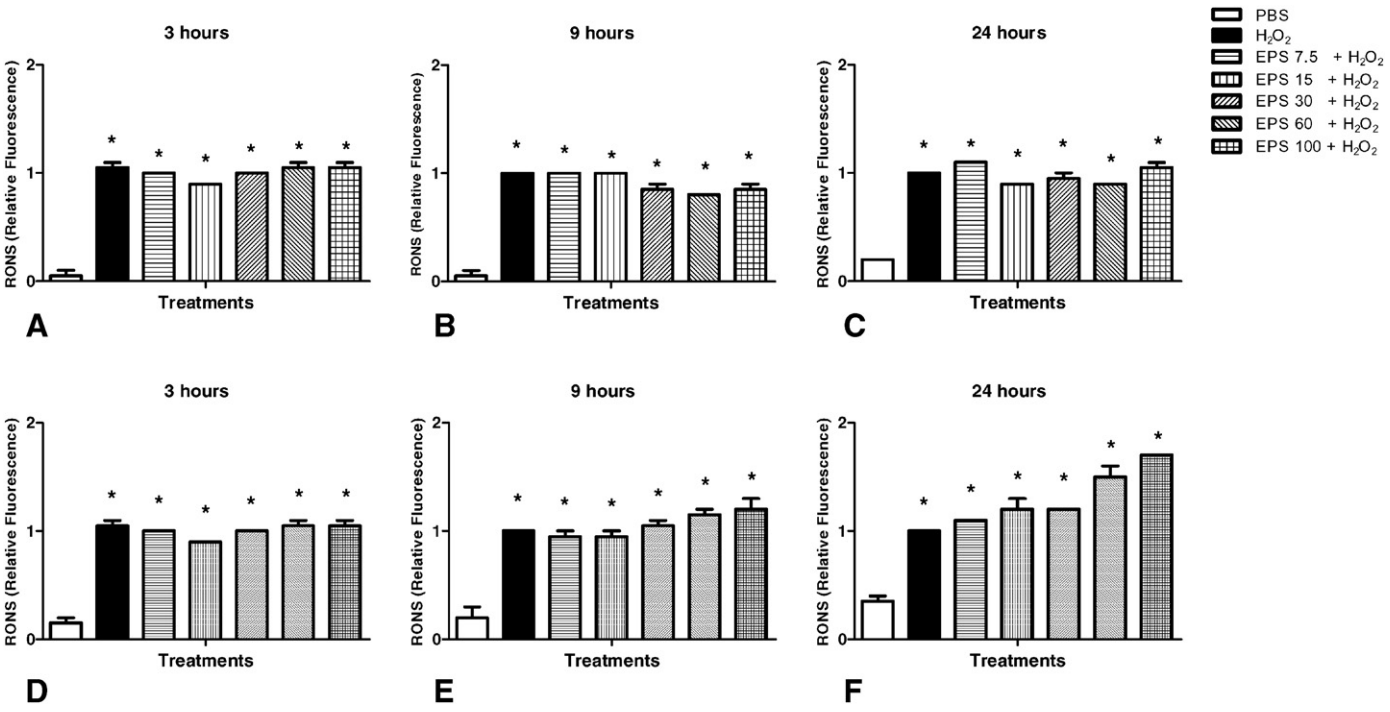

Fig. 2. Reactive oxygen and nitrogen species (RONS) production in relative fluorescence units after treatment of human leukemic lymphocytes (Jurkat) (A, B and C) and normal human lymphocytes (D, E and F) with botryosphaeran (EPS) at different concentrations for 3 h, 9 h and 24 h. The H_2O_2 concentration used was 100 μM in each of the samples examined. *Significant values different from the others at 5% level by ANOVA, followed by the Tukey test.

Table 3
Ames test - mutagenic activity expressed as the mean and standard deviation (X ± SD) of the number of bacterial revertants/plate and mutagenicity index (MI) of the TA98, TA100, TA102 and TA97 *Salmonella typhimurium* strains exposed to five different concentrations of botryosphaeran with (+S9) and without (−S9) metabolic activation.

Treatment (μg/plate)	Number of revertants/plate (X ± SD) and mutagenicity index (MI, values in parenthesis) *Salmonella typhimurium* strains							
	TA 98		TA 100		TA 102		TA 97a	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
0.00[a]	29 ± 2	24 ± 2	120 ± 6	90 ± 4	576 ± 13	599 ± 2	133 ± 21	119 ± 11
75	33 ± 2 (1.1)	29 ± 2 (1.2)	126 ± 1 (1.0)	112 ± 27 (1.2)	577 ± 23 (1.0)	575 ± 16 (1.0)	125 ± 11 (0.9)	126 ± 8 (1.1)
150	25 ± 1 (0.9)	25 ± 3 (1.1)	107 ± 3 (0.9)	90 ± 15 (1.0)	555 ± 27 (1.0)	604 ± 17 (1.0)	134 ± 4 (0.9)	136 ± 9 (1.1)
300	25 ± 1 (0.9)	27 ± 6 (1.1)	130 ± 9 (1.1)	91 ± 4 (1.0)	554 ± 8 (1.0)	600 ± 11 (1.0)	133 ± 5 (1.1)	133 ± 8 (1.1)
450	26 ± 2 (0.9)	31 ± 2 (1.3)	120 ± 3 (1.0)	97 ± 11 (1.1)	575 ± 6 (1.0)	627 ± 20 (1.0)	154 ± 21 (1.2)	123 ± 2 (1.0)
600	28 ± 3 (1.0)	34 ± 4 (1.4)	114 ± 2 (1.0)	89 ± 11 (1.0)	541 ± 6 (0.9)	654 ± 11 (1.1)	139 ± 8 (1.0)	115 ± 11 (1.0)[g]
C+	1455 ± 5[b]	292 ± 7[e]	2715 ± 54[c]	484 ± 9[e]	2171 ± 59[d]	702 ± 6[f]	1364 ± 4[b]	569 ± 25[e]

[a] Negative Control: Milli-Q water (200 μL/plate); C+.
[b] Positive Controls: 4-nitro-*o*-phenylenediamine (NPD − 10.0 μg/plate).
[c] Sodium azide (1.25 g/plate).
[d] Mitomycin C (0.5 g/plate).
[e] 2-Antramine (1.25 g/plate).
[f] 2-Aminofluorene (10.0 g/plate).
[g] Different values from the others at 5% level by ANOVA analysis followed by the linear regression test at the 5% significance level.

receptors. On the other hand, repression was observed in the *CCR5* gene transcripts when normal lymphocyte cells were treated with botryosphaeran alone, and in association with doxorubicin (DXR). This may indicate a possible mechanism of molecular action of botryosphaeran. It is likely that this β-glucan has binding affinity for the CCR5 receptor, thus inhibiting its gene product (Malini et al., 2015). According to Weitzenfeld and Ben-Baruch (2014), high *CCR5* gene expression is associated with pro-malignancy and tumor growth. Since DXR and MMS are agents that cause distinct damage in cells, the development of protective effects against MMS-induced damage in normal lymphocytes as well as the antitumor effect observed on Jurkat cells treated with DXR and in combination with botryosphaeran (Malini et al., 2015) - both under *post-treatment* conditions - demonstrated that botryosphaeran has the ability to exercise different forms of protection.

The antitumor activity of β-glucans may also be influenced by their antioxidant capacity; *i.e.*, oxidative stress. A study conducted on the free radical scavenging ability of botryosphaeran in an *in vitro* model was evaluated in the absence of mammalian cells and demonstrated total antioxidant activity (80%), as well as scavenging of hydroxyl radicals (90.6%) and nitric oxide (90%) (Giese et al., 2015). Botryosphaeran investigated in our study showed no antioxidant effect on normal and tumor lymphocytes treated with H_2O_2 under any of the concentrations evaluated. Thus, the present study did not corroborate the data gathered by Giese et al. (2015) who found antioxidative capacity in botryosphaeran in comparison to H_2O_2 (38%).

The lack of antioxidant activity in the lymphocyte cells studied in the present study may have been due to the low botryosphaeran concentrations in the co-treatment evaluated, and the short duration times (3, 9 and 24 h), in comparison to that in studies by Giese et al. (2015) who found more relevant activity at 3 g/L concentration. Although chemical *in vitro* tests are widely accepted in antioxidant evaluations, they are not representative of human cell conditions (Fukumoto and Mazza, 2000; Peris et al., 1997).

5. Conclusion

This study revealed that botryosphaeran was not mutagenic and genotoxic in normal and tumor human T lymphocytes as evaluated by the Ames and Comet assays, respectively. This carbohydrate biomacromolecule exhibited antigenotoxic activity against damage induced by the alkylating agent MMS in normal and tumorigenic lymphocytes. Although there are reports in the literature on the antioxidant capacity of fungal exopolysaccharides, botryosphaeran did not appear to reduce H_2O_2-induced RONS production in the human lymphocyte cells studied herein. The findings of this study reinforce the absence of genotoxic effects of botryosphaeran and helps validating the use of this β-glucan for commercial applications. Moreover, the findings reported here will encourage further investigations to foster a better understanding of the protective mechanisms of botryosphaeran as triggered in human lymphocytes following their exposure to alkylating agents.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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