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In vivo antimutagenic and antiatherogenic effects of the (1 → 3)(1 → 6)-β-D-glucan botryosphaeran

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ABSTRACT

The antimutagenic effect of botryosphaeran, an exocellular (1 → 3)(1 → 6)-β-D-glucan, from the ascomyceteous and plant-borne endophytic fungus, *Botryosphaeria rhodina* MAMB-05, was evaluated in young (6–8 weeks) and elderly (18 months) Swiss albino mice of both genders. The hypolipidemic, hypoglycemic and antiatherogenic potential was also evaluated in 18-month old male LDL receptor knockout (LDLr$^{-/-}$) mice. Administration of botryosphaeran by gavage (doses: 7.5, 15, 30 mg/kg b.w./day) in a 30-day pretreatment protocol (young mice), or 15-day protocol (older mice), did not cause genotoxicity as assessed by the micronucleus test in peripheral blood (PB) and bone marrow cells (BMCs). Furthermore, there was no cytotoxic effect of this β-D-glucan in the treatments. A lower frequency of micronuclei was observed in BMCs from young and old mice that received botryosphaeran, indicating its antimutagenic effect. Botryosphaeran (30 mg/kg b.w./day) promoted 102.22% (young) and 103.45% (elderly) reductions in cyclophosphamide-induced damage in male mice. Botryosphaeran also exerted chemoprotective effects in LDLr$^{-/-}$ and wild-type (C57BL/6) mice. Botryosphaeran treatment for 15 days at a dose of 30 mg/kg b.w./day improved the lipidic profile (reductions of 53.8–84.3%), and decreased aortic lipid deposition (32.8%) in the LDLr$^{-/-}$ atherosclerotic mice. The results indicate botryosphaeran has relevant biologic effects, making it a promising candidate for the development of new therapeutic agents.

1. Introduction

Over the past decades, there has been an increasing interest and consumption of natural medicinal plant and microbial products to alleviate or reduce the risks associated with cardiovascular disease, diabetes and cancer, which are responsible for significant morbidity and mortality [1]. Among these are macrofungal products such as mushrooms and brackets that have traditionally been used for millennia as folkloric medicines in the Orient to treat human disease conditions. They have also been recognized by the occidental world in clinical practice to treat cancers [2,3]. Among the bioactive compounds identified are the polysaccharides, and esp., the β-glucans that have been evaluated in animal models and cell lines [4]. These carbohydrate biopolymers act as biological response modifiers due to their

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immunomodulatory activities [5]. Other biological functions are also recognized and include antithrombosis, anti-inflammatory, anti-proliferation, hypolipidemia and hypoglycemia, and they also bear antioxidant properties [6,7]. Human clinical trials involving fungal polysaccharides have been conducted, but are few and the limited studies conducted were with small numbers of patients that were often poorly controlled [4].

The (1 → 3)(1 → 6)-β-D-glucan named botryosphaeran is an exocellular branched chain polysaccharide secreted by the fungus *Botryosphaeria rhodina* MAMB-05, and is composed of a main chain of (1 → 3)-linked β-D-glucose residues bound with a single branch comprising either glucose orgentiobiose linked by (1 → 6) bonds at every five glucose units along the backbone chain [8]. Botryosphaeran has been demonstrated from our research groups to possess a broad spectrum of activities with potential applications in the food, pharmaceutical and cosmetic sectors. For example, this mixed-linked β-D-glucan, has previously been shown in murine models that it lacked mutagenicity and exhibited strong anticlastogenic activity [9], and was demonstrated to possess hypoglycemic and hypocholesterolemic properties [10].

The antimutagenic and genotoxic, glucose and lipidic profiles and atheroprotective potential of botryosphaeran, however, has not been evaluated in a pre-treatment assay and experimental model ($LDLr^{-/-}$ mice) of atherosclerosis with 15-day and 30-day treatments using tests for genomic instability, and biochemical and morphologic parameters. Such studies are relevant because they may contribute to the development of innovative and promising strategies in the use of biomolecules to alleviate the effects of oxidative lesions in the genetic material that accompany the aging process and diseases, and to treat chronic degenerative diseases [11,12].

The objective of the present work was to investigate the (anti)genotoxic effect of botryosphaeran in Swiss albino mice of both genders, and of different age groups (young and elderly), as well as to assess its hypoglycemic, hypolipidemic, and antiatherogenic potential in older male mice (WT-C57BL/6 and $LDLr^{-/-}$ knockout) with a predisposition to atherosclerosis.

2. Material and methods

2.1. Microorganism and culture conditions

B. rhodina (isolate MAMB-05), an ascomyceteous endophytic fungus, was grown in Erlenmeyer flasks by submerged fermentation on sucrose as sole carbon source for 72 h at 28 °C under shaking conditions (180 rpm) as previously described [8].

2.2. Botryosphaeran production and solution preparation

After cultivating the fungus, the mycelium was removed by centrifugation (1250 × *g*/15 min), the supernatant was recovered, and the exopolysaccharide was precipitated from the solution by adding three volumes of isopropanol and leaving the solution stand overnight at 4 °C. The precipitate was recovered by centrifugation, dissolved in distilled water (gently heated at 60 °C for 2 h) and dialyzed exhaustively for 48 h, with frequent changes of water. The botryosphaeran-containing solution was then lyophilized and the dried powder was stored at −20 °C until use. For use in the *in vivo* assays, botryosphaeran was solubilized in isotonic saline solution (0.9%, w/v) at a concentration of 3 g/L (stock solution). A sample of this solution was used to quantify total sugars by the phenol-sulfuric acid method [13], and to confirm the concentration of the stock solution. The doses of botryosphaeran were chosen based on its solubility limit of 3 g/L in isotonic saline solution [9].

2.3. Experimental design

The experiments were conducted in strict accordance with the recommendations of the ethics principles and guidelines of the National Institutes of Health (USA) for the care and handling of laboratory animals. This study was approved by the Research Ethical Committee on Animal Use at Universidade Federal do Espírito Santo (CEUA/UFES case 017/2013). For the use and handling of experimental inbred animals (wild-type C57BL/6 and $LDLr^{-/-}$ knockout mice), the standards established by Comissão Técnica Nacional de Biossegurança (Brazil) were followed.

2.3.1. Experimental protocol to assess antimutagenic and anticytotoxic activities of botryosphaeran in young and aged mice

2.3.1.1. Animals and treatments. Swiss albino mice (*Mus musculus*) were obtained from Biotério do Centro de Ciências da Saúde, Universidade Federal do Espírito Santo, and housed in groups in standard plastic cages in a room set with constant temperature (22–23 °C), relative humidity (50 ± 10%), and a light-dark cycle of 12-h under fluorescent lighting. The animals received the classic standard commercial animal ration (Nuvilab CR1, Nuvital, Colombo-PR, Brazil) and water *ad libitum*.

Fifty young mice aged 6–8 weeks (25 males and 25 females) and 50 older mice aged 18 months (25 males and 25 females) were preconditioned on the standard commercial animal ration for one week prior to the start of the treatments.

Botryosphaeran was evaluated using the pre-treatment protocol in the following experimental groups: (i) botryosphaeran-treated groups with administered doses of 7.5, 15 and 30 mg/kg animal body weight (b.w.), (ii) positive control group treated with cyclophosphamide (Sigma-Aldrich, St. Louis, MO, USA), and (iii) negative control group treated with isotonic saline. Each experimental group consisted of 10 animals that were randomly selected and separated by gender. Young mice had body weights ranging from 39.5 ± 4.0 g (males) to 35.7 ± 1.4 g (females), and the aged mice had body weights ranging from 50.4 ± 3.4 g (males) to 42.1 ± 3.0 g (females).

The pre-treated groups of Swiss albino mice received each dose of botryosphaeran by gavage (7.5, 15 and 30 mg botryosphaeran/kg animal b.w.) once daily for 30 days for the young animals, and for 15 days for the older animals. At the end of each of the treatment periods with the three doses of botryosphaeran by gavage, the clastogenic agent cyclophosphamide was administered intraperitoneally to the young animals at a dose of 100 mg/kg b.w., and to the older animals at a dose of 50 mg/kg b.w. These doses and the different time intervals for treatments (15 and 30 days) were defined according to previous tests. The negative control group received isotonic saline solution by gavage, whereas the positive control group received only 100 and 50 mg/kg b.w. (intraperitoneally) to induce micronucleus formation, according to the age category of the animals.

Aged (18- month old) male, low-density lipoprotein-receptor knockout ($LDLr^{-/-}$) mice (LDL receptor-deficient mice show elevated plasma cholesterol levels and develop atherosclerosis on feeding a lipid-rich diet), which had a C57BL/6 (wild-type, WT) genetic background, were obtained from the Laboratórios de Fisiopatologia de Doenças Humanas e Animais, of Universidade Vila Velha (Vila Velha, Espírito Santo), and Fisiologia Translacional of Universidade Federal do Espírito Santo. The animals were maintained in the latter vivarium in groups housed in standard plastic cages under the same temperature, relative humidity and light-dark cycle conditions mentioned above, and were fed the classic standard commercial animal ration and water *ad libitum*.

When the $LDLr^{-/-}$ animals reached 18 months of age, they received a Western-type diet (Rhoster®, Araçoiaba da Serra, São Paulo, Brazil) to accelerate spontaneous hyperlipidemia and the development of atherosclerotic lesions. The composition of this atherogenic diet is shown in Table 1. After five weeks (18–month old animals plus five weeks), the $LDLr^{-/-}$ mice were divided into three groups: (i) animals that were treated with botryosphaeran (30 mg/kg b.w.) by gavage once

Table 1
Composition of Western-type (atherogenic) diet used to feed older LDLr$^{-/-}$ knockout mice.

Ingredients	% (g/100 g)
Cornstarch	15.00
Casein	19.50
DL-methionine	0.30
Sucrose	34.15
Lard	21.00
Cellulose	5.00
Cholesterol[a]	0.15
Mineral mixture[b]	3.50
Calcium carbonate	0.40
Vitamin mixture[b]	1.00
tert-Butylhydroquinone[a]	0.004

[a] Sigma-Aldrich (St. Louis, Missouri, USA).
[b] AIN–93 M [43].

daily, (ii) a negative control group (treated with isotonic saline) and (iii) a positive control group (treated with atherogenic diet (Table 1); and all were treated for 15 days. Similarly, wild-type (WT C57BL/6) male mice were divided into two groups and treated with (i) botryosphaeran (30 mg botryosphaeran/kg b.w.) and (ii) isotonic saline (negative control) by gavage for the same period of time.

At the end of the treatment period with botryosphaeran by gavage, cyclophosphamide was intraperitoneally administered to the WT mice at a dose of 50 mg/kg b.w. A third group of WT male animals, the positive control (iii), only received 50 mg/kg b.w. cyclophosphamide (intraperitoneally) to induce micronucleus formation. This dose was defined according to previous tests.

Each experimental group consisted of five randomly selected animals with body weights ranging from 30.5 ± 4.5 g (C57BL/6) and 28.4 ± 1.7 g (LDLr$^{-/-}$). All animals were euthanized 24 h after the last treatment. Fig. 1 shows the treatment protocol on young mice to evaluate the anti (genotoxicity) of botryosphaeran. The same protocol was adopted for older Swiss mice (WT-C57BL/6 and LDLr$^{-/-}$ knockout) except that in this case the treatment was for 15 days, and cyclophosphamide dose used was 50 mg/kg b.w.

2.3.1.2. Micronucleus assay in peripheral blood and bone marrow. The micronucleus assay on peripheral blood was performed using the method described by Hayashi et al. [14] with modifications. A small volume of peripheral blood was collected from the caudal artery of each mouse to prepare smears on two glass slides. This procedure was performed on days 0, 15 and 30 of the experiment, according to the time established for the young and old animals for the evaluation of the mutagenic potential of botryosphaeran. This assay was also conducted on the WT and LDLr$^{-/-}$mice at the end of the treatment (15 days). After air-drying, the glass slides containing peripheral blood cells were fixed with methanol (100%), and then stained with acridine orange (100 µg/L, Sigma-Aldrich, USA). Cytological analyses were conducted in duplicate under a fluorescent microscope at 40× magnification (Olympus BX40, Olympus Latin America INC., Miami, Florida, USA). Two thousand normochromatic erythrocytes (NCE) were analyzed, and the frequencies of micronucleated cells were noted.

The micronucleus test in bone marrow of mice was performed using the method described by Schmid [15] with some modifications to evaluate the antimutagenic effect of botryosphaeran in young and aged mice. This assay was also conducted on the WT and LDLr$^{-/-}$ mice, at the end of the treatment (15 days).

After the animals were euthanized, both femurs were immediately removed and the bone marrow was washed out with 1 mL of fetal bovine serum (Sigma-Aldrich, USA) into centrifuge tubes. The cellular suspension was mixed, then centrifuged (280 × *g*/10 min) and the supernatant was discarded, and the resulting pellet resuspended in fetal bovine serum. After air-drying the smears of bone marrow on glass slides, the cells were fixed with methanol (100%) and stained twice with two different concentrations of Leishman's stain (100% for three min, and then a mixture of Leishman's stain in distilled water (ratio of 1:6) for 15 min; New Prov, Produtos para Laboratório, Pinhais, Paraná, Brazil) to differentiate between polychromatic (PCE), normochromatic (NCE) and micronucleated polychromatic erythrocytes (MNPCE).

The antimutagenic effect of botryosphaeran against cyclophosphamide-induced damage was determined by analyzing 2000 PCEs, and noting the MNPCE frequency. The protective action of botryosphaeran against the cytotoxic effects was assessed at a ratio of PCE to 400 erythrocytes (PCE + NCE) using the formula: PCE/(PCE + NCE). These analyses were performed according to the criteria established by Krishna and Hayashi [16]. The slides were analyzed in duplicate using an optical microscope with 1000× magnification (Nikon E200-LED, Nikon Instruments INC., NY, USA).

The percent reduction in the number of cells with micronuclei in treatments (per sex and per group) with botryosphaeran that showed protective activity was calculated according to Waters et al. [17] using the formula:

$$\text{Damage Reduction}(\%) = \frac{\text{MNPCE (A)} - \text{MNPCE (B)}}{\text{MNPCE (A)} - \text{MNPCE (C)}} \times 100$$

where A is the group treated with cyclophosphamide (positive control); B is the group treated with the botryosphaeran solutions plus cyclophosphamide; and C is the group treated with isotonic saline (negative control).

The positive control of LDLr$^{-/-}$ mice received only the atherogenic diet (Table 1) and was evaluated for cytotoxic and mutagenic damage that occurred during the aging and pathological processes.

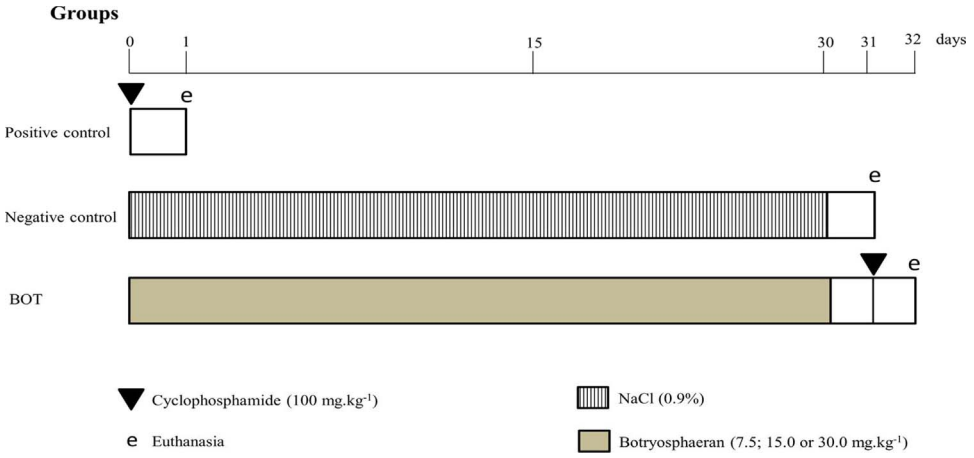

Fig. 1. Experimental protocol for assessing the (anti) genotoxicity of botryosphaeran in young mice.

Table 2
Frequency of micronucleated polychromatic erythrocytes (MNPCE) in 1000 PCE and the ratio of the number of polychromatic erythrocytes (PCEs) to normochromatic erythrocytes (NCEs), i.e., PCE:(PCE + NCE) in bone marrow of mice treated with botryosphaeran by gavage at different doses, and botryosphaeran plus cyclophosphamide (with the respective percentage of reduction).

Mice	Treatment	MNPCE/1000 PCE (P_{25}–P_{75})			Ratio PCE:(PCE + NCE) (P_{25}–P_{75})			Reduction (%)		
		Male	Female	Per group	Male	Female	Per group	Male	Female	Per group
Young	Negative Control	3.5 (2.00–4.75)***	1.00 (2.00–3.00)***	2.50 (1.00–4.75)***	0.56 (0.56–0.60)***†	0.65 (0.65–0.69)***†	0.60 (0.60–0.65)***			
	Positive Control Botryosphaeran	48.50 (43.75–57.50)	44.00 (41.75–47.25)	45.50 (41.50–57.50)	0.40 (0.36–0.44)†	0.55 (0.46–0.55) †	0.45 (0.40–0.55)			
	7.5 mg kg^{-1}	5.50 (4.25–12.75)**	10.00 (7.00–12.75)	7.50 (5.00–13.75)**	0.55 (0.48–0.59)	0.59 (0.55–0.60)	0.57 (0.55–0.60)*	95.56	81.15	88.61
	15 mg kg^{-1}	4.50 (2.25–10.00)**	8.00 (4.25–12.00)**	6.00 (2.25–11.75 ***	0.58 (0.55–0.60)***	0.55 (0.51–0.58)	0.55 (0.55–0.60)*	97.78	85.92	92.06
	30 mg kg^{-1}	2.50 (2.00–3.75)***	3.00 (2.00–7.50)***	3.00 (2.00–4.75)***	0.60 (0.55–0.60)***	0.60 (0.56–0.60)	0.60 (0.55–0.60)***	102.22	97.85	100.12
Aged	Negative Control	5.00 (3.25–6.75)***	4.00 (3.00–5.75)***	4.50 (3.00–6.75)***	0.59 (0.56–0.62)***†	0.65 (0.62–0.66)***†	0.62 (0.57–0.65)***			
	Positive Control Botryosphaeran	34.00 (26.00–41.25)	38.00 (35.5–39.00)	36.50 (28.75–39.00)	0.44 (0.42–0.47)	0.43 (0.41–0.45)	0.43 (0.42–0.47)			
	7.5 mg kg^{-1}	6.50 (4.00–9.00)**†	11.00 (10.00–13.75)†	9.5 (5.50–11.00)***	0.56 (0.55–0.56)**	0.55 (0.52–0.58)*	0.55 (0.52–0.57)***	94.83	79.41	86.51
	15 mg kg^{-1}	4.00 (3.00–6.50)***†	9.00 (8.00–12.25)**†	7.00 (4.00–9.00)***	0.54 (0.51–0.57)	0.58 (0.56–0.63)***	0.57 (0.53–0.60)***	103.45	85.29	93.65
	30 mg kg^{-1}	6.00 (1.5–6.75)***†	8.00 (6.25–19.5)**†	6.50 (3.25–11.00)***	0.55 (0.55–0.58)**	0.55 (0.53–0.61)**	0.55 (0.53–0.58)***	96.55	88.24	92.06
WT	Negative Control	5.50 (4.00 − 7.50)**	–	–	0.54 (0.53–0.56)**	–	–			
(18 months)	Positive Control Botryosphaeran	36.00 (17.25–40.00)	–	–	0.43 (0.42–0.44)	–	–			
	30 mg kg^{-1}	3.00 (2.25–3.00)***	–	–	0.56 (0.54–0.58)***	–	–	108.20	–	–
LDLr$^{-/-}$	Negative Control	4.00 (3.00–5.75)***	–	–	0.49 (0.47–0.52)	–	–			
(18 months)	Positive Control$^{\blacklozenge}$ Botryosphaeran	27.50 (21.00–28.00)	–	–	0.44 (0.42–0.46)	–	–			
	30 mg kg^{-1}	5.00 (4.25–6.75)**	–	–	0.59 (0.58–0.61)***	–	–	95.74	–	–

Values are medians (Percentile 25 – Percentile 75).Values followed by * in the column differ statistically from the positive control – Kruskal-Wallis post-hoc Dunn's test ($^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$); Mann-Whitney test ($^{\dagger}p < .05$). n = 10 (young and older mice); n = 5 (WT and LDLr$^{-/-}$ mice). $^{\blacklozenge}$Animals were fed a Western-type diet and without treatment with cyclophosphamide. Mann-Whitney test (and its corresponding symbol) was used to compare sexes.Young mice group was treated with botryosphaeran for 30 days. Aged mice, WT and LDLr-/- mice were treated with botryosphaeran for 15 days.Abbreviations: WT = Wild-type/C57BL/6; LDLr$^{-/-}$ = LDLr knockout; P_{25} = percentile 25; P_{75} = percentile 75.

2.3.2. Experimental protocol for assessing the hypoglycemic, hypolipidemic and atheroprotective effects of botryosphaeran in older WT (C57BL/6) and LDLr$^{-/-}$ male mice

2.3.2.1. Animals and treatments. When the male LDLr$^{-/-}$ animals reached 18 months of age, they received the Western-type diet using the conditions described above to accelerate spontaneous hyperlipidemia and the development of atherosclerotic lesions. After five weeks (18 months plus five weeks), the WT and LDLr$^{-/-}$ mice were divided into two groups: (i) WT and LDLr$^{-/-}$ B animals were treated with botryosphaeran (30 mg/kg animal b.w.) by gavage once daily for two weeks, and (ii) the WT and LDLr$^{-/-}$ V group were treated with isotonic saline (vehicle control) for two weeks. The dose of botryosphaeran was established according to the results obtained in the tests to assess mutagenicity, antimutagenicity and cytotoxicity, the number of inbred animals, and the cost of the atherogenic diet (Table 1).

2.3.2.2. Glucose and lipid measurements in blood plasma. After euthanizing the mice, blood samples were collected and immediately transferred into heparinized tubes, and then centrifuged (850 × *g*/10 min) to evaluate the hypoglycemic and hypolipidemic effects of botryosphaeran. The plasma fraction was separated and immediately stored at −20 °C until the time of analysis.

The levels of glucose, triglycerides, total cholesterol and high-density lipoprotein (HDL-cholesterol) were determined colorimetrically using commercial kits (Bioclin, Belo Horizonte, Minas Gerais, Brazil). The low-density lipoprotein (LDL-cholesterol) concentration could not be calculated from the triglyceride and HDL-cholesterol concentrations because the Friedewald formula is not accurate in mice [18]. Thus, the non-HDL-cholesterol [LDL-cholesterol plus VLDL-cholesterol (very low-density lipoprotein)] was determined by subtracting the HDL-cholesterol values from the total cholesterol values.

2.3.2.3. Morphological analysis and quantification of atherosclerotic lesions. The morphological analysis was performed using the method reported by Tonini et al. [19]. The left ventricle of the heart of the euthanized animals was perfused with phosphate-buffered saline (0.1 M at pH 7.4), followed by a solution of 4% (v/v) formaldehyde in phosphate-buffered saline at a pressure of 100 mm Hg. The aortic arch and ascending aorta was dissected, and the perivascular tissue was removed. Aortic *en face* samples were fixed and stained with Oil-Red-O marker (Sigma-Aldrich, USA; a lysochrome (fat-soluble diazo dye)) to identify the neutral lipids, and the images were captured using a digital high-resolution camera (Canon Power Shot A530, Canon USA INC., NY, USA). The analysis and quantification of lipid deposition was performed using the morphometric software: Image J from the National Institutes of Health (ImageJ 1:35 d, NIH, Bethesda, MD, USA, http://imagej.nih.gov/ij).

Table 3
Frequency of micronucleated normochromatic erythrocytes (MNNCE) in 1000 NCE of peripheral blood of young and older mice treated with botryosphaeran by gavage at different doses.

Mice	Treatment	MNNCE/1000 NCE (P_{25}–P_{75})								
		0th day			15th day			30th day		
		Male	Female	Per Group	Male	Female	Per group	Male	Female	Per group
Young	Negative Control	0.50 (0.00–1.00)	2.00 (1.00–2.00)	1.00 (0.00–2.00)	0.00 (0.00–1.00)	0.50 (0.00–1.00)	0.00 (0.00–1.00)	1.00 (0.25–2.00)	0.50 (0.00–1.00)	1.00 (0.00–1.00)
	Positive Control	0.50 (0.00–1.00)	2.00 (1.00–2.00)	1.00 (0.00–2.00)	2.50 (1.25–3.00)$^{\#\bullet}$	3.00 (2.00–3.00)$^{\#\#\bullet}$	3.00 (1.25–3.00)$^{\#\#\#\bullet\bullet}$	2.50 (1.25–3.00)$^{\#}$	3.00 (2.00–3.00)$^{\#\#}$	3.00 (1.25–3.00)$^{\#\#}$
	Botryosphaeran									
	7.5 mgkg^{-1}	2.00 (1.00–4.25)	2.00 (2.00–2.75)	2.00 (1.00–3.00)	0.50 (0.00–1.75)	1.50 (1.00–2.00)	1.00 (0.00–2.00)	0.00 (0.00–0.75)$^{\#\#}$	1.00 (0.00–1.00)	0.00 (0.00–1.00)
	15 mgkg^{-1}	1.00 (0.00–3.25)	1.50 (1.00–2.75)	1.00 (0.25–3.00)	0.00 (0.00–0.00)	1.00 (1.00–2.00)	1.00 (0.00–2.00)	1.00 (0.00–2.00)	1.00 (0.00–2.00)	1.00 (0.00–2.00)
	30 mgkg^{-1}	0.00 (0.00–1.50)	1.00 (0.25–1.00)	0.50 (0.00–1.75)	0.50 (0.00–1.00)	1.50 (0.25–2.00)	1.00 (0.00–2.00)	0.00 (0.00–0.00)	0.50 (0.00–1.00)	0.00 (0.00–1.00)
Aged	Negative Control	1.00 (0.00–1.75)	1.50 (1.00 − 2.00)	1.00 (0.00–2.00)	1.00 (0.25–1.00)	1.00 (0.00–1.00)$^{\#}$	1.00 (0.00–1.00)	–	–	–
	Positive Control	1.00 (0.00–1.75)	1.50 (1.00–2.00)	1.00 (0.00–2.00)	4.00 (4.00–4.75)$^{\#\bullet\bullet}$	3.50 (3.00–5.00)$^{\#\#\bullet\bullet\bullet}$	4.00 (3.00–5.00)$^{\#\#\#\bullet\bullet}$	–	–	–
	Botryosphaeran									
	7.5 mgkg^{-1}	0.00 (0.00–0.00)	1.00 (1.00–2.00)	0.00 (0.00 − 1.75)	0.00 (0.00–0.00)†	1.00 (1.00–2.00)$^{\#\#\dagger}$	0.50 (0.00–1.00)	–	–	–
	15 mgkg^{-1}	0.00 (0.00–0.00)	0.00 (0.00–1.00)	0.00 (0.00–0.75)	0.00 (0.00–0.00)	0.00 (0.00–0.75)	0.00 (0.00–0.00)	–	–	–
	30 mgkg^{-1}	0.00 (0.00–1.00)	1.00 (0.00–2.00)	0.50 (0.00–1.00)	0.00 (0.00–0.00)	0.00 (0.00–0.75)	0.00 (0.00–0.00)	–	–	–
WT	Negative Control	–	–	–	1.00 (0.00–1.25)	–	–	–	–	–
(18 months)	Positive Control	–	–	–	4.00 (3.00–5.25)$^{\#\#}$	–	–	–	–	–
	Botryosphaeran									
	30 mgkg^{-1}	–	–	–	00.00 (0.00–1.00)	–	–	–	–	–
LDLr$^{-/-}$	Negative Control	–	–	–	3.50 (2.00–5.00)	–	–	–	–	–
(18 months)	Positive Control$^{\blacklozenge}$	–	–	–	6.00 (4.75–7.50)$^{\#}$	–	–	–	–	–
	Botryosphaeran									
	30 mg kg^{-1}	–	–	–	3.50(2.75–4.25)	–	–	–	–	–

Values are medians (Percentile 25 – Percentile 75).Values followed by $^{\#}$ in the column differ statistically from the negative control – Kruskal-Wallis post-hoc Dunn's test ($^{\#}p < .05$; $^{\#\#}p < .01$; $^{\#\#\#}p < .001$); Values followed by $^{\bullet}$ in the line differ statistically from the negative control – Friedman test ($^{\bullet}p < .05$; $^{\bullet\bullet}p < .01$; $^{\bullet\bullet\bullet}p < .001$); Mann-Whitney test ($^{\dagger}p < .05$). n = 10 (young and older mice); n = 5 (WT and LDLr$^{-/-}$ mice). $^{\blacklozenge}$Animals were fed a Western-type diet and without treatment with cyclophosphamide. Mann-Whitney test (and its corresponding symbol) was used to compare sexes.Young mice group was treated with botryosphaeran for 30 days. Aged mice, WT and LDLr-/- mice were treated with botryosphaeran for 15 days. Abbreviations: M = Male; F = Female; WT = Wild-type/C57BL/6; LDLr$^{-/-}$ = LDLr knockout; P_{25} = percentile 25; P_{75} = percentile 75.

2.4. Statistical analysis

Statistical analysis was performed using GraphPad PRISM Software version 7.0 (GraphPad Software Inc., San Diego, California, USA, http://www.graphpad.com/) and significance was set at $p < .05$. The results were expressed as the median (Percentile 25 – Percentile 75) or mean ± standard error of mean. The normality of the data was verified. To analyze the anticlastogenic and genotoxic effects of botryosphaeran on the mice groups the following tests were used: Mann Whitney test, Kruskal-Wallis test, (followed by the Dunn test) and Friedman test. Two types of analyses were carried out: non-repeated values (groups analyzed – botryosphaeran doses *vs*. controls) and repeated values (differences over time – 0, 15 and 30 days of treatment). To compare the effects of botryosphaeran on blood glucose levels, lipid profiles and aortic lipid deposition between the different groups, the following were used: two-way ANOVA [(Wild-type *vs*. LDLr$^{-/-}$) x (Vehicle *vs*. botryosphaeran)], or one-way ANOVA and the Tukey test, when appropriate. For the analyses of the blood glucose and lipid levels, the data were $1og_{10}$ transformed to fit a normal distribution.

3. Results

The frequency of micronuclei, the ratio of the PCE to NCE, and the percentage of damage reduction in the bone marrow of young and old Swiss albino mice treated with botryosphaeran and their respective controls are shown in Table 2. All the groups of young (30 days) and old (15 days) mice that were treated with botryosphaeran showed a statistically significant reduction in the frequency of MNPCE compared to the positive control (comparison per group) (Table 2). This observation indicated that botryosphaeran exhibited antimutagenic activity. The numbers of micronuclei in PCE of the young male and female mice that received doses of 15 and 30 mg/kg b.w./day, were lower than the positive control. Among the older Swiss mice that received three different doses of botryosphaeran, the frequency of MNPCE in the group of males was lower than the positive control (Table 2).

When the response for the older Swiss mice was compared between the sexes, males and females exhibited different MNPCE frequencies following treatment with all doses of botryosphaeran; the frequency being lower among the male mice. All doses of botryosphaeran administered to the young mice for 30 days and the older mice (treated for 15 days) of both sexes reduced the percentage of cyclophosphamide-induced damage; the greatest reduction was observed in the male mice: at the dose of 30 mg/kg b.w./day in the young mice (102.22%) and at the dose of 15 mg/kg b.w./day in the older mice (103.45%) (see Table 2).

Based on these data, the protective effect of botryosphaeran was investigated in isogenic male (WT C57BL/6 and LDLr$^{-/-}$) animals at this dose (30 mg/kg b.w./day). Older wild-type mice (WT C57BL/6) that received botryosphaeran had fewer MNPCE, as well as a reduction in the percentage of cyclophosphamide-induced damage (Table 2). A significant increase in MNPCE frequency was observed in the older LDLr$^{-/-}$ knockout mice that received the atherogenic diet (positive control) compared to the group receiving 30 mg of botryosphaeran/kg b.w./day, and the negative control. These findings demonstrate that botryosphaeran was able to reduce the number of micronucleated cells.

The analysis of the effect of botryosphaeran on cyclophosphamide-induced cytotoxicity generally showed a significant increase in the PCE: (PCE + NCE) ratio in all groups (Table 2). Among the young and older Swiss mice, the PCE: (PCE + NCE) ratio in the mice (per group) that were treated with all doses (7.5, 15 and 30 mg/kg b.w./day) was statistically different to the positive control group (cyclophosphamide). These results demonstrated that botryosphaeran was not cytotoxic. In relation to gender, the highest values of the PCE: (PCE + NCE) ratio were for female mice. Furthermore, at the dose of 30 mg/kg b.w./day, this mixed-linked β-glucan was able to protect the LDLr$^{-/-}$ knockout animals from the cytotoxic damage arising from the atherogenesis process, as the highest values for PCE: (PCE + NCE) ratio were observed for the botryosphaeran-treated animals (0.59 (0.58–0.61)) compared to the group that received the lipid-rich diet (0.44 (0.42–0.46)) in Table 2.

Regarding possible genotoxicity, the micronucleus test in mouse peripheral blood demonstrated that botryosphaeran was not mutagenic under these experimental conditions, as shown in the results presented in Table 3. All the groups of young and aged mice that had been treated with botryosphaeran showed frequencies of MNNCE that were statistically similar to the negative control (male/female and per group) at the end of the 30- or 15-day treatment periods. Among the young and older animals, males and females did not differ in the frequency of MNNCE, except at time point 15 at a dose of 15 mg/kg b.w./day administered to older mice group (Table 3). Similarly, the responses did not vary with time. At time zero no significant difference in MNNCE frequency was found between young and older mice because the animals had not undergone any type of treatment.

Among the older isogenic animals (Table 3), the WT group that received 30 mg of botryosphaeran/kg b.w./day showed a micronucleus frequency that was similar to the negative control. At a dose of 30 mg/kg b.w./day for 15 days, botryosphaeran administration resulted in the frequency of MNNCE, in the aged LDLr$^{-/-}$ mice, statistically similar to the negative control even in the absence of cyclophosphamide-induced damage.

The antimutagenic effect associated with the lack of mutagenicity and cytotoxicity of botryosphaeran, and the percentage of reduction in cyclophosphamide-induced damage at a dose of 30 mg/kg b.w./day, was used as the basis to evaluate the hypocholesterolemic, hypoglycemic and antiatherogenic effects of this β-glucan in experiments conducted with older male WT and LDLr$^{-/-}$ knockout mice.

Table 4 summarizes the results of the glucose and lipidic profiles in the plasma of the experimental groups of wild-type animals and animals with genetic ablation of the LDL receptor that received the saline solution, Western-type diet and botryosphaeran for 15 days. The Western-

Table 4
Biochemical data of botryosphaeran-treated older male wild type and LDLr knockout mice compared with non-treated mice (control).

	Groups			
	Wild-type		LDLr$^{-/-}$	
Biochemical data (mg/dL)	Vehicle (V)	Botryosphaeran (B)	Vehicle (V)	Botryosphaeran (B)
Glucose	71.50 ± 13.97	83.04 ± 13.07	329.30 ± 32.19***	210.60 ± 28.76
Triglycerides	88.02 ± 8.98	87.82 ± 10.46	137.10 ± 12.62*	63.30 ± 9.66###
Total cholesterol	83.55 ± 4.57	83.69 ± 4.20	607.60 ± 33.65***	123.70 ± 29.17###
High-density lipoprotein	43.06 ± 4.27	41.01 ± 10.55	25.33 ± 2.01	32.51 ± 3.62
Non-high-density lipoprotein	45.75 ± 2.12	46.92 ± 3.66	582.20 ± 2.09***	91.46 ± 4.36###

Values are means ± SEM. Groups were compared after logarithmic transformation of these data. *$p < 0.05$ and *** $p < 0.001$ *vs.* WTV and ### $p < 0.001$ and ### $p < 0.001$*vs.* LDLr$^{-/-}$ V (two-way Anova). n = 5. Wild-type and LDLr-/- mice were treated with botryosphaeran for 15 days.Abbreviations: V = vehicle; B = botryosphaeran test; LDLr$^{-/-}$ = LDLr Knockout; SEM = standard error of the mean. Non-high-density lipoprotein = (LDL-cholesterol + VLDL-cholesterol).

type diet induced hyperglycemia, as the average glucose levels in the LDL$^{-/-}$ V group were significantly increased ($p < .001$) compared with the wild-type V group. At a dose of 30 mg/kg b.w., botryosphaeran decreased the glucose levels in the LDLr$^{-/-}$ B group, compared with the LDLr$^{-/-}$ V group, though no significant difference was observed. Significant increases in the plasma concentrations of triglycerides (~1.5x), total cholesterol (~7x) and non-HDL-cholesterol (~12x) were observed in the LDLr$^{-/-}$ V mice that were fed the Western-type diet for seven weeks compared to the wild-type V group ($p < .05$; $p < .001$). A reduction of 53.8% was observed in the triglyceride levels following treatment with botryosphaeran, as the average values of the LDLr$^{-/-}$ B group were smaller than the LDLr$^{-/-}$ V group, and were statistically similar to the wild-type V group. Similarly, hypercholesterolemic animals with atherosclerosis that received botryosphaeran at a dose of 30 mg/kg b.w. showed a decrease in the total cholesterol (79.6%) and non-HDL-cholesterol (84.3%) levels compared to the LDLr$^{-/-}$ V group. The total cholesterol data were also similar to the wild-type V group. The mean HDL-cholesterol levels, however, did not differ significantly in all of the groups examined.

With respect to lipid deposition, the aorta *en face* analysis demonstrated that the LDLr$^{-/-}$ V animals showed a greater deposition-area over the aorta compared to the wild-type V group, as shown in Fig. 2 (top panel). The aortic lipid deposition-area (graph) in the first group was significantly increased (~200%, $p < .05$) compared to the second group (20 ± 0.82 mm^2). At a dose of 30 mg/kg b.w./day for 15 days, botryosphaeran was able to reduce this deposition in the aortas of older male LDLr$^{-/-}$knockout animals by 32.8%.

4. Discussion

Fungal β-glucans constitute bioactive macromolecules with broad potential for biotechnological applications, considering that these carbohydrate biopolymerspresent functional properties that can reduce health risks [6].

We have previously demonstrated that a 15-day treatment regimen with botryosphaeran did not induce mutagenic activity and presented

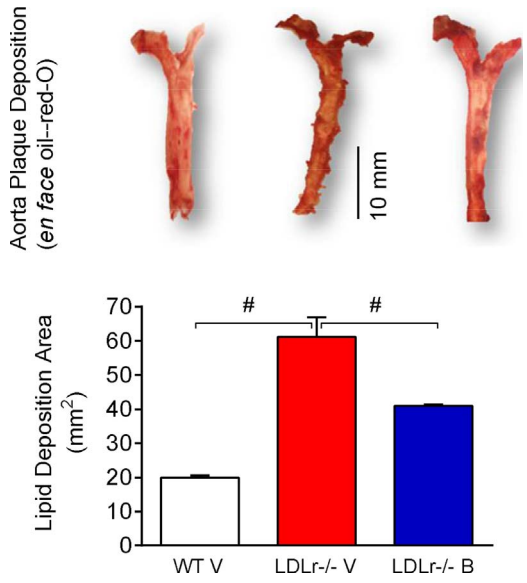

Fig. 2. The effects of botryosphaeran at 30 mg/kg b.w./day dose for 15 days treatment on vascular lipid deposition of older male LDLr$^{-/-}$ mice that received a Western-type diet. The top panel depicts representative aorta *en face* images of oil-red-O staining of each of the respective groups studied (vehicle wild-type – WTV; vehicle LDLr$^{-/-}$ that received a Western-type diet – LDLr$^{-/-}$V; LDLr$^{-/-}$ that received a Western-type diet and botryosphaeran- LDLr$^{-/-}$B). The bar graph depicts average vascular lipid deposition areas. Values are presented as the mean ± SEM for five animals per group. $^{\#}$ $p < 0.05$ *vs.* LDLr$^{-/-}$ V (one-way Anova).

antimutagenic activity against cyclophosphamide in mice [9]. Botryosphaeran also exhibited hypoglycemic and hypocholesterolemic properties in diabetic-induced rats and in rats receiving a lipid-rich diet, respectively [10].

In the present study, young mice were treated with 7.5, 15 and 30 mg of botryosphaeran/kg body weight for 30 days, which was demonstrated to be more efficient than treatment for 15 days in the earlier study because the 30-day treatment reduced damage by 88.6, 92.1 and 100.1% compared to the results obtained from the 15-day treatment: 45, 71 and 78%, respectively [9]. Also, when botryosphaeran was administered to older mice by gavage for 15 days, this treatment regime presented antimutagenic and hypolipidemic activities, did not induce mutagenicity and was not cytotoxic (Tables 2–4). These results were similar to the previously reported results obtained when botryosphaeran was offered to mice and rats [9],[10] for 15 days.

Cyclophosphamide is mainly metabolized in the liver by P_{450} cytochrome (CYPs) isozymes [20], and is used to induce genomic damage [21]. The administration of substances that can inactivate CYPs can also prevent or reduce the possible damage induced by mutagenic compounds [22]. Doxorubicin (a mutagen) was used to induce DNA damage *in vitro* in HepG2 cells that were treated with the unbranched (1 → 6)-β-D-glucan (lasiodiplodan) [23]. The results showed that lasiodiplodan had an antimutagenic effect, and there were no changes in CYP3A4 expression, suggesting that this kind of β-glucan does not interfere with the expression of the CYP family of genes.

Literature studies have shown similar results to those obtained in this work; for example, the mutagenicity analysis of 24 h after treatment with the biopolymer produced by *Agrobacterium radiobacter* showed that this molecule was not genotoxic [24]. Jonker et al. [25] also showed that a chitin-glucan complex derived from *Aspergillus niger* was not toxic to *Wistar* rats after 13 weeks of treatment.

Some studies on toxicity use a pre-treatment protocol similar to the one described in this work. The cytotoxic effects and DNA protection may be directly related to the interaction between the toxic agent and the bioactive compound evaluated. This protocol avoids the interaction of DNA with the mutagenic compound [26]. There are few reports that evaluated the impacts of β-glucans on detoxification systems, and our results are the first to describe the lack of cytotoxic effects of botryosphaeran *in vivo*.

In our study, botryosphaeran did not induce genotoxic or clastogenic damage, and this observation was similar to that reported by Oliveira et al. [27] for β-glucans in which they proposed that these polysaccharides may become an important adjunct in chemotherapy to decrease the adverse effects of clastogenic drugs.

With regard to differences or similarities between the sexes found in our study, Fenech et al. [28] demonstrated that gender is a parameter that needs to be considered in mutagenesis studies because of its influence on basal micronucleus production. That way, females may be more susceptible to the spontaneous micronucleus formation. However evidence suggests that sex hormones, especially estrogens may protect females from genotoxic damage because they have antioxidant activity [29,30]. Another possible explanation for these differences may be the influence of sex hormones on cytochrome P_{450} [31].

The hypocholesterolemic and hypoglycemic effects of (1 → 3)-β-glucans have been described [7,10]. Here, we observed significant increases in the plasma levels of glucose and lipids in the older group of hyperlipidemic male LDLr$^{-/-}$ mice. At the end of the 15-day period of treatment with botryosphaeran these parameters reached values that were similar to those in the wild-type mice (WT) studied, or lower than the LDLr$^{-/-}$ V group, indicating a health promoting benefit of this (1 → 3)(1 → 6)-β-glucan. When our results were compared with those described by Miranda-Nantes et al. [10], we observed that the total cholesterol levels in older mice were higher (607.60 ± 33.65) (Table 4) than those in adult rats (137.51 ± 6.70) [10]. This feature may be associated with the fact that aging is a natural process that leads to health vulnerability [12]. Furthermore, in the study by Miranda-

Nantes et al. [10], adult rats with induced hyperglycemia and hypercholesterolemia were treated with 12 mg of botryosphaeran/kg b.w. by gavage over 15 days, which resulted in a significant reduction in the blood glucose levels (52%), but only a modest decrease in the total cholesterol (18.6%) and LDL-cholesterol (27%) concentrations. In the study reported herein, when older hyperlipidemic LDLr$^{-/-}$ mice were treated with 30 mg of botryosphaeran/kg b.w. by gavage for 15 days, we observed a non-statistically significant difference in the plasma glucose levels. In contrast to the study of Miranda-Nantes et al. [10], significant reductions in the blood levels of triglycerides (53.8%), total cholesterol (79.6%) and LDL plus VLDL (non-HDL) (84.3%) in the mice were observed.

The observed differences may be related to the experimental animal models adopted, and the concentration of botryosphaeran that was administered. Similar results have been observed when rodents were treated for 15 days with an exopolysaccharide from *Auricularia polytricha*, which reduced the LDL-cholesterol levels by 70% [32], and the administration of a high molecular weight barley (1 → 3)(1 → 4)-linked β-D-glucan to humans was able to reduce the total cholesterol levels (8.5%) in blood circulation [33].

Fungal natural products have been used as antidiabetic agents in traditional Chinese medicines [34]. Other fungi, such as the mushroom *Phellinus vanilla*, have been used in Asian countries to treat pulmonary infections, atherosclerosis and diabetes mellitus. The predominant presence of β-glucans may explain its beneficial effects [35]. Unlike our results, the exopolysaccharide isolated from *Stropharia rugosoannulata* showed antihyperglycemic effects in *in vivo* assays [36].

In the present study, we observed that a 30 mg/kg b.w. dose of botryosphaeran was able to reduce the deposition of atherosclerotic plaques in the aortas of hyperlipidemic LDLr$^{-/-}$ mice. Studies have shown that antioxidants can slow the development of atherosclerotic plaques *in vivo*, and it is known that oxidation of LDL-cholesterol plays a role in atherogenesis [37,38]. The antioxidant property of botryosphaeran has also been reported by our research group [39]. This property would be an important alternative therapeutic strategy for the treatment and prevention of atherosclerosis, as the use of hypolipidemic drugs may trigger unwanted side-effects, such as severe myopathy.

Macrophage activity could be modulated by polysaccharides during the atherosclerotic plaque development process, resulting in the production of lower levels of nitric oxide (NO), and hence peroxynitrite, which attenuate the characteristic inflammatory process of this pathological condition, showing the possibility of pleiotropic effects exerted by β-glucans [40]. This hypothesis was reinforced by the findings of Rubel et al. [41], who reported that peritoneal macrophages produced lower levels of NO in hypercholesterolemic mice receiving a β-glucan from *Ganoderma lucidum*. NO has a protective role as an antioxidant and vasodilator, but at high levels induced by pathological conditions and cytokines, it was associated with endothelial dysfunction, lipoprotein oxidation, hypercholesterolemia and increased susceptibility to developing atherosclerosis [42]. The atheroprotective effect associated with the antimutagenic effects of botryosphaeran in the older group of hyperlipidemic LDL receptor knockout animals showed that botryosphaeran attenuated the oxidative process and protected the animals from the cytogenotoxicity observed in the atherosclerotic process, as described by Tonini et al. [19].

5. Conclusions

The present study demonstrated that the (1 → 3)(1 → 6)-β-D-glucan (botryosphaeran) from *B. rhodina* MAMB-05 acted as a chemoprotector under the experimental conditions conducted by modifying biological responses to prevent cyclophosphamide-induced mutagenicity and cytotoxicity. Botryosphaeran was not mutagenic or cytotoxic, regardless of mouse gender, lineage or life-cycle phase. Moreover, it was effective in improving the lipidic profile and reducing hyperglycemia and vascular lipid deposition in an *in vivo* model of atherosclerosis. Botryosphaeran therefore has promising health applications in the development of new products related to pharmaceuticals and foods.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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