EVALUATION OF CYTOTOXIC EFFECT AGAINST TUMOUR CELLS OF THE ACIDIC POLYSACCHARIDES OF THE FUNGUS *Nothophellinus andinopatagonicus*

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ABSTRACT

Fungal polysaccharides possess an important bioactive potential, including antioxidant and anticarcinogenic activity. The aim of this work was to determine the antioxidant activity and cytotoxicity against tumour and non-tumour cell lines acidic polysaccharides (NAAPs) of the fungus *Nothophellinus andinopatagonicus*. The effect of NAAPs on tumour cells lines was evaluated by MTT assay and flow cytometry. The analyses determined that glucose was the most abundant monomer and IR spectrum showed the typical peaks of β-glucans in the NAAPs. The cell viability assays revealed significant activity of NAAPs against HL-60, HCT-116 and MCF-7 tumour cell lines (IC_{50} = 767,16 µg mL^{-1}, 1256 µg mL^{-1} and 4241,7 µg mL^{-1}, respectively); but a much lower cytotoxicity against the non-tumour cell line HGF-1 (outside the range of the highest concentration tested (>10 mg mL^{-1})). NAAPs affected the cell cycle of HL-60 tumour cells, increasing the percentage of cells in the sub G1 phase and reducing it in the S/G2/M phases. Moreover, low concentrations of NAAPs also showed an effective cytotoxic activity against tumour cell lines while the non-tumour cell line was unaffected, maintaining a viability close to 100%. The antioxidant activity of the highest NAAPs concentration tested was 6.24% and 4.63%, for DPPH and ABTS method, respectively.

Keywords: Acidic polysaccharides; fungi; *Nothophellinus andinopatagonicus;* anticarcinogenic activity; antioxidants; cell cycle.

INTRODUCTION

Cancer is a disease exhibiting the second-largest mortality rate at a worldwide level, being surpassed only by cardiovascular diseases [1-3]. The development of cancer is mainly associated with exposure to different carcinogens, which generate genetic alterations and the loss of normal regulatory processes, impeding the antitumoral immune response [4-5].

Free radicals produced by the cell's metabolism play an essential role in the regulation and expression of genes. An imbalance in their production induces irreversible oxidative damage resulting in various diseases, such as cardiovascular diseases, Alzheimer's disease, reduced immune function, muscle degeneration, and cancer [6-7]. The treatments used to control the development and progresss of cancer cause secondary effects that deteriorate the patients`quality of life and have elevated cost [3,8]. The search for new compounds capable of protecting tissues from the oxidative stress and/or having the capacity to eliminate or control cancer cells require all possible attention [7].

Polysaccharides are macromolecules showing an elevated bioactive potential and their activity is determined by their structure, composition, and size [9-12]. They can be obtained from plants, animals, fungi, and microorganisms [2,12,13]. Polysaccharides produced by fungi, mainly *Basidiomycetes*, have been extensively studied because they are one of the most abundant fungal cell components, and they have potential biomedical use as antioxidant, immunomodulator, anticoagulant, anticarcinogenic, hypoglycaemic, anti-inflammatory, antimicrobial, and cytotoxic agents [2,7,9,10,12,14-18].

Acidic polysaccharides constitute an important group of fungal polysaccharides and usually their bioactivity exceeds that of neutral polysaccharides [19]. Acid polysaccharides produced by *Tremella fuciformis*, have shown an important anticarcinogenic activity, inhibiting up to 91.7% the growth of sarcoma S180 cells in affected mice [20-21]. Many fungi have been used during centuries by different cultures as a source of foodstuff and medication [11,17,22], including many species belonging to the genus *Phellinus sensu lato* (*Hymenochaetaceae*; Div. *Basidiomycota*) [2,12,18,23].

The polysaccharides of certain species belonging to the genus *Phellinus s.l.* have potential pharmacological use as immunomodulators, activating cells of the immune system (such as T lymphocytes, B lymphocytes, natural killer (NK) cells, and macrophages); as anti-inflammatory compounds, inhibiting the production of pro-inflammatory cytokines; as anticarcinogens, slowing the growth of tumours and metastasis, and as antioxidants [12-13,15,17,19,22,24-25]. *Nothophellinus andinopatagonicus* (J.E. Wright & J.R. Deschamps) Rajchenb. & Pildain (Hymenochaetaceae) Syn. *Phellinus andinopatagonicus* (J.E. Wright & J.R. Deschamps) Ryvarden is a fungus endemic from southern South America, belonging to a monotypic genus. It has been found associated with several *Nothofagus* species, such as *N. antarctica*, *N. dombeyi* and *N. pumilio* and it is described as an important pathogen causing wood decay [26-27]. Aqueveque et al. [28] reported that total extracts obtained from mycelial cultures of this species showed an important antimicrobial activity against bacteria of clinical interest [28]. However, no other biological activity has been reported for this species.

Therefore, considering, as mentioned above, the potential pharmacological uses of polysaccharides obtained from members of the *Phellinus* genus, including anticarcinogenic activity, and that some fungal acidic polysaccharides have shown better bioactivity than neutral of fungi, the aim of the present study was to characterize the acidic fraction of the polysaccharides obtained from an *in vitro* culture of *N. andinopatagonicus* and to evaluate their possible antioxidant and antitumor potential against human colorectal carcinoma, mammary adenocarcinoma and leukaemia cell lines.

MATERIALS AND METHODS

Biological material

The strain of *Nothophellinus andinopatagonicus* used in this study was collected at Coyhaique (Aysén Region, Chile) (46°12'13.3"S 72°48'46.5"W) in 2016. It was identified by Dr. Mario Rajchenberg (Centro de Investigación y Extensión Forestal Andino Patagónico, Chubut, Argentina) and assigned the code FQ1645 of the Laboratory of Chemistry of Natural Products (University of Concepcion, Concepcion, Chile). It was maintained by culturing it, at 20±2°C,

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in vitro using YMG agar medium (5 g yeast extract (BD Biosciences, San José, USA), 10 g malt extract (BD Biosciences, San José, USA), 15 g glucose and 20 g agar per litter of medium). To improve the yield of mycelial mass, fragments of mycelium were transferred to liquid YMG medium (1% w/v glucose, 1% w/v malt extract, 0.4% w/v yeast extract) at pH 5.8 and incubated at 20 °C for 1 to 2 months under constant agitation (120 rpm). Finally, the mycelia were lyophilized (Lyophilizer Cryodos, Telstar, Terrasa, Spain).

Extraction of polysaccharides

The acidic polysaccharides were obtained from lyophilized mycelia of the FQ1645 strain according to Abdala-Díaz et al. [29] and Figueroa et al. [30], with minor modifications. Briefly, 100 g of lyophilized mycelia was cut and boiled in sterile distilled water for 1 h with constant agitation. After cooling, the mixture was filtered using sterile muslin cloth. An acidic polysaccharide selective precipitation was prepared adding a cold solution of 2% (w/v) n-cetylpyridinium bromide (Cetavlon) (Merck, Darmstadt, Germany). Acidic polysaccharides were precipitated by centrifugation at 10000 g for 20 min. The pellet was purified using a 4 M NaCl solution and centrifuged at 4000 g for 15 min and the supernatant recovered. Polysaccharides were flocculated with 96% (v/v) ethanol (1:1 etanol:extract) and centrifuged at 4000 g for 20 min. Polysaccharides of the pellet were dialyzed using 2M NaCl solution and finally lyophilized (Lyophilizer Cryodos, Telstar, Terrasa, Spain). The *N. andinopatagonicus* acidic polysaccharides extracted will be henceforth referred as NAAPs.

Fourier-transform infrared spectroscopy

Infrared spectra were obtained using a Fourier transform infrared spectrophotometer (FT-IR spectra). To analyse the NAAPs, disks (16 mm diameter) were prepared with a polysaccharide and potassium bromide (KBr) (1% w/w) mixture, pressed at 15.0 T hydrostatic pressure for 5 min. Subsequently, the discs were measured using a Thermo Nicolet Avatar 360 IR spectrophotometer (Thermo Electron Inc., Waltham, USA) with a resolution of 4 cm^{-1}, a DTGS detector and the OMNIC 7.2 software (bandwidth 50 cm^{-1}, enhancement factor 2.6). The 4000–450 cm^{-1} region was analysed. Baseline adjustment was generated using the Nicolet OMNIC Software (Version 7.2, Thermo Nicolet, Waltham, USA) (bandwidth 50 cm^{-1}, enrichment factor 2.6), to flatten the baseline of each spectrum. The OMNIC correlation algorithm was used to compare the spectra of the sample with those of the spectra library (Thermo Fischer Scientific, Waltham, USA).

Characterization of NAAPs by HPLC-IR

NAAPs (100 mg) were subjected to acidic hydrolysis using H_2SO_4 (72% v/v), following the protocol described by Peredo et al. [31]. An aliquot of the hydrolysate was filtered (0,45µm sterile syringe filter) and the sample analysed by HPLC-IR (Young Lin YL Clarity 9000, Korea) with a 0.6 mL min^{-1} flow of the mobile phase (5 mM H_2SO_4) and 20 µL injection in an Aminex HPX-87H column (300 mm x 7.8 mm), at a temperature of 35 °C. According to the retention times, the spectra obtained were compared to standards of glucose, xylose, arabinose, rhamnose and mannose (Sigma-Aldrich, San Luis, USA).

Characterization of NAAPs by Gas Chromatography-Mass Spectrometry (GC-MS)

Polysaccharides previously hydrolysed with H_2SO_4 (72% v/v) and then dried were acetylated using acetic anhydride, as described by Meng et al. [32], with modifications. The hydrolysed sample (100 mg) was dissolved in 1 mL pyridine anhydrous, and an excess of acetic anhydride was added to obtain the acetylated derivatives and the mixture was agitated during 6 h at room temperature. Once the derivatization was completed, the mixture was diluted with a NaCl solution, and extracted with dichloromethane. The organic phases obtained were combined and dried adding anhydrous sodium sulphate. The solvent was eliminated by distillation using a Heidolph rotavapor (Heidolph Instruments GmbH & CO. KG Schwabach, Germany). GC-MS analysis was conducted with an Agilent Technologies 7890A/5975C instrument, using a HP- 5MS capillary column (30m × 0.250 mm × 0.25 µm). The initial column temperature was kept at 70 °C for 4 min, increased to 200 °C at 3 °C min^{-1}, then increased to 300 °C at 10 °C min^{-1}, and finally, the temperature was maintained for 5 min. The ionization potential was 70 eV and the temperature of the ion source was 280 °C. Peak assignments were made based on retention times and mass spectra with the aid of a NIST17 mass spectral library.

Cell lines

HCT-116 human colorectal carcinoma, MCF-7 human mammary adenocarcinoma, HL-60 human leukaemia cells and non-tumoral HGF-1 human gingival biopsy cells (all from ATCC, Manassas, Virginia, USA), were used in this study. The HCT-116, MCF-7 and HGF-1 cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Biowest, Nuaillé, France) supplemented with 10% fetal calf serum (FCS) (Biowest, South America Origin, S1810), 1% penicillin-streptomycin solution 100X (Biowest, Nuaillé, France) and 0.5% amphotericin B (Biowest, Nuaillé, France). HL-60 cells were cultured in RPMI-1640 medium (Biowest, Nuaillé, France) supplemented with 20% FCS, 1% 100X penicillin-streptomycin solution and 0.5% amphotericin B. Cells were kept sub-confluent at 37 °C in the presence of 5% CO_2. Adherent cells were collected when the confluence reached 75%. Suspended cells were collected by centrifugation at 600g during 5 min [29].

Anti-carcinogenic activity of NAAPs

To assay the antitumoral effect of NAAPs, HL-60, HCT-116 and MCF-7 tumour cells were independently incubated with serial dilutions of NAAPs including 10, 5, 2.5, 1.25, 0.625 or 0.3125 mg mL^{-1}. As control of cytotoxicity of NAAPs on a non-tumoral cell line, HGF-1 cells were subjected to the same concentrations of NAAPs mentioned above. A total of $1x10^4$ MCF-7 or HL-60 cells, $4x10^3$ HCT-116 cells or $5x10^3$ HGF-1 cells in 100 µL of the same culture media described in the previous section (including FCS and antibiotics) were placed in wells of a 96 wells microplate and subjected to the different concentrations of NAAPs during 72 h at 37 °C and 5% CO_2. Proliferation of the cell lines was evaluated by adding 10 µL of MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, San Luis, USA) solution (5 mg mL-1 MTT in phosphate buffered saline (PBS) pH 7.5) per well [33]. Microplates were incubated at 37°C for 4 h and the insoluble purple formazan crystals formed were dissolved by addition of sulphated isopropanol (150 µL 0.04 N HCl in 2-propanol) and measured spectrophotometrically at 550 nm (Micro Plate Reader 2001, Whittaker Bioproducts, Dauphin, USA). Finally, the relative cell viability was expressed as the mean percentage of viable cells compared to untreated cells. The same cell lines not exposed to NAAPs were used as positive controls. This experiment was made in triplicate, inoculating three wells of the microplate per variable analysed.

Cell cycle analysis by flow cytometry

The tumour cell line showing the greatest susceptibility to the cytotoxic effect of NAAPs was selected for this assay. Changes in the progression of the cell cycle of the selected cell line were analysed by flow cytometry, following the procedure of Afrin and co-workers [34] with modifications. A volume of 1.5 mL containing $5x10^5$ cells were placed in each well of 6 wells microplates and incubated at 37 °C in the presence of 5% CO_2 until reaching sub-confluence. Then, different NAAPs concentrations were added in fresh culture media. Three concentrations of NAAPs, the first one corresponding to the half maximal inhibitory concentration (IC_{50}), the second one equal to 4X IC_{50} and the third one equal to ¼ IC_{50}, were used. After incubating overnight in the presence of NAAPS, cells were harvested, centrifuged and the resulting pellets washed using PBS (pH 7.5) and fixed with 70% ethanol for 1 h, at -20 °C. Finally, cells were centrifuged at 600g during 5 min and washed twice using PBS before been suspended in the staining solution containing 40 µg mL^{-1} propidium iodide (Sigma-Aldrich, San Luis, USA) and 0.1 mg mL^{-1} RNase-A (Sigma-Aldrich, R6513) in PBS. This mixture was incubated for 30 min at 37°C in darkness. Samples were analysed in a FACS VERSETM (BD Biosciences, San José, USA) flow cytometer and the results analysed using the Kaluza analysis software version 2.1 (Beckman Coulter, Brea, USA). A 2-methoxyestradiol (20µM) (Sigma-Aldrich, San Luis, USA) solution was used as positive control for changes in the cell cycle.

Antioxidant activity of NAAPs

The antioxidant activity of NAAPs was evaluated following the procedure of Brand-Williams et al. [35], with modifications. The previously prepared DPPH (2,2-diphenyl -1-picryl-hydrazyl-hydrate) (Sigma-Aldrich, San Luis, USA) radical was adjusted to an absorbance of 0.3 at 517nm. Serial dilutions of the NAAPs sample were prepared from a 40 mg mL^{-1} stock solution in sterile distilled water. Mixtures of 180 µL DPPH radical plus 20 µL of each respective concentration of NAAPs were prepared in a 96 wells microplate.

Samples were incubated for 30 min in darkness and the absorbance read at 517nm using a microplate spectrophotometer (Epoch BioTek (BioTek Instruments Inc., Winooski, VT, USA)). A control including 180 µL DPPH radical plus 20 µL of sterile distilled water was used.

The antioxidant activity was calculated using the following equation and it was expressed in Trolox (µg mL^{-1}) (Thermo Fisher Scientific, Loughborough, United Kingdom) equivalents in accordance with a standard curve (concentrations from 0 to 100 µg mL^{-1} Trolox),

AA% = ((Abs (control) – Abs (sample)) / Abs (control)) x 100

Where, AA% is the percentage of antioxidant activity, Abs (control) is the absorbance of the DPPH radical plus water and Abs (sample) is the absorbance of the DPPH radical plus the different concentrations of NAAPs used.

The antioxidant activity was also determined by the ABTS method, as described by Re et al. [36] with some modifications. The ABTS (2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich, San Luis, USA) solution was adjusted to an absorbance of 0.7 at 725 nm. The antioxidant activity of the different concentrations of NAAPs was determined by adding 190 µL ABTS solution plus 10µL of NAAPs in wells of a 96 wells microplate at the respective concentration. A control containing 190 µL ABTS plus 10 µL sterile distilled water was used. The mixture was incubated for 8 min at room temperature and its absorbance measured at 725 nm using a microplate spectrophotometer (Epoch BioTek (BioTek Instruments Inc., Winooski, VT, USA)).

The percentage of antioxidant activity of the different NAAPs concentrations was calculated using the following equation and expressed in Trolox (µg mL^{-1}) equivalents in accordance with a standard curve (concentrations from 0 to 100 µg mL-1 Trolox)

AA% = ((Abs (control) – Abs (sample)) / Abs (control)) x 100

Where, AA% is the percentage of antioxidant activity, Abs (control) is the absorbance of the ABTS plus water and Abs (sample) is the absorbance of the DPPH radical plus the different concentrations of NAAPs.

Statistical analysis

All results were expressed as mean ± standard deviation of three experiments (n=3). One-way analysis of variance (ANOVA) with post-hoc Tukey HSD test was used to compare the differences of cell viability and antioxidant activity. Previous to these analyses, normality and homogeneity of variance assumptions were evaluated. Differences were considered as significant when $p<0.05$. All analyses were done using the Statistica 12.0 software (TIBCO Data Science, Palo Alto, USA). Graphs were obtained using the SigmaPlot software version 12.0, 2015 (Systat Software Inc., Chicago, USA).

RESULTS

NAAPs yield

The extraction of NAAPs from *N. andinopatagonicus* using a selective precipitation method with Cetavlon from the mycelial mass in aqueous solution yielded 20.22 mg of NAAPs per g of wet mycelia, corresponding to 20% of dry mycelia.

Infrared analysis

The IR spectrum of the polysaccharides showed absorption peaks characteristic for polysaccharides` compounds (Figure 1). In particular, a wide and strong peak was present at 3445.67 cm^{-1} associated to the stretching produced by O-H bonds and another small peak at approximately 2922 cm^{-1} attributed to the stretching vibration produced by C-H bonds. On the other hand, the contraction vibration detected at 1637.96 cm^{-1} can be ascribed to the C=O bond of carboxyl groups. The small peak at approximately 1431 cm^{-1} was attributed to the bending contraction of the C-H bond. The presence of a vibration at 1250 cm^{-1} allowed the assumption sulphate groups, while the absence of a peak at nearly 1730 cm^{-1} indicated the absence of uronic acid in the NAAPs. Furthermore, a peak between 1200 and 1000 cm^{-1} is characteristic of the presence of β-glucans, whose vibration can be attributed to the presence of β bonds in their glycosidic chain, as well as the presence of pyranose rings [9,11-12].

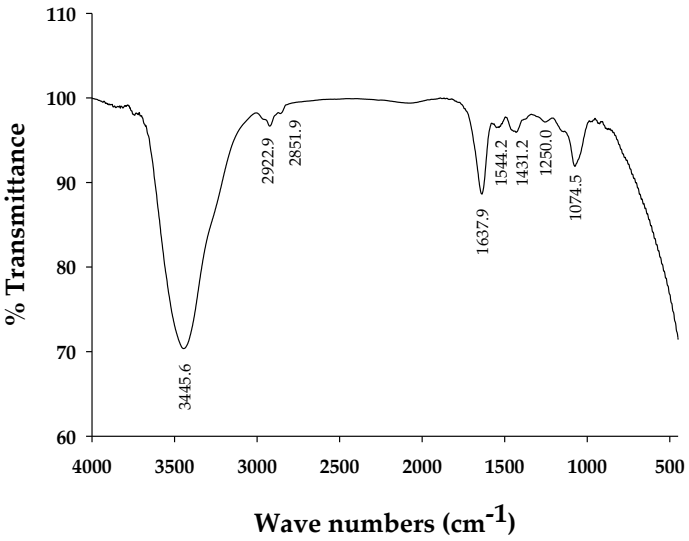

Figure 1. Fourier-transform infrared spectroscopy (FT-IR) of the acidic polysaccharides obtained from *N. andinopatagonicus* FQ1645 strain.

Characterization of monosaccharides of NAAPs

The retention times obtained by HPLC-IR analysis of a sample of NAAPs allowed the identification of at least three monosaccharides: arabinose, xylose and a derivative of glucose (Table 1), the last being the one present in the highest percentage. On the other hand, the GC-MS analysis allowed the elucidation of the presence of acetylated monomers in the NAAPs, which, according to their respective retention times, included arabinose, mannose, D-glucose, mannitol, myo-inositol and trehalose (Table 2).

Table 1. Monosaccharides identified in the acidic polysaccharides obtained from *Nothophellinus andinopatagonicus* FQ1645 strain by HPLC-IR analysis.

Compound	Retention Time (min)	Amount (%)
Glucose derivate	7.675	49.3
Xylose	9.342	30.7
Arabinose	10.225	20.0

Table 2. Acetylated derivatives of saccharides present in acidic polysaccharides of *Nothophellinus andinopatagonicus* FQ1645 strain determined by Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

Compound	Retention Time (min)	Amount (%)
Methyl 2,3,5-tri-O-acetyl-L-arabinofuranoside	12.40	0.27
α-D-Mannopyranoside-methyl tetraacetate	15.04	0.77
D-Glucose, 2,3,4,5,6-pentaacetate	15.89	7.42
Mannitol hexaacetate	16.78	0.51
Trehalose octaacetate	25.78	70.03

Effect of NAAPs on the cell viability of tumour cell lines

This assay evaluated the effect of the tested NAAPs concentrations on the cell viability of the three human tumour cells lines (HL-60 human leukaemia, HCT-116 human colorectal carcinoma and MCF-7 human mammary adenocarcinoma) and on the non-tumour cell line (HGF-1 human gingival biopsy), which was used as control. The results demonstrated that the cytotoxic activity of NAAPs on the tumour cell lines increased, observed as reduced survival percentages, when the cells were exposed to higher NAAPs concentrations (Figure 2A, B and C). The highest NAAPs concentration evaluated (10 mg mL^{-1}) resulted in a survival of 4.94±0.8% for HL-60 cells (Figure 2A), 5.30±1.45% for HCT-116 cells (Figure 2B) and 15.55±1.26% for MCF-7 cells (Figure 2C). When testing the 0.312 mg mL^{-1} concentration of NAAPs, the lower concentration evaluated, the survival of tumour cells achieved 74,3±3.5% for HL-60 cells, 74.5±0.4% for HCT-116 cells and 69.7±1% for MCF-7 cells (Figure 2).

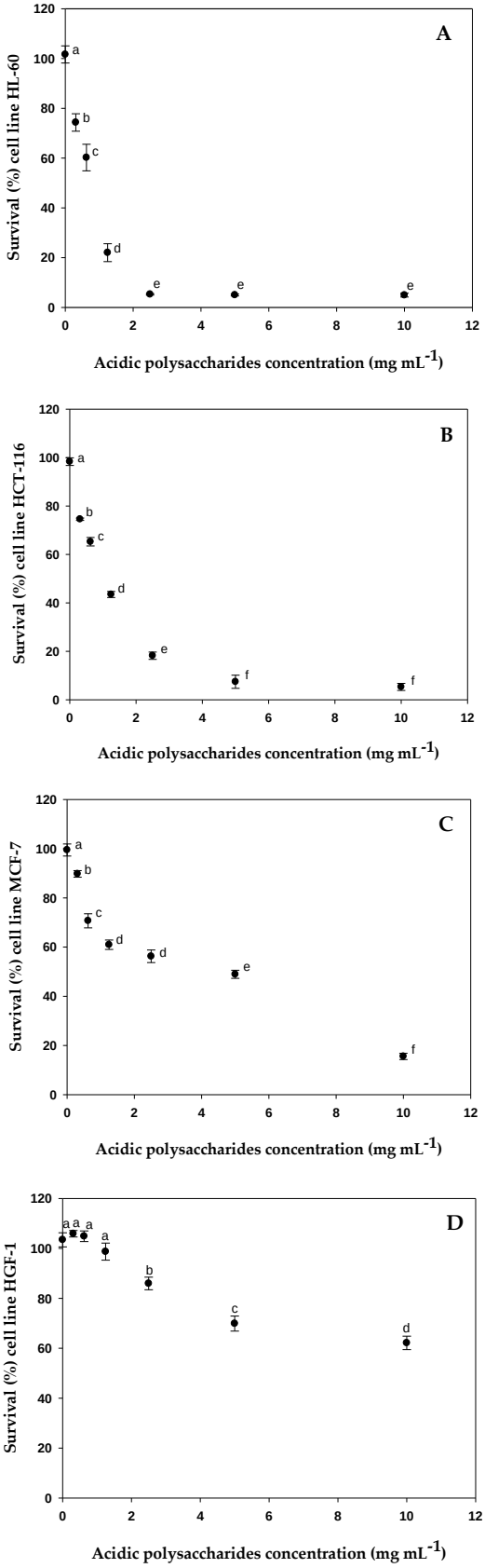

Figure 2. Cell survival (%) of cell lines exposed to 10, 5, 2.5, 1.25, 0.625, 0.3125 mg mL^{-1} of acidic polysaccharides of *Nothophellinus andinopatagonicus* (NAAPs) FQ1645 strain. (**A**) Survival of tumour cell line HL-60; (**B**) Survival of tumour cell line HCT-116; (**C**) Survival of tumour cell line MCF-7; (**D**) Survival of non-tumour cell line HGF-1. A same letter indicates that there are no significant differences between the different concentrations (Tukey, $p<0.05$).

With all the concentrations of NAAPs tested, including the lowest one (0.312 mg mL^{-1}), none of the three human tumour cell lines reached survivals over 75%. When comparing the above-mentioned figures obtained for the tumour cell lines with those of the non-tumour HGF-1 cell line, it was possible to observe that the cytotoxic effect of NAAPs affected to a lesser degree the non-tumour cell line (Figure 2D), which can be considered an encouraging result. The viability of the HGF-1 cell line in the presence of the highest NAAPs concentration tested (10 mg mL^{-1}) was 62.15±2.6% (equivalent to a mortality of 37.85±2.6%), a cell survival four times better than that of the tumour cell line MCF-7 (viability of 15.55±1.26%) when exposed to the same NAAPs concentration, the one showing the best survival of the three tumour lines tested. Regarding the viability of the non-tumour cell line HGF-1 when exposed to the lower NAAPs concentrations assayed, the viability of this cell line was approximately 100% in the presence of the three lower NAAPs concentrations (0.312, 0.625 and 1.25 mg mL^{-1}), a viability not significantly different to that in the absence of NAAPs. On the contrary, at the same low concentrations of NAAPs not affecting the viability of the non-tumour cell line, the viability of tumour cell lines was significantly reduced, ranging approximately from 20% to 60%. The actual viability figures for the tumour cell lines were 22.00±3.5% for HL-60 cells, 43.47±1.2% for HCT-116 cells and 60.95±1.9% for MCF-7 cells. The IC_{50} figures for the NAAPs isolated from *N. andinopatagonicus* FQ1645 strain were 767.16 µg $mL^{-1}$for HL-60 cells, 1256 µg mL^{-1} for HCT-116 cells and 4241.7 µg mL^{-1} for MCF-7 cells, while the IC_{50} for the non-tumour cell line HGF-1 could not be calculated because it is outside the range of highest concentration used (>10 mg/mL^{-1}). Therefore, NAAPs were capable to reduce the survival of tumour cell lines to a much greater extent than the non-tumour cell line.

Effect of NAAPs on the cell cycle of tumour cells

HL-60 cells were treated with different concentrations of de NAAPs. For positive control, 2-methoxyestradiol (20 µM) was used. The different NAAPs concentrations showed dose dependent responses. The highest NAAPs concentrations tested (3068.6 µg mL^{-1}) showed an increase of cells in apoptotic phase (sub. G1) of 46.30±1,99%, exceeding the negative control (sub G1 13,49±0,87%) by 32,81%. Regarding cells in the G0/G1 phase, there was a significant decrease from 54.45±1.01% for untreated cells to 28.87±1.82% for cells subjected to the highest NAAPs concentration. Similarly, the percentage of cells in the S/G2/M phases was reduced to a 24.83±0,17% in NAAPs treated cells as compared to 32.39±1.88% in the negative control (untreated cells) (Figure 3 A and C). Lower NAAPs concentrations caused non-significant differences in the distribution of cells in the different phases of the cell cycle when compared to the untreated cells (data not shown). On the other hand, 20 µM 2-methoxyestradiol caused, as expected, an increased number of cells in phases S/G2/M, reaching 45.65±3.35%; while figures for phases G0/G1 and sub G1 were 30.51±0.17% and 23.84±3.19% respectively (Figure 3B).

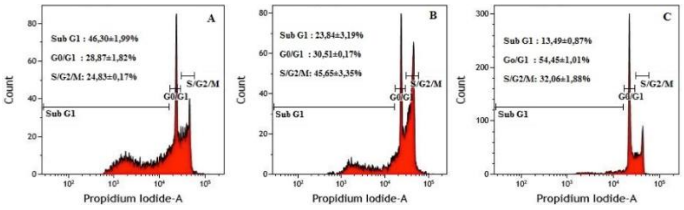

Figure 3. Subpopulations of tumour HL-60 cells at different cell cycle phases after 16 h treatment with (**A**) four-fold the IC_{50} concentration of acidic polysaccharides of *Nothophellinus andinopatagonicus* FQ1645 strain. (**B**) For the positive control 2-methoxyestradiol was used. (**C**) For the negative control the cells with RPMI-1640 was used. The percentages are the mean ± SD of three independent experiments.

Antioxidant activity of NAAPs

Results shown in Figure 4 demonstrated that the bioactivity of NAAPs against DPPH and ABTS radicals was directly related to the concentration of the NAAPs tested. The assays performed indicated that only the higher concentrations of NAAPs evaluated (20 and 40 mg mL^{-1}) exhibited a significant antioxidant activity. The results obtained for the highest NAAPs concentration tested (40 mg mL^{-1}) showed that, for the DPPH radical method, the figure was 6.24% while the ABTS radical method only showed an antioxidant activity at the same NAAPs concentration of 4.63%.

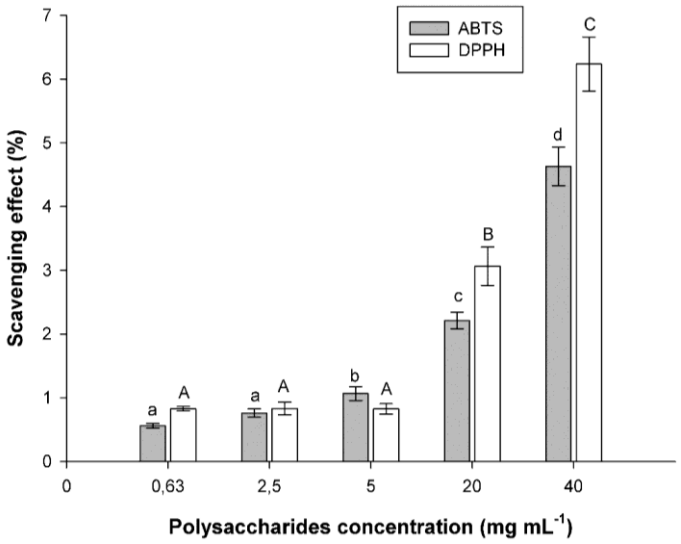

Figure 4. Scavenging effects (%) of acidic polysaccharides of *Nothophellinus andinopatagonicus* FQ1645 strain on ABTS and DPPH radical. The data are the mean of 3 replicates measurements ± standard error. Similar letters indicate that there are no significant differences between the different concentrations.

DISCUSSION

Polysaccharides are among the most important metabolites produced by representatives of genus *Phellinus s.l.*. So far, a number of polysaccharides having anticarcinogenic activity have been isolated from the fruiting body and from mycelial cultures [2,37-38]. Nevertheless, besides the antimicrobial activity reported by Aqueveque *et al.* [28] for the total extract of by *Nothophellinus andinopatagonicus*, there are no reports regarding their biological activity, either as total polysaccharides or some of their fractions, against any tumoral cell line or of any other cell type.

The biological activity of polysaccharides depends mostly on their structural characteristics, such as chemical composition and associated components (i.e. proteins or sulphate groups), and their physical properties (i.e. solubility and molecular weight) [10,16,18,38-39]. The IR spectra of the NAAPs isolated from *N. andinopatagonicus* FQ1645 strain included sulphate groups and β-glucans. Fungal β-glucans are polymers constituted by D-glucopyranosyl residues linked by β-1,3 and β-1,6 glycosidic bonds [40]. Several studies have demonstrated that β-glucans possess bioactivity, including antitumoral, immunomodulatory and anti-inflammatory activities, which are directly related to the length of the glucan chain and its interaction with functional groups [39,41]. Polysaccharides having sulphate groups in their composition considerably increase the anticarcinogenic activity of these polymers because they facilitate the interaction of the polysaccharide with the surface of tumour cells, reducing the IC_{50} when compared to non-sulphated glucan chains or achieving a synergistic effect with anticarcinogenic compounds against breast cancer (MCF-7), lung cancer (NCI-H460), colon cancer (HT-29) and leukaemia (CEM) cell lines [40,42].

According to Ferreira *et al.* [43], the polysaccharides constituting the cell wall of fungi can possess a variety of at least eleven monosaccharides, with D-glucose, N-acetylglucosamine and D-mannose the most frequently found [44]. The remaining monosaccharides, such as xylose, galactose, fucose, arabinose, ribose and rhamnose, can be found in lesser amounts. The HPLC-IR and GC-MS results showed that the NAAPs are heteroglycans constituted by at least glucose, arabinose, mannose and xylose, being D-glucose the most abundant monomers present in the sample. In fact, D-glucose is the main component of the polymers present in the cell wall of fungi [45-46]. On the other hand, the presence of myo-inositol and mannitol in the GC-MS spectrum may be explained because these poly-alcohols are present in the cell membrane of fungi or serve as storage of carbohydrates, respectively [45-46]. The GC-MS analysis allowed the identification of trehalose as the main component of the NAAPs. This glucose dimer (α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside) is synthetized by numerous organisms and serves as a cell membrane protecting agent against freezing. In fungi, it is involved in carbon translocation from mycelia to fruiting bodies [46-48].

The cell viability assays showed that the NAAPs significantly reduced the viability of the three tumour cell lines we assayed, reducing the survival of these cells up to more than 90% when subjected to the highest NAAPs concentration tested (10 mg mL^{-1}). On the other hand, the viability of the non-tumour cell line was reduced by only 37.85% when exposed to the same NAAPs concentration (10 mg mL^{-1}) and at lower NAAPs concentrations the viability of the non-tumour cell line reached survival figures close to 100%. A mortality close to 0% of the non-tumour cell line in the presence of NAAPs concentrations up to 1.25 mg mL^{-1} while the tumour cell lines showed significant mortalities at the same NAAPs concentrations probably deserves further analysis in the future.

Results of cell viability assays testing polysaccharides obtained from other species of *Phellinu* s.l. reported mortality rates of tumour cell lines in the order of 90-96% using concentrations under 200 µg mL^{-1} [12,18,24]. Nevertheless, most studies evaluated the bioactivity of crude polysaccharides; thus, not allowing a fair comparison with NAAPs. Analysis of the IC_{50} values evidenced that the cytotoxic bioactivity of NAAPs is dependent on the tumour cell line tested. The NAAPs IC_{50} values for the tumour cell lines HCT-116 and MCF-7 were two and six times, respectively, that of cell line HL-60. The molecular weight of the polysaccharides [1] plus particular characteristics of the cell lines evaluated, such as their adherence, cell morphology and the expression and/or mutation of specific oncogenes [49-51]; could be responsible for the difference in IC_{50} observed in cell lines HCT-116 y MCF-7, when compared to cell line HL-60.

In accordance with Yan *et al.* [2], the anticarcinogenic activity associated to fungal polysaccharides, particularly those obtained from *Phellinus s.l.* species, can be classified on the basis of their mechanism of action. It can be preventive (avoiding the development of oncogenes), inducing soluble mediators and activating the cells of the immune system; or by the direct activity on tumour cells, causing their apoptosis or inhibiting their metastasis. The results obtained by flow cytometry showed that leukaemia HL-60 cells treated with NAAPs increased the events in Sub. G1 phase and decreased the events in cell growth and division phases. It is possible that the mechanism of action of NAAPs causing a high "mortality" rate of these tumour cells could be associated to a cytotoxic event, resulting in a decrease of cells capable to divide by mitosis. The alteration of the cell cycle is one of the causes of carcinogenesis, which induces cell proliferation; thus, mechanisms effecting cell viability may play a key role in the control of cancer progression [34]. Kudo *et al.* [47] and Allavena *et al.* [45] reported that trehalose possesses antiproliferative activity against several human tumour cell lines, which is attributed to a blocking effect on phases G2/M of the cell cycle and activation of apoptosis. Since one of the main components obtained from our *N. andinopatagonicus* culture was trehalose, the same antiproliferative activities reported by those authors may be present in our NAAPs. Moreover, previous studies reported that polysaccharides of *Phellinus s.l.* species can induce the cell cycle arrest of tumour cell lines at different stages of the cycle and/or induce their apoptosis [2,10]. One example is polysaccharides obtained from *Phellinus baumii* and *Phellinus linteus*, which are capable of suppressing proliferation and stimulating apoptosis of murine melanoma cells and induce apoptosis of sarcoma (S-180) cells [12,24]. The anticarcinogenic activity of these fungi is mostly due to a combination of mechanisms acting directly or indirectly on tumour cells. Polysaccharides containing glucose or mannose could show immunomodulator activity besides having direct antitumoral activity because human innate immunity cells have receptors highly specific for these two monomers [2,35,41,52]. Therefore, it is possible to suggest that *N. andinopatagonicus* polysaccharides may induce a cell mediated anticarcinogenic activity in addition to reducing the viability of tumour cell lines observed in our *in vitro* assays. Future analyses may contribute to bring light into thus issue.

Oxidative stress has been identified as a crucial factor of oncogenesis proliferation. This idea is based on the finding that carcinogenic cells have greater amounts of reactive oxygen species (ROS) when compared to healthy cells and that ROS are responsible for maintaining the carcinogenic phenotype overexpressing certain genes [6,9,53]. According to both methods used in the present study (DPPH and ABTS) the NAAPs obtained from *N. andinopatagonicus* showed significant antioxidant activity only at the highest concentrations tested (40 and 20 mg mL^{-1}) inhibiting less than 10% of the radical, equivalent to <7.3 µg mL^{-1} Trolox for the DPPH assay and <16 µg mL^{-1} Trolox for the ABTS assay. These concentrations are very high when compared with polysaccharides previously studied from other species of *Phellinus s.l.* genus.

Those studies demonstrated a strong capacity to capture free radicals (i.e. being potent antioxidant agents), even at low concentrations (IC_{50} in the order of 2-5 mg mL^{-1}), when assayed using DPPH and ABTS assays, the same methods used in this study [7,13,54]. According to Wang *et al*. [55], several studies postulate that proteins or other compounds (such as phenolic compounds) present in crude extracts of fungal polysaccharides could be responsible for trapping free radicals, making crude extracts better antioxidants than purified fractions. Thus, the low free radicals trapping activity exerted by NAAPs of the strain studied could be the consequence of its purification process.

CONCLUSIONS

The present study constitutes the first report regarding the biological activity of the acidic fraction of the polysaccharides produced by *N. andinopatagonicus*. On the basis of the results provided by different assays, it is possible to conclude that NAAPs possess anticarcinogenic activity, reducing the cell viability of leukaemia (HL-60), colon (HCT-116) and breast (MCF-7) tumour cell lines. Moreover, NAAPs were shown to be less cytotoxic to the non-tumour cell line (HGF-1) than for the three human tumour cell lines (HL-60, HCT-116 and MCF-7), a result encouraging further studies on the bioactivity of the acidic polysaccharides produced by *N. andinopatagonicus*.

DECLARATION OF CONFLICTING INTERESTS

The authors declare no conflict of interest.

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