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Isolation and characterization of extracellular polysaccharide Thelebolan produced by a newly isolated psychrophilic Antarctic fungus *Thelebolus*

Sourav K. Mukhopadhyay[a], Soumya Chatterjee[a], Samiran S. Gauri[a], Shibendu S. Das[a], Abheepsa Mishra[a], Moumita Patra[a], Ananta K. Ghosh[a], Amit K. Das[a], Shiv M. Singh[b], Satyahari Dey[a,*]

[a] *Plant Biotechnology Laboratory, Department of Biotechnology, Indian Institute of Technology Kharagpur, Kharagpur 721302, West Bengal, India*
[b] *National Centre for Antarctic and Ocean Research, Goa 403804, India*

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ABSTRACT

The present investigation is on a newly isolated psychrophilic Antarctic filamentous Ascomycetous fungus that has been identified as *Thelebolus* sp. and given the designation of *Thelebolus* sp. IITKGP-BT12. The culture was primarily identified through morphological studies, and was further confirmed by 18S rRNA sequencing (GenBank Accession No. KC191572), which revealed its close relatedness with *Thelebolus microsporus*. The exopolysaccharide (EPS) produced (1.94 g L^{-1}) by the fungus was isolated, purified and characterized as glucan having an average molecular mass of 5×10^5 Da. The structure of EPS was determined by gas chromatography with tandem mass spectrometry (GC–MS/MS), Fourier transform infrared spectroscopy (FT-IR) and nuclear magnetic resonance (NMR) studies (^{1}H, ^{13}C and HSQC). NMR analysis indicated the presence of (1 → 3)-linked β-D-glucan backbone with (1 → 6)-linked branches of β-D-glucopyranosyl units. Antiproliferative activity of EPS was demonstrated in B16-F0 cells, with IC_{50} of 275.42 μg mL^{-1}. Flow cytometry analysis and DNA fragmentation studies revealed that the cytotoxic action of the EPS mediated apoptosis in cancer cells. This is the first ever report on bioactive EPS thelebolan from *Thelebolus* sp.

1. Introduction

More than three-quarters of the earth's surface is occupied by cold ecosystem that has been successfully colonized by the psychrophiles (Feller & Gerday, 2003). Psychrophiles are predominant in cold environments and have adapted themselves for efficient growth at sub-zero temperatures. Major features in adaptation include: higher ratio of polyunsaturated fatty acids (PUFAs) in membranes, cold acclimation proteins (CAPs), anti-freeze proteins (AFPs), anti-nucleating proteins (ANPs), cold-adapted enzymes and extracellular polymeric substances (EPSs) (Fendrihan & Negoita, 2012; Selbmann, Stingele, & Petruccioli, 2002). Generally, EPSs produced by Antarctic microbes are high molecular weight polysaccharides that provide cryoprotection to the organism in harsh conditions (Selbmann, Onofri, et al., 2002). Exopolysaccharide (EPS) production by psychrophilic fungi has been traditionally investigated (Pavlova, Panchev, Krachanova, & Gocheva, 2009; Pavlova et al., 2010; Selbmann, Onofri, et al., 2002). However, bioactive properties of EPS from *Thelebolus* sp. are yet to be reported.

Microbial polysaccharides are a diverse class of polymers that have acquired immense importance in recent times due to their possible bioactive roles (Kim, Shim, Kim, & Jang, 1999), rheological properties (Sutherland, 1994), high stability (Wang & McNeil, 1996) at wide range of temperature and because they possess great applicability in cosmetic, food and pharmaceutical industries (Kumar, Mody, & Jha, 2007; Schulz, Rau, & Wagner, 1992). Fungi are abundant sources of polysaccharides along with bacteria and plants. The cell wall of fungi is mainly composed of chitin and glucan [(1 → 3)-β-glucan, (1 → 6)-β-glucan] (Adams, 2004), the latter being long-chain polysaccharides found also in the cell wall of certain bacteria and plants (Gannam & Schrock, 2001; Rice et al., 2005). Natural β-glucans, useful in treating and/or preventing various diseases, have great demand.

The incidence of cancer is gradually increasing and the spectrum of cancer-prone organs is changing each year. Yeast and mushroom based (1 → 3)-β-D-glucans are well-established biological response modifiers (BRM) that have been reported to modulate various aspects of immunity (Williams, 1997). Fungal derived

* Corresponding author. Tel.: +91 9434018791; fax: +91 3222 278707.
E-mail addresses: satyahari01@yahoo.com, sdey12.iitkgp@gmail.com (S. Dey).

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polysaccharides are attracting huge attention for their potent biological and pharmacological activities (Burns, Yeo, Keshavarz, Roller, & Evans, 1994; Cheung, 1996; Chihara, Hamuro, Maeda, Arai, & Fukuoka, 1970; Sutherland, 1990). Fungal polysaccharides, particularly (1 → 3)-β-D-glucans have many therapeutic uses. The effect of such fungal β-glucans as lentinan, sonifilan, curdlan, and scleroglucan has been clinically proven in cancer therapy (Bohn & BeMiller, 1995; Ohno, Miura, Nakajima, & Yadomae, 2000). Most polysaccharides, with undeciphered mode of action, are classified as nonspecific bioactive substances where the exact conformation of the active components remains undefined. The indirect pathway, in which the 'bioactive' polysaccharide acts only as a mediator of the immune response, has been studied in detail (Adachi, Okazaki, Ohno, & Yadomae, 1994; Chihara, 1992; Ho, Konerding, Gaumann, Groth, & Liu, 2004), while the direct pathway, where the polysaccharide itself inhibits the tumorous growth, is quite complex and until now has been only partially characterized (Chen, Zhao, Chen, & Li, 2008; Li et al., 2004; Xie et al., 2006; Zaidman, Yassin, Mahajna, & Wasser, 2005; Zhou et al., 2007).

In the present study, an EPS from *Thelebolus* has been characterized and its antiproliferative effect has been evaluated.

2. Materials and methods

2.1. Sampling site

The unidentified fungal isolate was provided by National Centre for Antarctic and Ocean Research, Goa 403804, India. Sampling was done at McLeod Island, Larsemann Hills (69°20′ S to 69°30′ S Lat: 75°55′ E to 76°30′ E Long) of Prydz Bay area in East Antarctica.

2.2. Microorganism and growth medium

The fungal strain was maintained on potato dextrose agar (PDA) plates at 18 °C for 5 days and then stored at 4 °C. The seed culture was grown in a 250 mL Erlenmeyer flask containing 50 mL of potato dextrose broth (PDB) medium with agitation (150 rpm) for 3 days at 18 °C.

2.3. Morphological study

Morphology of fungus was examined using light and scanning electron microscopy (SEM). In brief, fungal culture was mounted in lactophenol cotton blue (Riddell, 1950) and observed under bright field microscopy. For scanning electron microscopy, fungal culture was placed on glass cover slips and the fixed cells were dried at a critical point by a drying apparatus and kept in desiccators until use. Samples were then fixed onto a graphite stub and kept in an auto-sputter coater (E5200, Bio-Rad) under vacuum up to 150 s for gold coating. Images were captured using field emission scanning electron microscope (FESEM) of Carl Zeiss, model SUPRATM 40, with an accelerated voltage 5–20 kV.

2.4. Genomic DNA extraction, amplification and sequencing of partial DNA fragments of 18S rRNA gene

The selected strain was identified by 18S rRNA gene sequencing. Total genomic DNA was extracted from the isolate following the method of Doyle and Doyle (1990). Amplification of 18S rRNA gene was performed by PCR using universal fungal specific primers (Cappa & Cocconcelli, 2001) TR1 [5′-GTTTCTAGGACCGCCGTA-3′] and TR2 [5′-CTCAAACTTCCATCGACTTG-3′]. The PCR conditions were maintained as an initial denaturation step of 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s and a final extension step at 72 °C for 7 min. PCR products were analyzed by 1.0% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV-transilluminator (UST-20M-8R, Biostep, Germany). Gel eluted PCR product was purified by QIAquickgel extraction kit (QIAGEN) as per manufacturer's protocol. The amplicon was sequenced and was submitted to NCBI database.

2.5. Submerged mycelial culture for EPS production

Thelebolus sp. IITKGP-BT12 was found to produce EPS when grown in basal salt medium. The composition of modified basal salt medium (Selbmann, Onofri, et al., 2002) was (in g L^{-1}): $NaNO_3$, 3.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; yeast extract, 1.0; glucose, 50.0, pH 6. Batch fermentation was performed in 50 mL of basal salt medium in 250 mL Erlenmeyer flasks and incubated (150 rpm) at 18 °C for 10 days.

2.6. Isolation of EPS

EPS was isolated from culture filtrate following the method of Gauri, Mandal, Mondal, Dey, and Pati (2009). In brief, EPS was recovered by precipitation at 4 °C from culture filtrate by the addition of one volume of isopropanol. After centrifugation (11,000 × *g* for 10 min) the resulting precipitate was collected. Crude EPS was found to be insoluble in water even after boiling and sonication. In order to solubilize crude EPS, it was boiled in 1 M NaOH (Bacon, Farmer, & Jones, 1969). Alkali soluble EPS was then dialyzed and lyophilized. Total sugar content of polysaccharide was determined by the phenol–sulfuric acid assay (Dubois, et al., 1956). Schematic representation of the isolation and characterization processes of EPS has been described in Fig. 1.

2.7. Purification of EPS

2.7.1. Ion exchange chromatography (IEC)

EPS was subjected to anion exchange chromatography on a column of DEAE–Sephadex A-25 (18 × 1.8 cm, Sigma), pre-equilibrated using 0.01 M Tris–Cl (pH 7.8) (Christensen, Kjosbakken, & Smidsrod, 1985). The column was washed with equilibration buffer followed by successive elution with sodium chloride solution of increasing molarities in a stepwise manner. Fractions (10 mL) were collected and monitored spectrophotometrically by phenol–sulfuric acid assay (Dubois et al., 1956). The major polysaccharide fractions eluted at 200 mM NaCl were pooled, dialyzed, freeze-dried and used for further purification by gel permeation chromatography.

2.7.2. Gel permeation chromatography (GPC) and molecular weight determination of EPS

The IEC purified polysaccharide (45 mg) was purified by GPC on Sepharose CL-6B column (90 × 2.1 cm, GE Healthcare) with water as eluant (0.75 mL min^{-1}) using Redifrac fraction collector (Ghosh et al., 2008). Ninety fractions (2 mL each) were collected and monitored spectrophotometrically at 490 nm with phenol–sulfuric acid reagent. One homogeneous fraction (test tubes 13–34) was pooled and freeze-dried, yielding 20 mg of material. The Column Sepharose CL-6B was standardized by loading different dextran molecular weight markers (range 40–2000 kDa). From the separation pattern of dextran marker, a standard curve was plotted by taking log of molecular weight against fraction number. GPC purified EPS was used for further chemical characterization and biological activity study.

2.8. Chemical analysis

2.8.1. Thin layer chromatography (TLC) analysis of EPS

EPS (5 mg) was hydrolyzed with 4 M TFA (1 mL) at 100 °C in oil bath for 5 h (Blakeney, Harris, Henry, & Stone, 1983). The excess

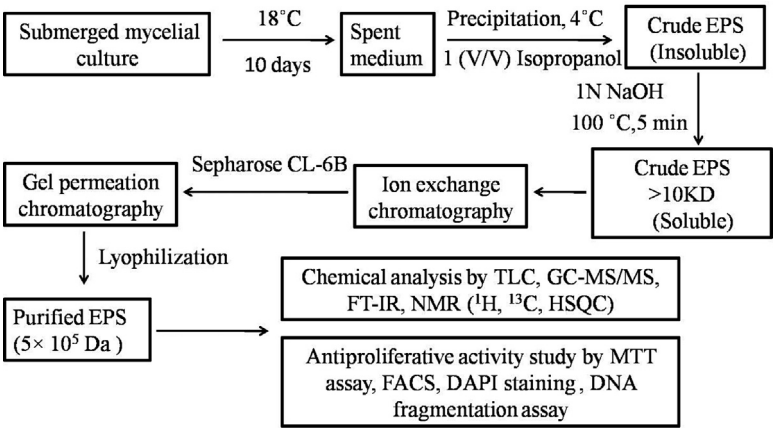

Fig. 1. Schematic representation of isolation and characterization of EPS from *Thelebolus* sp. IITKGP-BT12.

acid was completely removed by co-distillation with water and methanol. Monosaccharide composition of TFA digested EPS was determined by TLC. Isopropanol:ethyl acetate:acetic acid:water (4:2:2:1) was used as mobile phase on TLC silica gel 60 F_{254} plates (MERCK, Germany) with orcinol–sulfuric acid solution as staining reagent (Waksmundzka-Hajnos, Sherma, & Kowalska, 2008).

2.8.2. Monosaccharide analysis of EPS by GC–MS/MS

The monosaccharide composition was determined following alditol acetate method (Blakeney et al., 1983). After reduction and acetylation of TFA digested EPS, resulting alditol acetates were analyzed by GC–MS/MS (Thermo Scientific, USA). Chromatography was performed in a TR-5MS column (30 m × 0.25 mm ID, 0.25 μM, Thermo Scientific, USA) with helium as carrier gas. Detection was done by mass detector and identification was based on matching with a laboratory made mass spectral library/database using NIST as well as by comparing with standard sugars.

2.8.3. FT-IR analysis of EPS

Infrared spectra (IR) of EPS film (2%) was recorded with a Nicolet 170SX FT-IR spectrometer (Spectrum One, Perkin–Elmer Corp, USA) in the range 4000–400 cm^{-1}.

2.8.4. NMR study of EPS

EPS was kept over P_2O_5 in vacuum for 7 days, and exchanged with deuterium by lyophilizing with D_2O (99.96% atom $^2H_1$, Aldrich) (Dueñas Chaso et al., 1997). ^{13}C, ^1H and HSQC NMR spectra were recorded in DMSO-d_6 at 30 °C with a Bruker Avance DPX-500 spectrometer.

2.9. Antiproliferative activity assays

2.9.1. Cell lines

B16-F0 (mouse skin carcinoma cell line) and L929 (normal mouse fibroblast cell line) were obtained from National Centre for Cell Science (NCCS) Pune, India and maintained in DMEM medium supplemented with 10% fetal bovine serum.

2.9.2. Cytotoxicity assay

The effect of EPS treatment in different concentrations (187.5–1500 μg mL^{-1}) on viability of cancer cell (B16-F0 melanoma) and normal cell (L929) (1×10^5 cells/well of a 96-well plate) were evaluated by MTT assay (Mosmann, 1983). The cells were treated for 48 h at 37 °C in a 5% CO_2 incubator and cell viability was determined by measuring absorbance at 570 nm using a micro plate reader (Bio-Rad 550). Cell viability was calculated using the formula: Inhibition (%) = 100 × A1 − A2/A1 [where A1 – absorbance of treated cells and A2 – absorbance of control cells].

2.9.3. Cell cycle analysis

Cell cycle distribution in cells treated with the tested agents was analyzed by propidium iodide DNA staining according Pilatova et al. (2010) with slight modification. Briefly, B16-F0 cells (1×10^6) after EPS treatment (187.5–1500 μg mL^{-1}) for 24 h were harvested and washed twice with PBS (pH 7.4) and fixed overnight in 70% ethanol at 4 °C. Then, cells were washed with PBS, incubated for 30 min with 1 mg mL^{-1} RNase A and 10 μg mL^{-1} propidium iodide at 37 °C. This was then analyzed for the distinct cell cycle phases on a FACS Vantage SE flow cytometer using Cell Quest Pro software (Becton Dickinson, USA). Ten thousand cells were counted per analysis. PI fluorescence was detected in the pulse-processed FL2 channel. Percentage of cells corresponding to G_0/G_1, S and G_2/M phases of the cell cycle was analyzed from DNA histogram. A sub-G_0/G_1 fraction of cells was identified as an apoptic population.

2.9.4. DAPI staining

The assay was performed as described previously (Yamada, Ichimura, & Doorn, 2006). Briefly, 1×10^5 B16-F0 cells after EPS treatment (750 μg mL^{-1}) for 16 h were rinsed twice with PBS and fixed in 4% formaldehyde solution for 25 min. Thereafter, cells were washed once with PBS and permeabilized with 0.1% Triton-X. Cells were again washed with PBS and were incubated for 30 min with 0.4 μg mL^{-1} of DAPI (Sigma) staining solution. DNA alterations were observed by fluorescence microscopy (IX 51, Olympus) using high-performance CCD camera with the appropriate filter using Image-Pro discovery 5.1 software.

2.9.5. DNA fragmentation assay

EPS treated and untreated B16-F0 cells (1×10^5 cells/well) were washed twice with 1× PBS. DNA was isolated from both treated and untreated cells by quick Apoptotic DNA Ladder detection Kit (Invitrogen), as per manufacturer protocol. Test samples along with 100 bp DNA ladder were loaded on to 1.2% agarose gel and run with 40 V for 2 h using 1× TBE buffer. DNA fragments were visualized using UV transilluminator (UST-20M-8R, Biostep, Germany) (Wyllie, 1980).

2.10. Statistical analysis

All statistical data were represented as mean ± standard deviation (SD) with significance level evaluated using *t*-test statistics. *P*-value < 0.05 was considered to be of significant level.

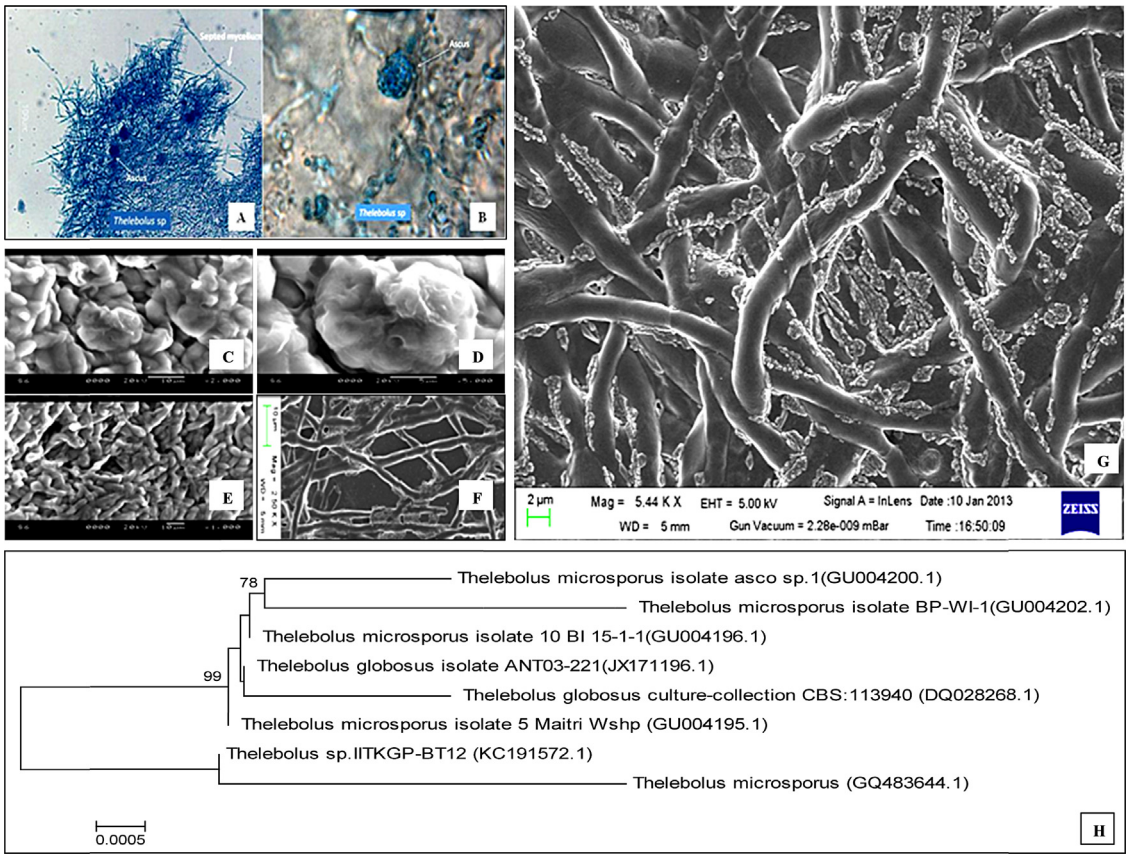

Fig. 2. *Thelebolus* sp. IITKGP-BT12. (A) Fungal mycelium, (B) ascus with multiple spores, (C) and (D) ascus, (E) conidia, (F) septed mycelium, (G) Fungal mycelium extracellularly secrete EPS. (A and B: light microscopic view after lactophenol cotton blue staining; C–G: SEM view). (H) Phylogenetic neighbor joining tree obtained with the 18S rRNA gene sequences of strain *Thelebolus* sp. IITKGP-BT12 and members of other related fungus. Numbers at branch points are bootstrap values for a parsimony-based analysis.

3. Results and discussion

3.1. Morphological study and molecular identification of EPS producing fungal isolate

Microscopic observation revealed that the fungal hyphae were highly septate (Fig. 2A and F); asci were oval with multiple spores inside (Fig. 2B–D). Conidia were present (Fig. 2E). The 18S rRNA gene sequence based identification strategy was used to reveal the phylogenetic status of the fungal isolate. The PCR amplification of the genomic DNA using TR1/TR2 primer yielded an amplicon of ~540 bp (data not shown). Phylogenetic analysis of the obtained sequence (GenBank Accession No. KC191572) and morphological studies (Fig. 2A–G) showed close relationship of *Thelebolus* sp. IITKGP-BT12 with *Thelebolus microsporus* (Fig. 2H).

3.2. EPS isolation, purification and chemical characterization

Thelebolus sp. IITKGP-BT12 was found to produce EPS of 1.94 g L^{-1} in basal salt medium. EPS production by fungus was also shown by scanning electron micrograph (Fig. 2G). Crude EPS was found to be insoluble in water; this may be due to the formation of compact triple-stranded helix structure in glucans as reported earlier (Han, Han, Hyun, & Shin, 2008; Manners, Masson, & Patterson, 1973). Water insoluble crude EPS was solubilized by boiling with 1 M NaOH (Bacic, Fincher, & Stone, 2009) and further purified by anion exchange chromatography. Major fraction eluted at 200 mM NaCl concentration (Fig. S1A) was taken for purification and molecular weight determination using gel permeation chromatography (Fig. S1B). Gel permeation chromatographic separation using Sepharose CL 6B (Bead size, 45–165 µm; fractionation ranges 1×10^4 to 4×10^6 Da) has been traditionally used for purification and molecular weight (MW) determination of high MW polysaccharides (Cherniak, Reiss, & Turner, 1982; Ghosh et al., 2008). The molecular weight of the EPS was determined from standard curve as approximately 5×10^5 Da and the purified EPS was used for further chemical characterization. Evidence suggests that the diverse array of properties of the so-far reported 'bioactive' fungal polysaccharides might depend on their molecular weight, with high molecular weight fractions being most functional, while fractions from the same source with lesser molecular weights showing no activity of interest (Blaschek, Kasbauer, Kraus, & Franz, 1992; Fabre, Bruneteau, Ricci, & Michel, 1984; Kojima, Tabata, Itoh, & Yanaki, 1986). The high molecular weight of the presently isolated EPS from *Thelebolus* is suggestive that it might possess some bioactive properties. This indication has been duly explored through extensive experimentation.

Monosaccharide composition of purified EPS was identified as glucose from TLC (Fig. S1C) and confirmed by GC–MS/MS (Fig. S1D).

Table 1
^{13}C NMR and ^{1}H chemical shifts (in ppm) for the glucan isolated from *Thelebolus* sp. IITKGP-BT12.

Carbon assignment		Proton assignment	
C1	103.4	H1	4.21, 4.52, 4.55
C2	73.2	H2	2.95, 3.24
C3	77.1, 86.1, 86.6	H3	3.07, 3.5
C4	70.4	H4	3.02
C5	76.6	H5	3.12, 3.15
C6	61.3, 68.9	H6	3.39, 3.5, 3.65, 4.18

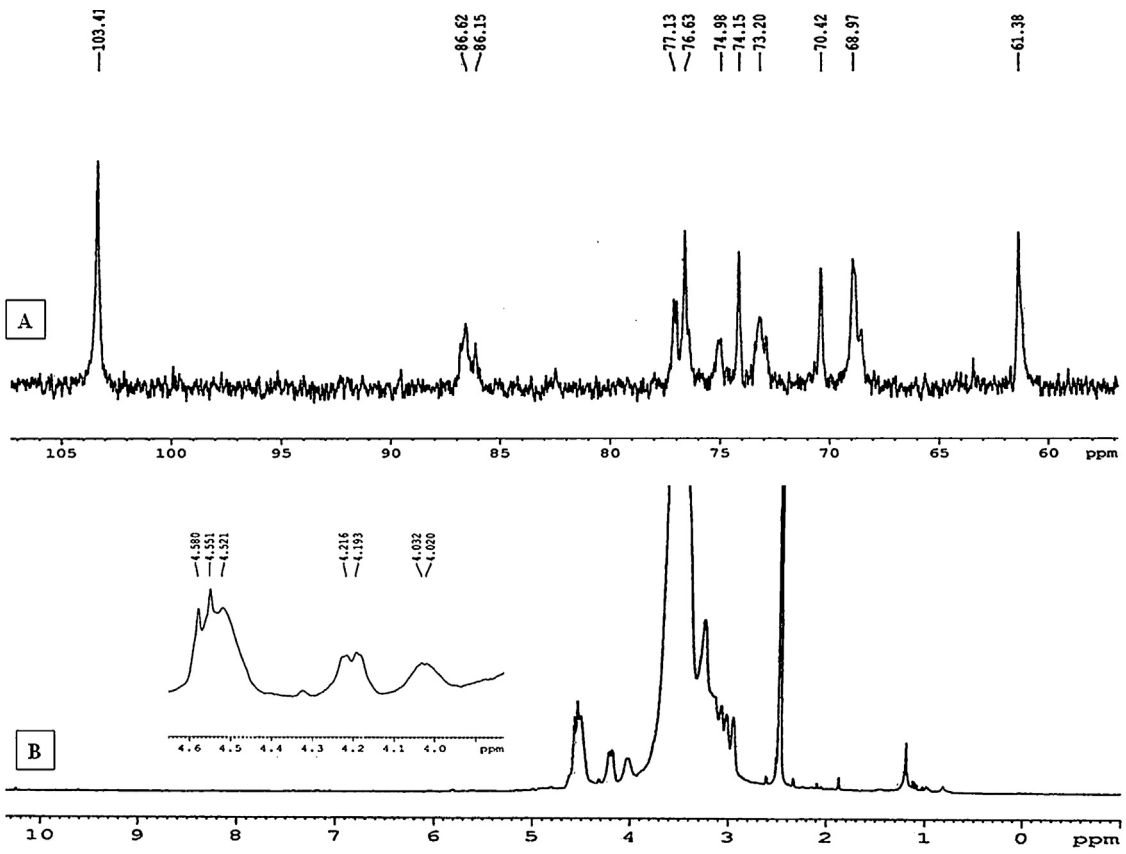

Fig. 3. (A) ^{13}C NMR spectra of EPS (125 MHz, DMSO-d_6, 30 °C) and (B) ^{1}H NMR spectra of EPS (500 MHz, DMSO-d_6, 30 °C) isolated from *Thelebolus* sp. IITKGP-BT12.

The GC profile of EPS fractions showed the presence of a single sugar monomer as the main building block of this polysaccharide fraction (Fig. S1D). The mass chromatogram of EPS was identical with that of standard glucose. Thus, the purified EPS was identified as a glucan. Previously, Pavlova et al. (2010) have reported a hetropolysaccharide producing Antarctic fungal strain, *Cryptococcus laurentii AL100* having the composition: arabinose, mannose, glucose, galactose and rhamnose. Another hetropolysaccharide producing yeast *Cryptococcus flavus A51*, from Antarctica was also reported (Pavlova, Panchev, Krachanova, & Gocheva, 2009). But very few reports are available on Antarctic fungus producing glucan and mannan-like EPS (Poli et al., 2010; Selbmann, Onofri, et al., 2002).

The absolute configuration of the glucose residue in EPS was determined as D by the method of Gerwig, Kamerling, and Viliegenthart (1978). Glycosidic linkage configuration for EPS was exclusively of the β-type as determined from the FT-IR spectrum (Fig. S2) with absorption at 893 cm^{-1} which is characteristic of the β anomer of glucopyranose (Devi, Roy, Patra, Sahoo, Islam, & Maiti, 2013).

The ^{13}C NMR spectrum (125 MHz) of EPS at 30 °C in DMSO-d_6 reflected only one anomeric carbon signal at δ 103.4 (Fig. 3A), a characteristic feature of β-linkage (Barbosa, Steluti, Dekker, Cardoso, & Corradi da Silvac, 2003; Bhanja et al., 2012; Bubb, 2003). The ^{1}H NMR (500 MHz) spectrum (Fig. 3B) of EPS at 30 °C in DMSO-d_6 showed three anomeric proton signals at δ 4.55, δ 4.52, δ 4.21 in a ratio of nearly 1:1:0.5. The $^{1}J_{C\text{-}1,\ H\text{-}1}$ value of approximately 160 Hz, determined from HSQC (Fig. S3), indicated the moiety to have β-linkage (Bubb, 2003; Sen et al., 2013). The downfield shift for C-3 (δ 86.6) and C-6 (δ 68.9) with respect to standard value and small upfield chemical shift of C-5 (δ 76.6), C-4 (δ 70.42) and C-2 (δ 73.2) with respect to standard value indicated the presence of glycosidic linkages at C-3 and C-6 position (Table 1) (Agrawal, 1992; Bhanja et al., 2012; Ojha, Chandra, Ghosh, & Islam, 2010; Sen et al., 2013; Ukawa, Ito, & Hisamatsu, 2000). Thus, it may be inferred that the EPS comprised of (1 → 3; 1 → 6)-linked β-D-glucopyranosyl moiety. Although, some literature papers have reported a similar kind of glucan-like polysaccharides from different fungal sources (Barbosa et al., 2003), specifically from mushroom (Lee, Tan, Fung, Tan, & Ng, 2012), having potential as bioactive immunomodulatory molecule (Weng et al., 2011), yet there were no report on antitumor and antiproliferative polysaccharide from Antarctic fungus. The glucan EPS, being the first and the only one reported from *Thelebolus* sp., we prefer to name it as Thelebolan.

3.3. Antiproliferative activity

In recent years, MTT assays have been routinely used for preliminary screening of antiproliferative compounds. It is a quick and effective method for testing mitochondrial impairment and correlates quite well with cell proliferation. Polysaccharide from diverse sources is proven to inhibit the growth of many different types of tumor cells (Wasser, 2002; Xue, Sun, Zhao, Zhang, & Lai, 2011; Zhang, Cheung, & Zhang, 2001). Additionally, a water-soluble β-glucan isolated from *Poria cocos* was shown to have antiproliferative effects on human breast carcinoma MCF-7 cells so mediated by cell-cycle arrest and apoptotic induction (Zhang, Chiu, Cheung, & Ooi, 2006). Also, Lee et al. (2012) have reported that polysaccharide extracted from sclerotia of *Lignosus rhinocerus* demonstrated cytotoxicity against human breast carcinoma (MCF 7) and human lung carcinoma (A549) with IC_{50} of 96 μg mL^{-1} and 466 μg mL^{-1} respectively. Presently, in order to check the antiproliferative effect of Thelebolan, mouse skin carcinoma, B16-F0 was chosen. The cytotoxic effect against B16-F0 and L929 cells were determined by treating cells with various concentrations of the

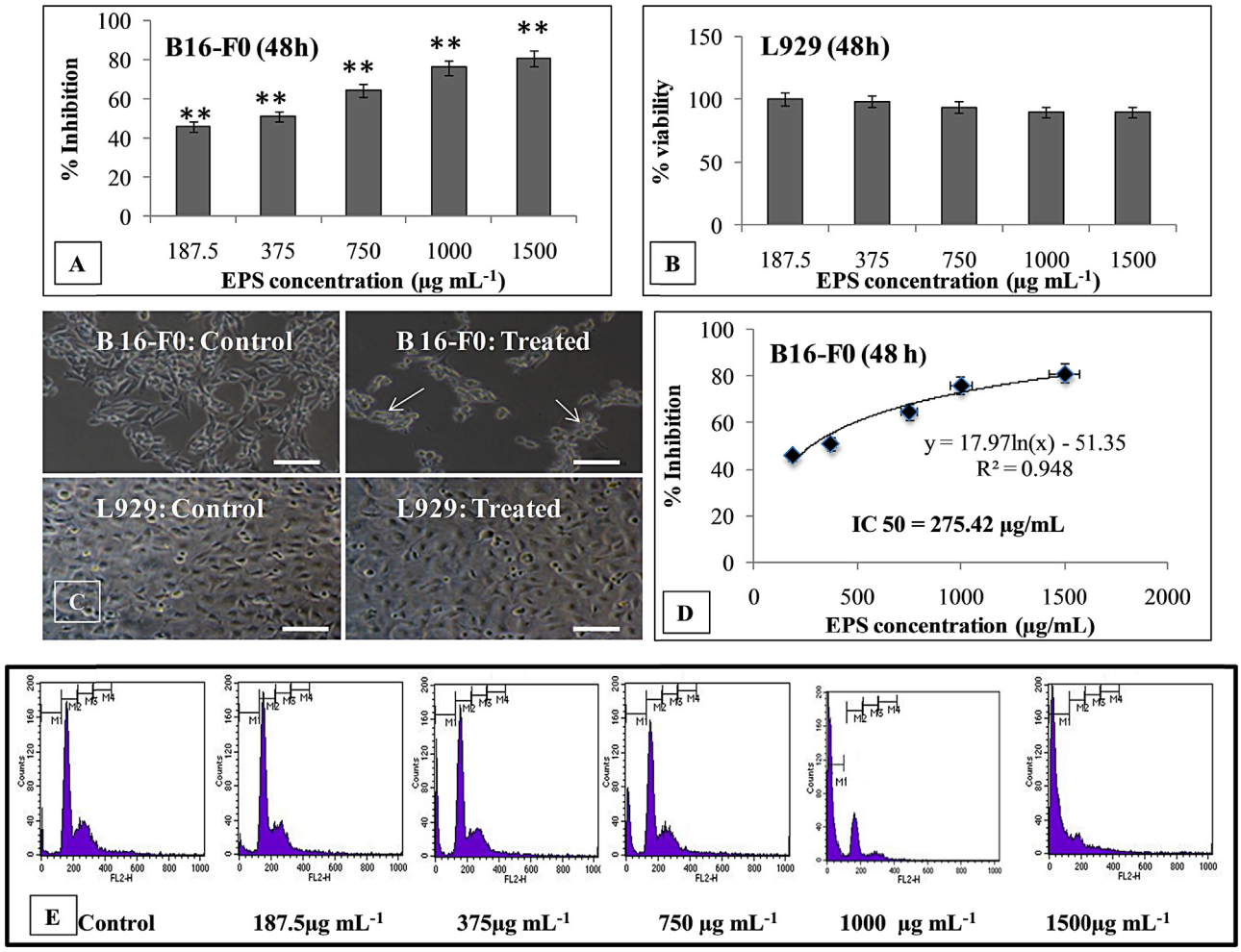

Fig. 4. (A) Antiproliferative activity of EPS in B16-F0 skin cancer cell line. EPS at 187.5–1500 µg mL^{-1} showed significant cytotoxicity with IC_{50} of 275.42 µg mL^{-1}. Data are expressed as percent of inhibition of growth compared to the control. (B) Cytotoxic activity of EPS against normal cell line, L929. EPS at (187.5–1500 µg mL^{-1}) exhibit less significant cytotoxicity on L929 when compared with B16-F0 cell line. (C) Effect of EPS on cancer cell and normal cells (phase contrast microscopic view; scale bar – 100 µm). (D) IC_{50} value was determined from standard curve. Data are the mean ± SD ($n = 3$) from three independent experiments. *, $P < 0.05$, **, $P < 0.01$. (E) Cell cycle distribution detected by flow cytometric analysis. B16-F0 was treated with EPS (187.5–1500 µg mL^{-1}) for 24 h. Symbol M1, M2, M3, M4 represented sub-G0/G1 peak, G0/G1 phase, S phase and G2/M phase, respectively. Each histogram is representative of three experiments.

EPS (187.5–1500 µg mL^{-1}) for 48 h of incubation. As shown in Fig. 4A and D, EPS exhibited strong antiproliferative activity against B16-F0 cell line, with IC_{50} 275.4 µg mL^{-1}. In addition, EPS did not exhibit any significant cytotoxicity on normal fibroblast cell line at concentration 187.5– 1500 µg mL^{-1} (Fig. 4B). The morphological characteristics of EPS treated (750 µg mL^{-1}) B16-F0 and L929 cells using inverted microscopy were presented in Fig. 4C. B16-F0 cells treated with EPS (750 µg mL^{-1}) for 24 h showed cell shrinkage, significant decrease in the cell density showing round-shaped apoptotic bodies while remaining cells showed swelling and damage of plasma membrane integrity compared to the control cells (Fig. 4C).

Flow cytometry offers some important advantages for the study of apoptosis. This powerful technique allows cell subpopulations to be identified and permits the simultaneous measurement of several cellular parameters (Dive et al., 1992). It has already been reported that mushroom polysaccharides exhibited direct inhibitory effects on cancer cell proliferation, by modulating cell-cycle progression and inducing apoptosis (Wang et al., 2002). Current study showed that the decrease in cancer cell viability by EPS was associated with induction of cell cycle arrest at the G2/M phase followed by an increase in the fraction of cells with sub-G0/G1 DNA content which is considered a marker of apoptic cell death (Fig. 4E, Table 2). Nuclear condensation and fragmentation, one of the special features of apoptotic cell death (Liu, Yao, Kirschenbaum, & Levine, 1998), was seen in EPS (750 µg mL^{-1}) treated B16-F0 cells for 16 h using DAPI staining procedure (Fig. 5A and B). Apoptotic DNA fragmentation was qualitatively analyzed by DNA fragmentation assay (Fig. 5D). Fig. 5C (16 h) and D (24 h) showed the presence of DNA ladder fragments on the EPS treated B16-F0 cells, indicating that cell death was mediated by apoptosis (Lee et al., 2012). A single band was observed on the untreated (control) cells, and there was no DNA ladder formation (Fig. 5C and D). This

Table 2
Flow cytometric analysis of cell cycle distribution in B16-F0 cells treated with EPS.

	M1 (SubG0-G1)[a]	M2 (G0-G1)[a]	M3 (S)[a]	M4 (G2-M)[a]
Control	3.05 ± 0.05	69.7 ± 0.1	22.66 ± 0.07	4.59 ± 0.01
187.5 µg mL^{-1}	5.26 ± 0.09	72.36 ± 0.02	19.26 ± 0.02	3.12 ± 0.01
375 µg mL^{-1}	12.67 ± 0.1	65.05 ± 0.16	18.66 ± 0.09	3.62 ± 0.03
750 µg mL^{-1}	16.12 ± 0.01	62.93 ± 0.03	17.74 ± 0.02	3.21 ± 0.01
1000 µg mL^{-1}	41.46 ± 0.13	44.4 ± 0.02	8.95 ± 0.13	4.33 ± 0.04
1500 µg mL^{-1}	78.9 ± 0.11	14.27 ± 0.09	4.32 ± 0.01	2.15 ± 0.02

[a] Values are represented as mean ± SD ($n = 3$).

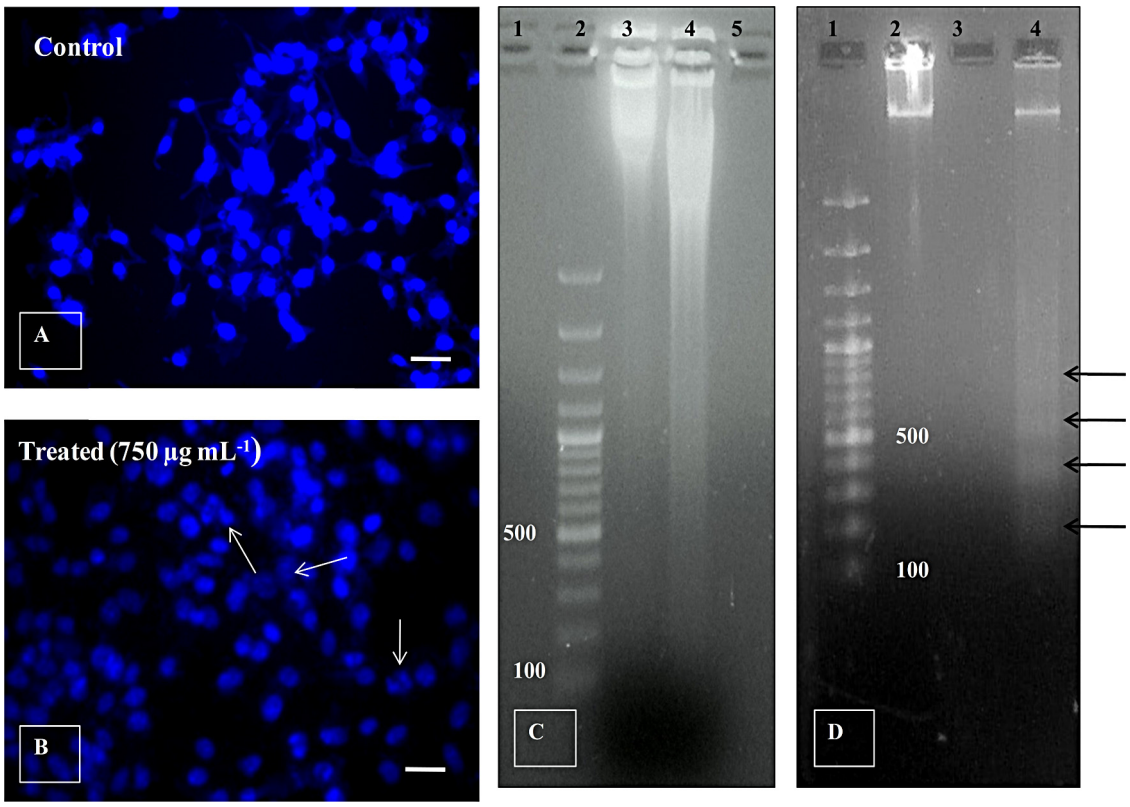

Fig. 5. Antiproliferative activity of EPS (A) DAPI staining: B16 F0 cells without EPS treatment (control) shows intact nucleus. (B) B16 F0 cells treated with EPS (750 μg mL^{-1}) shows nuclear fragmentation (C) DNA fragmentation assay for 16 h. Lane 2: 100 bp DNA ladder (Fermentas); Lane 3: control B16-F0 cell DNA; Lane 4: EPS (750 μg mL^{-1}) treated B16-F0 cell DNA. (D) DNA fragmentation assay for 24 h. Lane 1: 100 bp DNA ladder (Fermentas); Lane 2: control B16-F0 cell DNA; Lane 4: EPS (750 μg mL^{-1}) treated B16-F0 cell DNA. Scale bar – 10 μm.

finding established *in vitro* antiproliferative and apoptosis-inducing abilities of EPS on cancer cell line which is in good agreement with previously available reports on fungal β-glucans (Sen et al., 2013), specifically (1 → 3; 1 → 6)-β-glucans (Bohn & BeMiller, 1995). Hence, the effects of Thelebolan in cancer cells, *in vitro*, are suggestive of putative effects of these molecules in *in vivo* models.

4. Conclusion

The isolated psychrophilic fungus belongs to the genus of *Thelebolus*, the phylogenetic study indicating its close relationship with *Thelebolus microsporus*. This newly isolated fungus is able to produce the EPS, Thelebolan. Chemical characterization of EPS reveals the presence of (1 → 3; 1 → 6)-linked β-D-glucopyranosyl moiety as building block molecules. Promising antiproliferative and apoptosis-inducing abilities of this EPS against cancer cell line is indicative of its potential use in healthcare applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.01.034.

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