

ANTINEOPLASTIC ACTIVITY OF EXTRACTS OF THE
ANTARCTIC YEAST *SPOROBOLOMYCES ROSEUS* AL₁₀₃
ON URINARY BLADDER CANCER CELLS

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Received on February 21, 2023
Presented by B. Petrunov, Member of BAS, on March 28, 2023

Abstract

Currently, malignant diseases are one of the leading causes of mortality worldwide. Antarctic yeasts represent a poorly explored source of novel bioactive compounds with antineoplastic activity and expected favourable toxicological profile. The aim of the work was the evaluation of the antiproliferative and proapoptotic potential of extracts obtained from the psychrophilic strain *Sporobolomyces roseus* AL₁₀₃. Data from MTT dye reduction assay showed that urothelial cancer cell line T-24 was more sensitive (85–101 µg/mL) than the non-tumour cell line (CCL-1- 343 to 432 µg/mL). Apoptosis analysis after treatment with different yeast extracts revealed strong potential to inhibit various antiapoptotic factors as well as selective induction of apoptosis in tumour cells as confirmed by flow cytometry, too.

Key words: antarctic yeast extracts, bioreactor, in vitro antiproliferative effect, apoptosis

Introduction. Cancer is one of the top three causes of morbidity and mortality worldwide, especially in developed countries. Worldwide neoplastic deceases

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This research was funded by the Bulgarian Ministry of Education and Science through the National Centre for Polar Studies, and Sofia University “St. Kliment Ohridski” in the frame of the National Programme for Polar Studies 2022–2025, grant No. 70-25-40/23.01.2023.

DOI:10.7546/CRABS.2023.05.14

caused over 9.6 million deaths in 2018. Urinary bladder carcinoma (urothelial cancer) is a heterogeneous malignant disease. It is among the most common types of cancer in men (on the fourth place in developed countries) [1]. This carcinoma can be muscle-invasive or muscle-noninvasive, with 70% of patients having the muscle-noninvasive variant. With them, life expectancy is longer and the probability of metastases is lower, but unfortunately the relapses are frequent [2]. There are many ways of treatment. Because of high toxicity of the classic chemotherapeutics (e.g. mitomycin C and anthracyclines) there is a growing need to find new antitumour drugs with lower toxicity and minimal side effects. During the last decade, the inhibition of cancer cells by natural compounds and extracts has attracted much attention. Microbial metabolites as antineoplastic agents and tools for modulation of apoptosis signal transduction represent an attractive therapeutic concept due to the specific targeting of cancer cells and unlike chemical inhibitors, they do not lead to significant toxic side effects. Additionally, microbial/yeast production can be performed constantly without significant climate influence and can be regulated by optimization of the cultivation parameters. Anticancer activity has only been reported for dozens of the unique metabolites produced by fungi, because for a long time, chemical and pharmacological investigations of these living organisms have been neglected [3].

Psychrophilic microorganisms have successfully colonized all permanently cold environments from the deep sea to the mountains in Polar Regions of Earth. They survive and multiply at low temperatures due to their unique ability to adapt their metabolism to extreme environmental conditions [4]. The biosynthesis of intracellular metabolites from psychrophilic yeast demonstrated a great variety of metabolic profiles [5]. Some of them are characterized by photoprotective and antioxidant activities (e.g. carotenoids and CoQ₁₀) [6] and have rich lipid content. However, pharmaceutical products from psychrophilic fungi are quite rare and to the best of our knowledge, they are not yet available for pharmaceutical application [7]. During the last several years, intensive investigations on Antarctic microflora revealed an ability of psychrophilic yeasts to synthesize new substances with intriguing functional properties. The scarcity of information about the synthesis of biologically active substances with antineoplastic effects from Antarctic yeasts and the social significance of malignant tumours, affecting millions of people worldwide, generated our interest in evaluating the antiproliferative and proapoptotic potential of several extracts from the psychrophilic strain *Sporobolomyces roseus* AL₁₀₃ obtained under submerged cultivations.

Materials and methods. Biological material. The psychrophilic strain *Sporobolomyces roseus* AL₁₀₃ was isolated from soil samples collected in Antarctica (Livingston Island). It was preserved in the Antarctic collection of Laboratory Cellular biosystems at The Stephan Angeloff Institute of Microbiology – BAS. The sequence was deposited in the National Center for Biotechnology Information (NCBI) under number ON567313.

Media and growth conditions. The yeast culture medium contained the following components (g/L): sucrose, 40.0; $(\text{NH}_4)_2\text{SO}_4$, 2.5; KH_4PO_4 , 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; NaCl, 0.01; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01; and yeast extract, 1.0. The initial pH was adjusted to 5.3, and the medium was sterilized for 30 min at 115 °C. The cultivation was performed in a volume of 50 mL in 500 mL flasks on a rotary shaker with 220 rpm mechanical stirring at 22 °C for 120 h. Cell growth (in a volume of 5 L) was obtained under bioreactor cultivation conditions. We used Sartorius bioreactor with a working capacity of 5 L, equipped with a turbine stirrer, oxygen (Hamilton, Bonaduz AG, Switzerland) and pH (Hamilton, Bonaduz AG, Switzerland) electrodes. The temperature, agitation speed, air flowrate, foam, and levels in the vessel were monitored by the software program (BioPAT[®]MFCS/DA 3.0). Cultivation was performed for 96 h at 22 °C, 400 rpm and an air rate of 1.00 L/L/min. The cultivation temperature was in line with a possible transfer of the process to an industrial scale. Biomass was obtained from both cultivation methods, and extracts for cytotoxicity and metabolic fingerprinting were prepared from each type of cultivation. The yeast extract was made using 96 h culture in a stationary growth phase. The inoculums were prepared in 500 mL flasks. The cultivation was made in a rotary shaker with 220 rpm mechanical stirring at 22 °C for 48 h.

Biomass production and extracts preparation. Biomass was separated by centrifugation at $6000 \times g$ for 30 min, washed twice with distilled water and lyophilized. Freeze-dried biomass samples (1 g) were re-suspended in 20 mL methanol (100%) and placed in an ultrasonic cleaner for 20 min. The samples were filtered through Whatman[®] filter paper. After double extraction, the collected extract was evaporated to dryness using a rotary evaporator. The dry extract was dissolved in DMSO prior to application.

Cell lines and culture maintaining. Cell lines T-24 (ATCC[®] HTB-4[™], urinary bladder carcinoma) and CCL-1 (NCTC clone 929 [L cell, L-929, derivative of Strain L], ATCC[®] CCL-1[™], non-tumour cell line) were delivered from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cell cultures were maintained at cell density as follows: T-24: 5.4×10^5 viable cells/mL, CCL-1: 5×10^5 viable cells/mL, under standard conditions (37 °C, 5 CO₂, humidified atmosphere). T-24 cells were cultured in 90% RPMI-1640 (#RPMI-HA, Capricorn, 164 Germany) supplemented with 10% (v/v) fetal bovine serum (#FBS-HI-12A, Capricorn, 165 Germany). The CCL-1 cell line was maintained in MEM (#MEM-A, Capricorn, Germany) supplemented with 10% horse serum (#HOS-1A, Capricorn, Germany) and 2 mM L-glutamine (#GLN-B, Capricorn, Germany). Both adherent cell lines were passaged by trypsinization 2–3 times a week.

MTT-assay. The in vitro cytotoxicity was determined with the MTT-dye assay according to Annex C, ISO 10993-5 [8]. Briefly, prior treatment cells were seeded in 96-well plates (1.5×10^5 cells/mL for T-24 and 2×10^5 cells/mL for CCL-1) under sterile conditions (Laminar Air Flow Telstar Bio 2 Advance, Terrassa,

Spain) for 24 h until entering the *log*-phase of the cell growth curve. Thereafter, they were treated with the yeast extracts (from yeasts cultivated in flasks or bioreactor system) at final concentrations ranging from 25 to 400 µg/mL as twofold serial dilutions. The treated and untreated samples were incubated for 72 h. At the end of the incubation period 10 µL MTT-solution (10 mg/mL) were added to each well and the plates were left for 180 min at 37 °C. The formazan crystals formed as a consequence of the reducing activity of mitochondrial dehydrogenases in the vital cells were dissolved with addition of an equivalent volume of organic solvent (2-propranolol acidified with 5% formic acid). The absorbance was measured at 550 nm on ELISA Reader ELx800 (Bio-Tek Instruments Inc., USA) against blank solution (organic solvent with medium and MTT). The results were analyzed using the GraphPad Prizm software and experimental points were fitted to respective concentration-response curves, which served for extrapolation of IC₅₀ values.

Apoptosis assay for protein expression. The pro-apoptotic capacity of the yeast extracts was investigated on the bladder cancer cell line T-24. The analysis was performed with the Proteome Profiler Array Human Apoptosis Array Kit (#ARY009, R&D Systems, Inc, USA) according to the manufacturer's instructions. Briefly, cells were treated with both *S. roseus* extracts (obtained from flasks and bioreactor) and cultivated for 24 h. Thereafter, cells were washed in PBS and centrifuged for 5 min at 2000 rpm. Cell pellets were lysed in a Ripa buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA; 0.5 mM EGTA; 1% Triton X-100; 0.1% Sodium Deoxycholate; 0.1% SDS; 140 mM NaCl; Diluted with dH₂O). Lysates were boiled 10 min and centrifuged at 13 000 rpm for 10 min at 4 °C. Before use Human Apoptosis Detection Antibody Cocktail was dissolved in 100 µL deionized or distilled water. All buffers (Lysis, Array, Wash buffer) were prepared before use. Thereafter, were pipetted 2 mL of Array buffer. This buffer serves as a blocking buffer. After incubation for 1 h, 1.5 mL Lysis Buffer17 was added, followed by Array buffer. The membranes were washed with deionized or distilled water three times. For each membrane were mixed 15 µL of the prepared Detection Antibody Cocktail with 1X Array Buffer 2/3 to 1.5 mL. From prepared Detection Antibody Cocktail were pipetted 1.5 mL to each well. The chemiluminescent signal was acquired with Azure 600 Imaging System (Azure Biosystems, Sierra Court, CA, USA).

Flow cytometry. For flow cytometric analysis 10 000 cells per sample were sufficient. Cells were loaded into a 6-well plate at a starting density that fell within the range of $0.2\text{--}0.5 \times 10^6$ cells/ml and corresponded to the selected cell density. In each well 5 ml of this cell suspension were distributed and the plate was left for 24 h in an incubator at T = 37 °C, 5% CO₂ and maximum humidity. The next day, the cells were treated with the intended cytostatics, and the concentrations of the stock solutions were calculated so that no more than 10–15 µl of the respective stock solution was added per well. The cells were left for the

intended incubation time in an incubator at $T = 37^{\circ}\text{C}$, 5% CO_2 and maximum humidity. Flow cytometric analysis was performed immediately after the incubation time of the cell line with the applied cytostatic. Cells were treated with the extracts and incubated for the indicated period of time. After its outflow, cells were trypsinized and were centrifuged at 1500 rpm, 5 min. Cells from each sample were resuspended in 1 ml PBS, centrifuged and resuspended in 0.5 ml PBS until a cell suspension containing single cells was obtained. In the next step the supernatant was discarded, 4.5 ml of ice-cold 70% ethyl alcohol was added to each sample to fix/permeabilize the cells and incubate at 4°C overnight. The next day, cells were centrifuged at 3000 rpm/5min and resuspended in PBS (5 ml). They were counted and a volume containing the required number of cells was taken. The cell suspension was centrifuged and the cells were resuspended in PBS containing RNAs (concentration 1 mg/ml), following the rule of thumb to resuspend 300 000 cells in 150 μl of PBS. Samples were incubated for 30 min at 37°C . An additional 150 μl of PBS containing propidium iodide (PI) was added to each sample to reach a final PI concentration of 50 $\mu\text{g}/\text{ml}$ and 0.1% Triton X-100. The samples were stored in dark at a temperature of 4°C for 30 min before FACS analysis.

Results. Antiproliferative activity of yeast extracts on malignant and non-tumour cell lines. Cell lines T-24 and CCL-1 were exposed for 72 h to extracts from *S. roseus* AL₁₀₃, cultivated in flasks and bioreactor. Final concentrations ranged between 25 and 400 $\mu\text{g}/\text{mL}$. The median inhibitory concentration (IC_{50}) of the extract determined by the MTT test was used as a parameter for evaluating the antiproliferative effect and for comparison of the cell's sensitivity. The results are summarized in Table 1 and Fig. 1. The IC_{50} values determined for the non-tumour CCL-1 cell line were almost three times higher (343.0 $\mu\text{g}/\text{mL}$ for the flask's and 432.0 $\mu\text{g}/\text{mL}$ for the bioreactor's culture, respectively) than the

T a b l e 1

Median inhibitory concentration of methanol extract from *S. roseus* AL₁₀₃ cultivated in flasks and bioreactor on urinary bladder carcinoma cells and normal mice fibroblasts after 72 h of exposure

Cell line	Parameters measured							
	<i>S. roseus</i> (YExF)				<i>S. roseus</i> (YExB)			
	IC_{50} ($\mu\text{g}/\text{mL}$)	95% CI	<i>R</i>	SI	IC_{50} ($\mu\text{g}/\text{mL}$)	95% CI	<i>R</i>	SI
CCL-1	343	319.7–368.1	0.94		432	343.7–543.1	0.88	
T-24	85.81	72.6–101.4	0.95	3. 99	101	83.65–121.9	0.95	4.28

Legend: IC_{50} – inhibitory concentration 50% (median); 95% CI – confidence interval 95%; *R* – correlation coefficient; SI – selectivity index; YExF – yeast extract, cultivated in flasks; YExB – yeast extract, cultivated in bioreactor

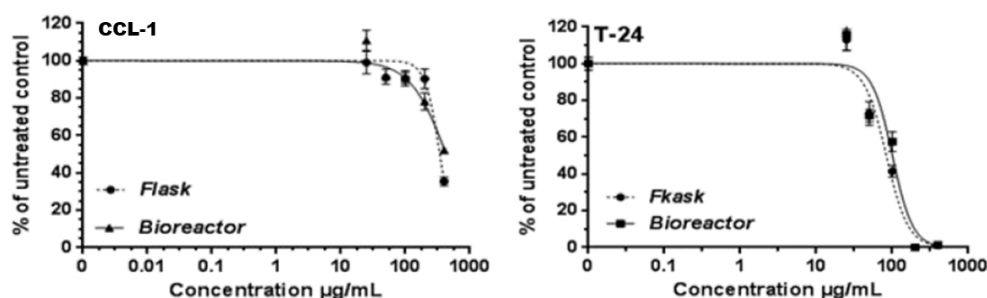


Fig. 1. Antiproliferative effect of methanol extracts obtained from *S. roseus* AL₁₀₃ after 72 h exposure – dose-inhibition curves

IC₅₀ values calculated for cell line T-24 (85.81 µg/mL for the flask's and 101.0 µg/mL for the bioreactor's culture, respectively).

The selectivity index (SI) is a ratio that measures the window between cytotoxicity and antiviral activity by dividing the given AVA value into the TOX value (AVA/TOX). The higher the SI ratio, the theoretically more effective and safe a drug would be during in vivo treatment for a given viral infection. The SI at extract, cultivated in flasks, is higher. This is the reason why we could conclude that this extract is slightly more effective.

Apoptotic effect of yeast extracts. A panel of antiapoptotic proteins were investigated for expression level after treatment with extracts obtained from flask

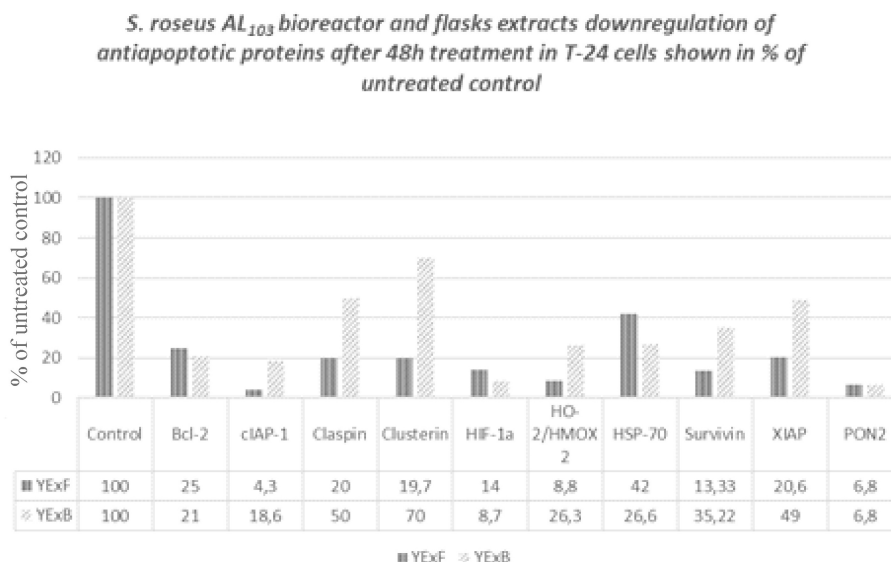


Fig. 2. Expression levels of anti-apoptotic proteins after 48 h treatment of T-24 cells with *S. roseus* AL₁₀₃ extracts obtained from flask or bioreactor culture

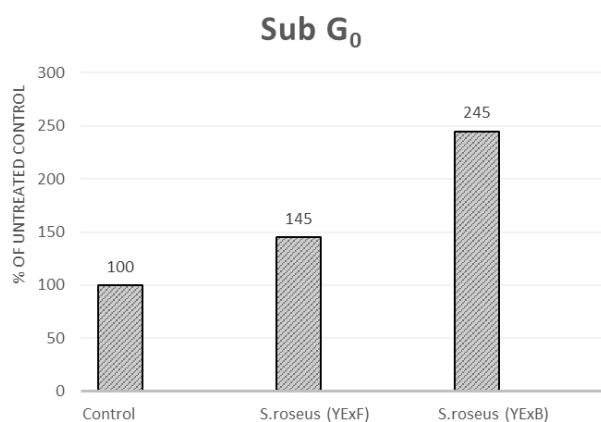


Fig. 3. Graphical representation of the value of SubG₀ after treatment of T-24 with extracts of yeast *S. roseus* AL₁₀₃

or bioreactor culture. The expression levels of the antiapoptotic proteins Bcl-2, cIAP-1, Clapsin, Clusterin, HIF-1a, PON2 and Survivin were suppressed with 65% (Survivin, bioreactor culture) to 95% (cIAP-1, flask culture). The percentage of the downregulation varied among the different signalling molecules independent of the type of the culture. The extract obtained from flask culture was more effective than the extract obtained from the bioreactor culture (Fig. 2). Samples were repeated and no deviation greater than 2% was observed.

Flow cytometry-Nicoletti assay. Flow cytometric analysis showed some increase in the G1-fraction and accumulation of cells in the S-phase. In the control, the S-phase accounted for 8% of the cell cycle, while in the treated with yeast extracts cells this phase occupied 9.2% and 9.58% for the extracts obtained in flasks and bioreactor, respectively. This effect was observed due to a probable G2/M arrest. A typical apoptotic subG₀-fraction is formed. It also showed a relative increase in values – from 0.020% for the untreated control up to 0.029% and 0.049% after treatment with extracts from yeasts cultivated in flasks and bioreactor, respectively (Fig. 3).

Discussion. The extracts of *S. roseus* AL₁₀₃ possessed a diverse spectrum of active substances. Their composition was determined by Gas Chromatography/Mass Spectrometry and the detected substances could be divided into several groups – esters, organic and inorganic acids, carbohydrates. Some of these substances possessed cytotoxic activity. Indole-3-acetic (IAA) acid is recognized as an auxin-type plant hormone. IAA toxicity has been described in T24 bladder carcinoma, breast carcinoma, nasopharyngeal FaDu carcinoma, G361 melanoma cells, pancreatic cancer, and others. A dose-dependent and time-dependent effect of IAA on apoptosis induction in human hematopoietic tumours has been demon-

strated [9]. We can speculate that the results in our study could be due at least in part to these active ingredients.

For our study we used a cell line from urinary bladder carcinoma. This cell line was chosen for detailed investigation of the mode of action of the yeast extracts because one important treatment approach of this malignant disease represents the local administration of appropriate antineoplastic drug which allows at certain conditions the use of concentrations higher than those suitable for systematic therapy (intravesical instillation). The cytotoxic activity of both extracts was dose-dependent. The results from MTT-assay showed that the yeast extracts possessed stronger effect on the cells of the T-24 line than the non-tumour cell line, which is indicative for the selectivity of the yeast extracts investigated. The bioreactor culture extract was more cytotoxic for cells of T-24 ($IC_{50} = 101 \mu\text{g/mL}$) than cells of CCL-1 ($IC_{50} = 432 \mu\text{g/mL}$). The extract of *S. roseus* AL₁₀₃ cultivated in flasks was more effective on the urothelial cancer cell line T-24 ($IC_{50} = 86 \mu\text{g/mL}$) than on the non-tumour cell line CCL-1 ($IC_{50} = 343 \mu\text{g/mL}$).

The propidium iodide (PI) flow cytometric Nicoletti assay after cell membrane permeabilization is being widely used to easily quantify apoptosis in various in vitro experimental models. It is based on the principle that apoptotic cells, in addition to other typical features, are characterized by fragmented nuclear DNA and consequently cells/cell fragments (apoptotic bodies) are formed which contain lower amounts of genomic DNA as compared with cells in the G1 phase of the cell cycle and were therefore referred to as apoptotic subG0 fraction increase [10]. Only one DNA labelling fluorochrome was used, namely propidium iodide (PI). This test allows a quick and relatively precise assessment of the DNA content of the analyzed particles, for example when identifying hypodiploid cells. These flow cytometry data showed that yeast extracts of *S. roseus* AL₁₀₃ lead to a specific increase of the G1-fraction and an accumulation of T-24 cells in the S-phase due to a probable G2/M arrest, too. Most importantly, we observed the formation of a small but typical apoptotic subG0-fraction after 24 h of treatment.

The apoptosis assay revealed a strong inhibition of a number of anti-apoptotic proteins involved in the extrinsic and intrinsic apoptotic pathways after exposure of the bladder cancer cell line T-24 for 24 h to IC_{50} of both extracts obtained from flask or bioreactor culture. The anti-apoptotic protein Bcl-2 was inhibited after exposure to the bioreactor yeast extract which pointed to the ability of the extract to potentiate the induction of the intrinsic apoptotic pathway as far as Bcl-2 was one of the pro-survival proteins that prevent the release of cytochrome-C and the subsequent induction of the initiator caspase-9 [11]. The hypoxia inducible factor (HIF)-1 α is related to potentiation of the tumour migration and invasion [12]. Both extracts inhibited its expression by more than 80% and revealed a possible anti-metastatic potential. Cellular inhibitor of apoptosis 1 and 2 (cIAP-1 and -2) often over-expressed in cancers [13] was also among the proteins inhibited by the yeast extracts tested in the present study with more than 90%. Clusterin expression

has been associated with tumourigenesis and progression of numerous malignancies including bladder cancer [13]. The extract from flasks inhibited the expression levels of Clusterin by about 80%, whereas the bioreactor extract lowered this expression by appr. 30%. First data on the impact of Survivin on the prognosis of patients with urinary bladder cancer indicated that its expression indicates worse prognosis [14]. Interestingly, both extracts tested in our study down-regulated the expression of this protein – by more than 70% for the bioreactor extract, and more than 85% for the flasks extract. Claspin is an unstable protein whose degradation occurs via the proteasome and is regulated via ubiquitination by ubiquitin ligases and deubiquitinases. As a consequence, Claspin levels change during the cell cycle and play a key role in terminating the check-point-mediated cell cycle arrest [15]. Accelerated degradation of Claspin was observed in genetically unstable cells in relation to cancerogenesis. Recently, it was shown that Claspin protects cancer cells from replication stress independently of the checkpoint regulation [16]. Claspin expression was reduced to a greater extent in cells treated with the extract of the flasks culture (appr. 80%) than the bioreactor culture (appr. 50%). The protein Paraoxonase 2 (PON2) which plays pleiotropic roles in different diseases has been shown to be overexpressed in malignant tissues in comparison to corresponding normal tissues in many types of cancers. It modulates the mitochondrial superoxide anion production and the endoplasmic reticulum (ER) stress-induced apoptosis [17]. PON2 was strongly down-regulated by the *S. roseus* AL₁₀₃ extracts only after 24 h incubation (> 90% after treatment with both extracts). The data from the apoptosis assay show that both yeast extracts possess strong potential to inhibit various anti-apoptotic proteins which reveal a mode of action related to the induction of apoptosis and inhibition of the cellular proliferation and migration. Taken together the experimental findings indicate that the active ingredients in the extracts rather normalize the otherwise overexpressed anti-apoptotic proteins in tumour cells.

Conclusions. In our study, Antarctic yeast extracts showed significant antitumour effects in vitro with an expected good safety profile and therefore could be used in the complex treatment of urinary bladder cancer. The antineoplastic potential of the investigated psychrophilic yeast extracts, analyzed for the first time by us, possessed a noteworthy therapeutic potential, which needs further detailed pharmacological and toxicological characterization of the extract individual constituents.

Acknowledgments. We thank the staff of the Bulgarian Antarctic Expedition for their logistic support. Special thanks are due to Assoc. Prof. Maya Zaharieva from The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Department of Infectious Microbiology, for her valuable contribution to carrying out the experimental work, as well as for the consultation in writing the article.

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