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Cantharellus cibarius branched mannans inhibits colon cancer cells growth by interfering with signals transduction in NF-κB pathway

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ABSTRACT

In order to evaluate the therapeutic potential of polysaccharides obtained from the widely consumed *Cantharellus cibarius* mushroom on NF-κB pathway involved in cancer cells proliferation, survival, and metastasis, their antiproliferative and cytotoxic properties in human colon cancer cells LS180 and human colon epithelial cells CCD841 CoN were studied. BrdU and LDH assays results show that a branched mannan isolated from *C. cibarius* (CC2a) was selective against colon cancer cells, suppressing their proliferation and destroying membrane integrity but at the same time not adversely affecting colon epithelial cells. Results of Western blotting, immunofluorescence, RealTime PCR revealed that CC2a anticancer abilities were accompanied by disorders in signals transduction in NF-κB pathway in particular inhibition of IκBα degradation, attenuation of activated NF-κB phosphorylation and the subsequent decrease of NF-κB nuclear level as well as significant down-regulation of NF-κB target genes *BAX*, *BCL2*, *CCND1*, *MMP9*, *MYC*, *BIRC5* and the corresponding proteins (except Bax). Furthermore, CC2a treatment of LS180 cells resulted in perturbation in G0/G and S phases of the cell cycle also associated with a marked increase of DNA fragmentation as well as inhibition of cancer cells motility. Obtained results indicated *C. cibarius* branched mannans as a new option in fighting with colon cancer.

1. Introduction

More than 1 million new cases of colorectal cancer (CRC) are diagnosed each year, being the 3rd most common malignancy and 4th most common cause of cancer mortality worldwide [1]. Despite medical advances, the incidence of this disease is systematically increasing and therefore it is necessary to seek new and more effective therapeutic strategies. These new strategies should take into account the following facts: 1) the largest number of CRC cases has been linked to environmental causes rather than to inherited genetic changes. Food-borne mutagens play a dominant role in the development of CRC [2]; 2) Similar to other types of cancer, CRC use the body's natural processes for its growth and metastasis. It has been proved that one of these processes is inflammation. The ability of cancer to induce inflammation and use the associated phenomena's for its progression is attributed to the transcription factor NF-κB [3–7]. Also due to its pro-inflammatory action, NF-κB also promotes neoplastic transformation by stimulation of cell proliferation and angiogenesis, inhibition of apoptosis and increase in the invasiveness of tumor cells [5,8,9]. It has also been proved that NF-κB is responsible for the resistance of cancer cells to chemotherapy and radiotherapy [10]; 3) Colorectal carcinogenesis is a multistage process which, when diagnosed early, increases the chance of patients to recover. As tumor development takes many years and early diagnosis is extremely difficult, primary lesions counteraction seems to play a fundamental role in cancer protection [11].

In view of these data, CRC prevention by changing unhealthy eating habits and increasing the intake of beneficial components of the daily diet able to prevent the transduction of signals in the NF-κB pathway seems to be one of the most effective strategies for decreasing the incidence of CRC [12]. The assumption of this strategy is compatible with compounds derived from edible mushrooms, the beneficial effect on health and usefulness in the treatment of cancer have been reported recently [13,14]. The anticancer properties of edible mushrooms are mainly attributed to polysaccharides and their derivatives, which are able to prevent carcinogenesis and inhibit the development of existing tumor lesions [13–18]. Their beneficial effect is mainly based on immunestimulation [15,17,18]; nevertheless, several direct anticancer mechanisms were also reported. One of them is a modulation of the NF-κB pathway in which the inhibition of phosphorylation and/or degradation of IKK and/or IκBα are noted. This allows inactivation of the

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NF-κB and NF-κB-dependent genes responsible for the induction of inflammation, stimulation of proliferation and inhibition of apoptosis [19,20].

An interesting and promising source of new anticancer polysaccharides appears to be *Cantharellus cibarius* (golden chanterelle), one of the widely-occurring edible mushrooms. Its anticancer properties against Sarcoma 180 and Ehrlich solid cancer were reported in 1973 by Ohtsuka et al. in mice studies [21]. Despite promising results, for many years nobody made any effort to isolate and characterize polysaccharides from this fungus, and elucidate the mechanism responsible for their anticancer properties. The latest studies by Nowacka-Jechalke et al. indicate that *C. cibarius* crude polysaccharides inhibited the proliferation of colon cancer cells (LS180), with the simultaneous absence of toxicity towards colon epithelial cells (CCD841 CoTr) [22]. Unfortunately, the mentioned studies did not explore the molecular mechanism of the observed anticancer properties, primarily because the observed antiproliferative effect was very weak (concentration causing proliferation inhibition at 50% compared to the control "IC_{50}" calculated for MTT test conducted in LS180 cells after 96 h of treatment was 624 μg/ml). Our team was also able to isolate and purify different polysaccharide-rich fractions from *C. cibarius* [23], which revealed greater antiproliferative abilities against LS180 cells than the polysaccharides described by Nowacka-Jechalke et al. [22]. IC_{50} values calculated for three fractions with the highest content of polysaccharides were as follows: 7531.9 μg/ml (36.9% of polysaccharides); 65.7 μg/ml (69.2% of polysaccharides); 71.4 μg/ml (53.2% of polysaccharides) [24]. Despite very promising results, the molecular mechanism of the anticancer activity of the investigated polysaccharides/polysaccharide-rich fractions was not explored. Therefore, the presented study was undertaken to examine the anticancer effect of *C. cibarius* polysaccharides/polysaccharide-rich fractions in an in vitro model of CRC, and investigate their impact on the transduction of signals in the NF-κB pathway as a possible mechanism responsible for their beneficial effects.

2. Materials and method

2.1. Reagents

Unless otherwise indicated chemical reagents used in the study were purchased from Sigma-Aldrich Co. LLC. Fractions stock solutions (10 mg/ml) were prepared in PBS (buffered saline solution) and stored at 4 °C.

2.2. Fungal origin

The wild-grown fruitbodies of *C. cibarius* were collected in a mixed forest stand of pine and oak (Vila Real, 41°28′N; 7°73′W; 430 m). Representative samples of collected *C. cibarius* fruitbodies were deposited at the mycological herbarium of the University of Trás-os-Montes e Alto Douro, Portugal.

2.3. Extraction and purification of C. cibarius biopolimers

For the preparation of the alcohol insoluble residue (AIR), water soluble material (WSM) and water soluble biopolymers (WSB) by anion-exchange chromatography the method described by Lemieszek et al. [24–26] was used. Sugar composition of the AIR, WSM, WSB and purified fractions were determined after hydrolysis with sulfuric acid 1 M at 100 °C during 2.5 h [24–26], by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD, ICS-3000, Dionex) using a CarboPac PA-20 column (150 mm × 3 mm) with a CarboPac PA20 pre-column (Dionex) equipped with an electrochemical detector together with an Au working electrode, Ag/AgCl reference electrode and Ti counter electrode. Linkage position analysis of sugars was performed by methylation using the method described by Coimbra et al. [27], followed by a remethylation to ensure complete methylation of the polysaccharides [28]. Protein content was determined by the Bradford colorimetric assay using bovine serum albumin (BSA) as the protein standard [29]. The fourier transform infrared (FTIR) single-reflection ATR spectrum using a diamond crystal of the CC2a fraction was recorded using a Shimadzu IRAffinity-1S in the range 4000 to 500 cm^{-1} at a resolution of 8 cm^{-1}. The spectrum resulted from the co-addition of 128 scans.

2.4. Cell lines

Human colon adenocarcinoma cell line LS180 was purchased from the European Collection of Cell Cultures (ECACC, Centre for Applied Microbiology and Research, Salisbury, UK). Human colon epithelial cells CCD841 CoN was purchased from the American Type Culture Collection (ATCC, Menassas, VA, USA). LS180 cells were grown on Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham. CCD841 CoN cells were grown on Dulbecco's Modified Eagle's Medium. The media were supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO2 and 95% air.

2.5. Lactate dehydrogenase (LDH) based cytotoxicity assay

LS180 and CCD841 CoN cells were plated on a 96-well microplates at a density of 1×10^5 cells/ml. The following day, the culture medium was exchanged with fresh medium containing the tested fractions at concentrations 10, 50, 100 and 250 μg/ml. Fractions cytotoxicity was examined after 24 h using the in vitro Toxicology Assay Kit Lactate Dehydrogenase Based. LDH release were then quantified spectrophotometrically at 450 nm using a ELx800 Microplate Reader (BioTek Instruments Inc., Highland Park, Winooski, Vermont, USA).

2.6. BrdU incorporation assay

LS180 and CCD841 CoN cells were seeded on 96-well microplates at a density 5×10^4 cells/ml. Next day, the culture medium was removed and cells were exposed to tested fractions (10, 50, 100 and 250 μg/ml) for 48 h. Assessment of cells proliferation was performed by immunoassay using the Cell Proliferation ELISA BrdU (Roche Diagnostics GmbH, Penzberg, Germany) protocol. Absorbance was measured at 450 nm wavelength using ELx800 Microplate Reader.

2.7. May-Grünwald-Giemsa staining

A density of 5×10^4 cells/ml of LS180 and CCD841 CoN cells were plated on a Lab-Tek Chambers Slide (Nunc). After 24 h the culture

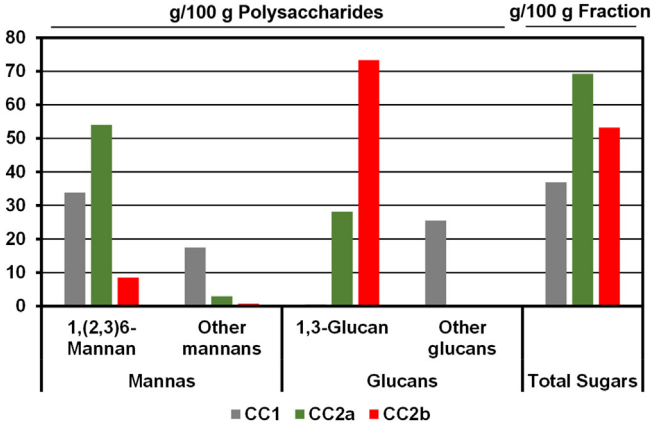

Fig. 1. Chemical composition of polysaccharides isolated from *C. cibarius* mushrooms and purified by anion-exchange chromatography. CC1 - non-retained fraction; CC2a and CC2b - retained fractions.

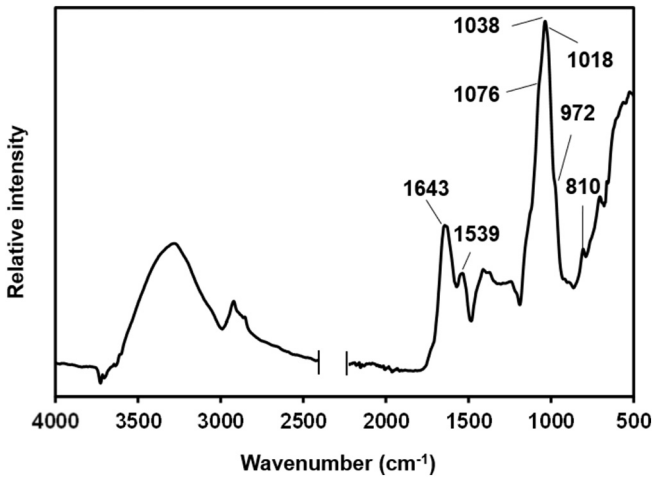

Fig. 2. FTIR spectra of the CC2a fraction in the 500–4000 cm^{-1} region.

medium was replaced with fresh medium supplemented with 50 and 100 μg/ml of fraction CC2a. After 48 h, cells were stained with the May-Grünwald-Giemsa method. Observations were performed by light microscope, Olympus BX51 System Microscope (Olympus Optical Co., Japan) at magnification 200×. The images acquired were analyzed using analySIS software (Soft Imaging System GmbH, Münster, Germany).

2.8. Western blot

LS180 cells were seeded on a 6-well microplates at 2×10^5 cells/ml density. Next day, the culture medium was removed and cells were incubated with 50 and 100 μg/ml fraction CC2a for 6 and/or 24 h. Cells were then washed with ice-cold PBS, harvested and lysed using NE-PER Nuclear and Cytoplasmic Extraction (Thermo Fisher Scientific, Rockford, USA) protocol. Protein concentration in obtained cytoplasmic and nuclear lysates was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, USA). Expression of selected proteins was examined by Western blotting method as described by Lemieszek et al. [25]. The research was performed with primary antibodies selective for: IKKβ, p-IKKβ (Ser177), IκBα, p-IκBα (Ser32), NF-κB, p-NF-κB (Ser536), Bax, Bcl2, Survivin, Cyclin D1, cMyc, MMP9 (1:1000) (Cell Signaling Technology, Beverly, MA, USA). Densitometric analysis of obtained western blots was performed using ImageJ software.

2.9. Immunocytochemistry

A density of 1×10^5 cells/ml of LS180 cells were plated on Lab-Tek Chambers Slide (Nunc). On the following day, cells were treated with fraction CC2a at concentrations of 50 and 100 μg/ml. After 24 h of treatment cells were fixed with 3.7% paraformaldehyde, permeabilized with 0.2% Triton X-100, and incubated overnight with primary antibodies specific for: p-IKKβ (Ser177), p-IκBα (Ser32), NF-κB, p-NF-κB (Ser536) (1:100) (Cell Signaling Technology, Beverly, MA, USA). Cells were then exposed to secondary FITC-conjugated antibodies for 2 h at

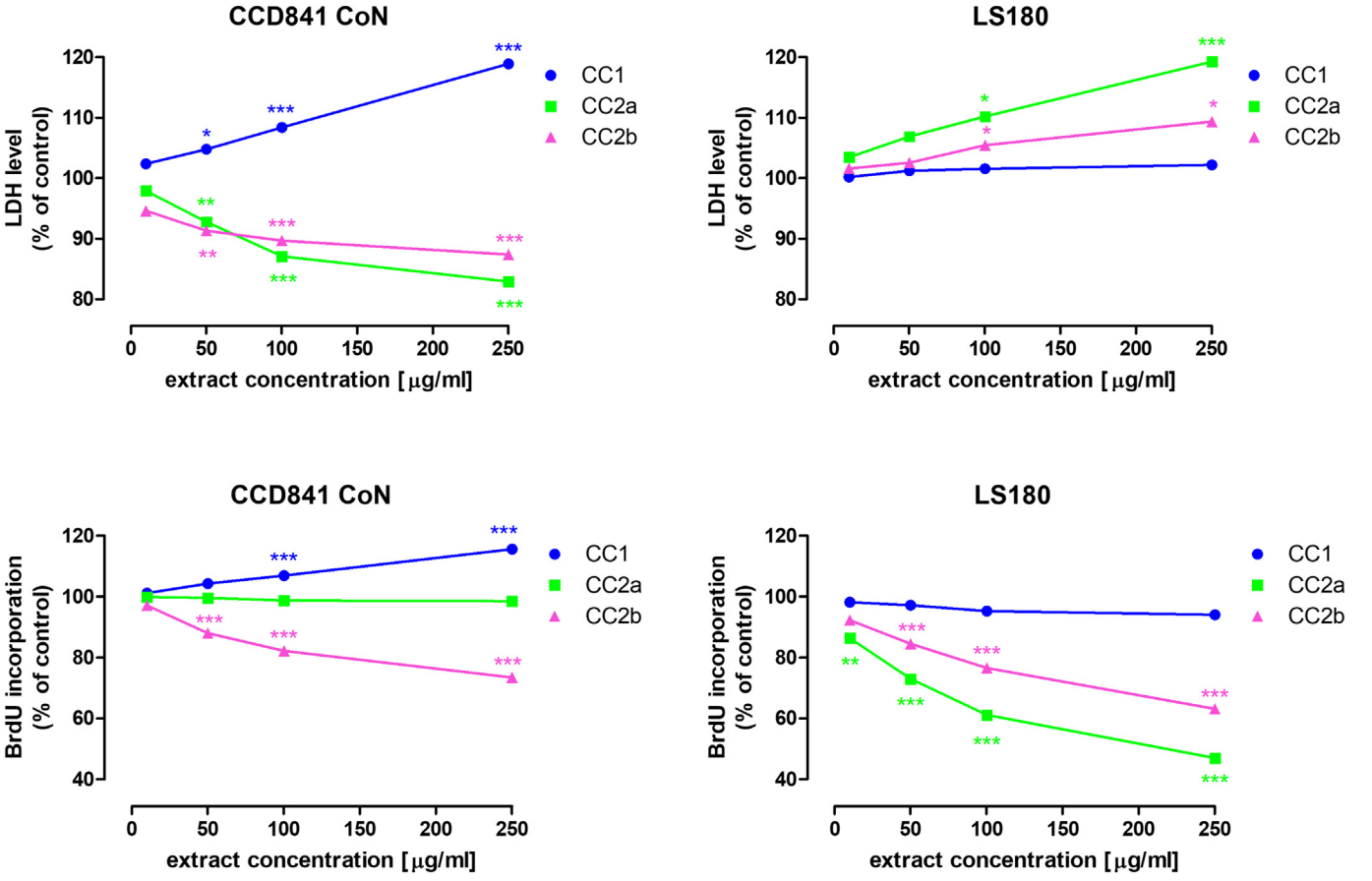

Fig. 3. Influence of *C. cibarius* polysaccharides fractions on cytotoxicity and proliferation of human colon epithelial cells CCD841 CoN and human colon adenocarcinoma cells LS180. Cells were exposed to investigated polysaccharides at concentrations ranging from 10 to 250 μg/ml. Cytotoxicity was measured after 24 h of cells treatment with use of LDH assay. Antiproliferative activity was assessed after 48 h of cells treatment using BrdU assay. Results are presented as the mean of 5 measurements. *** $p < 0.001$ vs. control; ** $p < 0.01$ vs. control; * $p < 0.05$ vs. control; one-way ANOVA test; post hoc test: Tukey.

room temperature. Cell images were captured with fluorescence microscopy Olympus BX51 System Microscope at magnification 400×. Obtained images were analyzed using the analySIS software.

2.10. Real Time PCR

LS180 cells were seeded on 6-well microplates at a density of 2×10^5 cells/ml. The following day, cells were exposed to 50 and 100 μg/ml of fraction CC2a. After 24 h cells were washed with ice-cold PBS and harvested. Then the total RNA was extracted using HighPure RNA Isolation Kit (Roche Diagnostics GmbH) and 2 μg of obtained RNA was transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in TProfessional Basic Thermocycler (Biometra) under the following conditions: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min and 4 °C for 5 min. Real Time PCR analysis was conducted using the following: TaqMan Gene Expression Assays (Hs00414514_m1 for BAX (Bax); Hs00608023_m1 for BCL2 (Bcl2); Hs00765553_m1 for CCND1 (Cyclin D1); Hs00234579_m1 for MMP9 (MMP9); Hs00905030_m1 for MYC (c-Myc); Hs00153353_m1 for BIRC5 (Survivin); Hs00357333_g1 for ACTB (β-actin) and TaqMan Fast Universal PCR MasterMix (Applied Biosystems, Foster City, CA, USA). The relative quantification of selected genes was performed using a 7500 Fast Real-Time PCR Systems (Applied Biosystems) under the following conditions: 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Gene expression was calculated using the relative advanced quantification and normalized to *ACTB* expression (SDS 1.4 Software for the 7500 Fast System, Applied Biosystems).

2.11. Flow cytometry

LS180 cells were plated on 6-well plates at 2×10^5 cells/ml density. The following day, cells were treated with fraction CC2a in concentrations of 50 and 100 μg/ml. After 24 h cancer cells were harvested and fixed with 70% ethanol and stored at −20 °C until further analysis. In the next step, cells were incubated in 0.1% Triton X-100 supplemented with 100 μg/ml of RNase and 10 μg/ml of propidium iodide on ice for 30 min. Distribution of cells at the different stages of the cell cycle was analyzed using a BD FACS Calibur (Becton-Dickinson). Obtained results were analyzed using the BD's Cell Quest software (Becton-Dickinson).

2.12. Cell death detection ELISA

A density of 2×10^5 cells/ml of LS180 cells were seeded on 96-well plates. After 24 h the culture medium was replaced with fresh medium containing 50 and 100 μg/ml of fraction CC2a and further incubated for 24 h. Apoptosis induction was then assessed using the Cell Death Detection ELISAPLUS Kit (Roche Diagnostics, Mannheim, Germany). Absorbance was measured at 405 nm wavelength using the ELx800 Microplate Reader.

2.13. Wound assay

LS180 cells were seeded at 4×10^5 cells/ml density on 3 cm culture dishes (Nunc). The next day, the cell monolayer was scratched by a pipette tip creating the "wound" (cell-free gap). One part of dishes were immediately stained with the May-Grünwald-Giemsa method and as "wound" they determined area of cell counting. Cells growing on the second part of dishes were exposed to culture medium alone (control) or supplemented with fraction CC2a (50 and 100 μg/ml). After 24 h of treatment, cells were stained with the May-Grünwald-Giemsa method and the number of cancer cells migrated into the wound area was estimated, as previously described [30].

Table 1
Cytotoxic concentration 50% (CC_{50}) and inhibition concentration 50% (IC_{50}) values of *Cantharellus cibarius* polysaccharide fractions.

	CCD841 CoN CC_{50} [μg/ml]	LS180 CC_{50} [μg/ml]	CCD841 CoN IC_{50} [μg/ml]	LS180 IC_{50} [μg/ml]
CC1	1212	386,218	Not converged	121,236
CC2a	Not converged	1957	180,264	206
CC2b	Not converged	6110	1098	560

2.14. Statistical analysis

Data are presented as the mean value and standard error of the mean (SEM). Statistical analysis was performed by one way-ANOVA with Tukey *post-hoc* test. Statistical significance was accepted at $p < 0.05$. The CC_{50} value (concentration causing induction of cytotoxicity at 50% compared to the control) was calculated based on results obtained from LDH test. The IC_{50} value (concentration causing proliferation inhibition at 50% compared to the control) was calculated based on results obtained from the BrdU test.

3. Results

3.1. Structural features of C. cibarius polysaccharides

The alcohol insoluble and water soluble high molecular weight polysaccharides isolated from *C. cibarius* and purified by anion-exchange chromatography were composed by three distinct polysaccharide/polysaccharide-rich fractions, one of which was not retained in the anion-exchange column (CC1, 36.9% sugar content), and two fractions retained and eluted with sodium chloride from the anion-exchange column (CC2a and CC2b with 69.2% and 53.2% sugar content) [23]. These fractions were free from ribonucleic acids, demonstrated by the absence of ribose, and presented different amounts of the two most abundant

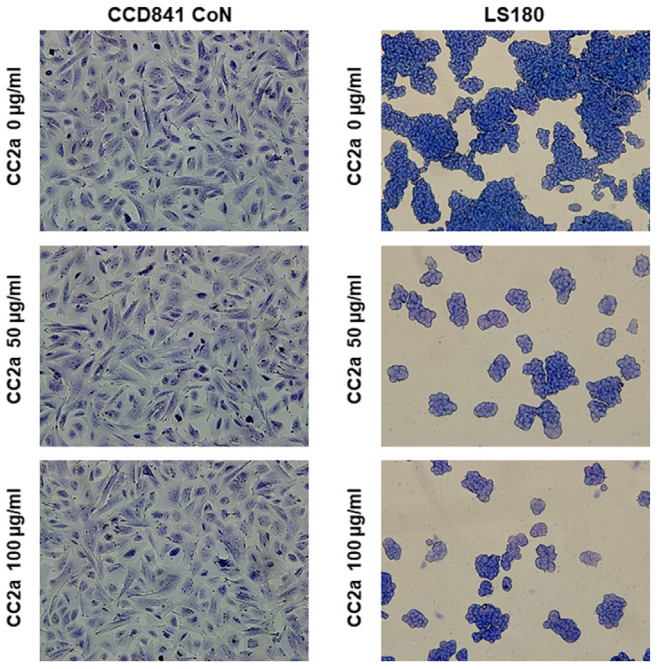

Fig. 4. Impact of *C. cibarius* polysaccharide fraction CC2a on morphology of human colon epithelial cells CCD841 CoN and human colon adenocarcinoma cells LS180. Cells were incubated for 48 h alone, or in the presence of CC2a fraction at concentrations 50 and 100 μg/ml and then staining with May-Grünwald-Giemsa and examined under light microscopy. Representative pictures were obtained from two independent experiments. Magnification 200×.

polysaccharides present in the extract: an O-2 and O-3 branched 1,6-linked mannan and a linear 1,3-linked glucan (Fig. 1) [23]. The two retained fractions richer in polysaccharides had a distinct polysaccharide composition, with CC2a being mainly composed of the O-2 and O-3 branched 1,6-linked mannan (54%), although also containing linear 1,3-glucan (28%), and CC2b being almost exclusively composed of a linear 1,3-linked glucan (73%). CC2a branched mannan enriched fraction was further characterized by FTIR spectroscopy to inspect for other components (Fig. 2). The broadband occurring at the region from 3567 to 3057 cm^{-1} was assigned to stretching vibration of O—H carbohydrate groups and hydrogen bonds [31]. Structural information was obtained from the carbohydrate region (1200–950 cm^{-1}), containing highly overlapping intense bands of C—O and C—C stretching vibrations in glycosidic bonds and pyranoside ring and from the anomeric region (950–750 cm^{-1}), assigned to weak bands of complex skeletal vibrations sensitive to anomeric structure [32]. The band at 810 cm^{-1} is characteristics of mannans and has been used to differentiate mannans from other polysaccharides [33]. The spectral features of the carbohydrate region is similar to the mannans of *Sacharomyces cerviseae* and *Candida albicans* [33–35]. It can also be seen clearly in the spectrum the amide I protein band (1643 cm^{-1}) assigned mainly to the stretching vibrations of the C═O with a lesser contribution from C—N groups and the amide II (1539 cm^{-1}) protein band assigned mainly to the in-plane N—H bending and to a lesser extent to the C—N and C—C stretching vibrations [36] Quantification of the protein present in CC2a fraction using the Bradford colorimetric assay with BSA as protein standard allowed to estimate a protein content of 4.6 ± 0.4 g/100 g. Taking into account that CC2a fraction was retained in the anion-exchange column and eluted with increasing salt concentrations, no uronic acids could be detected by sugar analysis and additionally there was observed a co-elution of the carbohydrate peak and absorbance at 280 nm when CC2a was analyzed by SEC [23], strongly suggests that CC2a is a branched mannan glycoprotein.

3.2. Therapeutic potential of C. cibarius polysaccharide/polysaccharide-rich fractions in colon cancer in vitro model

The influence of *C. cibarius* branched mannan (CC2a) and polysaccharide-rich fractions (CC1 and CC2b fractions) on cytotoxicity and proliferation of human colon epithelial cells CCD841 CoN and human colon adenocarcinoma cells LS180, were examined after 24 and 48 h of treatment by LDH and BrdU assays, respectively (Fig. 3, Table 1).

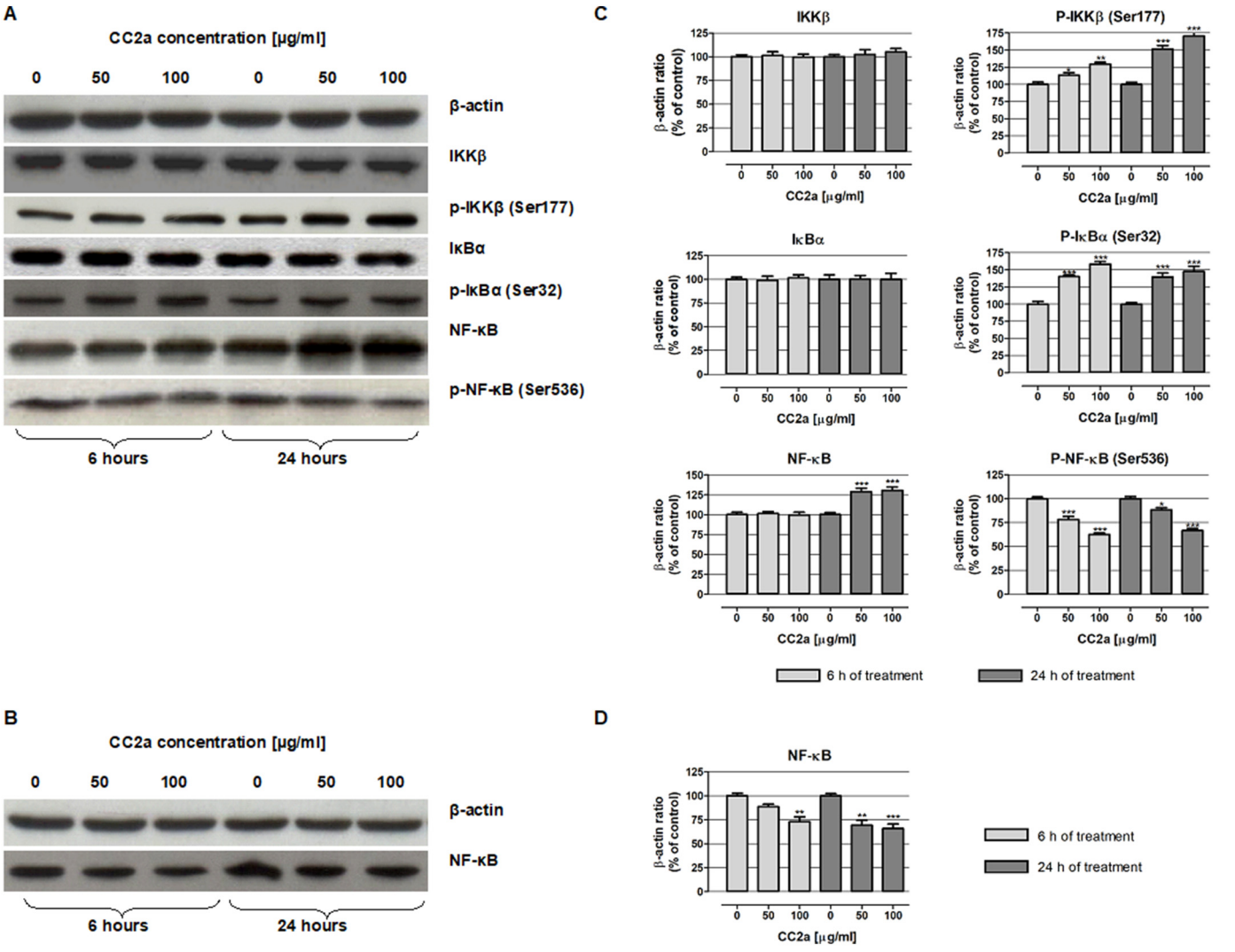

Fig. 5. Expression of components of NF-κB pathway in human colon adenocarcinoma cells LS180 treated with *C. cibarius* polysaccharide fraction CC2a. Cells were exposed for 6 h and 24 h to culture medium alone, and tested fraction at concentrations 50 µg/ml and 100 µg/ml. Protein expression was examined in cytoplasmic (A, C) and nuclear (B, D) cells lysates by Western blotting using target specific antibodies. Examination of ß-actin expression level was used as internal control. Representative Western blots with densitometric analyses of 3 independent experiments are presented. *** $p < 0.001$ vs. control; ** $p < 0.01$ vs. control; * $p < 0.05$ vs. control; one-way ANOVA test; post hoc test: Tukey.

Results of the LDH test showed a significant decrease in lactate dehydrogenase release from colon epithelial cells after treatment with CC2a and CC2b (50, 100, 250 μg/ml), while fraction CC1 showed a cytotoxic effect under the same conditions. At the same time, fraction CC2a and CC2b in concentrations of 100 and 250 μg/ml damaged the cell membranes of colon cancer cell line LS180, causing an increase in LDH level. CC_{50} values calculated for the fractions assayed were 1957 μg/ml and 6110 μg/ml, respectively. Fraction CC1 in the whole range of tested concentrations did not affect the integrity of colon cancer cells; nevertheless, the calculated CC_{50} value was 386,218 μg/ml.

Results of the BrdU test demonstrated significant inhibition in the proliferation of both normal and cancer cells after exposure to fraction CC2b used in concentrations of 50, 100, 250 μg/ml; however, LS180 cells were more sensitive to the tested fraction than epithelial cells. IC_{50} value was 1098 μg/ml for CCD841 CoN cells and 560 μg/ml for LS180 cells. A different effect was observed for CC1. This fraction in concentrations of 100 and 250 μg/ml stimulated proliferation of epithelial cells, without affecting DNA synthesis in colon cancer cells. On the contrary, fraction CC2a in the whole range of tested concentrations effectively inhibited proliferation of colon cancer cells (IC_{50} value - 206 μg/ml), without influencing DNA synthesis in epithelial cells (IC_{50} value - 180,264 μg/ml).

Because of the greatest anticancer properties indicated by the highest efficiency in inhibition of colon cancer cells proliferation (IC_{50} = 206 μg/ml) and disintegration of colon cancer cell membranes (CC_{50} = 1957 μg/ml) as well as beneficial effect on colon cancer cells (decrease of LDH release; no influence on cells proliferation) (Table 1), branched mannan fraction CC2a was selected for additional studies.

The beneficial anticancer properties of fraction CC2a and its selectivity were further demonstrated by May-Grünwald-Giemsa staining of CCD841 CoN and LS180 cells after 48 h treatment with 50 and 100 μg/ml fraction CC2a. The tested fraction did not cause any changes in the morphology of colon epithelial cells while at the same time significantly inhibited the proliferation of colon cancer cells and interrupting the integrity of cell membranes (Fig. 4).

3.3. C. cibarius branched mannan fraction CC2a disturbs signals transduction in NF-κB pathway in colon cancer cells

The next step in the study was to examine the influence of *C. cibarius* branched mannan fraction CC2a on the expression and phosphorylation of key proteins involved in the transduction of signals in the classical NF-κB pathway. Changes were recorded after 6 and 24 h of LS180 cells treatment with fraction CC2a at concentrations of 50 and 100 μg/ml using Western Blotting method.

The tested fraction did not affect the concentration of IKKβ and IκBα in LS180 cells but at the same time significantly increased their phosphorylation (Fig. 5). Treatment of LS180 cells with 100 μg/ml of CC2a increased phosphorylation of IKKβ (Ser177) to 130% (6 h) and 171% (24 h), while phosphorylation of IκBα (Ser32) under the same conditions reached the levels of 158% and 140%, respectively. It needs to be highlighted that changes in the concentration of p-IKKβ (Ser177) were dependent on both dose and time while intensity of IκBα phosphorylation at residue Ser32 was only dose dependent. The concentration of NF-κB was investigated in two cell compartments, i.e. cytoplasm and nucleus. The results obtained revealed a significant accumulation of NF-κB up to 28% (CC2a concentration of 50 μg/ml) and 30% (concentration of 100 μg/ml) in the cytoplasm of LS180 cells after 24 h of treatment. At the same time a decrease in NF-κB phosphorylation at residue Ser536 was observed, however a more significant effect was observed after 6 h of cancer cells treatment with 50 μg/ml of CC2a (73%) and 100 μg/ml of CC2a (60%). The changes observed in the expression of NF-κB in the cytoplasm of LS180 cells was accompanied by a decrease of NF-κB level in the nucleus, 24 h exposure to the tested fraction inhibited NF-κB translocation by 31% and 34%, respectively. Observed alterations in phosphorylation of IKKβ, IκBα, NF-κB as well as inhibition of NF-κB translocation from cytoplasm to nucleus indicated that CC2a was an effective modulator of signals transduction in the classical NF-κB pathway.

Immunocytostaining was applied to visualize the changes in the phosphorylation of IKKβ, IκBα and NF-κB, as well as the localization of

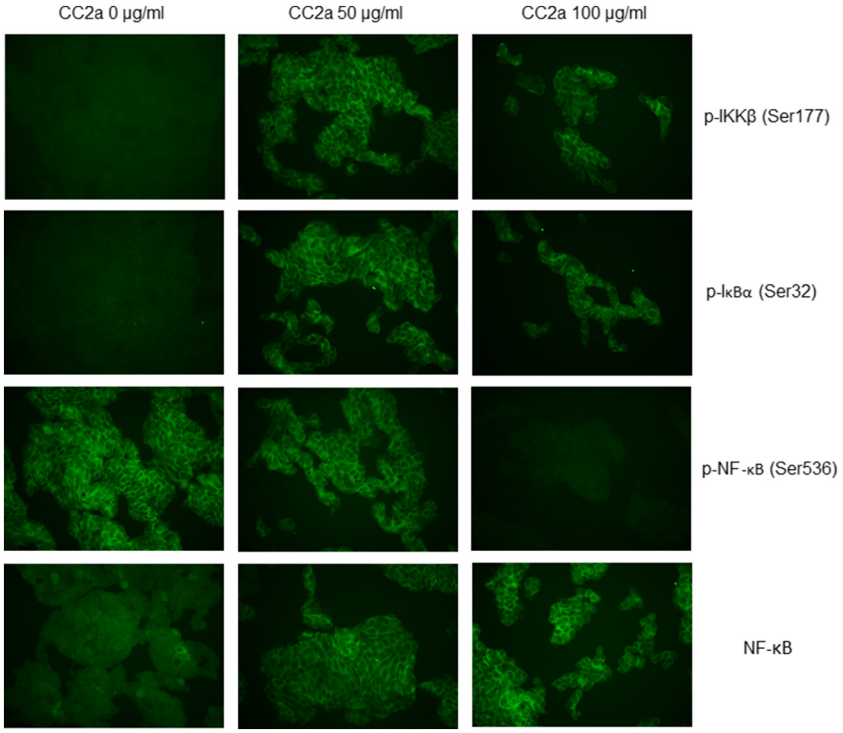

Fig. 6. Influence of *C. cibarius* polysaccharide fraction CC2a on the phosphorylation of selected components of NF-κB pathway and translocation of NF-κB in human colon adenocarcinoma cells LS180. Cells were exposed for 24 h to culture medium alone, and tested fraction at concentrations 50 and 100 μg/ml. Cells were then stained with target specific antibodies and examined under fluorescence microscopy. Representative pictures were obtained from 2 independent experiments. 400× magnification.

NF-κB within the LS180 cells exposed to fraction CC2a at concentrations 50 and 100 μg/ml for 24 h. An increased levels of phosphorylation of IKKβ, at residues Ser177 and IκBα at residues Ser32, were clearly observed (Fig. 6). At the same time a significant decrease in NF-κB phosphorylation at residue Ser536 was noted. Furthermore, cell localization of NF-κB was changed due to the treatment with fraction CC2a, and target protein was accumulated in the cytoplasm of treated cells. These results are in accordance with those from Western Blotting.

3.4. C. cibarius branched mannan fraction CC2a inhibits expression of NF-κB target genes and their protein products in colon cancer cells

Since we found that fraction CC2a inhibited NF-κB translocation from cytoplasm to nucleus, its influence on the expression of NF-κB target genes and their protein products was investigated after 24 h of LS180 cells treatment with 50 and 100 μg/ml of CC2a by Real Time PCR and Western Blotting, respectively.

Results of Real Time PCR revealed, that fraction CC2a down-regulated expression of all the investigated genes; the observed effect was dose-dependent (Fig. 7A). The most significant inhibition was noted in *CCND1*, the expression of which decreased by 27% and 41% in response to 50 and 100 μg/ml of fraction CC2a. Equally strong changes were observed in *MYC*, the expression of which after CC2a treatment reached 77% and 64%, respectively, compared to untreated cells. The least sensitive to CC2a treatment proved to be *BAX*, the expression of which decreased only by 7% and 15%, respectively. It has to be noted that the *BAX*/*BCL2* ratio calculated for both investigate CC2a concentrations was the same and amounted to 1.16.

Western Blotting evaluation has shown significant dose-dependent decrease of expression of all the examined proteins, except Bax, which level was not affected by the tested fraction (Fig. 7B). The strongest inhibition in response to 50 and 100 μg/ml of fraction CC2a was noted in case of Cyclin D1, the expression of which decreased by 42% and 51%, respectively. The significant reduction of cMyc and Bcl2 levels was also observed, their expression reached 73% and 75% after treatment with 50 μg/ml of CC2a as well as 64% and 58% in response to 100 μg/ml of CC2a. Alterations in the expression of MMP9 and Survivin after CC2a treatment were at a quite similar level. Performed studies also revealed evident increase of Bax/Bcl2 ration which amounted 1.34 and 1.72 for the subsequent analyzed CC2a concentrations.

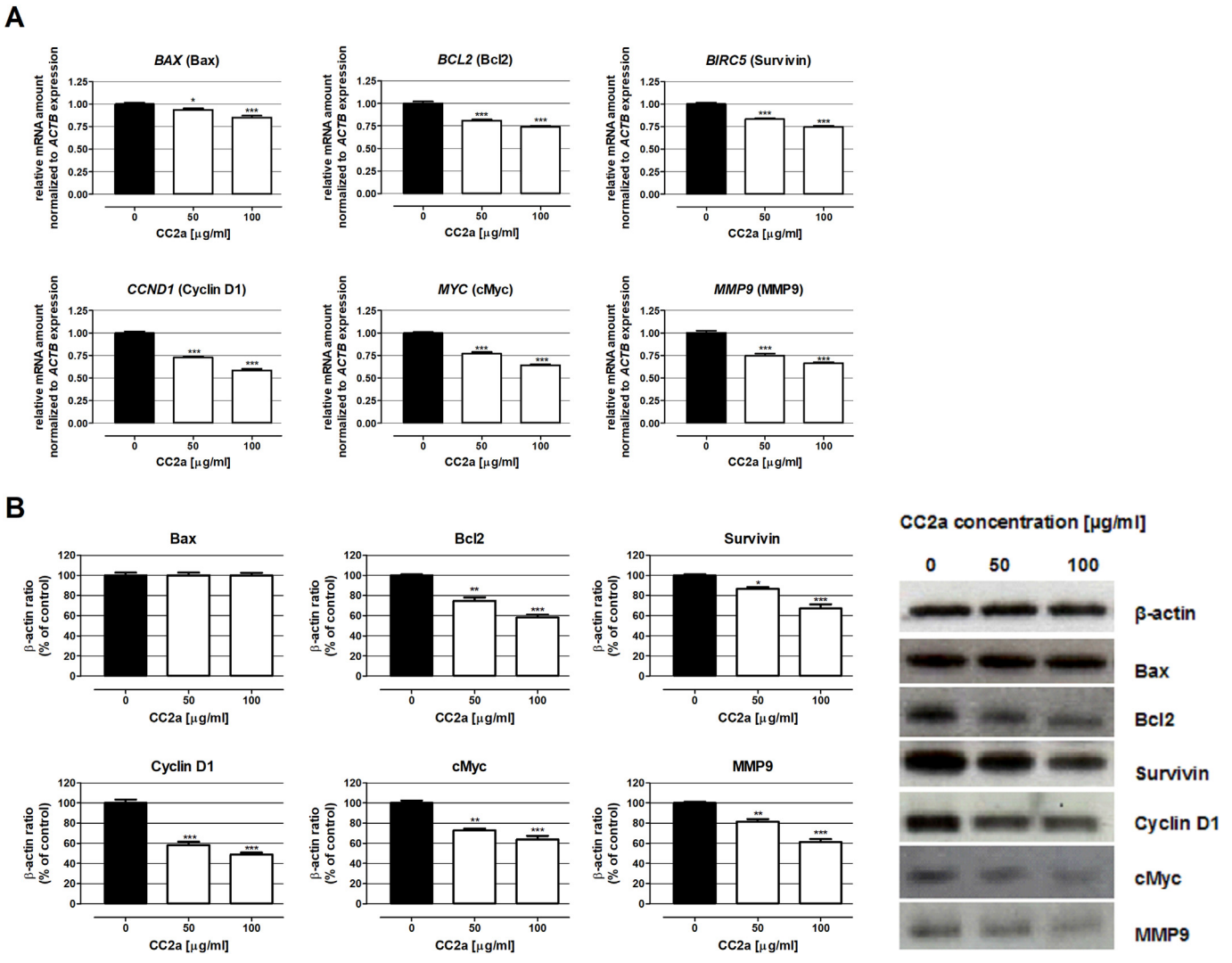

Fig. 7. Expression of selected NF-κB target genes and their proteins products in human colon adenocarcinoma cells LS180 treated with *C. cibarius* polysaccharide fraction CC2a. Cells were exposed for 24 h to culture medium alone, and tested fraction at concentrations 50 μg/ml and 100 μg/ml. A) Gene expression was analyzed by Real Time PCR using target specific primers. Results of Real Time PCR represent the mean of relative mRNA amount ± SEM of 6 measurements. *** $p < 0.001$ vs. control; ** $p < 0.01$ vs. control; * $p < 0.05$ vs. control; one-way ANOVA test; post hoc test: Tukey. Names of proteins encoded by the analyzed genes are given in brackets, next to the names of the genes. B) Protein expression was examined in cytoplasmic cells lysates by Western blotting using target specific antibodies. Examination of ß-actin expression level was used as internal control. Representative Western blots with densitometric analyses of 3 independent experiments are presented. *** $p < 0.001$ vs. control; ** $p < 0.01$ vs. control; * $p < 0.05$ vs. control; one-way ANOVA test; post hoc test: Tukey.

3.5. C. cibarius branched mannan fraction CC2a influence on NF-κB target processes: cell cycle progression, apoptosis induction and migration of colon cancer cells

Due to the inhibition of NF-κB target genes involved in proliferation, apoptosis and metastasis by CC2a fraction its impact on these processes was determined in LS180 cells after 24 h of incubation with 50 and 100 μg/ml of CC2a by flow cytometry, Cell Death Detection ELISA and Wound assay, respectively.

Flow cytometry allows observing a significant accumulation of LS180 cells treated with CC2a in G0/G1 and S phases of cell cycles and this effect was dose dependent. Compared to control conditions the number of cells in G0/G1 and S phases increased after 24 h of exposure to 100 μg/ml of CC2a by 11.3% and 4.8%, respectively. At the same time the incubation of LS180 cells with CC2a fraction decrease the number of cells in the G2-M phase up to 18.6% (50 μg/ml) and 14.3% (100 μg/ml) compared to untreated cells (33.3%). These results suggest the induction of both G0/G1 and S cell cycle arrest by CC2a fraction (Fig. 8A).

The changes observed in cell cycle progression were accompanied by apoptosis induction. LS180 cells exposed to 50 and 100 μg/ml of CC2a increased DNA fragmentation (nucleosomes enrichment) up to 121% and 132%, showing the proapoptotic properties of CC2a fraction (Fig. 8B).

CC2a treatment also inhibited colon cancer cells migration, as demonstrated in Fig. 8C. CC2a in concentration of 50 μg/ml inhibited cancer cells migration by 65.3% vs. control, while 100 μg/ml of CC2a decreased cells number by 72.3% vs. control. Obtained data proved antimigrative properties of CC2a fraction enriched with branched mannans (Fig. 8C).

4. Discussion

Our earlier studies on the anticancer activity of polysaccharides/polysaccharide-enriched fractions isolated from *C. cibarius* revealed significant antiproliferative activity of these fractions on human colon adenocarcinoma LS180 cells [37]. Nevertheless, evaluation was limited to MTT test, which only allows indirect evaluation of the antiproliferative activity of these fractions. As a result, in the present study, the antiproliferative ability of obtained polysaccharide-rich fractions (CC1 and CC2b) and polysaccharide/glycoprotein (CC2a) fraction was examined using the more specific and sensitive BrdU test. The results obtained showed inhibition of DNA synthesis in LS180 cells treated with fractions CC2a and CC2b (characterized by a higher polysaccharides content: 69.2% and 53.2%, respectively), while fraction CC1 (36.9% of polysaccharides) did not show effect. Additional studies on human colon epithelial CCD841 CoN cells revealed that only fraction CC2a did not affect cell proliferation while the other two fractions stimulated (CC1) or inhibited

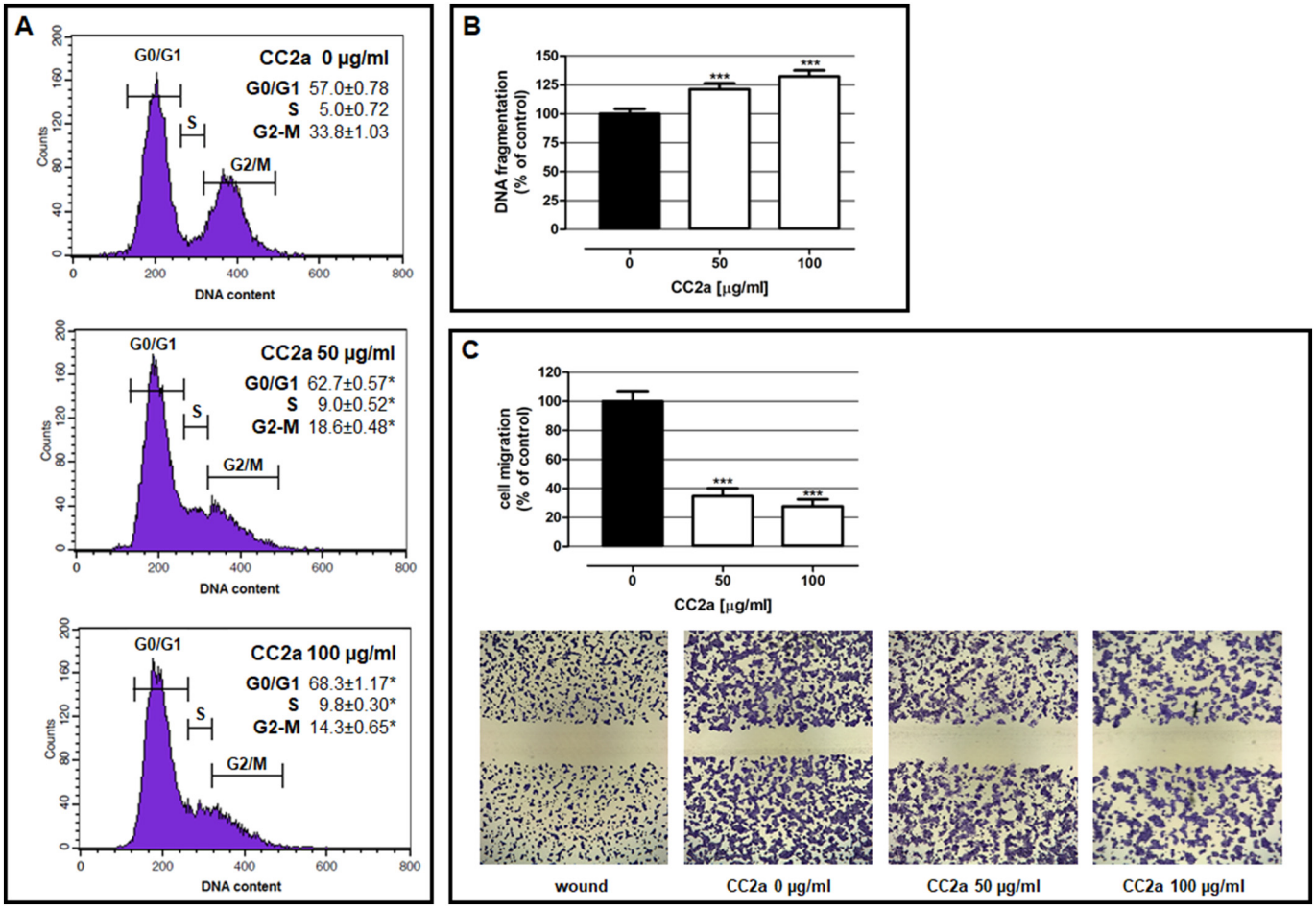

Fig. 8. Alterations in cell cycle progression, survival and motility of human colon adenocarcinoma cells LS180 induced by fraction CC2a. Cells were exposed for 24 h to culture medium alone, and tested fraction at concentrations 50 μg/ml and 100 μg/ml. A) Nuclear DNA content was determined with PI staining by flow cytometry. The percentage of cells in G1/G0, S, G2-M phases was analyzed in each sample. The data are representative of at least three separate experiments. * $p < 0.001$ vs control; one-way ANOVA test; post hoc test: Tukey. B) DNA fragmentation (nucleosomes enrichment) was determined by Cell Death Detection ELISA. Results are presented as the mean ± SEM of 6 measurements. *** $p < 0.001$ vs. control; one-way ANOVA test; post hoc test: Tukey. C) Number of cancer cells migrated into the wound (cell-free gap) was determined under light microscopy after CC2a treated cells staining with May-Grünwald-Giemsa. The area of cell counting was determined by the surfaces of the wound. Results are expressed as a mean number of cells present in wound ± SEM of 4 measurements. *** $p < 0.001$ vs. control; one-way ANOVA test; post hoc test: Tukey. Representative pictures of LS180 cells migrated to the wound area before and after treatment with CC2a are presented. Magnification 40×.

(CC2b) DNA synthesis. To deepen the screening of *C. cibarius* anticancer polysaccharide/polysaccharide-rich fractions, LDH assay, considered the gold standard for cytotoxicity evaluation, was conducted. A beneficial effect from the cancer therapy point of view was observed only in the case of CC2a and CC2b fractions, which were nontoxic to colon epithelial cells, and simultaneously damaged cell membranes of colon cancer cells. Based on the results obtained from both the BrdU and LDH tests, CC2a revealed the greatest anticancer potential which not only suppressed the majority of proliferation of colon cancer cells but also most effectively destroyed the integrity of their membranes and at the same time did not adversely affect normal colon epithelial cells. The anticancer properties and high selectivity of fraction CC2a was demonstrated by the May-Grünwald-Giemsa staining of normal and cancer colon cells treated with these polysaccharides. It should be noted that our earlier studies revealed neuroprotective properties of fraction CC2a and also described its chemical nature in detail [23].

In mammals, the NF-κB family is composed of five related transcription factors: p65/RelA, RelB, c-Rel, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100) [38]. The most common combination, also in colon cancer cell lines, is heterodimer, composed of p50 and p65 subunits [39]. Furthermore, this combination is characteristic for the classical NF-κB activation pathway, which is responsible for cell survival, inflammation, innate immunity, and as shown in recent studies, is associated with harmful features of cancer cells, such as antiapoptotic effects, uncontrolled cell proliferation, and metastasis [39,40].

Under physiological conditions, NF-κB is expressed in the cytoplasm where its activity is controlled by the inhibitor molecule IκBα. During classical activation, IκBα is phosphorylated at Ser32 and Ser36, resulting in the ubiquitination-dependent degradation of IκBα and the release of p50-p65 heterodimer [38,41]. Freed NF-κB is translocated after phosphorylation at Ser536 to the nucleus where it regulates transcription of various target genes [42,43]. The above-mentioned phosphorylation of IκBα is mediated through the activation of the IκBα kinase (IKK). IKK is composed of the catalytic IKKα and IKKβ subunits and a regulatory IKKγ subunit [38,41]. Although IKKα and IKKβ cooperate for IκBα phosphorylation, they differ in the type of transduced signals. The IKKβ is essential for signals mediated via the classical NF-κB pathway. It needs to be highlighted that IKKβ catalytical activity also requires phosphorylation at Ser177 and Ser181 in the activation loop [44].

Our studies revealed that polysaccharide fraction CC2a, mainly composed of an O-2 and O-3 branched 1,6-linked mannan and protein, did not affect the level of IKKβ; however, it was able to increase its activated phosphorylation at Ser177. The observed effect corresponded with the stimulation of IκBα phosphorylation at Ser36 in response to CC2a treatment. Despite the increase in IκBα phosphorylation, its subsequent degradation was not observed, suggesting that fraction CC2a disturbs this process. Similar properties were observed for chemically-sulfated polysaccharide obtained from *Grifola frondosa* (S-GFB) in human liver cancer cells HepG2 [45].

Our results also revealed the accumulation of NF-κB in the cytoplasm of LS180 cells treated with CC2a fraction which seems to be the consequence of disorders in the IκBα degradation induced by CC2a. Nevertheless, the observed significant decrease in NF-κB phosphorylation at Ser536, which is responsible for protein translocation to nucleus could also contribute to the increase in NF-κB cytoplasmic level as well as explaining the decrease in its concentration in the cell nucleus [42]. A similar mechanism of action was described previously by Li et al. in murine bone marrow-derived dendritic cells treated with polysaccharides purified from *Pholiota nameko* [46]. Induced by CC2a inhibition of NF-κB phosphorylation at Ser536 and the subsequent decrease in the nuclear level of NF-κB, shows that the branched mannans isolated from *C. cibarius* are inhibitors of NF-κB nuclear translocation. Furthermore, the changes observed suggest Ser536 phosphorylation in p65 subunit as the next molecular target for fraction CC2a. The discovered decrease in NF-κB transactivation in response to *C. cibarius* CC2a fraction seems to be a very common molecular mechanism for the anticancer activity of mushroom polysaccharides. Studies performed by Volman et al. on other human colon cancer cells (Caco-2) also revealed a decrease in NF-κB transactivation in response to polysaccharides isolated from the fruit bodies of *Agaricus bisporus*, *Agaricus blazei* Murill, *Coprinus comatus*, *Ganoderma lucidum*, as well as from the spores of *Ganoderma lucidum* [47]. A similar mechanism of anticancer activity was also observed in human liver cancer cells HepG2 after treatment with *Grifola frondosa* sulfated polysaccharide [45] and *Agaricus blazei* Murill polysaccharides [48]. Inhibition of NF-κB translocation causing inhibition of human colon cancer cell proliferation and suppression of tumor growth in athymic nude mice was also observed in response to *Lentinus edodes* water-extracted polysaccharides [49].

The importance of NF-κB is due to its ability to influence the expression of numerous genes that facilitate cancer cells proliferation, immortality, survival and drug resistance as well as genes involved in the promotion of cancer development, such as inflammation or angiogenesis [5,9]. Since we observed that *C. cibarius* polysaccharides retain NF-κB in the cytoplasm, as well as inhibiting its phosphorylation at Ser536, which is responsible for NF-κB nuclear localization and transcriptional activity, CC2a influence on the expression of selected NF-κB target genes and their proteins products was investigated. The results revealed that the tested fraction significantly inhibited expression of all investigated genes and almost all tested proteins (except Bax) involved in proliferation (Cyclin D1, c-Myc), apoptosis (Survivin, Bcl-2) and metastasis (MMP-9) [5,9]. The most significant changes were observed in the expression of both *CCND1* and *MYC* genes and the corresponding proteins Cyclin D1 and cMyc. Because of the fact that mentioned proteins are responsible for cell proliferation [50,51], this may explain the observed inhibition of DNA synthesis in LS180 cells treated with fraction CC2a. The suggested mechanism of CC2a anticancer action are in accordance with results obtained by Jedinak et al., which showed that *Pleurotus ostreatus* extract containing α- and β-glucans, suppressed the expression of Cyclin D1 in colitis-related colon carcinogenesis in mice [52]. Other studies also indicated a decrease of NF-κB activity as an important mechanism of cancer cells growth inhibition by the mushroom polysaccharides, which observed for example in human breast cancer cells (MCF-7 and MDA-MB-231) and human prostate cancer cells (PC-3) treated with *Ganoderma lucidum* polysaccharides [53–55], or colitis-associated colon carcinogenesis in mice exposed to glucans obtained from *Pleurotus pulmonarius* [56].

Since CC2a revealed inhibition of NF-κB target genes and their proteins products involved in proliferation, apoptosis and metastasis the fraction impact on these processes was determined. Cell cycle examination showed a significant accumulation of LS180 cells in G0/G1 and S phases of cell cycle after exposure to CC2a fraction. Furthermore, decrease of cells number in G2/M phase was also observed. These results are in accordance with the data obtained from BrdU assay as well as Real Time PCR and proved the antiproliferative properties of CC2a fraction. Changes in cell cycle progression especially induced by CC2a cell cycle arrests in G0/G1 and S were accompanied by significant increase of DNA fragmentation. Discovered proapoptotic properties of CC2a are in accordance with the down-regulation of both *BCL2* and *BIRC5* and the corresponding proteins Bcl2 and Survivin, however were in contradiction to *BAX* inhibition as well as cell membranes damage observed in LS180 cells after CC2a exposure. Nevertheless induced by tested fraction increase of *BAX*/*BCL2* ratio (1.16 the same for both 50 and 100 μg/ml CC2a) and especially Bax/Bcl2 ration (1.34 for 50 μg/ml CC2a and 1.72 for 100 μg/ml CC2a) indicated enhanced sensitivity/susceptibility of LS180 cells to apoptosis induction as a result of CC2a treatment [57]. Performed studies also revealed that CC2a treatment evidently inhibited colon cancer cells migration in accordance with the significant decrease of both *MMP9* gene and MMP9 protein expression in LS180 cells exposed to the investigated fraction. Disorders in the course of

the G1 and S cell cycle phases, as well as apoptosis induction, are common mechanisms of anticancer activity of mushroom polysaccharides, however there are only two studies on mushroom polysaccharides, which also showed inhibition of cancer cells migration. Such mechanism of action was observed in the case of *Antrodia cinnamomea* sulfated polysaccharide in human lung cancer cell lines A549 and LLC1 [58] and *Pleurotus nebrodensis* alkali extractable polysaccharide in human hepatic cancer cell line HepG2 [59]. Nevertheless, contrary to our results, previously mentioned studies did not associate the described anticancer activities with the inhibition of signals transduction in NF-κB pathway, nevertheless this aspect was not investigated and thus was not excluded.

In summary, this work revealed for the first time high selectivity of *C. cibarius* branched mannan polysaccharides, which were able to significantly suppress proliferation of colon cancer cells and effectively destroy their membranes integrity, but at the same time, did not adversely affect normal colon epithelial cells. The discovered antiproliferative ability of these polysaccharides was accompanied by disorders in signals transduction in NF-κB pathway, in particular the inhibition of IκBα degradation, attenuation of activated NF-κB phosphorylation, and the subsequent decrease in NF-κB nuclear level, as well as a significant down-regulation of NF-κB target genes *BAX*, *BCL2*, *CCND1*, *MMP9*, *MYC*, *BIRC5* and the corresponding proteins (except Bax). Furthermore, CC2a treatment of LS180 cells resulted in perturbation in G0/G and S phases of the cell cycle that was associated with a marked increase of DNA fragmentation as well as inhibition of cancer cells motility. Because of that, we postulate that CC2a-mediated down-modulation of NF-κB activity could lead to inhibition of colon cancer cell proliferation, induction of apoptosis and inhibition of cancer cells metastatic ability. The obtained results indicate *C. cibarius* branched mannan polysaccharide as a new, safe and effective therapeutic/preventive option in fighting colorectal cancer.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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