

The Apoptotic Effect of Polysaccharides with Specific Molecular Weight Extracted from *Inonotus Obliquus* on HT-29 Colon Cancer Cells

Xue Han (✉ xhan@hit.edu.cn)

Harbin Institute of Technology

Sainan Zhao

Harbin Institute of Technology

Yu Wang

Harbin Institute of Technology

Jialei Sun

Harbin Institute of Technology

Shiwei Chen

Harbin Institute of Technology

Research

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Abstract

Background: Recently, much attention has been paid to natural products owing to their effective anticancer effects with relatively low toxicity, especially *Inonotus obliquus* (*I. obliquus*).

Results: Polysaccharides with different molecular weights were filtered from water extract of *I. obliquus* using grading membrane filtration method. IOP60b (10 kDa \leq molecular weight \leq 30 kDa) was found to have the highest yield and the highest inhibition ratio of HT-29 cancer cells, therefore it was chosen to evaluate the apoptotic effect of HT-29 cancer cells. After treated with different concentrations of IOP60b, morphological changes including cell shrinkage and nuclear condensation and DNA fragmentation in HT-29 cancer cells were observed, and cells in early apoptosis and late apoptosis significantly ($p < 0.05$) increased in a dose-dependent manner by arresting cell cycle at G0/G1 phase. To further explore the underlying mechanism, RT-PCR and Western blotting were applied. Results indicated that both Bcl-2 family and Caspase family were involved in the process of apoptosis regulation and IOP60b induced cellular apoptosis via upregulation of Bax/Bcl-2 ratio and activation of Caspase-3.

Conclusion: These data suggested that IOP60b might be the potential candidate for the clinical prevention and treatment of colorectal cancer.

1. Introduction

Colorectal cancer is one of the most common global gastrointestinal malignant tumors, which has caused great concern worldwide particularly in western and developed nations due to its great threat to human being health¹. In fact, the incidence of colon cancer is closely related to the personal lifestyle. As far as we know, with the enhancement of the living standard and the change of the dietetic habit of people, the morbidity and mortality of colon cancer have been on the rise during the past few decades². At present, surgical resection, radiotherapy and chemotherapy are the dominant methods applied for colon cancer treatment. However, there still exist some drug resistance and severe side effects including hair loss, bleeding, diarrhea and immunosuppression³, which significantly limits their application. Thus, natural product offers a potential alternative approach for tumor therapy owing to their effective anticancer effects with relatively low toxicity⁴.

Inonotus obliquus (*I. obliquus*), a white rot fungus, has been widely used as a folk remedy in Russia since the 16th century⁵ for its non-toxic effects in treating gastrointestinal cancers and digestive system diseases⁶. Polysaccharides are considered to be the main bioactive constituents of *I. obliquus*⁷, and have triggered much attention in recent years due to their biological activities, including anti-cancer⁸, anti-inflammatory⁹, anti-oxidation¹⁰, hypolycemic effect¹¹, and immuno-stimulating¹². Moreover, it has been reported that polysaccharides from *I. obliquus* have cytotoxicity on multiple tumor cells, such as human stomach carcinoma^{13,14}, human hepatoma^{14,15}, human lung carcinoma^{14,16,17}, kidney adenocarcinoma¹⁴, human breast adenocarcinoma¹⁴, human ovary adenocarcinoma¹⁴, human

endometrial epithelial cells¹⁴, murine melanoma cells^{14,16,18}, human T lymphadenoma jurkat cells¹⁵ et al..

Whereas it has been mentioned that the extraction of *I. obliquus* by water and ethanol could directly inhibit the proliferation of colon cancer cells which was mainly composed of polysaccharides and proteins, among which polysaccharides accounted for the most proportion^{7,19,20}. A preliminary deduction is that polysaccharides from *I. obliquus* play a major role in its anticancer effects on colon carcinoma. Few studies were regarding the effect of polysaccharides with specific molecular weight extracted from *I. obliquus* on colon cancer. In addition, studies on the mechanism of anti-colon cancer of *I. obliquus* showed that the aqueous extract of *I. obliquus* induced apoptosis of colon cancer cells by down-regulating Bcl-2 protein expression and up-regulating Bax and Caspase-3 protein expression²¹. While Lee et al.²² found that the ethanol extract of *I. obliquus* inhibited colon cancer by arresting the cell cycle G1phase. The mechanism of polysaccharides from *I. obliquus* on HT-29 colon cancer is still unclear. Therefore, the current study was carried out to further explore the anticancer activities of polysaccharides with specific molecular weight isolated from *I. obliquus* and the related mechanism.

2. Materials And Methods

2.1. Materials and reagents

The fruiting bodies of *I. obliquus* were collected from Changbai Mountain, Jilin, China. Human colon carcinoma cells HT-29 were obtained from Cancer Hospital Chinese Academy of Medical Sciences, Beijing, China. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO₂. All reagents used for cell culture were from Hyclone (USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Japan). Tissue DNA Kit was purchased from Solarbio (Beijing, China). Annexin V-FITC Apoptosis Detection Kit was purchased from Njjcbio (Nanjing, China). Antibodies to Bcl-2, Bax, Caspase-3 and β-actin were obtained from Wanleibio (Shenyang, China).

2.2. Extraction, purification and fraction of polysaccharides

The crude water-soluble polysaccharides (CIOP) were extracted from *I. obliquus* samples with distilled water at 60 °C according to the method outlined by Xu et al²³. After removal of the protein by using the Sevag reagent, the CIOP solution was mixed with 95% ethanol to reach a final concentration of 60% ethanol (v/v), and then the mixture was stored at 4 °C overnight. After centrifugation, dialysis and freeze drying, the precipitate was termed IOP60. 10 mg/mL IOP60 solution was prepared, then passed through a 0.22 μm filter, and assembled with ultrafiltration centrifuge tubes (Merck Millipore Amicon™). After centrifugation and freeze drying, the fractions were collected and named as IOP60a (3 kDa ≤ molecular weight ≤ 10 kDa), IOP60b (10 kDa ≤ molecular weight ≤ 30 kDa), IOP60c (30 kDa ≤ molecular weight ≤ 50 kDa), IOP60d (50 kDa ≤ molecular weight ≤ 100 kDa), and IOP60e (molecular weight ≥ 100 kD),

respectively. The yield was calculated based on the weight of each fraction to the weight of the total IOP60.

2.3. Cell viability analysis

HT-29 cells were seeded for 12 h in 96-well plates (2×10^6 cells/mL), and then treated with 1.25 mg/mL polysaccharide fraction (IOP60a, IOP60b, IOP60c, IOP60d, IOP60e) for 24 h, respectively. CCK-8 assay was used to detect the cell viability according to the manufacturer's protocol²⁴. All determinations were done in six duplicates. Inhibition ratio of tumor cell proliferation was calculated according to the formula below:

$$\text{Inhibition ratio(\%)} = [(Ac - As)/(Ac - Ab)] \times 100 \quad (1)$$

Where A_s , A_c and A_b were the absorbance of treated cells, untreated cells and control group, respectively.

2.4. Observation of cell morphology

HT-29 cells (2×10^6 cells per well) were seeded in 6-well plates for 24 h and then treated with different concentrations of IOP60b (0, 0.625, 1.25, 2.5, 5, 10 mg/mL) for 48 h. After that, cells were fixed with 4% formaldehyde in PBS for 10 min, and then subjected to optical microscopy (Olympus BX51, Japan).

2.5. DNA fragmentation analysis

IOP60b-treated (48 h) HT-29 cells were washed and resuspended in 1 mL PBS. Cell total DNA was extracted according to the manufacturer's instructions. The extracted DNA samples were run on the 1.2% agarose gel in $1 \times$ TAE buffer. After that, the gel was visualized with a UV light by Gel Imaging System, and photographed.

2.6. Cell apoptosis and Cell cycle analysis by flow cytometry

The percentage of specific apoptotic cells was determined by flow cytometry according to the manufacturer's procedure. Briefly, HT-29 cells were plated in a 6-well plate (1×10^5 cells/mL) and treated with different concentrations of IOP60b (0.625, 1.25, 2.5, 5, 10 mg/mL) for 48 h. The cells were collected and washed twice with cold PBS, and then resuspended in 500 μ L binding buffer containing 5 μ L Annexin V-FITC and 5 μ L propidium iodide (PI) for 10 min in the dark, finally analyzed using Millipore guava easy Cyte™ flow cytometer (Millipore, USA) and Guavasoft 3.1.1 software.

Similarly, HT-29 cells were first plated in a 6-well plate (1×10^5 cells/mL) and treated with different concentrations of IOP60b (0.625, 1.25, 2.5, 5, 10 mg/mL) for 48 h and then used for cell cycle analysis. Cells were centrifuged at $12000 \times g$ for 5 min and then fixed with 70% (v/v) ethanol and stored at 4 °C overnight. Cells precipitate were washed with PBS and treated with 100 μ L RNase, incubating for 30 min

at 37 °C. After centrifugation, cells were incubated with PI at 4 °C for 30 min in the dark. Cell cycle distribution was detected by flow cytometry and analyzed using ModFit LT 5.0 software.

2.7. Real-time quantitative RT-PCR analysis

HT-29 cells (5×10^5 cells/mL) were seeded in 6-well plates and treated with 5 mg/mL IOP60b for 0 h, 24 h, 48 h, 72 h, respectively. Total RNA was extracted using total RNA extraction kit (GeneMark™) and then reversed to cDNA by using the All-in-One™ First-Strand cDNA Synthesis Kit (GeneCopoeia, USA) according to the manufacturer's protocol. Real-time PCR was performed with 2 × Es Taq MasterMix (Dye) (CW BIO, Beijing, China). The following primers (Sangon Biotech, Shanghai, China) were used for amplification: Bcl-2 forward 5'-CAG CTG CAC CTG ACG CCC TT-3' and reverse 5'-GCC TCC GTT ATC CTG GAT CC-3'; Bax forward 5'-TTC TGA CGG CAA CTT CAA CTG-3' and reverse 5'-TGA GGA GGC TTG AGG AGT CT-3'; Caspase-3 forward 5'-ACA CAG TAT GGC GGC AGA G-3' and reverse 5'-AGA CAG GCA ACA GAG CAC AT-3'; β-actin forward 5'-CCT CTA TGC CAA CAC AGT GC-3' and reverse 5'-ATA CTC CTG CTT GCT GAT CC-3'.

2.8. Western blotting

HT-29 cells were harvested and lysed after treated with IOP60b for desired time (0 h, 24 h, 48 h, 72 h). Total proteins were extracted by using a Total Protein Extraction Kit (Wanleibio, China), and the protein concentration was determined by using a BCA Protein Assay Kit (Wanleibio, China) according to the manufacturer's protocol. Equal amounts of proteins were boiled for 5 min and separated by SDS-PAGE, then transferred onto a PVDF membrane. Blocking of non-specific binding was achieved by placing the membrane in a dilute solution of 5% skim milk powder in tris-buffered saline (TBS) for 1 h. After blocking, a dilute solution of primary antibody (Bcl-2, Bax, Caspase-3 and β-actin) at a concentration of 1:500 was incubated with the membrane overnight at 4 °C under gentle agitation. The primary antibody was then diluted with TBST wash buffers. After rinsing the membrane to remove unbound primary antibody, the membrane was incubated with a dilute solution of secondary antibody (HRP-labeled goat anti-rabbit Ig G) at a concentration of 1:5000 for 45 min at 37 °C. The membrane was washed with TBST buffers again. The Electrochemiluminescence (ECL) reagent was used to measure the chemiluminescence intensity. Finally, the band intensities were quantitated using Gel-Pro-Analyzer software.

2.9. Statistical analysis

All the measurements and analyses were carried out in triplicate. The experimental results were presented as means of three determinations ± SD (standard deviation). Origin (version 8.5) and SPSS (version 16.0) with Tukey's multiple comparisons were used for the statistical and graphical evaluation. The statistical significance of mean differences was based on *p*-Values of < 0.05.

3. Results And Discussion

3.1. Yields and inhibition ratios of polysaccharides with different molecular weights

Results in Fig. 1 showed that the yield of IOP60b (10 kDa \leq molecular weight \leq 30 kDa) significantly higher than that of any other polysaccharide fraction ($P < 0.05$). As for the inhibition ratio of HT-29 colon cancer cells, IOP60b (10 kDa \leq molecular weight \leq 30 kDa) was the highest, reaching 61.9%, which was followed by IOP60a (3 kDa \leq molecular weight \leq 10 kDa), while IOP60e with the highest molecular weight (≥ 100 kDa) was the lowest (28.3%). As was reported, polysaccharides with lower molecular weight generally had higher biological activity^{14,25}, which was consistent with our results, that probably because polysaccharides with lower molecular weight were more likely to pass free radicals. Therefore, IOP60b was chosen to explore its effects on apoptosis of HT-29 colon cancer cells and the underlying mechanism.

3.2. IOP60b induced morphological changes and DNA fragmentation

Optical microscopy was used to observe morphological changes in HT-29 cells after treated with IOP60b (0, 0.625, 1.25, 2.5, 5 and 10 mg/mL) for 48 h. It can be seen from Fig. 2 that cells in the control group adhered to the wall and grew vigorously. The nuclei and cell bodies were large, and they were fusiform or polygonal. The cytoplasm was uniform and transparent with high transmittance. After 48 hours of IOP60b treatment, the number of adherent cells decreased and the cell contents increased which reduced the transmittance, the cells shrunk or even shattered, the nuclei were concentrated, and the cell volume became smaller, rounded and deformed. In addition, as the concentration of IOP60b increased, the changes in cell morphology became more pronounced.

Agarose gel electrophoresis was used to detect the nuclear DNA from HT-29 cells. As can be seen in Fig. 3, the untreated cells represented by Lane A showed normal chromosomal DNA with no DNA ladder, while DNA isolated from HT-29 cells treated with different concentrations of IOP60b for 48 h (Lane B-F) was all degraded into giant DNA fragments, and the DNA ladder phenomenon became more apparent as the increasing concentrations of IOP60b. As we all know, apoptosis is an extremely important process to maintain cellular homeostasis, accompanying with specific changes in cell morphology such as cell shrinkage, membrane blebbing, nuclear condensation and internucleosomal DNA fragmentation¹. Therefore, combined the cell morphological changes with nuclear DNA fragmentation, it is speculated that IOP60b may induce colon cancer HT-29 cell death through apoptotic pathway.

3.3. IOP60b induced apoptosis in HT-29 cells

To evaluate whether the IOP60b treatment (0, 0.625, 1.25, 2.5, 5 and 10 mg/mL) for 48 h in HT-29 cells was associated with apoptosis, annexin V-FITC/PI apoptosis assay²⁶ was conducted by flow cytometry. Results in Table 1 demonstrated that as the concentration of IOP60b increased from 0.625 mg/mL to 10 mg/mL, the percentages of cells in early apoptosis compared with the negative control group increased by 3.15%-6.97%, and the percentage of cells in late apoptosis significantly increased ranging from 10.20–28.46% ($P < 0.05$). In addition, the total number of apoptotic cells in HT-29 treated with 0.625, 1.25, 2.5, 5 and 10 mg / mL IOP60b increased by 13.35%, 21.64%, 27.68%, 34.74% and 35.43%, respectively. It was apparent that IOP60b induced cells in early apoptosis and late apoptosis increased in

a dose-dependent manner. Thus, we can conclude that IOP60b can significantly inhibit cell proliferation by inducing apoptosis of colon cancer HT-29 cells.

Table 1
Effects of IOP60b on apoptosis and cell cycle of HT-29 cells

Group (mg/mL)	Apoptotic cells (%)			Cell cycle distribution (%)		
	Live	Early apoptosis	Late apoptosis	G0/G1	G2/M	S
0(control)	92.42 ± 0.23 ^f	0.40 ± 0.03 ^a	0.35 ± 0.03 ^a	73.54 ± 0.40 ^a	8.00 ± 0.00 ^c	18.46 ± 0.40 ^e
0.625	80.16 ± 0.47 ^e	3.55 ± 0.21 ^b	10.55 ± 0.01 ^b	76.42 ± 0.5 ^b	8.00 ± 0.00 ^c	15.58 ± 0.5 ^d
1.25	73.33 ± 0.63 ^d	4.34 ± 0.67 ^b	18.05 ± 0.02 ^c	79.86 ± 0.82 ^c	8.00 ± 0.00 ^c	12.14 ± 0.82 ^c
2.5	67.17 ± 0.50 ^c	5.32 ± 0.22 ^c	23.11 ± 0.18 ^d	83.61 ± 0.6 ^d	6.27 ± 0.62 ^b	10.12 ± 0.2 ^b
5	54.75 ± 1.60 ^b	6.89 ± 0.83 ^d	28.60 ± 0.16 ^e	85.01 ± 0.6 ^d	5.46 ± 0.39 ^b	9.53 ± 0.82 ^b
10	51.50 ± 0.44 ^a	7.37 ± 0.70 ^d	28.81 ± 0.11 ^e	90.03 ± 0.40 ^e	3.31 ± 0.63 ^a	6.66 ± 0.40 ^a

The data are average values of triplicate ± standard deviation. ^{a-f} Mean values with different letters are significantly different ($P < 0.05$)

3.4. IOP60b triggers cell cycle arrest in HT-29 cells

The cell-division cycle is a vital process which consists of four distinct phases: G1 phase, S phase (synthesis), G2 phase (collectively known as interphase) and M phase (mitosis or meiosis). Deregulation of the cell cycle is the most common abnormality in human cancer. The cells which are actively undergoing cell cycle are targeted in cancer therapy as the DNA is relatively exposed during cell division and hence susceptible to damage by drugs or radiation²⁷. In the present study, flow cytometry was used to investigate the cell cycle distribution treated with different concentrations (0.625, 1.25, 2.5, 5 and 10 mg/mL) of IOP60b for 48 h. In the presence of IOP60b, we observed a dose-dependent increase in the percentage of cells in G0/G1 phase accompanied by a corresponding reduction in the percentages of cells in S and G2/M phases (Table 1). These data suggest that IOP60b induced G0/G1 phase arrest in HT-29 cells, which was in agreement with the results of Youn et al²⁸. The mechanism by which polysaccharides from *I. obliquu* inhibits cell proliferation and arrests cell cycle can be explained as follows: On the one hand, polysaccharides can inhibit ribosome synthesis in G0/G1 phase, thereby inhibiting cell protein synthesis and reducing mitosis, leading to cell proliferation. On the other hand, the

polysaccharides interferes with the synthesis of DNA by inhibiting the synthesis of RNA and protein, thereby arresting the cells in the G0/G1 phase and ultimately inhibiting cell proliferation²⁹.

3.5. Effects of IOP60b on Bcl-2, Bax, and Caspase-3 expressions in HT-29 colon cancer cells

Increasing evidences have identified natural product might control cancer via the direct or indirect modulation of apoptosis-related genes³⁰. RT-PCR was used to investigate the expression of caspase-3, Bax, Bcl-2, and Bad at different time points. As shown in Fig. 4, after 0 h, 24 h, 48 h and 72 h treatment with 5 mg/mL IOP60b, the expression level of Bcl-2 gene in the drug-exposed groups was significantly decreased, whereas the level of Bax gene was dramatically increased in a time-dependent manner compared to the negative control ($P < 0.05$). As we all know, Bcl-2 plays a role of anti-apoptosis in the Bcl-2 family, while Bax play a role of pro-apoptotic³¹, and the Bax/Bcl-2 ratio was often used as an index of apoptosis³². Therefore, we deduced that IOP60b promote HT-29 colon cancer cells apoptosis by regulating Bax/Bcl-2 ratio. Moreover, we examined whether caspase-3 activity would change with increasing concentrations of IOP60b. As was reported, caspases-3 protease, can target structural substrates and induce cancer cell breakdown and DNA fragmentation⁴ and its activation marks the irreversible stage of apoptosis³³. Results in Fig. 4 showed that caspase-3 activity increased significantly at all time-points ($P < 0.05$) which proved that caspase-3 also played an important role in the process of HT-29 colon cancer cells apoptosis induced by IOP60b. This conclusion was consistent with the point mentioned by Cheng-Chih Tsaia et.al.³⁴ who investigated apoptosis effects of *I. obliquus* extract on HCT-116 cell line.

3.6. Effects of IOP60b on Bcl-2, Bax, and Caspase-3 expressions in HT-29 cells

To further determine the role that the Bcl-2 families and caspase-3 played in IOP60b mediated apoptosis, western blot analysis was used to detect their effects on the protein levels of Bcl-2, Bax and a caspase-3. The expression of β -action was used as a loading control. As shown in Fig. 5, similar to the RT-PCR results, there was a significant decrease in the expression levels of Bcl-2 protein ($P < 0.05$), while significant increase in Bax and Caspase-3 in the IOP60b-treated groups compared to the negative control ($P < 0.05$). Besides, these trends were more pronounced as the treatment time increased from 0 h, to 72 h which indicated that IOP60b induced cell apoptosis of HT-29 cells in a time-dependent manner.

An article by Lee et al.²¹ mentioned that aqueous extracts from the fruiting bodies of *I. obliquus* could inhibit HT-29 colorectal cancer cells, and the anti-apoptotic protein Bcl-2 was found to be inhibited and the pro-apoptotic protein Bax was promoted, at the same time, the levels of procaspase-3 showed a decreasing trend implying that procaspase-3 underwent cleavage and activation into caspase-3, which in turn affected apoptosis in HT-29 cells. Youn et al.^{35,36} employed Western blotting to analyze the protein expression of procaspase-3 in HepG2 liver cancer cells which were treated with aqueous extracts of *I. obliquus*, results showed that as the concentration of *I. obliquus* increases, there was a significant

decrease in procaspase-3. It was explained that extracts from *I. obliquus* fruiting bodies could aid in procaspase-3 activation into caspase-3. Nomura et al.³⁷ also found that inotodiol, a lanostane triterpenoid from *I. obliquus* can inhibit P388 leukemia cells through caspase-3 activation.

In this case, our results are in agreement with these previous studies wherein extracts of *I. obliquus* have been reported to exhibit anticancer effects via upregulating Bax, Caspase-3 activity and downregulating Bcl-2 activity^{19,20}. That was to say, polysaccharides from *I. obliquus* induce apoptosis in HT-29 cells through the mitochondrial pathway.

4. Conclusions

In the present study, polysaccharides with different molecular weights were successfully purified from IOP60 with ultrafiltration centrifuge tubes. IOP60b (10 kDa \leq molecular weight \leq 30 kDa) was selected to verify the effects and mechanisms of apoptosis because it had the highest yield and inhibition ratio of HT-29 colon cancer cells. It was observed that IOP60b induced morphological changes and DNA ladder phenomenon of HT-29 colon cancer cells. The results of flow cytometry indicate that IOP60b induced cells in early apoptosis and late apoptosis increased in a dose-dependent manner and significant G0/G1 phase arrest in HT-29 cells. Moreover, according to the RT-PCR and western blot results, it could be preliminarily inferred that, IOP60b induced cellular apoptosis of HT-29 cells through the mitochondrial-mediated apoptosis pathway in which the expression of Bax and Caspase-3 were upregulated and the expression of Bcl-2 was downregulated. These data suggested that polysaccharides with specific molecular weight (10 kDa \leq molecular weight \leq 30 kDa) could significantly inhibit the growth of HT-29 cells and induce apoptosis. IOP60b might be the potential candidate for the clinical prevention and treatment of colorectal cancer, while the underlying mechanism still needs further study.

List Of Abbreviations

abbreviations	Full name
<i>I. obliquus</i>	<i>Inonotus obliquus</i>
FBS	fetal bovine serum
CCK-8	Cell Counting Kit-8
CIOP	crude water-soluble polysaccharides
PI	propidium iodide
TBS	tris-buffered saline
ECL	Electrochemiluminescence

Declarations

Ethics approval and consent to participate:

This manuscript does not report on or involve any animals, humans, human data, human tissue or plants.

Consent for publication:

This manuscript does not contain data from any individual person.

Availability of data and materials:

Not applicable.

Competing interests:

The authors declare that they have no competing interests in this section.

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Authors' contributions:

The author of Xue Han design the experience and revise the manuscript.

The author of Sainan Zhao analyzed data regarding the hematological disease and the transplant.

The author of Yu Wang and Jialei Sun do the experiment

The author of Shiwei Chen is a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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References

1. W. Cheng, J. Ren, D. Jing and C. Wang, Anti-tumor role of *Bacillus subtilis* fmbJ-derived fengycin on human colon cancer HT29 cell line, *Neoplasma*, 2016, **63**.
2. H. Yuan, X. Zhu, D. Chen, W. Wang, S. Meng and J. Wang, Effects of dual modified resistant indica rice starch on azoxymethane-induced incipient colon cancer in mice, *Exp. Ther. Med.*, 2017, **13**, 2036.
3. E. Bagheri, F. Hajiaghaalipour, S. Nyamathulla and N. A. Salehen, Ethanolic extract of *Brucea javanica* inhibit proliferation of HCT-116 colon cancer cells via caspase activation, *Rsc Advances*, 2018, **8**, 681-689.
4. H. Zhu, G. Lv, Q. Qu, J. Xu, L. Zhang and Y. Zhu, Thunder god vine extract exerts antiproliferative effects on growth of human colon cancer cells and inhibits colon cancer growth in xenograft mice models, *South African Journal of Botany*, 2018, **115**, 18-23.
5. H. Niu, D. Song, H. Mu, W. Zhang, F. Sun and J. Duan, Investigation of three lignin complexes with antioxidant and immunological capacities from *Inonotus obliquus*, *Int. J. Biol. Macromol.*, 2016, **86**, 587-593.
6. Y. O. Kim, S. B. Han, H. W. Lee, H. J. Ahn, Y. D. Yoon, J. K. Jung, H. M. Kim and C. S. Shin, Immunostimulating effect of the endo-polysaccharide produced by submerged culture of *Inonotus obliquus*, *Life Sci.*, 2005, **77**, 2438-2456.
7. H. S. Lee, E. J. Kim and H. K. Sun, Ethanol extract of *Inonotus obliquus* (Chaga mushroom) induces G1 cell cycle arrest in HT-29 human colon cancer cells, *Nutr. Res. Pract.*, 2015, **9**, 111.
8. X. Ning, Q. Luo, C. Li, Z. Ding, J. Pang and C. Zhao, Inhibitory effects of a polysaccharide extract from the Chaga medicinal mushroom, *Inonotus obliquus* (higher Basidiomycetes), on the proliferation of human neurogliocytoma cells, *Int. J. Med. Mushrooms*, 2014, **16**, 29.
9. S. K. Mishra, J. H. Kang, D. K. Kim, S. H. Oh and M. K. Kim, Orally administered aqueous extract of *Inonotus obliquus* ameliorates acute inflammation in dextran sulfate sodium (DSS)-induced colitis in mice, *J. Ethnopharmacol.*, 2012, **143**, 524-532.
10. Y. Hu, S. Shi, L. Lu, C. Teng, S. Yu, X. Wang, M. Yu, J. Liang and J. Qu, Effects of selenizing modification on characteristics and antioxidant activities of *Inonotus obliquus* polysaccharide, *Macromolecular Research*, 2017, **25**, 1-9.
11. J. Wang, C. Wang, S. Li, W. Li, G. Yuan, Y. Pan and H. Chen, Anti-diabetic effects of *Inonotus obliquus* polysaccharides in streptozotocin-induced type 2 diabetic mice and potential mechanism via PI3K-Akt signal pathway, *Biomed. Pharmacother.*, 2017, **95**, 1669-1677.
12. C. W. Wold, C. Kjeldsen, A. Corthay, F. Rise, B. E. Christensen, J. Ø. Duus and K. T. Inngjerdigen, Structural characterization of bioactive heteropolysaccharides from the medicinal fungus *Inonotus obliquus* (Chaga), *Carbohydrate Polymers*, 2018, **185**, 27.
13. L. Fan, S. Ding, L. Ai and K. Deng, Antitumor and immunomodulatory activity of water-soluble polysaccharide from *Inonotus obliquus*, *Carbohydrate Polymers*, 2012, **90**, 870-874.
14. Y. O. Kim, H. W. Park, J. H. Kim, J. Y. Lee, S. H. Moon and C. S. Shin, Anti-cancer effect and structural characterization of endo-polysaccharide from cultivated mycelia of *Inonotus obliquus*, *Life Sci.*, 2006, **79**, 72-80.

15. Y. Chen, Y. Huang, Z. Cui and J. Liu, Purification, characterization and biological activity of a novel polysaccharide from *Inonotus obliquus*, *Int. J. Biol. Macromol.*, 2015, **79**, 587-594.
16. K. R. Lee, J. S. Lee, Y. R. Kim, I. G. Song and E. K. Hong, Polysaccharide from *Inonotus obliquus* inhibits migration and invasion in B16-F10 cells by suppressing MMP-2 and MMP-9 via downregulation of NF- κ B signaling pathway, *Oncol. Rep.*, 2014, **31**, 2447-2453.
17. K. R. Lee, J. S. Lee, J. E. Song, S. J. Ha and E. K. Hong, *Inonotus obliquus*-derived polysaccharide inhibits the migration and invasion of human non-small cell lung carcinoma cells via suppression of MMP-2 and MMP-9, *Int. J. Oncol.*, 2014, **45**, 2533.
18. K. R. Lee, J. S. Lee, S. Lee, Y. K. Son, G. R. Kim, Y. C. Sim, J. E. Song, S. J. Ha and E. K. Hong, Polysaccharide isolated from the liquid culture broth of *Inonotus obliquus* suppresses invasion of B16-F10 melanoma cells via AKT/NF- κ B signaling pathway, *Mol. Med. Report.*, 2016, **14**, 4429.
19. S. H. Lee, H. S. Hwang and J. W. Yun, Antitumor activity of water extract of a mushroom, *Inonotus obliquus*, against HT-29 human colon cancer cells, *Phytotherapy Research Ptr*, 2009, **23**, 1784.
20. C. C. Tsai, Y. S. Li and P. P. Lin, *Inonotus obliquus* extract induces apoptosis in the human colorectal carcinoma's HCT-116 cell line, *Biomed. Pharmacother.*, 2017, **96**.
21. S. H. Lee, H. S. Hwang and J. W. Yun, Antitumor activity of water extract of a mushroom, *Inonotus obliquus*, against HT-29 human colon cancer cells, *Phytotherapy research : PTR*, 2009, **23**, 1784-1789.
22. H. S. Lee, E. J. Kim and S. H. Kim, Ethanol extract of *Innotus obliquus* (Chaga mushroom) induces G1 cell cycle arrest in HT-29 human colon cancer cells, *Nutrition research and practice*, 2015, **9**, 111-116.
23. X. Xu, Y. Wu and H. Chen, Comparative antioxidative characteristics of polysaccharide-enriched extracts from natural sclerotia and cultured mycelia in submerged fermentation of *Inonotus obliquus*, *Food Chem.*, 2011, **127**, 74-79.
24. Z. Wang, Z. Xu, Z. Niu, B. Liang and J. Niu, Epericalyxin A Induces Cell Apoptosis Through JNK and ERK1/2 Signaling Pathways in Colon Cancer Cells, *Cell Biochemistry & Biophysics*, 2015, **73**, 559-564.
25. I. B. Jeff, E. Fan, M. Tian, C. Song, J. Yan and Y. Zhou, In vivo anticancer and immunomodulating activities of mannogalactoglucan-type polysaccharides from *Lentinus edodes* (Berkeley) Singer, *Central-European Journal of Immunology*, 2016, **41**, 47-53.
26. G. Niu and X. Chen, Apoptosis imaging: beyond annexin V, *Journal of Nuclear Medicine Official Publication Society of Nuclear Medicine*, 2010, **51**, 1659-1662.
27. S. C. Tsaniras, N. Kanellakis, I. E. Symeonidou, P. Nikolopoulou, Z. Lygerou and S. Taraviras, Licensing of DNA replication, cancer, pluripotency and differentiation: An interlinked world?, *Seminars in Cell & Developmental Biology*, 2014, **30**, 174.
28. M. J. Youn, J. K. Kim, S. Y. Park, Y. Kim, S. J. Kim, S. L. Jin, K. Y. Chai, H. J. Kim, M. X. Cui and S. S. Hong, Chaga mushroom (*Inonotus obliquus*) induces G0/G1 arrest and apoptosis in human hepatoma HepG2 cells, *World Journal of Gastroenterology*, 2008, **14**, 511-517.

29. X. H. Zhong, L. B. Wang and D. Z. Sun, Effects of inotodiol extracts from inonotus obliquus on proliferation cycle and apoptotic gene of human lung adenocarcinoma cell line A549, *Chinese Journal of Integrative Medicine*, 2011, **17**, 218-223.
30. H. Suo, J. Song, Y. Zhou, Z. Liu, Y. I. Ruokun, K. Zhu, J. Xie and X. Zhao, Induction of apoptosis in HCT-116 colon cancer cells by polysaccharide of *Larimichthys crocea* swim bladder, *Oncol. Lett.*, 2015, **9**, 972-978.
31. P. Fatourachi, S. M. Mohammadi, B. Valipour, A. Dehnad and H. N. Charoudeh, Extracted metabolite from *Streptomyces Levis* ABRIINW111 altered the gene expression in colon cancer, *Gastroenterology & Hepatology from Bed to Bench*, 2018, **11**, 34-41.
32. C. Li, Y. Jeong and M. Kim, *Mammea longifolia* Planch. and *Triana* Fruit Extract Induces Cell Death in the Human Colon Cancer Cell Line, SW480, via Mitochondria-Related Apoptosis and Activation of p53, *J. Med. Food*, 2017, **20**, 485-490.
33. Jin, Yang, Ma, Ma, Ren, Guo, Wang, Zhang, Zhao and Cui, In vivo and in vitro induction of the apoptotic effects of oxysophoridine on colorectal cancer cells via the Bcl-2/Bax/caspase-3 signaling pathway, *Oncol. Lett.*, 2017, **14**, 8000.
34. C. C. Tsai, Y. S. Li and P. P. Lin, *Inonotus obliquus* extract induces apoptosis in the human colorectal carcinoma's HCT-116 cell line, *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie*, 2017, **96**, 1119-1126.
35. M. Youn, J. Kim, Sy, Y. Kim, S. Kim, J. Lee, K. Chai, H. Kim, M. Cui, H. So and K. Kim, Chaga mushroom (*Inonotus obliquus*) induces G0/G1 arrest and apoptosis in human hepatoma HepG2 cells, *World Journal of Gastroenterology*, 2008, **14**, 511-517.
36. M. J. Youn, J. K. Kim, S. Y. Park, Y. Kim, C. Park, E. S. Kim, K. I. Park, S. S. Hong and R. Park, Potential anticancer properties of the water extract of *Inonotus obliquus* by induction of apoptosis in melanoma B16-F10 cells, *Journal of Ethnopharmacology*, 2009, **121**, 221-228.
37. M. Nomura and T. A. Takahashi, Inotodiol, a lanostane triterpenoid, from *Inonotus obliquus* inhibits cell proliferation through caspase-3-dependent apoptosis, *Anticancer Research*, 2008, **28**, 2691-2696.

Figures

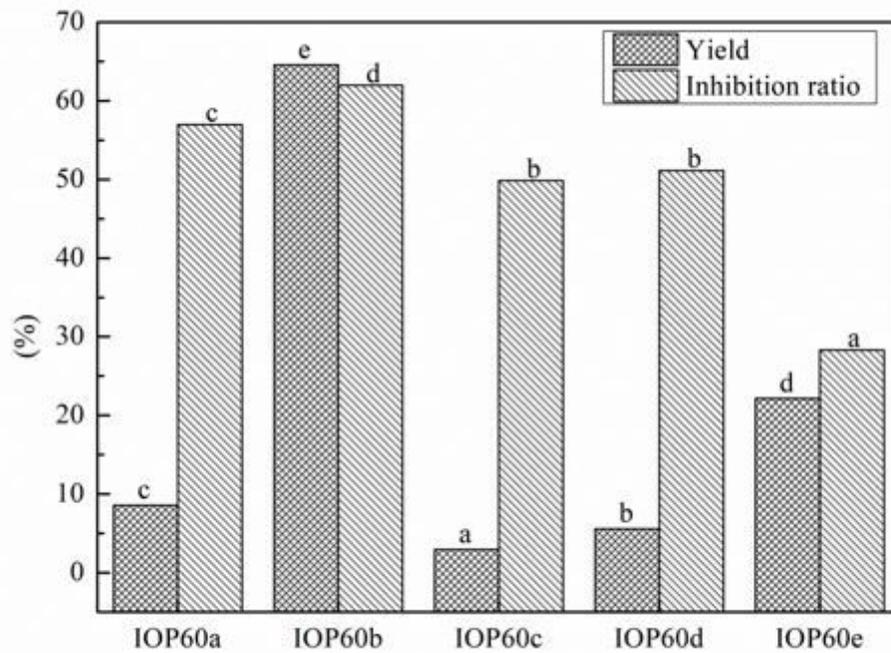


Figure 1

Yields of polysaccharides with different molecular weight extracted from *I. obliquus* and their inhibition ratios of HT-29 cells. Superscripts (a, b, c, d, and e) represent significant differences between the substrates ($P < 0.05$).

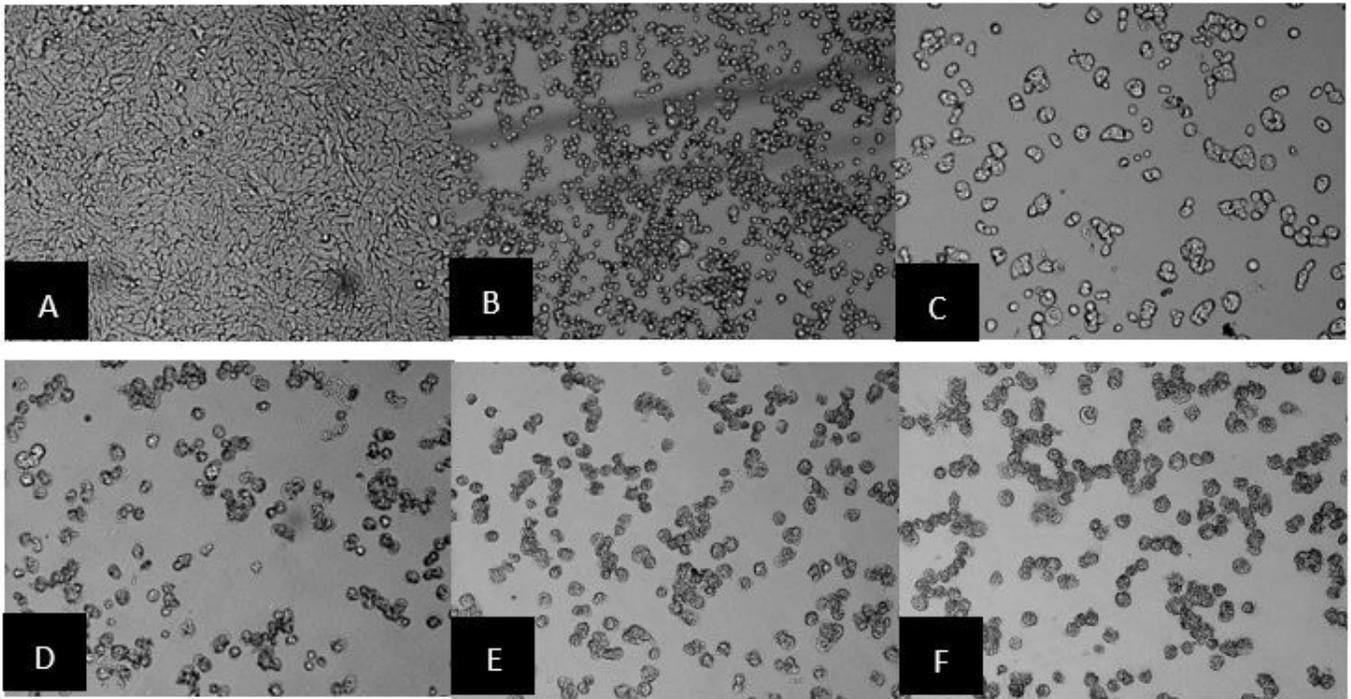


Figure 2

Effects of IOP60b on cellular morphology in HT-29 cells: Cellular morphology was observed under an optical microscopy ($\times 100$). A, B, C, D, E, F represent the morphology of HT-29 cells after treatment with IOP60b at 0, 0.625, 1.25, 2.5, 5 and 10 mg/mL concentrations for 48 h, respectively.

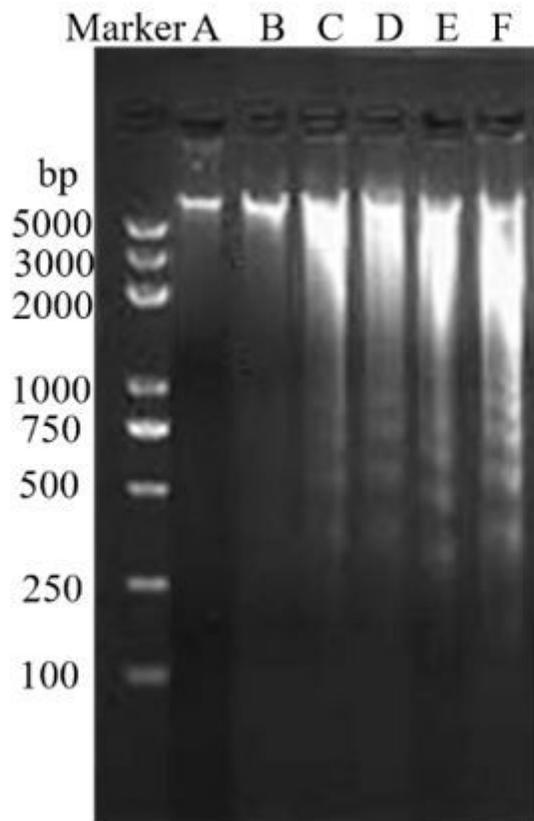


Figure 3

Effects of IOP60b with different concentration on DNA fragmentation in HT-29 cells. Lane A: Control; Lane B: 0.625 mg/mL; Lane C: 1.25 mg/mL; Lane D: 2.5 mg/mL; Lane E: 5 mg/mL; Lane F: 10 mg/mL.

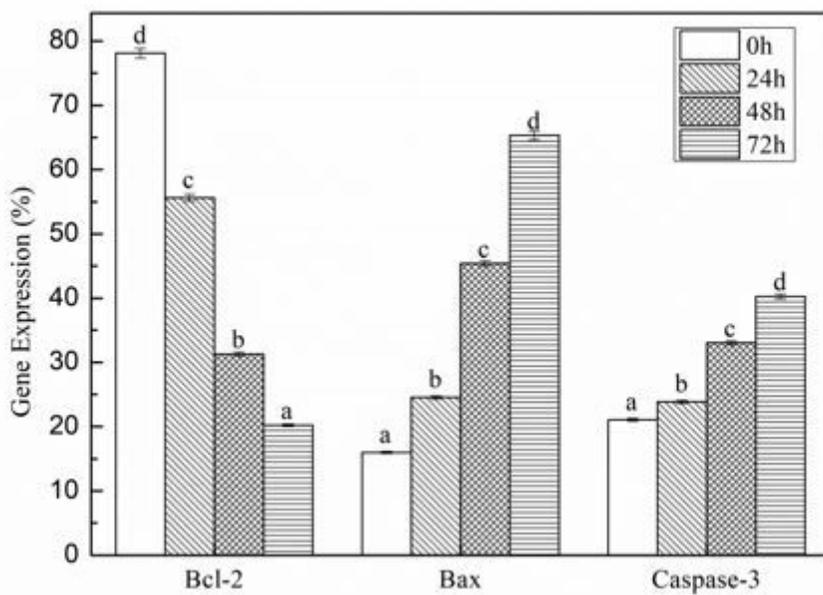
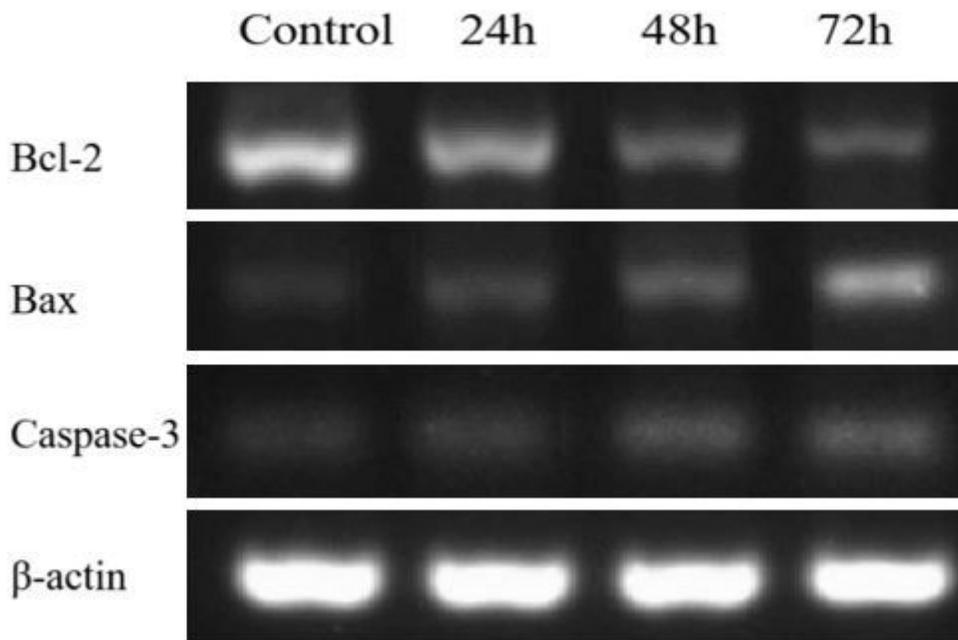


Figure 4

Effects of IOP60b on mRNA of Bcl-2, Bax and Caspase-3 in HT-29 colon cancer cells. Superscripts (a, b, c, and d) represent significant differences between the time-points ($P < 0.05$).

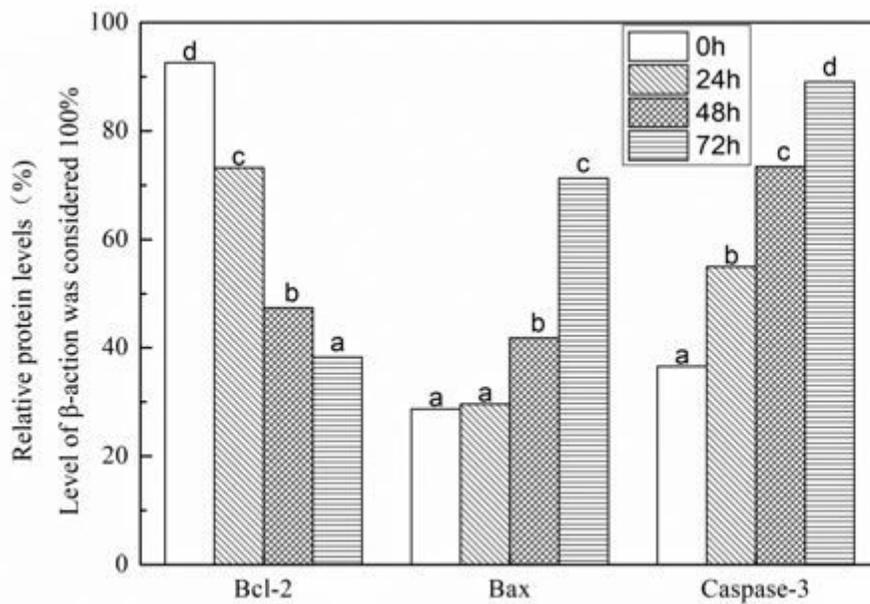
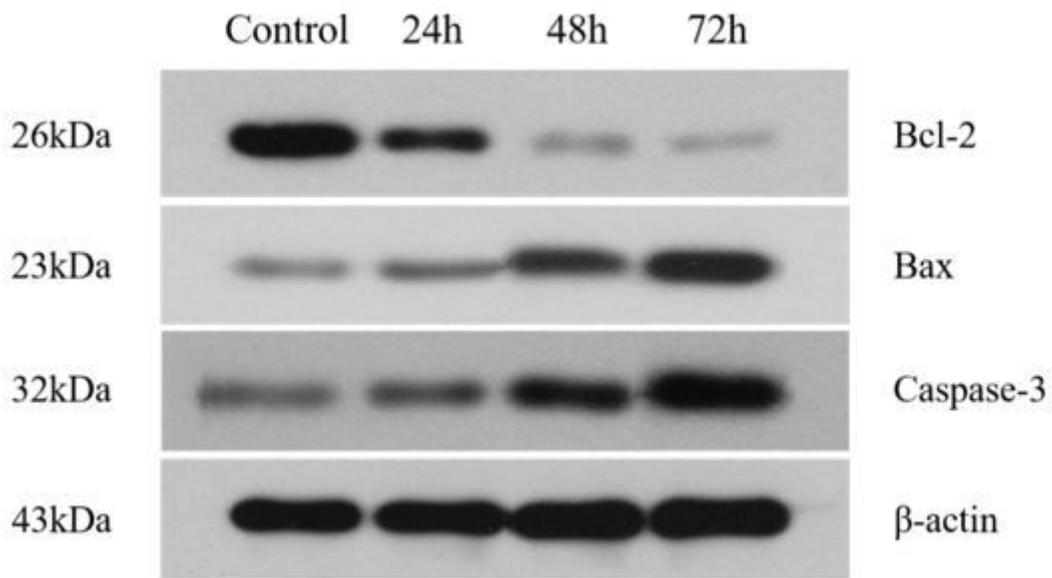


Figure 5

Effects of IOP60b on protein expression of Bcl-2, Bax and Caspase-3 in HT-29 colon cancer cells. Superscripts (a, b, c, and d) represent significant differences between the time-points ($P < 0.05$).