Original article

**Inonotus obliquus** extract induces apoptosis in the human colorectal carcinoma's HCT-116 cell line

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**ABSTRACT**

Because of irregular dietary habits and lifestyle in Taiwan, the incidence and mortality rate of colorectal cancer have been increasing rapidly these years. This study investigated the inhibitory activity against the proliferation of human colorectal cancer HCT-116 cells by **Inonotus obliquus** extracts obtained from submerged fermentation. Cell viability was measured by the reduction of MTT and cell membrane integrity was determined by lactic dehydrogenase (LDH) release. The mRNA expression of proapoptosis and antiapoptosis mediators was assayed by real-time PCR, and the levels of p53 and NF-κB p65 were assessed using Western blot analysis. Furthermore, the influences of **I. obliquus** extracts to HCT-116 cells were evaluated by caspase-3 activity. The results can be summarized as, for the mitochondrial apoptotic pathway, quantitative RT-PCR data showed up-regulation of proapoptotic genes (Bax, bad, and caspase-3) and increased Bax/bcl-2 ratio by **I. obliquus** extracts. Moreover, treating with 20 mg/mL **I. obliquus** extracts augmented caspase-3 activity in HCT-116 cells. Induction of cell cycle G0/G1 phase arrest: **I. obliquus** extracts up-regulated the mRNA expression of proapoptotic genes (p53, p21WAF1/CIP1) and down-regulated antiapoptotic gene (CyclinD1), while extracts of **I. obliquus** mycelia increased the expressions of p53 protein in HCT-116 cells. **I. obliquus** extracts decreased the expression of NF-κB p65 protein and COX-2 gene in HCT-116 cells. Taking together, **I. obliquus** extracts may be used as a potentially novel food material for health care to improve the treatment of colorectal cancer.

1. **Introduction**

In Taiwan, according to the statistical data reported by the Health Promotion Administration, Ministry of Health and Welfare, colorectal carcinoma (CRC) was again ranked number one in incidence and prevalence in Taiwan, while its mortality rate had risen to number three. This was mainly attributed to CRC and colitis-associated cancer (CAC) caused by diet and living environment becoming more Westernized. Every year, there are over 1 million new CRC cases globally. CRC is also the third most common malignant tumor, and its mortality rate is the fourth largest cause of cancer-related deaths [1]. Only about 20% of CRC cases are inherited while the majority of remaining cases are caused by the environment [2].

**Inonotus obliquus** is also known as chaga mushroom. Since the beginning of the 20th century, researchers gradually began to study **I. obliquus**. Currently, its known effects include inhibition of HIV-1 protease activity [3], immune regulation and clearance of free radicals, and inhibition of lipid peroxidation [4], inhibition of acute colitis symptoms [5], decreasing the production of aging cells and wrinkles on the skin of hairless mice [6], regulation of secretion of Th1/Th2 hormones, and inhibition of IgE production [7]. The fruting body and mycelium of **I. obliquus** showed inhibitory effects towards HT-29 human colorectal cancer cells, A-549 human lung cancer cells, MCF-7 human breast cancer cells, AGS human gastric cancer cells, SGC-7901 human gastric cancer cells, HepG2 human liver cancer cells, Hep3B human liver cancer cells, B16-F10 mouse melanoma cells, and Sarcoma-180 mouse sarcoma cells [8–12].

Zheng et al. classified the physiological active ingredients of **I. obliquus** into triterpenoids, ergosterol, ergosterol peroxide [13], sesquiterpene [14], benzoic acid derivatives [15], hispidin-like compounds, melanins [16], and polysaccharides [17]. These polysaccharides include β-glucans and heteroglucans [13,18]. Studies have also shown that polyphenols and polysaccharides in **I. obliquus** aqueous extracts can inhibit proliferation and metastasis of cancer cells [19–21].

The evolution of CAC is intimately associated with oncogenes and tumor suppressors, particularly NF-κB. NF-κB has been found to over-express in many colorectal cancer patients, confirming that NF-κB is intimately associated with the development of colorectal cancer.

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NF-κB can also promote the activation of Bcl-2, Bcl-xL, Cflip, and other anti-apoptotic proteins. Inhibition of NF-κB activity has been shown to inhibit proliferation, promote apoptosis, and inhibit chemotherapy-induced drug resistance in colorectal cancer cells [24,25].

In recent studies, it was found that use of 5-FU (5-Fluorouracil) in treatment of colorectal cancer will stimulate the production of NF-κB. This is also the major cause of poor prognosis and development of drug resistance in cancer patients [26]. This study used I. obliquus powder made from liquid-state fermentation and freeze-drying to investigate whether extracts of I. obliquus mycelium could inhibit mitochondrial-mediated apoptosis pathway, and genes as well as proteins associated with cytokine and cell cycle regulation in colorectal cancer cells.

2. Materials and methods

2.1. Preparation of Inonotus obliquus extracts and cell culture

The lyophilized I. obliquus powder used in this study was provided by the manufacturer and stored in a −80 °C freezer. To prepare a concentration 50 mg/mL, we weighted 5 g of the I. obliquus powder and added the McCoy’s 5A medium to quantify to 100 mL in the volumetric flask, as well as sonication for 30 min on ice before filtration (sample solution A). Then we took 50 mL sample solution A (50 mg/mL) and diluted it with 50 mL McCoy’s 5A medium to get the concentration 25 mg/mL (sample solution B) and use sample solution B to dilute the concentration of 5, 10, 15, 20, 25, 50 mg/mL. The solution was then stored in a −80 °C freezer until for use.

The HCT-116 cell line was obtained from the Bioresources Collection and Research Center (BCRC), Hsin-Chu, Taiwan. HCT-116 cells were grown in McCoy’s 5A Medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum, 1% nonessential amino acid (NEAA), and 1.1 g sodium bicarbonate.

2.2. Analysis of cell viability

This experiment focused on analysing the ability of I. obliquus to inhibit colorectal cancer cells. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), a colourless, transparent tetrazolium salt, is reduced to yield a purple formazan crystal by mitochondrial dehydrogenase in living cells. In total, 500 μL (3 × 10^4 cells/mL) of cells was seeded into a 24-well plate, and the cells were subjected to overnight culture at 37 °C in a CO2 incubator, which made the cells attach, divide and grow in the 24 wells. After gentle washing twice with 1 × PBS, and after discarding PBS, 1 mL of mixed solutions of 100 μL of I. obliquus (0, 5, 10, 15, 20, 25 and 50 mg/mL), and following 24 h cultures at 37 °C in the CO2 incubator, the cells were centrifuged at 1100 rpm for 5 min. Next, 50 μL of supernatants were aspirated and transferred to a new 96-well plate and then according to the manufacturer’s instructions. The resulting absorbance in each well was measured at 490 nm. Cytotoxicity % = (OD experimental/OD positive control) × 100; OD, optical density.

2.3. Analysis of lactate dehydrogenase (LDH)

LDH stably exists in the cytoplasm and is released from cells with a damaged membrane; therefore, LDH activity in the cell culture medium is positively correlated with the number of necrotic cells. The CytoScan™LDH cytotoxicity assay kit (G-Biosciences, St. Louis, MO, USA) was used to measure LDH release: the HCT-116 cells (10^4 cells/100 μL) were seeded in 96 wells and cultured overnight to ensure that the cells attached and grew in the wells. After the removal of the old culture medium, the cells were washed twice with PBS (including 1% BSA) and then cultured with a series of 100 μL mixed solutions of I. obliquus in different concentrations and cell culture media for 24 h at 37 °C in the CO2 incubator. Alternatively, the HCT-116 cells were cultured with mixed solutions of 100 μL of I. obliquus (0, 5, 10, 15, 20, 25 and 50 mg/mL), and following 24 h cultures at 37 °C in the CO2 incubator, the cells were centrifuged at 1100 rpm for 5 min. Next, 50 μL of supernatants were aspirated and transferred to a new 96-well plate and then according to the manufacturer’s instructions. The resulting absorbance in each well was measured at 490 nm. Cytotoxicity % = (OD experimental/OD positive control) × 100; OD, optical density.

2.4. RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was isolated from cell lines using TRIzol reagent (Invitrogen, Carlsbad, USA). Quantitative RNA was using Qubit® RNA BR Assay Kit (Invitrogen, Carlsbad, USA). The cDNAs were prepared with the use of MMLV First-Strand Synthesis Kit (Gene Direx X, USA) as the manufacturer’s protocol. Quantitative cDNA was using Qubit® ssDNA Assay Kit (Invitrogen, Carlsbad, USA). QRT-PCR was performed in 7500 system real-time PCR (Applied Biosystems, Carlsbad, CA, USA) in triplicate and normalized with β-actin as an endogenous control. Real-time PCR was carried out by using the 7500 (Applied Biosystems) according to the manufacturer’s instructions. The relative expression was calculated by the 2^ΔΔCt method [29]. The real-time PCR primers were as Table 1 [30–37].

2.5. Quantitative measurements of NF-κB p65 proteins involved in the HCT-116 cells

Apoptosis-associated protein expression in the HCT-116 cells was determined by western blotting. The HCT-116 cells were added to a 175 flask and cultured overnight at 37 °C in the CO2 incubator to make the cells attach, divide and grow in the wells. The solutions of I. obliquus in various concentrations mixed with the cell culture media were added to the wells, and the cells were cultured for 24 h at 37 °C in the CO2 incubator, which was followed by the addition of I. obliquus (0, 5, 10, 15 and 20 mg/mL) mixed with the cell culture media. After a 6-h culture in the CO2 incubator at 37 °C, the cells were collected and then lysed in radioimmunoprecipitation assay buffer for 30 min on ice using Qubit®

### Table 1

<table>
<thead>
<tr>
<th>Q-PCR primers used in this study.</th>
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<tr>
<td><strong>Target</strong></td>
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<tr>
<td>p53</td>
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<tr>
<td>p21</td>
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<tr>
<td>Bax</td>
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<tr>
<td>Bad</td>
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<tr>
<td>Bcl-2</td>
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<tr>
<td>Caspase-3</td>
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<td>Cox-2</td>
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<tr>
<td>Cyclin E</td>
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<td>Cyclin D1</td>
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<td>β-actin</td>
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Protein Assay Kit (Invitrogen, Carlsbad, USA). Next, the cells were centrifuged at 12,000 rpm for 5 min at 4 °C. The resulting were subjected to quantitative protein analysis using the Qubit® fluorometer (Invitrogen, Carlsbad, USA).

Cellular protein extracts were mixed with 5 × loading dye, heated at 95 °C for 5 min and loaded in each well for SDS-PAGE. Following electrophoresis, proteins on the gel were transferred to a PVDF membrane for an hour at 37 °C with the addition of 5% skim milk as the blocking buffer. Subsequently, the membrane was washed in TBST [20 mM Tris–HCl, pH 8 and 137 mM NaCl containing 0.1% (v/v) Tween-20] three times and was incubated with the primary antibody (monoclonal antibodies of NF-κB p65) overnight at 4 °C. After three washes with TBST, the secondary antibody (HRP-conjugated goat anti-mouse IgG) was added and incubated with the membrane for 1 h, which was followed by TBST washes. The resulting gel images were captured using a luminometer, and bands were observed [27,38].

2.6. Statistical analysis

Statistical analysis of the study data was performed using the SAS 9.4 (Statistical Analysis System) or SPSS 19.0 (Statistical Package for the Social Sciences) statistical software. One-way analysis of variance (ANOVA) or independent sample Duncan’s Multiple Range Test was used to determine the statistical significance; p < 0.05 indicates significant difference.

3. Results and discussion

3.1. Analysis of CRC cell viability and membrane integrity after treatment with extracts of I. obliquus mycelium

This experiment employed the MTT method and the concentrations of I. obliquus extracts used were 5, 10, 15, 20, and 25 mg/mL. Results showed that I. obliquus showed the best inhibitory effect on HCT-116 cells and this was dose-dependent. After calculation, the IC_{50} was found to be 10 mg/mL (Table 2).

On the other hand, analysis of cell membrane integrity was carried out using the LDH release assay. Even though LDH is generally used to determine cell necrosis, but LDH levels also shows a rising trend in late apoptosis stages [39,40]. This study used HCT-116 cells to determine the membrane integrity of colorectal cancer cells under different concentrations of extracts of I. obliquus mycelium. Results showed that when concentrations of extracts of I. obliquus mycelium were 20 and 25 mg/mL, there was a slight increase in LDH (Fig. 1). However, relative to MTT results, there was massive cell death, suggesting that cells had already entered the late stages of apoptosis.

I. obliquus also showed significant inhibitory effects on cell survival in other cancer cells. Chung et al. [8] used I. obliquus extracts to inhibit A-549 human lung cancer cells, MCF-7 human breast cancer cells, AGS in other cancer cells. Chung et al. [8] relative to MTT results, there was massive cell death, suggesting that cells and this was dose-dependent. After calculation, the IC_{50} was found to be 10 mg/mL (Table 2).

Table 2

<table>
<thead>
<tr>
<th>I. obliquus Concentration (mg/mL)</th>
<th>Incubation time (hours)</th>
<th>HCT-116 Cell Survival (%)</th>
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<tbody>
<tr>
<td></td>
<td>12h</td>
<td>24h</td>
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<tr>
<td>25</td>
<td>0.00 ± 0.00 a</td>
<td>0.00 ± 0.00 a</td>
</tr>
<tr>
<td>20</td>
<td>23.97 ± 4.68 b</td>
<td>1.47 ± 0.90 b</td>
</tr>
<tr>
<td>15</td>
<td>45.75 ± 1.64 c</td>
<td>16.04 ± 8.77 d</td>
</tr>
<tr>
<td>10</td>
<td>74.88 ± 14.98 d</td>
<td>41.30 ± 14.42 c</td>
</tr>
<tr>
<td>5</td>
<td>82.84 ± 6.16 b</td>
<td>68.90 ± 7.16 b</td>
</tr>
<tr>
<td>0</td>
<td>100 ± 0.00 a</td>
<td>100 ± 0.00 a</td>
</tr>
</tbody>
</table>

Each value is expressed as mean ± standard deviation (n = 4).

3.2. Extracts of I. obliquus mycelium regulates cell cycle in colorectal cancer cells

This study used lyophilized I. obliquus powder after liquid-state fermentation and 10mg/ml (the concentration refers from IC_{50} result) were added to human colorectal cancer cells. RT-PCR was used to determine the mRNA expression of p53, p21^{Waf1/Cip1}, Cyclin D1, and Cyclin E. Results showed that at 18 h, there was significant increase in p53 expression (Fig. 2A), while at 3, 12, and 18 h, there was a significant decrease in Cyclin D1 expression (Fig. 2B). There were no significant changes in Cyclin E expression (Fig. 2C). It was found that there were significant increases in p21^{Waf1/Cip1} at 3, 6, 18, and 24 h (Fig. 2D). Summarizing the above results, there were significant increases in p53 and p21^{Waf1/Cip1} but cyclinD1 showed a decreasing trend. This suggests that I. obliquus could affect cell cycle by regulating p53 and p21^{Waf1/Cip1}. Cyclin D1 is a protein that is needed for cells to transit from the G0 to the G1 phase during cell cycle progression while p21^{Waf1/Cip1} is responsible for regulation of cyclin-dependent kinases (CDK), and can inhibit cyclins and CDKs from forming complexes. On
the other hand, p53 can regulate p21\textsubscript{Waf1/Cip1}. p53 is a type of tumor suppressor and it can inhibit the tumor development. p53 can also inhibit cell cycle progression, resulting in stagnation of cancer cell growth. p53 can also regulate cellular apoptosis and inhibit the activity and proliferation of cancer cells. p21\textsubscript{Waf1/Cip1} will in turn inhibit the activity of CDKs, resulting in inhibition of cell cycle progression and cells will be stalled at the G1 checkpoint. Currently, there is still a lot of controversy on the p53 regulation by \textit{I. obliquus} as up till now, it is still not confirmed whether the \textit{I. obliquus} increases or decreases p53 protein expression \cite{42}. Recent studies have also found that p21\textsubscript{Waf1/Cip1} is not completely regulated by p53. Zhong et al. \cite{43} used inotodiol from \textit{I. obliquus} fruiting bodies to inhibit A549 human lung cancer cells and investigated the pathways involved. They found that there were increases in pro-apoptotic proteins p53 and Bax, while anti-apoptotic proteins such as Bcl-2 were decreased \cite{43}. However, a study by Youn et al. \cite{11} used fruiting body extracts of \textit{I. obliquus} to inhibit HepG2 human liver cancer cells and induced G0/G1 arrest. However, the levels of p53, pRb, p27, cyclin D1, Cdk2 all decreased \cite{11}. A study by Müller et al. found that lingzhi (\textit{Ganoderma lucidum}) could inhibit lymphoma cells through p21\textsubscript{Waf1/Cip1} activation \cite{44}.

3.3. \textit{Extracts of I. obliquus mycelium affect genes related to mitochondrial-mediated apoptosis}

In this study, RT-PCR was used to investigate the expression of caspase-3, Bax, Bcl-2, and Bad at different time points. Bcl-2 plays a role of anti-apoptosis in the Bcl-2 family but in this study, Bcl-2 shows a slight decrease only at the 24-hour time-point (Fig. 3A). On the other hand, the pro-apoptotic member of the Bcl-2 family, Bax, was significantly increased at 6 h (Fig. 3B). Generally, determination of cellular apoptosis is through the Bax/Bcl-2 ratio. When Bax/Bcl-2 ratio increases, this means that apoptosis becomes more and more evident (Fig. 3C). In this study, we also quantitated another Bcl-2 pro-apoptotic family member, Bad. Results found that there was significant increase in Bad at 12 and 18 h but there was negative regulation at 6 h. Currently it is still unclear why negative regulation occurred at 6 h (Fig. 3D). From the perspective of the terminal protein in mitochondrial-mediated apoptosis (caspase-3), RT-PCR results showed that there were no significant changes in caspase-3 levels at 1, 3, 6, and 12 h, and significant
Fig. 3. Real-time PCR analysis of HCT-116 cells stimulated with 10 mg/mL of \textit{I. obliquus} DNA for 1, 3, 6, 12, 18, and 24 h. Relative expression was determined with the \( E^{-\Delta\Delta Ct} \) method using the PCR efficiencies determined with the standard curve included in each run. Expression of target genes (A) Bcl-2, (B) Bax, (C) Bax/Bcl-2 ratio, (D) Bad, and (E) Caspase-3, are normalized to \( \beta \)-actin and is presented as mean \( \pm \) the standard error.

increases were only observed at 18 and 24 h (Fig. 3E).

In this study, we also examined whether caspase-3 activity will change with increasing concentrations of I. obliquus. Results showed that there was significant increase in caspase-3 activity at 20 mg/mL and 25 mg/mL I. obliquus (Fig. 4A). In order to verify that caspase-3 affects HCT-116 colorectal cancer cells and cause apoptosis, we added a caspase-3 inhibitor (DEVD-FMK) at a concentration of 20 μM and found that at concentrations of 20 mg/mL and 25 mg/mL I. obliquus, caspase-3 activity was decreased to levels similar to the control group. Using the MTT method to analyze cell viability, we found that after addition of caspase-3 inhibitor, there was a significant increase in viability of HCT-116 colorectal cancer cells and this result was very significant. This shows that after caspase-3 inhibition, there was a large decrease in apoptosis in cancer cells (Fig. 4B).

Lee et al. [9] used aqueous extracts from the fruiting bodies of I. obliquus to inhibit HT-29 colorectal cancer cells. Through Western blot analysis, they found that these extracts could inhibit the anti-apoptotic protein Bcl-2 and promote the pro-apoptotic protein Bax. In addition, compared with the control group, the levels of procaspase-3 showed a decreasing trend. This showed that procaspase-3 undergoes cleavage and activation into caspase-3, which in turn affects apoptosis in HT-29 cells [9]. Youn et al. found that aqueous extracts of I. obliquus fruiting bodies can inhibit melanoma B16-F10 cells and upregulate caspase-3 activity [11,12]. They also employed Western blotting to analyze the protein expression of procaspase-3 in HepG2 liver cancer cells. Results found that as the concentration of I. obliquus increases, there was a significant decrease in procaspase-3. This could be explained as extracts from I. obliquus fruiting bodies could aid in procaspase-3 activation into caspase-3 [11,12]. Nomura et al. found that inotodiol, a lanostane triterpenoid from I. obliquus can inhibit P388 leukemia cells through caspase-3 activation [10]. Hwang et al. also found that the I. obliquus fruiting bodies can increase caspase-3 production in HT-29 colorectal cancer cells and SNU484 gastric cancer cells [45].

3.4. Regulation of NF-κB p65 protein levels and COX-2 gene expression by extracts of I. obliquus mycelium

This study uses different concentrations of extracts of I. obliquus mycelium on the HCT-116 human colorectal cancer cells. Results showed that NF-κB p65 protein expression was inhibited at all concentrations (Fig. 5A). COX-2 is generally used as a marker of cancer treatment, and COX-2 and NF-κB show an upstream-downstream relationship [46]. In our experiments, COX-2 mRNA expression at all time-points was decreased, particularly at 3 h (Fig. 5B).

Many researchers believed that mushroom metabolites could decrease NF-κB activity [47]. Kim et al. stimulated RAW macrophages with LPS before adding I. obliquus fruiting bodies then found that I. obliquus fruiting bodies could inhibit NF-κB activity, along with COX-2 and iNOS activity [48]. Park et al. also found that alcohol extracts from I. obliquus can inhibit the expression of COX-2, iNOS, and NF-κB [49]. A study by Silva et al. found that lyophilized lingzhi powder can effectively decrease NF-κB activity in breast cancer and prostate cancer cells and inhibit these two types of cancer [50]. An article by Petrova et al. mentioned that NF-κB has already been recognized as a major target for inhibition in treatment for many cancers [51]. In breast cancer, I. obliquus fruiting bodies were found to inhibit breast cancer through NF-κB inhibition [51]. Yang et al. published a paper showing that Antrodia camphorata could inhibit breast cancer through regulation of multiple pathways, including downregulation of NF-κB [52]. In cancer therapy, many researchers believe that p53 activation and NF-κB inhibition are the major methods of inhibiting cancer cells [53].

4. Conclusions

In the mitochondrial-mediated apoptosis pathway, extracts of I. obliquus mycelium can induce apoptosis in HCT-116 cells by upregulating pro-apoptotic genes Bax, Bad, TNFSF10, caspase-3) and increasing the Bax/Bcl-2 ratio. Also, addition of 20 mg/mL and 25 mg/mL of extracts of I. obliquus mycelium could significantly increase caspase-3 activity in HCT-116 cells. Western blot was used to analyze the protein expression of the tumor suppressor p53 and NF-κB p65 that results showed that extracts of I. obliquus mycelium can increase p53 protein expression and decreased NF-κB p65 protein expression in HCT-116 cells. As well as the COX-2 mRNA expression was decreased. In inducing stalling of cell cycle progression from G0 to G1 phase, I. obliquus can upregulate the pro-apoptotic genes (p53 and p21WAF1/CIP1) and inhibit the anti-apoptotic gene CyclinD1 in HCT-116 cells. This resulted in cells being stalled at the G0/G1 checkpoint, and increased the probability of apoptosis induction.

Conflict of interest

The authors declare no conflict of interest.
Fig. 5. (A) Effect of I. obliquus on the levels of NF-κB p65 in human colorectal carcinoma HCT-116 cells by western. (B) Real-time PCR analysis of HCT-116 cells stimulated with 10 μg/mL of I. obliquus DNA for 1, 3, 6, 12, 18, and 24 h. Relative expression was determined with the E-ΔΔCt method using the PCR efficiencies determined with the standard curve included in each run. Expression of target genes (COX-2) is normalized to β-actin and is presented as mean ± the standard error.

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References


