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# Ethanol extract of *Innotus obliquus* (Chaga mushroom) induces $G_1$ cell cycle arrest in HT-29 human colon cancer cells

Hyun Sook Lee[1*], Eun Ji Kim[2*] and Sun Hyo Kim[3§]

[1]Department of Food Science and Nutrition, Dongseo University, Busan 617-716, Korea
[2]Research Institute, Adbiotech Co. Ltd., Gangwon 200-957, Korea
[3]Department of Technology and Home Economics Education, Kongju National University, 56 Kongjudaehak-ro, Chungnam 314-701, Korea

**BACKGROUND/OBJECTIVES:** *Inonotus obliquus* (*I. obliquus*, Chaga mushroom) has long been used as a folk medicine to treat cancer. In the present study, we examined whether or not ethanol extract of *I. obliquus* (EEIO) inhibits cell cycle progression in HT-29 human colon cancer cells, in addition to its mechanism of action.
**MATERIALS/METHODS:** To examine the effects of *Inonotus obliquus* on the cell cycle progression and the molecular mechanism in colon cancer cells, HT-29 human colon cancer cells were cultured in the presence of 2.5 - 10 μg/mL of EEIO, and analyzed the cell cycle arrest by flow cytometry and the cell cycle controlling protein expression by Western blotting.
**RESULTS:** Treatment cells with 2.5 - 10 μg/mL of EEIO reduced viable HT-29 cell numbers and DNA synthesis, increased the percentage of cells in $G_1$ phase, decreased protein expression of CDK2, CDK4, and cyclin D1, increased expression of p21, p27, and p53, and inhibited phosphorylation of Rb and E2F1 expression. Among *I. obliquus* fractions, fraction 2 (fractionated by dichloromethane from EEIO) showed the same effect as EEIO treatment on cell proliferation and cell cycle-related protein levels.
**CONCLUSIONS:** These results demonstrate that fraction 2 is the major fraction that induces $G_1$ arrest and inhibits cell proliferation, suggesting *I. obliquus* could be used as a natural anti-cancer ingredient in the food and/or pharmaceutical industry.

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**Keywords**: *Inonotus obliquus,* cell cycle, Rb, colon cancer, anti-cancer

## INTRODUCTION

*Inonotus obliquus* (*I. obliquus*), known as chaga mushroom, is a white rot fungus [1]. *I. obliquus* can be made into tea decoctions, extracts, syrup, injections, hip bath agent, and aerosol and has been used as a folk medicine for treating cancer in many areas such as Russia, Asia, and North America [2,3].

Prior studies have reported that *I. obliquus* contains bioactive compounds such as polysaccharides, and polyphenols, which include triterpenoids, steroids, ergosterol peroxides, inotodial, and 3β-hydroxy-lanosta-8,24-dien-21-al, a lignin-like substance. *I. obliquus* has also been shown to possess biological activities, including antioxidant, anti-viral, anti-inflammatory, hepatoprotective, platelet aggregation inhibitory, and anti-tumor effects [4-18]. However, the molecular mechanisms responsible for the anti-cancer effects of *I. obliquus* are not well understood, despite its increasing usage.

Cell proliferation and death are involved in maintenance of homeostasis in normal cells, however, in cancer cells, homeostasis is often disrupted due to deregulation of cell cycle mechanisms [19]. Anti-tumor effects can be attributed to changes in biochemical mechanisms, such as inhibition of proliferation, induction of cell cycle arrest at various cell cycle checkpoints, induction of apoptosis, and regulation of signal transduction pathways, all of which are related to altered expression of key enzymes [20]. The mammalian cell cycle is divided into 4 separate phases: $G_1$, S, $G_2$, and M phases. During $G_1$ phase, cells respond to extracellular signals by either advancing toward another division or withdrawing from the cell cycle into a resting state ($G_0$) [21]. Cyclin-dependent kinases (CDKs), CDK inhibitors (CDKIs), and cyclins are all important regulators of mammalian cell cycle progression [22]. Each phase of the cell cycle is controlled by different CDKs, each of which is associated with their individual regulatory cyclin. The $G_0/G_1$ phases of the cell cycle is regulated by CDK4 and CDK 6 associated with cyclin D, late $G_1$ into early S phase by CDK2 with cyclin E, S phase by CDK2 with cyclin A, and $G_2$/M phase by CDK1 (CDC2 kinase) with cyclin A or B [23]. Increased

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§ Corresponding Author: Sun Hyo Kim, Tel. 82-41-850-8307. Fax. 82-41-850-8300, Email. shkim@kongju.ac.kr
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* These authors contributed equally to this work.

expression of CDKs and cyclins accompanied by enhanced CDK activity has been observed in cancer cells, and could be associated with uncontrolled cell proliferation [24]. Since autonomous cell proliferation is a hallmark of cancer cells, cell cycle arrest has become a major anti-cancer effect indicator [15].

In this study, we investigated the effects of ethanol extract of *I. obliquus* on cell cycle progression of HT-29 cells, as well as its molecular mechanism of action.

## MATERIALS AND METHODS

*Materials*

The reagents used in this study were purchased from the following suppliers: Dulbecco's modified Eagle's medium/Ham's F12 nutrient mixture (DMEM/F12) and selenium from Gibco BRL (Gaithersburg, MD, USA); fetal bovine serum (FBS), trypsin-EDTA, and penicillin/streptomycin from Cambrex Bio Technology (Walkersville, MD, USA); 3-[4,5-dimetjylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), anti-β-actin, RIA-grade bovine serum albumin (BSA), and transferrin from Sigma-Aldrich Co. (St. Louis, MO, USA); antibodies against cyclin D1 and phospho-Rb (Ser807/811) from Cell Signaling Technology (Beverly, MA, USA); antibodies against $p21^{CIP1/WAF1}$ (c-19), $p27^{KIP1}$, p53, CDK2 (M-2), CDK4 (c-22), E2F-1 (C-20), and Rb (c-15) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Unless otherwise noted, all other materials were obtained from Sigma-Aldrich Co.

*Preparation of extract and fractionation of Inonotus obliquus*

Dried *I. obliquus* derived from Russia was purchased from a local drug store in Chuncheon, Korea. The fruiting body of *I. obliquus* was pulverized to about 30 mesh with a disintegrator. The powder was extracted with 95% ethanol (100g of powder/L 95% ethanol) by heating at 70°C for 12 h. The extract was filtered through Whatman filter paper, after which the filtrate was evaporated in a rotary vacuum evaporator and subsequently freeze-dried at -70°C. The resulting powder was used as ethanol extract of *I. obliquus* (EEIO) and stored at -20°C until further use. EEIO (20 g) was suspended in water (1 L), and fractionated sequentially with n-hexane (1 L), dichloromethane (1 L), and ethyl acetate (1 L), as described in Fig. 1. Each resulting supernatant was filtered, concentrated by a rotary vacuum evaporator, and then freeze-dried. These fractions were stored -20°C until use.

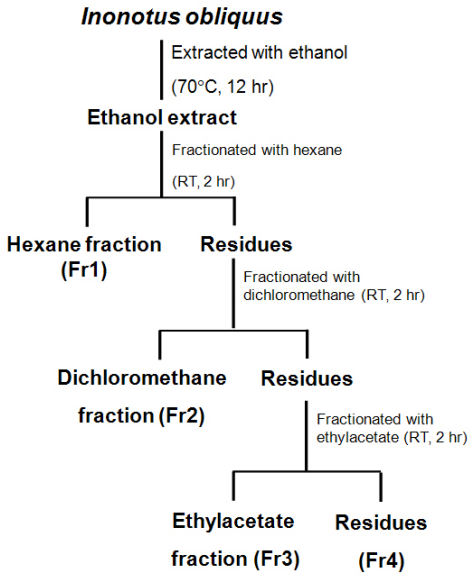


**Fig. 1.** Preparation of extract and various fractions of *Inonotus obliquus*

*Cell culture*

HT-29 human colon cancer cell line was acquired from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM/F12 containing 100 mL/L of FBS, 100,000 U/L of penicillin, and 100 mg/L of streptomycin, and maintained in an incubator with a humidified atmosphere of 5% $CO_2$ at 37°C. To examine the effect of EEIO, we plated cells with DMEM/F12 containing 10% FBS. Before EEIO treatment, we rinsed cell monolayers and serum-starved them for 24 h, with DMEM/F12 supplemented with 5 mg/L of transferrin, 5 μg/L of selenium, and 0.1 g/L of BSA (serum-free medium). After serum starvation, fresh serum-free medium with or without the indicated concentrations of EEIO was replaced. The abilities of EEIO and each fraction to reduce HT-29 cell viability were assessed using MTT assay, as described previously [25].

*5-bromo-2′-deoxyuridine (BrdU) incorporation*

To estimate DNA synthesis, we plated cells in 96-well plates at a density of 50,000 cells/well, serum-starved, and then treated with EEIO for 48 h in the manner described above. Next, we added 10 μM 5-bromo-2′-deoxyuridine (BrdU) to each well and continued incubation for an additional 5 h at 37°C. We then determined BrdU incorporation into DNA using a cell proliferation enzyme-linked immunosorbent BrdU assay kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

*Cell cycle analysis by flow cytometry*

Cells were plated in 24-well plates at a density of 50,000 cells/well in DMEM/F12 containing 10% FBS. Cells were serum-starved and treated with EEIO for 48 h as described above. Cells were separated by trypsin-EDTA and treated with RNase, after which cellular DNA was stained with propidium iodide [26]. The percentages of cells in the $G_1$, S, and $G_2$/M phases of cell cycle were analyzed by flow cytometry. Data were analyzed using Modfit version 1.2 software (Becton Dickinson, Franklin Lakes, NJ, USA).

*Western blot analysis*

Cells were lysed after EEIO or each fraction treatment for 48 h, as described previously [27]. Protein contents of the cell lysates were measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). Western blot analyses were conducted as described previously [28]. Signals were detected based via an enhanced chemiluminescence method using Immobilon™ Western Chemiluminescent HRP Substrate (Pierce). The expressions were normalized to β-actin.

*Statistical analysis*

Data were expressed as the means ± SEM and analyzed using ANOVA. We analyzed differences across treatment groups by

analysis of variance and Duncan's multiple-range test, using SAS system software for Windows, version 9.1 (SAS Institute, Cary, NC, USA). A $P$ value of $< 0.05$ was considered to indicate statistical significance.

## RESULTS

*EEIO inhibits growth and DNA synthesis of HT-29 cells*

EEIO decreased viable HT-29 cell numbers, in a dose-dependent manner, within 72 h of 10 μg/mL of EEIO addition (Fig. 2). DNA synthesis was significantly decreased in HT-29 cells treated with 10 μg/mL of EEIO (Fig. 3).

*EEIO induces $G_1$ arrest in HT-29 cells*

To determine whether or not EEIO regulates cell cycle progression in HT-29 cells, cells were treated with or without 10 μg/mL of EEIO. In the presence of EEIO, we observed a dose-dependent increase in the percentage of cells in $G_1$ phase accompanied by a corresponding reduction in the percentages of cells in S and $G_2$/M phases (Fig. 4).

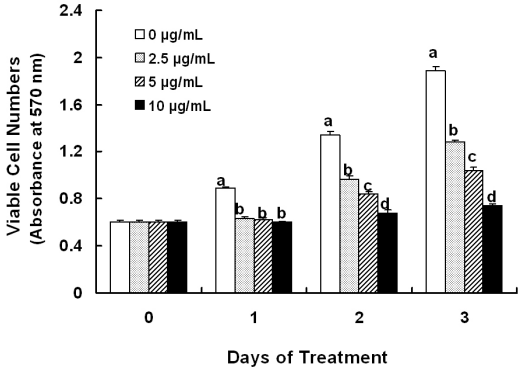


**Fig. 2. Effect of EEIO on HT-29 cell growth.** HT-29 cells were plated in 24-well plates at a density of 50,000 cells/well in DMEM/F12 supplemented with 10% FBS. One day later, the monolayers were serum-starved with serum-free DMEM/F12 supplemented with 5 mg/L transferrin, 0.1 g/L BSA, and 5 μg/mL selenium for 24 h. After serum starvation, cells were incubated in serum-free medium in the absence or presence of various concentrations of EEIO. Cell numbers were estimated by MTT assay. Each bar represents the mean ± SEM (n = 6). Bars with different letters are significantly different at $P < 0.05$ by Duncan's multiple range test at each time point.

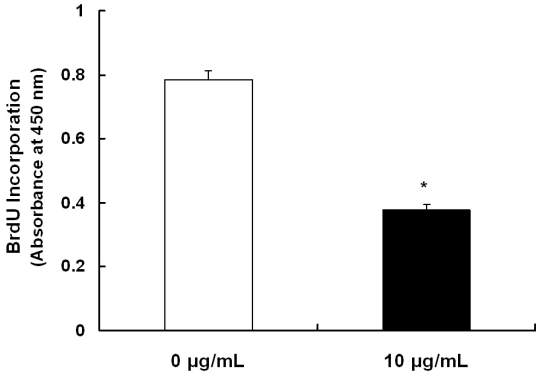


**Fig. 3. Effect of EEIO on 5-bromo-2′-deoxyuridine (BrdU) incorporation in HT-29 cells.** HT-29 cells were plated and treated with EEIO for 2 days as described in Fig. 2. After BrdU was added, incubation was continued for another 5 h to measure incorporation into DNA. Each bar represents the mean ± SEM (n = 6). * Different from 0 μg/mL, $P < 0.05$.

*EEIO decreases phosphorylation of Rb in HT-29 cells*

Based on our observation that $G_1$ arrest results from EEIO treatment in HT-29 cells, we next examined the effects of EEIO on proteins controlling the $G_1$/S cell cycle transition by Western blot analysis. As shown in Fig. 5, EEIO decreased protein levels of CDK2 and CDK4. The level of cyclin D1 also decreased in HT-29 cells treated with EEIO. EEIO markedly increased the protein expression levels of p21, p27, and p53 after treatment with EEIO. Western blot analysis of total cell lysates revealed a decreased level of phosphorylated Rb levels. Immunoblotting using total Rb antibody showed that EEIO treatment reduced hyperphosphorylated Rb and increased hypophosphorylated Rb levels. These data indicate that EEIO induced $G_1$ phase arrest by inhibition of CDK2, CDK4, cyclin D1, and Rb, activation of CDK inhibitors such as p21 and p27, and activation of tumor suppressor protein such as p53.

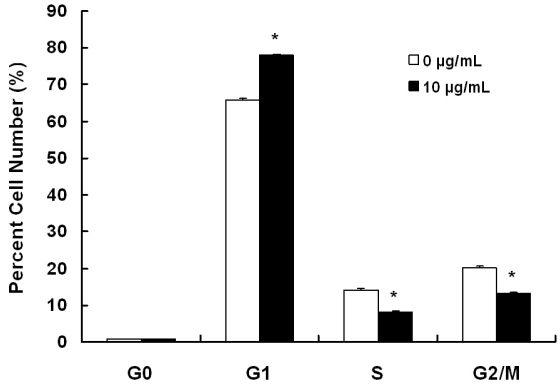


**Fig. 4. Effect of EEIO on cell cycle progression in HT-29 cells.** Cells were plated and treated with EEIO for 2 days as described in Fig. 2. Cells were trypsinized and collected. Cells were then fixed and digested with RNase, after which cellular DNA was stained with propidium iodide and analyzed by flow cytometry. Each bar represents the mean ± SEM (n = 6). * Different from 0 μg/mL, $P < 0.05$.

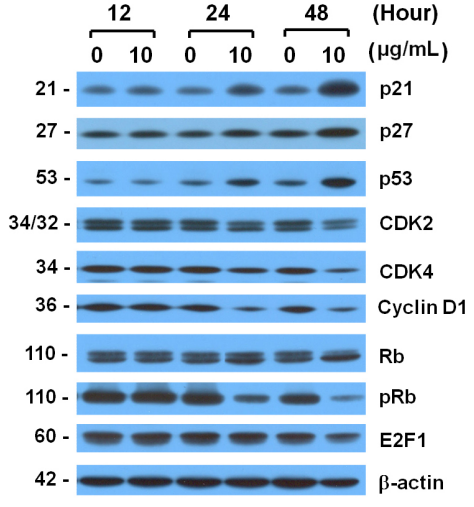


**Fig. 5. Effect of EEIO on expression of various cell cycle-regulating proteins in HT-29 cells.** Cells were cultured and treated with EEIO for 2 days as described in Fig. 2. Cell lysates were analyzed by immunoblotting with an antibody against p21, p27, p53, CDK2, CDK4, cyclin D1, Rb, pRb, E2F1, or β-actin. A photograph of chemiluminescent detection of a blot, which is representative of 3 independent experiments, is shown.

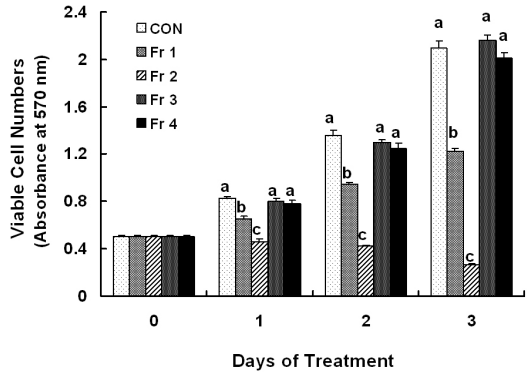


**Fig. 6.** Effects of various fractions of EEIO on HT-29 cell growth. HT-29 cells were plated and treated with 0 or 10 μg/mL of various fractions for 2 days as described in Fig. 2. Cell numbers were estimated by MTT assay. Each bar represents the mean ± SEM (n = 6). Bars with different letters are significantly different at $P < 0.05$ by Duncan's multiple range test at each time point.

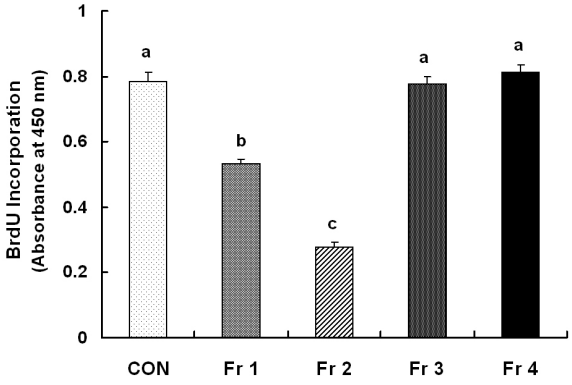


**Fig. 7.** Effects of various fractions of EEIO on 5-bromo-2′-deoxyuridine (BrdU) incorporation in HT-29 cells. HT-29 cells were plated and treated with 0 or 10 μg/mL various fractions for 2 days as described in Fig. 2. BrdU was added and incubation was continued for another 5 h to measure incorporation into DNA. Each bar represents the mean ± SEM (n = 6). Bars with different letters are significantly different at $P < 0.05$ by Duncan's multiple range test.

*Dichloromethane fraction is an active fraction of EEIO*

To identify the active fraction of EEIO, we separated EEIO fractions using different solvents and determine the ability of each fraction to reduce HT-29 cell viability. As shown in Fig. 6, the dichloromethane fraction (Fr2) significantly inhibited cell proliferation compared to the other fractions. Further, BrdU incorporation assay showed that DNA synthesis was significantly decreased in HT-29 cells treated with hexane fraction (Fr1) and Fr2 compared to cells treated with other fractions (Fig. 7).

*Dichloromethane fraction of EEIO induces $G_1$ arrest and alteration of cell cycle progress-regulating protein levels in HT-29 cells*

To determine whether or not dichloromethane fraction inhibits cell cycle progressions in HT-29 cell, cells were treated with or without 10 μg/mL of each fraction. Among the EEIO fractions, Fr2 treatment increased the percentage of cells in $G_1$ phase and decrease in the percentages of cells in S and $G_2$/M phases compared to others (Fig. 8). The protein levels of CDK2, CDK4, cyclin D1, pRb, and E2F1 were reduced, whereas those of p21, p27, and p53 were increased in Fr2-treated cells (Fig 9). Based on these results, we suggest that Fr2 is the major fraction that induces $G_1$ cell cycle arrest and inhibits cell proliferation.

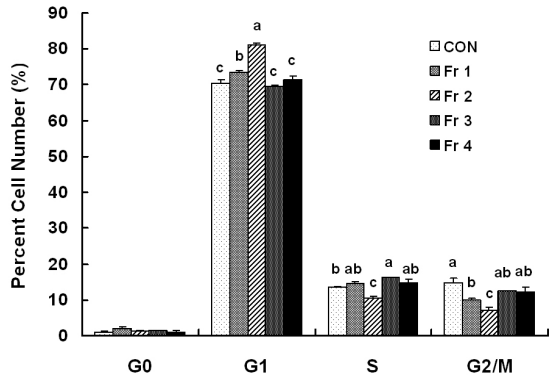


**Fig. 8.** Effects of various fractions of EEIO on cell cycle progression in HT-29 cells. Cells were plated and treated with various fractions for 2 days as described in Fig. 2. Cells were trypsinized and collected. Cells were then fixed and digested with RNase, after which cellular DNA was stained with propidium iodide and analyzed by flow cytometry. Each bar represents the mean ± SEM (n = 6). Bars with different letters are significantly different at $P < 0.05$ by Duncan's multiple range tests.

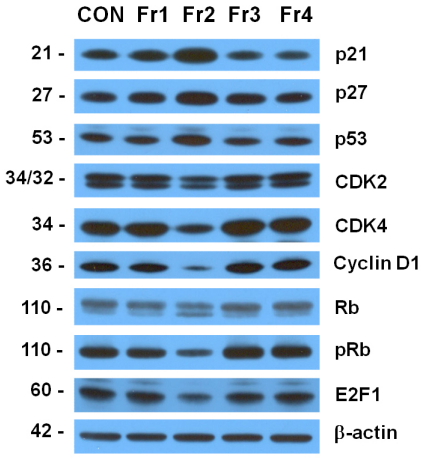


**Fig. 9.** Effects of various fractions of EEIO on expression of various cell cycle-regulating proteins in HT-29 cells. Cells were cultured and treated with various fractions for 2 days as described in Fig. 2. Cell lysates were analyzed by immunoblotting with an antibody against p21, p27, p53, CDK2, CDK4, Cyclin D1, Rb, pRb, E2F1,or β-actin. A photograph of chemiluminescent detection of a blot, which is representative of 3 independent experiments, is shown.

## DISCUSSION

Prior studies with various cells have reported that extract of *I. obliquus* from several types of solvents, including-water, ethanol, methanol, and hexane, prevents proliferation and metastasis of cancer cells [10,11,15,18,29-31]. This is the first study to report the anti-tumor effect of the dichloromethane fraction of EEIO in HT-29 cells.

Deregulation of the cell cycle is the most common abnormality in human cancer. Therefore, cell cycle arrest and induction of apoptosis has become the major target of anti-cancer drugs to prevent cancer cells proliferation. In the present study, EEIO inhibited proliferation and DNA synthesis in HT-29 cells, and

the dichloromethane fraction showed greater inhibition of cell proliferation. EEIO and the dichloromethane fraction induced $G_1$ arrest in HT-29 human colon cancer cells and led to reduced expression of CDK2 and CDK4. CDK hyperactivation is a common characteristic of most human cancers. The levels of cyclin proteins fluctuate temporally during the cell cycle, leading to activation of their respective CDKs. In this study, EEIO treatment decreased cyclin D1 levels in HT-29 cells, which might have contributed to the decreased expression of CDK2 and CDK4.

$p21^{CIPI/WAF1}$ protein, a potent CDK inhibitor, binds to and inhibits the activity of cyclin-CDK2 complexes, and thus functions as a regulator of cell cycle progression in $G_1$ and S phases [32]. Expression of this gene is tightly controlled by the tumor suppressor protein, p53 [33]. $p27^{Kip1}$ is another CDK inhibitor that binds to and prevents activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls cell cycle progression at $G_1$ phase. In this study, we observed that EEIO-treated HT-29 cells increased p21, p27, and p53 expression. Overexpression of p21and p27 has been shown to inhibit proliferation of mammalian cells [32,34]. Our data indicate that EEIO is capable of inducing sufficient expression of p21 and p27 to inhibit CDK activity and progression from $G_1$ into S phase.

Retinoblastoma protein (Rb) is a tumor suppressor protein that binds to and inhibits transcription factors of E2F family [35]. When Rb is bound to E2F, this complex acts as a growth suppressor and prevents progression through the cell cycle. Rb is initially phosphorylated to pRb by cyclin D/CDK4/CDK6 and followed by additional phosphorylation by cyclin E/CDK2 [36,37]. One function of pRb is to prevent excessive cell growth by inhibiting cell cycle progression through $G_1$ into S. pRb is unable to complex E2F and therefore, unable to restrict progression from $G_1$ to S phase of the cell cycle [38]. In the present study, EEIO decreased pRb level, indicating that induction of p21 by EEIO leads to inhibition of CDK activity. Youn *et al*. [15] reported that water extract of *I. obliquus* inhibited the viability and proliferation in human hepatoma HepG2 cells as well as induced apoptotic cell death. In detail, HepG2 cells were arrested by water extract of *I. obliquus* at $G_0/G_1$ phase of the cell cycle, and cyclin D1 and CDK2, and CDK6. HepG2 cells that had a functional p53 were more sensitively damaged by water extract *I. obliquus* than Hep3B cells lacking a functional p53 [10]. In another study by Youn *et al*. [39], water extract of *I. obliquus* inhibited growth of melanoma B16/F10 cells by causing cell cycle arrest at $G_0/G_1$ phase and apoptosis. These effects were shown to be associated with the down-regulation of pRb, p53, p27, cyclin E/D1 and CDK2/4 expression [39]. These results collectively show that both water and ethanol extracts of *I. obliquus* inhibit cancer cell proliferation in various cells through cell cycle arrest. In addition, in the present study, the dichloromethane fraction of EEIO was shown to contain compounds that induce cell cycle arrest.

The main chemical constituents of organic solvent and water extracts of *I. obliquus* are the lanostane-type triterpenoid compounds, especially inotodiol that have potent anti-cancer abilities [6,15,29]. Petroleum ether and ethyl acetate fractions of *I. obliquus* inhibited the proliferation of PC3 human prostatic cancer cells and MDA-MD-231 breast cancer cells, and it was reported that ergosterol peroxide and trametenolic acid were the main compounds of these two fractions with cytotoxicity in cancer cells [18]. Chung *et al*. [40] reported that three pure compounds (3β-hydroxy-lanosta-8,24-dien-21-al, inotodiol, and lanosterol) separated from *I. obliquus* extracts have anti-cancer effects on both human cancer cells and BALB/c mice bearing sarcoma-180 cells. Sun *et al*. [31] reported that the principle compounds of ethanol extract of *I. obliquus* were lanosterol, inotodiol and ergosterol. As the present study, we did not analyze the compound profiles of the fractions, the effective materials remain known. However, the compounds contained in EEIO and the dichloromethane fraction showed strong anti-cancer effects. In future, there is a need for the more study analyzing the effective compounds in EEIO and the dichloromethane fraction.

In conclusion, the present study shows that EEIO and the dichloromethane fraction induced cell cycle arrest at $G_1$ phase. An increase in the level of p21 and p27 in EEIO or the dichloromethane fraction treated cells led to the inhibition of the CDK, which resulted in a decrease in Rb phosphorylation. These effects caused an arrest in the $G_1$ into S cell cycle progression, followed by inhibition of HT-29 cell proliferation. Among the *I. obliquus* fractions, the dichloromethane fraction is the major fraction that induces $G_1$ cell cycle arrest and inhibits the cell proliferation in HT-29 cells. Thus, EEIO and the dichloromethane fraction may be useful as ingredients in functional anti-cancer foods.

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