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Bioactivity-based analysis and chemical characterization of cytotoxic constituents from Chaga mushroom (*Inonotus obliquus*) that induce apoptosis in human lung adenocarcinoma cells

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ABSTRACT

Ethnopharmacological relevance

Inonotus obliquus, also known as Chaga mushroom, is one of the most widely appreciated wild edible mushrooms in Russia and northern European countries and is renowned for its use in cancer treatment. Indeed, recently published *in vitro* and *in vivo* studies have demonstrated its anticancer activity in various types of cancer and support its potential application for therapeutic intervention in cancer. However, its activity against lung cancer, the most commonly diagnosed cancer and the leading cause of cancer death worldwide, and the underlying molecular basis of its action remain to be fully elucidated.

Objective

This study aimed to evaluate the cytotoxic activity of *I. obliquus* in four human lung adenocarcinoma cell lines with different p53 status (A549, H1264, H1299, and Calu-6) and identify its active constituents by bioactivity-based analysis and the underlying molecular basis of their cytotoxicity on lung cancer cells.

Materials and methods

Bioactivity-guided fractionation and preparative/semi-preparative HPLC purification were used with LC/MS analysis to separate the bioactive constituents. Cell viability and apoptosis in human lung cancer cell lines (A549, H1264, H1299, and Calu-6) were assessed using the WST-1 assay and TUNEL staining, respectively. Caspase activation was assessed by detecting its surrogate markers, cleaved poly (ADP-ribose) polymerase (PARP) and caspase-3, using an immunoblot assay.

Results

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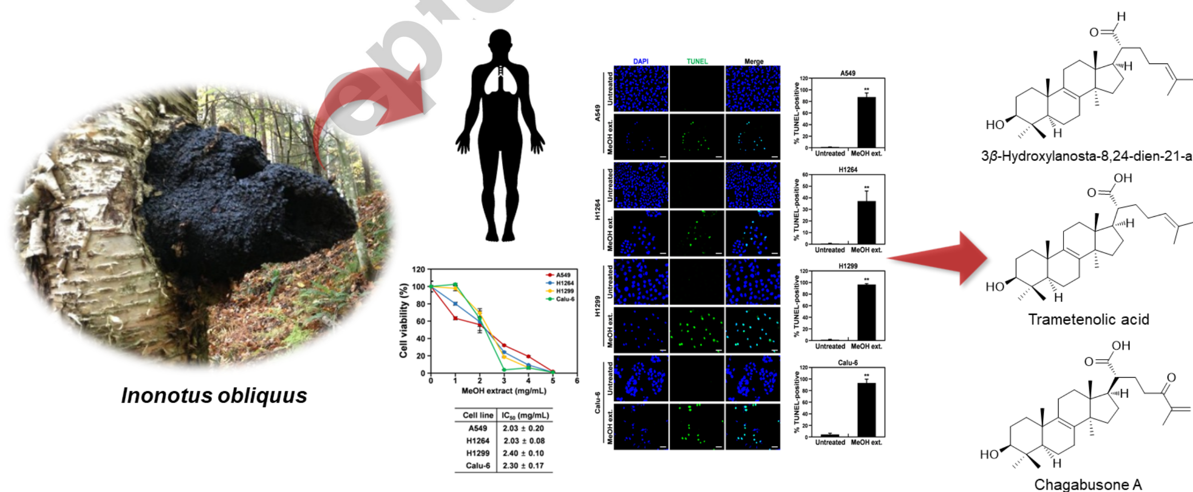
The MeOH extract of *I. obliquus* reduced cell viability in all lung cancer cell lines tested through induction of apoptosis accompanied by caspase-3 cleavage. Bioactivity-guided fractionation of the MeOH extract and chemical investigation of its cytotoxic hexane-soluble and CH_2Cl_2-soluble fractions led to the isolation of eight triterpenoids (**1-8**), including a new lanostane-type triterpenoid named chagabusone A (**7**). The structures of the isolates were elucidated based on spectroscopic analysis, including 1D and 2D NMR and high-resolution ESIMS. Among isolated compounds, compounds **1**, **6**, and **7** showed the most potent cytotoxic activity in all human lung cancer cell lines examined, with IC_{50} values ranging from 75.1 to 227.4 μM. Cytotoxicity of these compounds was mediated by apoptosis with caspase-3 activation.

Conclusion

These findings provide experimental evidence supporting the potential application of *I. obliquus* in lung cancer treatment and reveal the molecular basis underlying its cytotoxic activity against human lung cancer cells.

Graphical abstract



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KEYWORDS: *Inonotus obliquus*; Chaga mushroom; Hymenochaetaceae; triterpenoids; cytotoxicity; lung cancer; apoptosis

Chemical compounds studied in this article

3*β*-hydroxylanosta-8,24-dien-21-al (PubChem CID: 180709); inonotsutriol E (PubChem CID: 102424675); inotodiol (PubChem CID: 44422314); trametenolic acid (PubChem CID: 16226646);

1. Introduction

Cancer is a group of diseases involving uncontrolled proliferation and growth of abnormal cells followed by their invasion into surrounding tissues and possible eventual metastasis to distant organs and is now the second leading cause of death globally after cardiovascular disease (Hanahan and Weinberg, 2011; Fitzmaurice et al., 2017). Among all cancers, lung cancer is the most frequently diagnosed type as well as the most common cause of cancer-related morbidity and mortality worldwide, causing approximately 1.69 million deaths in 2015 (Fitzmaurice et al., 2017). However, despite recent advances in the early diagnosis and therapy of cancer, the prognosis of lung cancer patients remains unsatisfactory, with a 5-year overall survival rate of approximately 16% (Jemal et al., 2010).

Mushrooms, especially medicinal edible mushrooms, are emerging as plentiful sources of bioactive molecules with potent medicinal properties useful for cancer treatment, such as antioxidant, angiostatic, and immunomodulatory activities as well as cytotoxicity toward cancer cells (Wasser, 2011; Paterson and Lima, 2014). Therefore, screening for mushrooms

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with potential cytotoxicity against lung cancer and delineating the underlying molecular basis has significant implications for therapeutic intervention. However, although numerous research efforts have focused on pharmacological and biochemical characterization of mushrooms in relation to cancer, most of these sources remain untapped (Paterson and Lima, 2014).

Inonotus obliquus, also well known as Chaga mushroom, belongs to the family Hymenochaetaceae and is commonly distributed in Russia, China, Japan, Eastern and Northern Europe, and Alaska. It parasitizes on birch (*Betula platyphylla* var. *japonica*), usually after the host tree is dead, and has the appearance of burnt charcoal (Balandaykin and Zmitrovich, 2015; Lee et al., 2008; Shashkina et al., 2006). In several countries, including Russia, northeastern European countries, and North America, Chaga mushroom is renowned as a medicinal mushroom and has been extensively used to treat various human disorders such as gastrointestinal diseases, diabetes, tuberculosis, and cardiovascular disorders since ancient times (Balandaykin and Zmitrovich, 2015; Song et al., 2013). Indeed, published studies have demonstrated the biological and pharmacological activities of *I. obliquus* underlying the medicinal effects, such as antibacterial, antiallergic, anti-nociceptive, antithrombotic, and anti-inflammatory functions, and identified the bioactive constituents responsible for those activities, supporting its use for medicinal purposes (Park et al., 2005; Hyun et al., 2006; Kim et al., 2007; Yoon et al., 2013; Glamočlija et al., 2015).

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Of note, *I. obliquus* has been used to treat neoplastic diseases in Russia and northern European countries. In the Russian folk medicine, this fungus is used for the treatment of various cancers including breast, oral, gastrointestinal, lung, and skin cancers (Pilz, 2004). Indeed, in recent *in vitro* and *in vivo* studies, its crude extract was shown to possess potent

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antineoplastic properties such as antioxidant, antimutagenic, and immunomodulatory activities (Cui et al., 2005; Kim et al., 2005; Ham et al., 2009; Mishra et al., 2012). In addition, the crude extract exhibits cytotoxicity through induction of apoptosis in various types of cancer cell *in vitro*, including breast adenocarcinoma, colorectal carcinoma, cervical carcinoma, hepatocellular carcinoma, lymphoma, leukemia, and lung adenocarcinoma cells, and suppresses the growth of implanted murine lung tumor and 7,12-dimethylbenz(a)anthracene (DMBA)-induced spontaneous breast tumor in rodents (Song et al., 2013; Debnath et al., 2013; Youn et al., 2008; Hu et al., 2009; Lee et al., 2009; Zhong et al., 2011; Arata et al., 2016; Zhang et al., 2018). Phytochemical investigations of *I. obliquus* have identified triterpenoids, polysaccharides, small phenolic compounds, and ergosterol peroxide as bioactive constituents contributing to those anticancer activities (Yoshikawa et al., 2002; Kim et al., 2006; Nakajima et al., 2007; Taji et al., 2008; Kim et al., 2011; Tanaka et al., 2011; Mu et al., 2012; Liu et al., 2013; Zhao et al., 2015; Kang et al., 2015). However, the biological activity of *I. obliquus* against cancer, especially lung cancer, and the underlying molecular basis should be further addressed and fully elucidated to support its therapeutic application in the treatment of lung cancer.

In this study, as part of our ongoing efforts to screen wild mushrooms exhibiting anticancer potential toward lung cancer and to characterize the responsible compounds (Kim et al., 2017; Lee et al., 2018), we examined the biological effects of the MeOH extract of *I. obliquus* fruiting bodies in four different human lung adenocarcinoma cell lines, A549, H1264, H1299, and Calu-6, and found that the extract decreased cell viability in all lung cancer cell lines tested through induction of apoptosis. Bioactivity-based analysis and chemical investigation of the extract led to successful isolation of its main cytotoxic constituents, compounds **1**, **6**,

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and **7**, which showed potent pro-apoptotic activity in these lung cancer cells. Of note, among the eight isolated compounds, compound **7** was identified as a novel lanostane-type triterpenoid based on structural analysis using spectroscopic techniques including 1D and 2D nuclear magnetic resonance (NMR) and high-resolution electrospray ionization mass spectrometry (HR-ESIMS). This study reports the bioactivity-guided isolation of eight triterpenoids from *I. obliquus* fruiting bodies, structural elucidation of the triterpenoids, and bioactivity of the isolates with regards to their cytotoxic effect on lung cancer cells.

2. Materials and methods

2.1. General experimental procedures

HR-ESI mass spectra were measured on a Waters Xevo G2 QTOF mass spectrometer using an analytical Acquity UPLC (2.1 × 100 mm, 1.7 μm; Waters MS Technologies, Manchester, UK). Ultraviolet (UV) spectra were determined on an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Infrared (IR) spectra were acquired on a Bruker IFS−66/S FT-IR spectrometer, and NMR spectra were measured on a Bruker Avance III HD 800 NMR spectrometer with a 5 mm TCI Cryoprobe (Bruker, Karlsruhe, Germany). Preparative high-performance liquid chromatography (HPLC) and semi-preparative HPLC were performed using an analytical Agilent Eclipse XDB-C18 column (250 × 21.2 mm, 7 μm) (Waters Corporation, Milford, CT, USA) and Phenomenex Luna Phenyl−hexyl 100 A column (250 × 10 mm, 10 μm), respectively, with a Waters 1525 binary HPLC pump equipped with a Waters 996 photodiode array detector. LC/MS analysis was performed on an Agilent 1200 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector and a 6130 Series ESI mass spectrometer

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using an analytical Kinetex C18 100 Å column (100 × 2.1 mm i.d., 5 μm; Phenomenex, Torrance, CA, USA). Column chromatography was performed with a silica gel 60 (Merck, 230–400 mesh) and an RP-C18 silica gel column (Merck, 230–400 mesh). The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia, Uppsala, Sweden). First-grade solvents (Samchun pure chemicals Co., Ltd., Pyeongtaek, Korea) were used for fractionation and isolation. Merck precoated silica gel F_{254} plates and reverse-phase (RP)-18 F_{254s} plates were used for thin-layer chromatography (TLC). Spots were detected on TLC under UV light (dual wavelength 254/365 nm) or by heating after spraying with anisaldehyde-sulfuric acid.

2.2. Sample material

The fruiting bodies of Russian Chaga mushroom (*I. obliquus*) were purchased at Kyungdong herbal market, Seoul, Korea, in July 2015, and were identified by one of the authors (K. H. Kim). A voucher specimen (SKKU CG-2017-07) has been deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

2.3. Extraction and isolation

Dried fruiting bodies of *I. obliquus* (461.5 g) were partially chopped and extracted with 80% MeOH for 2 days twice at room temperature. The extracts were then filtered, and the filtrate was concentrated under vacuum pressure, generating a crude MeOH extract (20.3 g). The crude extract was dissolved as a stock solution at 25 mg/mL in RPMI-1640 medium (WelGENE, Seoul, Korea) and stored at −80ºC until use. For isolation of compounds contributing to the cytotoxicity of the MeOH extract against human lung cancer cells, a

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suspension of crude extract (20.3 g) in distilled water (800 mL) was further subjected to solvent partition using hexane (HX), CH_2Cl_2 (MC), EtOAc (EA), and *n*-BuOH (BuOH), which yielded residues of 1.7 g, 1.7 g, 0.5 g, and 0.9 g, respectively. The HX and MC soluble fractions were prepared as stock solutions at 100 mg/mL in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA). The EA and BuOH soluble fractions were dissolved as stock solutions at concentrations of 10 and 25 mg/mL, respectively, in RPMI-1640 medium. All stock solutions were aliquoted and stored at −80ºC until use.

To isolate the main cytotoxic constituents of the fruiting bodies of *I. obliquus*, the HX soluble fraction (1.7 g), which displayed significant cytotoxicity in all the human lung cancer cell lines examined, was fractionated on silica gel column chromatography with a gradient solvent system from hexane-EtOAc (10:1, v/v) to CH_2Cl_2-MeOH (1:1, v/v) to give six sub-fractions (H1 – H6). Sub-fraction H2 (48.4 mg) was purified with semi-preparative HPLC (93% MeOH, flow rate: 2 mL/min) using the Phenomenex Luna phenyl-hexyl column (250 × 10 mm, 10 μm) to yield compound **1** (12.0 mg, t_R = 27.5 min). Sub-fraction H3 (520.7 mg) was separated in a silica gel column using a gradient solvent system from hexane-EtOAc (7:1, v/v) to CH_2Cl_2-MeOH (1:1, v/v) to give five sub-fractions (H31 – H35). Compound **4** (7.6 mg, t_R = 37.5 min) was obtained from sub-fraction H33 (31.7 mg) by semi-preparative HPLC (85% MeOH, flow rate: 2 mL/min) using the Phenomenex Luna phenyl-hexyl column (250 × 10 mm, 10 μm). Sub-fraction H34 (51.9 mg) was purified with semi-preparative HPLC (93% MeOH, flow rate: 2 mL/min) using the Phenomenex Luna phenyl-hexyl column (250 × 10 mm, 10 μm) to give compounds **2** (8.4 mg, t_R = 20.5 min), **3** (4.4 mg, t_R = 22.5 min), and **5** (4.4 mg, t_R = 29.5 min).

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The MC-soluble fraction (1.7 g), which also exhibited significant cytotoxic activity against all human lung cancer cell lines tested, was fractionated using a Sephadex LH-20 column [CH_2Cl_2-MeOH (1:10, v/v)] to obtain six sub-fractions (M1 – M6). Sub-fraction M3 (1.0 g) was subjected to separation in a silica gel column using a gradient system from CH_2Cl_2-MeOH (200:1, v/v) to CH_2Cl_2-MeOH (1:1, v/v) to give six sub-fractions (M31 – M36). Sub-fraction M33 (358.0 mg) was separated in a silica gel column using a gradient system from CH_2Cl_2-MeOH (40:1, v/v) to CH_2Cl_2-MeOH (1:1, v/v) to yield six sub-fractions (M331 – M336). Sub-fraction M334 (341.0 mg) was further separated with preparative HPLC with a gradient system (60 – 80% MeOH, flow rate: 5 mL/min) using an Agilent Eclipse XDB-C18 column (250 × 21.2 mm, 7 μm) to give six sub-fractions (M3341 – M3346). Compound **8** (1.9 mg, t_R = 24.5 min) was obtained from sub-fraction M3346 (37.3 mg) using semi-preparative HPLC [Phenomenex Luna phenyl-hexyl column (250 × 10 mm, 10 μm), 85% MeOH, flow rate: 2 mL/min]. Sub-fraction M335 (50.2 mg) was purified with semi-preparative HPLC (82% MeOH, flow rate: 2 mL/min) using a Phenomenex Luna phenyl-hexyl column (250 × 10 mm, 10 μm) to yield compound **7** (1.6 mg, t_R = 32.0 min). Finally, sub-fraction M6 (26.1 mg) was purified with semi-preparative HPLC (90% MeOH, flow rate: 2 mL/min) using a Phenomenex Luna phenyl-hexyl column (250 × 10 mm, 10 μm) to obtain compound **6** (4.1 mg, t_R = 26.0 min).

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All resultant compounds were prepared as stock solutions at a concentration of 50 mM in DMSO and tested for cytotoxicity toward human lung cancer cell lines.

*2.3.1. Chagabusone A (**7**)*

White powder. $[\alpha]_D^{25}$ +36.7 (*c* 0.001, MeOH); UV (MeOH) λ_{max} (log ε): 215 (3.8) nm; IR (KBr) ν_{max}: 3388, 2946, 2832, 1721, 1029 cm^{-1}; ^1H NMR (CD_3OD, 800 MHz) and ^{13}C NMR

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(CD_3OD, 200 MHz) spectroscopic data, see **Table 1**; HR-ESIMS (negative-ion mode) *m/z*: 469.3324 $[M – H]^-$ (calcd. for $C_{30}H_{45}O_4$, 469.3318).

2.4. Cell culture

Human lung adenocarcinoma cell lines A549, H1264, H1299, and Calu-6 were kindly provided by Dr. Steven M. Albelda (University of Pennsylvania School of Medicine, Philadelphia, PA, USA) and grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA, USA), 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (WelGENE).

2.5. Cell viability analysis

Human lung cancer cell lines A549, H1264, and H1299 (5×10^3 cells/well) and Calu-6 (7.5×10^3 cells/well) were seeded in triplicate in 96-well tissue culture plates (Thermo Scientific, Waltham, MA, USA) and grown overnight. Cells were then treated with the MeOH extract of *I. obliquus* fruiting bodies, its fractions, and the isolated compounds at various concentrations. As vehicle controls, cells were also incubated in growth medium containing DMSO at concentrations ranging from 0 to 0.6% DMSO. After treatment for 48 hr, cell viability was assessed by WST-1 cell proliferation assay (Daeil Lab Service, Seoul, Korea) as previously described (Lee et al., 2017) and determined as a percentage of the corresponding vehicle control. IC_{50} values of the MeOH extract, its fractions, and the isolated compounds were estimated by a non-linear regression analysis of the dose-response curve using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

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2.6. TUNEL assay

A549, H1264, and H1299 (7.5 × 10^3 cells) and Calu-6 (1.0 × 10^4 cells) were plated overnight in triplicate on 12-mm glass coverslips (Marienfeld GmbH, Lauda-Königshofen, Germany) and treated with the MeOH extract of *I. obliquus* fruiting bodies, its fractions, and the isolated compounds for 48 hrs. Cells incubated in medium only or medium containing DMSO were used as vehicle controls. Apoptotic cells were assessed by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining using the Dead-End labeling kit (Promega, Madison, WI, USA) as previously described (Lee et al., 2017). The cells were then counterstained with 0.5 μg/mL 4',6-diamidino-2-phenylindole (DAPI, Sigma) to visualize cell nuclei and observed under a fluorescence microscope (Carl Zeiss, Jena, Germany). The percentage of apoptotic cells was determined as the ratio between TUNEL-positive and DAPI-stained nuclei counted in six randomly selected high-power fields (400×) on each slide.

2.7. Immunoblotting

Calu-6 cells (5 × 10^5 cells/dish) were plated in 60-mm tissue culture dishes (Thermo Scientific), grown overnight, and treated with the MeOH extract of *I. obliquus* fruiting bodies, HX and MC fractions, and the isolated compounds. Cells incubated in growth medium containing 1 μM doxorubicin (Sigma) and DMSO were used as positive and vehicle controls, respectively. After treatment for 48 hrs, whole cell lysates were prepared by lysing the cells in RIPA buffer containing 10 mM NaF, 1 mM Na_3VO_4, 1 μM DTT, 1 mM PMSF (Sigma), and a protease inhibitor cocktail (Roche, Mannheim, Germany); separated on SDS-PAGE; and probed for poly (ADP-ribose) polymerase (PARP), caspase-3 (Cell Signaling Technology,

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Danvers, MA, USA), and β-actin as a loading control, as previously described (Lee et al., 2017).

2.8. Statistical analysis

The statistical significance between vehicle control and treated groups was evaluated using two-tailed unpaired Student's *t* test. All data are presented as the mean ± standard error of the mean (SEM), and *p* values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Cytotoxicity of the MeOH extract of I. obliquus fruiting bodies in human lung adenocarcinoma cells

In previously published studies, the crude extract of *I. obliquus* was found to exhibit cytotoxicity against A549 cells, human lung adenocarcinoma cells expressing wild-type *p53* (Zhong et al., 2011). However, considering that the tumor suppressor *p53* is mutated in more than 50% of human cancers and confers chemoresistance in human cancer patients (Wang and Sun, 2010), the biological effects of *I. obliquus* on human lung cancer cells and the underlying mechanisms should be further examined in cells with different *p53* status.

We prepared the MeOH extract of *I. obliquus* fruiting bodies and investigated its effects on cell viability in four different human lung adenocarcinoma cell lines: A549 cells with wild-type *p53*, H1264 cells expressing mutant *p53*, and H1299 and Calu-6 cells null for *p53* (Mitsudomi et al., 1992; Rämet et al., 1995) (**Fig. 1**). Consistent with previous reports (Zhong et al., 2011), assessment of cell viability using WST-1 assay revealed that the MeOH extract exhibited cytotoxicity against A549 cells in a dose-dependent manner, with an IC_{50} value of

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2.03 mg/mL (**Fig. 1A**). Of note, the MeOH extract also decreased cell viability in the human lung cancer cell lines with *p53* mutant or null phenotype, with IC_{50} values ranging from 2.03 to 2.40 mg/mL (**Fig. 1A**), implying that cytotoxicity of *I. obliquus* toward human lung cancer cells is unaffected by their *p53* status.

Upon treatment with the MeOH extract, the lung cancer cells showed the typical morphological features of apoptotic cells (Elmore, 2007) including cell rounding, cell shrinkage, detachment from the substratum, and blebbing of the cell membrane (**Figure 1B**), suggesting that the cytotoxicity of the MeOH extract against human lung cancer cells is mediated by apoptosis. To verify that the MeOH extract triggers apoptosis in human lung cancer cells, apoptotic cells were assessed using TUNEL staining after treatment with the extract (**Fig. 2**). As expected, after 48 hrs, the apoptotic cell population was significantly increased in all human lung cancer cell lines after treatment with the MeOH extract compared with their untreated controls, demonstrating that the cytotoxicity of the extract against human lung cancer cells is attributable to its pro-apoptotic activity.

Taken together, our data suggest that *I. obliquus* exhibits cytotoxic activity through induction of apoptosis in human lung cancer cells irrespective of their *p53* status and further support its potential therapeutic application in human lung cancer.

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3.2. Cytotoxic activity of fractions from the MeOH extract of I. obliquus fruiting bodies in human lung cancer cell lines

To identify the main constituents of *I. obliquus* fruiting bodies responsible for cytotoxicity against human lung cancer cells, we first fractionated the MeOH extract into four different fractions, hexane (HX), CH_2Cl_2 (MC), EtOAc (EA), and *n*-BuOH (BuOH) soluble fractions,

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and examined which fractions were enriched with cytotoxic compounds using human lung adenocarcinoma cell lines (**Fig. 3**). Cell viability assessed using WST-1 assay revealed that HX and MC fractions of the extract showed the most significant cytotoxicity in all human lung cancer cell lines examined in a dose-dependent manner, with IC_{50} values ranging from 95.3 to 225.1 μg/mL and from 134.1 to 173.0 μg/mL, respectively (**Fig. 3A-3D** and **Table 1**). However, the BuOH extract failed to show any substantial effects on cell viability up to a concentration of 600 μg/mL in all human lung cancer cell lines tested. In addition, although treatment with the EA fraction decreased viability of the human lung cancer cells, its cytotoxicity was relatively moderate compared with that of the HX and MC fractions, with IC_{50} values ranging from 292.5 to 513.5 μg/mL. Of note, similar to the cytotoxicity observed for the MeOH extract, the cytotoxic effects of HX and MC fractions did not show any correlation with *p53* status in human lung cancer cells. These observations indicate that the cytotoxic effects of *I. obliquus* fruiting bodies on human lung cancer cells are attributable to constituents enriched in the HX and MC fractions.

Upon treatment with the HX and MC fractions, the human lung cancer cells underwent morphological changes similar to those observed in cells treated with the MeOH extract of *I. obliquus* fruiting bodies (**Fig. 3E-3F**). In addition, detection of apoptotic cells using TUNEL staining showed that both fractions dramatically increased the TUNEL-positive cell population compared with the vehicle controls in all human lung cancer cell lines examined after 48 hrs of treatment (**Fig. 4**). These results demonstrate that, similar to the MeOH extract of *I. obliquus* fruiting bodies, the cytotoxic effects of the HX and MC fractions on human lung cancer cells are mediated by their pro-apoptotic activity.

Taken together, our data indicate that cytotoxic and pro-apoptotic activities of *I. obliquus*

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fruiting bodies observed in human lung cancer cells are attributable to bioactive constituents enriched in the HX and MC fractions.

3.3. Chemical identification of the bioactive compounds from the HX and MC fractions

To identify the main constituents responsible for the cytotoxicity of *I. obliquus* fruiting bodies against human lung cancer cells *in vitro*, further chemical investigation was performed on the HX and MC fractions. Repeated column chromatography and HPLC purification led to the isolation of eight triterpenoids (**1-8**), including a new lanostane-type triterpenoid (**7**) (**Fig. 5**). The known compounds were identified as 3β-hydroxylanosta-8,24-dien-21-al (**1**) (Taji et al., 2008), (+)-fuscoporianol C (**2**) (Zhao et al., 2015), inonotsutriol E (**3**) (Tanaka et al., 2011), inotodiol (**4**) (Liu et al., 2013), inonotsutriol A (**5**) (Taji et al., 2008), trametenolic acid (**6**) (Zhao et al., 2015), and saponaceoic acid I (**8**) (Yoshikawa et al., 2002), by comparing their spectroscopic and physical data with previously reported values, together with LC/MS analysis.

*3.4. Structural elucidation of the new compound, chagabusone A (**7**)*

Compound **7** was isolated as a white powder. The molecular formula was established as $C_{30}H_{46}O_4$ from the molecular ion peak $[M - H]^-$ at *m/z* 469.3324 (calcd. for $C_{30}H_{45}O_4$, 469.3318) in the negative-ion mode of HR-ESIMS. The IR spectrum showed absorption of hydroxyl (3388 cm^{-1}) and conjugated carbonyl (1721 cm^{-1}) groups. The UV absorption bands at 215 nm also suggested a conjugated carbonyl group. The ^{1}H NMR spectrum of **7** (**Table 2**) showed six tertiary methyl [δ_H: 1.86 (s), 1.02 (s), 1.00 (s), 0.94 (s), 0.82 (s) and 0.81 (s)], an exomethylene [δ_H: 6.06 (s), 5.84 (s)], and oxygenated methine proton [δ_H: 3.17 (dd, J = 11.0,

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5.5 Hz)] signals. ^{13}C NMR (**Table 2**) exhibited a total of 30 carbon signals, which were comprised of six methyls, 11 methylenes including an exomethylene group [δ_C: 126.6], an oxygenated methine [δ_C: 80.4], a ketone [δ_C: 204.4], a tetrasubstituted double bond [δ_C: 136.8, 136.3], and a carboxyl group [δ_C: 177.7], which were determined by the aid of HSQC analysis, as well as eight quaternary carbons. Considering that lanostane-type triterpenoids are the main components of *I. obliquus* (Kim et al., 2011; Liu et al., 2013; Zhao et al., 2015), these NMR data suggest that compound **7** is most likely a lanostane-type triterpene with a carboxylic acid, a ketone, and an exomethylene group. Detailed analysis of the NMR data of **7** revealed that the NMR spectra were very similar to those of inonotusol G (Liu et al., 2013), except for the area of substructure C-21/C-20/C-22. The structure of compound **7** was determined by 2D NMR analysis comprised of HSQC, HMBC, and ^{1}H-^{1}H COSY experiments (**Fig. 6**). The location of the double bond was confirmed to be C-8/C-9 by HMBC correlations of H_2-11/C-8, H_3-30/C-8, H_3-19/C-9, and H_2-12/C-9. The carboxylic acid, ketone, and exomethylene groups were assigned to C-21, C-24, and C-25, respectively, by the HMBC correlations of H_2-22/C-21, H_2-22/C-24, H_2-26/C-24, H_2-26/C-27, H_3-27/C-24, H_3-27/C-25, and H_3-27/C-26 and contiguous proton correlation of the ^{1}H-^{1}H COSY spectrum including H-17/H-20, H-20/H_2-22, and H_2-22/H_2-23. The complete gross structure of **7** was further confirmed by the cross-peaks in the ^{1}H-^{1}H COSY and HMBC spectra (**Fig. 6**). The relative configuration of **7** was established by analysis of NOESY experiments (**Fig. 7**) together with comparison of the coupling constants and chemical shifts with reported ^{1}H NMR data of inonotusol G (Liu et al., 2013), which turned out to have an identical configuration to compound **7**. Particularly, the β-orientation of OH-3 was deduced from correlations of H-3/H-5, H-3/H_3-28, and H-5/H_3-28 in the NOSEY spectrum (**Fig. 7**). Based

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on the above evidence, the structure of compound **7** was designated as 3*β*-hydroxy-5*α*-lanosta-8,25-dien-21-oic acid. Compound **7** was named chagabusone A.

*3.5. Cytotoxic effects of isolated compounds **1** to **8** in human lung adenocarcinoma cells*

Our chemical investigation on the cytotoxic fractions, the HX and MC fractions, led to the identification of eight triterpenoids, compounds **1** to **8**, including a novel lanostane-type triterpenoid, chagabusone A, as the main constituents of the MeOH extract of *I. obliquus* fruiting bodies. To determine the major bioactive constituents contributing to the cytotoxic activity of *I. obliquus* fruiting bodies against human lung cancer cells *in vitro*, we evaluated the effects of the isolated compounds on cell viability in human lung adenocarcinoma cell lines (**Fig. 8**). After treatment for 48 hrs, compounds **3**, **4**, and **8** failed to show any significant cytotoxicity at concentrations up to 250 μM, whereas compounds **1**, **6**, and **7** exhibited profound cytotoxic effects on all human lung cancer cell lines examined in dose-dependent manner, with IC_{50} values ranging from 75.1 to 128.0, 184.1 to 227.4, and 82.0 to 141.2 μM, respectively (**Fig. 8A-8D** and **Table 3**). Compound **2** also decreased cell viability in H1264 and Calu-6 cell lines, with IC_{50} values of 203.7 and 162.1 μM, respectively, but failed to show substantial cytotoxicity against A549 and H1299 cells. In addition, compound **5** showed considerable cytotoxicity only in H1299 cells, with an IC_{50} value of 239.3 μM, but reduced the viability of other lung cancer cell lines by less than 50% compared to vehicle-treated control cells at concentrations up to 250 μM. These observations strongly suggest that compounds **1**, **6**, and **7** are the main constituents contributing to the *in vitro* cytotoxic effects of *I. obliquus* fruiting bodies in human lung cancer cell lines.

Triterpenoid and its derivatives have been shown to induce apoptosis in various types of

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human cancer cells, including lung cancer cells (Patlolla and Rao, 2012). Concordant with these previous findings, compounds **1** and **6**, identified as triterpenoid derivatives, also induced morphological changes typical of apoptosis in all human lung cancer cells tested (**Fig. 8E**). In addition, the human lung cancer cells treated with compound **7**, a novel triterpenoid derivative, showed typical morphological characteristics of apoptotic cells. To verify the pro-apoptotic activities of compounds **1**, **6**, and **7**, human lung cancer cells were treated with these compounds, and apoptosis was assessed by TUNEL staining (**Fig. 9**). As expected, quantitation of TUNEL-positive cells revealed that all three compounds dramatically increased the apoptotic cell populations in all human lung cancer cell lines compared to vehicle-treated controls after 48 hrs of treatment. These data demonstrate that the cytotoxicity of compounds **1**, **6**, and **7** against human lung cancer cells is mediated by induction of apoptosis and further support our notion that these compounds are the main constituents responsible for the cytotoxic and pro-apoptotic activities of *I. obliquus* fruiting bodies observed in human lung cancer cells *in vitro*.

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3.6. Induction of caspase-3 activation in human lung adenocarcinoma cells by I. obliquus fruiting bodies and their cytotoxic constituents

Crude extracts of *I. obliquus* have been shown to induce apoptosis via activation of caspase-3 in cancer cells, including human colon cancer cell lines (Lee et al., 2009). However, the mechanisms underlying the pro-apoptotic activities of *I. obliquus* in human lung cancer cells remain to be examined. In addition, although inotodiol, a lanostane triterpenoid, from *I. obliquus* was reported to exhibit cytotoxicity in murine leukemia cell lines through induction of apoptosis in a caspase-3-dependent manner (Nomura et al., 2008), the mechanisms of the

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pro-apoptotic activities of 3*β*-hydroxylanosta-8,24-dien-21-al (**1**) and trametenolic acid (**6**), triterpenoids identified as main cytotoxic constituents of *I. obliquus* in human lung cancer cells, have never been investigated.

Therefore, to explore the molecular mechanisms underlying apoptosis induced by *I. obliquus* and its main cytotoxic constituents in human lung cancer cells, we examined the effects of the MeOH extract of *I. obliquus* fruiting bodies, its cytotoxic fractions, and its main cytotoxic compounds on activation of caspase-3 and cleavage of its substrate PARP (Kaufmann et al., 1993) in Calu-6 cells (**Fig. 10**). Detection of caspase-3 and PARP by immunoblotting showed that cleavage of both caspase-3 and PARP was significantly increased upon treatment with the MeOH extract and its cytotoxic HX and MC fractions, compared with DMSO treatment as a vehicle control. In addition, compounds **1** and **6** as well as compound **7** (identified as a novel lanostane-type triterpene, chagabusone A) increased levels of the cleaved form of caspase-3 and PARP in human lung cancer cells compared with vehicle-treated control cells. These observations suggest that the cytotoxicity of *I. obliquus* and its main cytotoxic triterpenoid derivatives, 3*β*-Hydroxylanosta-8,24-dien-21-al (**1**), trametenolic acid (**6**), and chagabusone A (**7**), is mediated by apoptosis accompanied by caspase-3 activation in human lung cancer cells.

Overall, in this study, we demonstrated the *in vitro* cytotoxic activity of *I. obliquus* fruiting bodies via induction of apoptosis in four human lung cancer cell lines with different *p53* status. In addition, bioactivity-guided fractionation and chemical analysis led to the isolation and identification of a novel lanostane-type triterpene, 3*β*-hydroxy-5*α*-lanosta-8,25-dien-21-oic acid (**7**) (named chagabusone A), as well as seven previously reported triterpenoids (compounds **1** to **6**, and **8**) as main constituents of the most cytotoxic fractions of the MeOH

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extract of *I. obliquus* fruiting bodies. Furthermore, evaluation of cytotoxicity of the isolated triterpenoid derivatives revealed 3*β*-hydroxylanosta-8,24-dien-21-al (**1**), trametenolic acid (**6**), and chagabusone A (**7**) as the major constituents contributing to the cytotoxic effects of the MeOH extract of *I. obliquus* fruiting bodies in human lung cancer cells *in vitro*.

The aqueous extract of *I. obliquus* fruiting bodies has been shown to induce apoptosis in a caspase-3-dependent manner in various types of cancer cells, including human colon cancer cell, human liver cancer cells, human gastric cancer cells, and murine melanoma cells (Youn et al., 2008; Lee et al., 2009; Youn et al., 2009; Hwang et al., 2003). In addition, triterpenoids derived from *I. obliquus* have been shown to exhibit cytotoxicity against various types of cancer cells, including the human lung cancer cell line A549 (Tanaka et al., 2011; Liu et al., 2013; Zhao et al., 2015; Patlolla and Rao, 2012; Nomura et al., 2008). However, with the exception of inotodiol (Nomura et al., 2008), the mechanisms underlying the cytotoxicity of *I. obliquus*-derived triterpenoids against cancer cells remain to be elucidated. In this study, apoptosis induced by the MeOH extract of *I. obliquus* fruiting bodies was accompanied by increased cleavage of caspase-3 and its substrate, PARP, in human lung cancer cells, consistent with previously published studies. In addition, the three main cytotoxic compounds of the extract were found to trigger activation of caspase-3 and cleavage of PARP in human lung cancer cells. Taken together, these observations not only suggest that the cytotoxicity of *I. obliquus* fruiting body against human lung cancer cells is mediated by induction of apoptosis in a caspase-3-dependent manner, but also provide the molecular basis underlying its cytotoxic and pro-apoptotic activities in human lung cancer cells. In addition, this is the first demonstration of caspase-3 activation in cancer cells treated with *I. obliquus*-derived triterpenoids other than inotodiol. These findings are further supported by previously

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published studies reporting induction of caspase-3-dependent apoptosis by various triterpenoid derivatives derived from natural resources, including mushrooms, in cancer cells (Patlolla and Rao, 2012; Ríos et al., 2012).

Loss of functional *p53*, a tumor suppressor, through various mechanisms such as mutational inactivation or loss of heterozygosity is observed in more than 50% of all human cancers, and *p53* status affects drug resistance not only in human cancer cell lines *in vitro*, but also in cancer patients in the clinic (Wang and Sun, 2010). However, our evaluation of the cytotoxic effects of *I. obliquus* fruiting body and its main constituents, compounds **1**, **6**, and **7**, in human lung cancer cell lines with differential *p53* status revealed no significant correlation between either cytotoxicity or pro-apoptotic activity toward human lung cancer cells and *p53* status of those cells. These findings indicate that *I. obliquus* and its main cytotoxic compounds can exert their cytotoxic effects on human lung cancer cells irrespective of their *p53* status, thus broadening their potential application in lung patients with various *p53* expression levels and mutation status.

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4. Conclusions

Our study demonstrates for the first time the cytotoxic and pro-apoptotic activities of *I. obliquus* and its main cytotoxic triterpenoids in human lung cancer cells with different *p53* status. In addition, our chemical investigation led to the isolation and identification of a novel cytotoxic triterpenoid, chagabusone A, from *I. obliquus* fruiting bodies. Although the anticancer activity of *I. obliquus* and its main cytotoxic triterpenoids should be further validated using *in vivo* models of lung cancer, our findings provide a molecular explanation

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for the anticancer properties of *I. obliquus* and further support its potential application for therapeutic intervention in lung cancer.

Conflict of interest

The authors declare that there are no conflicts of interests.

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Authors' contributions

J.B. and H.S.R. performed most of the experimental work. K.H.B. and K.H.K. conceived the project and designed the experiments. J.B. and S.L. designed and implemented the separation and purification protocols. H.S.R., S.L., S.S.S., and K.H.B. designed and implemented the biological test protocols. J.B., K.H.B., and K.H.K. drafted and revised the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://

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Figure legends

Fig. 1. The MeOH extract of *I. obliquus* fruiting bodies decreases cell viability in human lung adenocarcinoma cells. (**A**) Cell viability in four human lung adenocarcinoma cell lines, A548, H1264, H1299, and Calu-5, as assessed by the WST-1 assay after 48-hr treatment with the MeOH extract of *I. obliquus* fruiting bodies at the indicated concentrations (*upper*) and IC_{50} values of the extract in these cell lines (*lower*). (**B**) Representative bright-field images (200× total magnification) of human lung adenocarcinoma cells treated with MeOH extract at the indicated concentrations for 48 hr. Data are presented as means ± SEMs. Scale bar: 100 μm.

Fig. 2. The MeOH extract of *I. obliquus* fruiting bodies induces apoptosis in human lung adenocarcinoma cells. Representative immunofluorescence images (400× total magnification) of TUNEL staining (*left*) and quantitation of TUNEL-positive cells (*right*) in A548, H1264, H1299, and Calu-5 cells treated with 2.5 mg/mL of the MeOH extract of *I.*

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obliquus fruiting bodies or growth medium only (untreated) as a negative control for 48 hr. Data are presented as means ± SEMs. Scale bar: 50 μm. ** *p < 0.01*.

Fig. 3. Cytotoxicity of four fractions separated from the MeOH extract of *I. obliquus* fruiting bodies toward human lung adenocarcinoma cells. (**A-D**) Viability of A549 (**A**), H1264 (**B**), H1299 (**C**), and Calu-6 (**D**) cells as assessed by WST-1 assay after 48-hr treatment with BuOH, EA, HX, and MC fractions prepared from the MeOH extract of *I. obliquus* fruiting bodies at the indicated concentrations. (**E**-**F**) Representative bright-field images (200× total magnification) of human lung adenocarcinoma cells treated with HX (**E**) and MC (**F**) fractions at the indicated concentrations or with 0.6% DMSO as vehicle control. Data are presented as means ± SEMs. Scale bar: 100 μm.

Fig. 4. The HX and MC fractions of the MeOH extract of *I. obliquus* fruiting bodies increase apoptotic cell death in human lung adenocarcinoma cells. Representative immunofluorescence images (400× total magnification) of TUNEL staining (*left*) and quantitation of TUNEL-positive cells (*right*) in A548, H1264, H1299, and Calu-5 cells treated with HX (200 μg/mL) and MC (150 μg/mL) fractions of the MeOH extract of *I. obliquus* fruiting bodies for 48 hr. Cells incubated in growth medium containing 0.2% DMSO were used as vehicle controls. Data are presented as means ± SEMs. Scale bar: 50 μm. ** *p < 0.01*.

Fig. 5. Chemical structures of compounds 1-8.

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Fig. 6. Key ^{1}H-^{1}H COSY ( ) and HMBC ( ) correlations of compound 7.

Fig. 7. Key NOESY correlations of compound 7.

Fig. 8. Compounds 1, 6, and 7 exhibit significant cytotoxicity against human lung adenocarcinoma cells. (**A-D**) Cell viability determined by WST-1 assay in A549 (**A**), H1264 (**B**), H1299 (**C**), and Calu-6 (**D**) cells after treatment with the eight compounds isolated from the HX and MC fractions of the MeOH extract of *I. obliquus* fruiting bodies at the indicated concentrations for 48 hr. (**E**) Representative bright-field images (200× total magnification) of human lung adenocarcinoma cells after 48-hr treatment with the compounds at the indicated concentration or with 0.4% DMSO as vehicle control. Data are presented as means ± SEMs. Scale bar: 100 µm.

Fig. 9. Pro-apoptotic effects of compounds 1, 6, and 7 on human lung adenocarcinoma cells. Representative immunofluorescence images (400× total magnification) of TUNEL staining (*left*) and quantitation of TUNEL-positive cells (*right*) in A548, H1264, H1299, and Calu-5 cells treated with compounds **1**, **6**, and **7** at 100, 250, and 150 µM, respectively, for 48 hr. Cells treated with 0.5% DMSO were used as vehicle controls. Data are presented as means ± SEMs. Scale bar: 50 µm. ** $p < 0.01$.

Fig. 10. MeOH extract of *I. obliquus* fruiting bodies, its cytotoxic fractions, and its main cytotoxic triterpenoids induce cleavage of caspase-3 and its substrate PARP in human lung adenocarcinoma cells. Calu-6 cells were treated with 2.5 mg/mL MeOH extract of *I.*

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obliquus fruiting bodies (*left*), 200 μg/mL HX fraction and 150 μg/mL MC fraction (*middle*), and compounds **1**, **6**, and **7** at 100, 250, and 150 μM, respectively (*right*), for 48 hrs. Cells were also treated with 1 μM doxorubicin (Doxo) as positive control and growth medium only (untreated) or 0.2 to 0.5% DMSO as negative and vehicle controls, respectively. Whole cell lysates were prepared and subjected to immunoblot analysis of caspase-3, PARP, and β-actin as a loading control.

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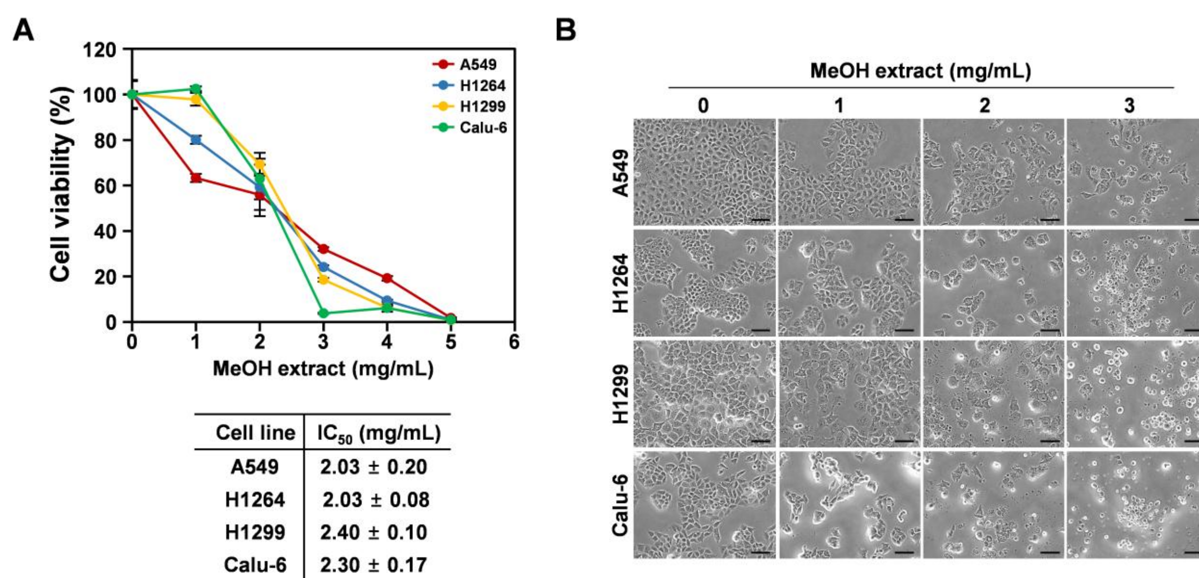

Cell line	IC_{50} (mg/mL)
A549	2.03 ± 0.20
H1264	2.03 ± 0.08
H1299	2.40 ± 0.10
Calu-6	2.30 ± 0.17

Fig. 1.

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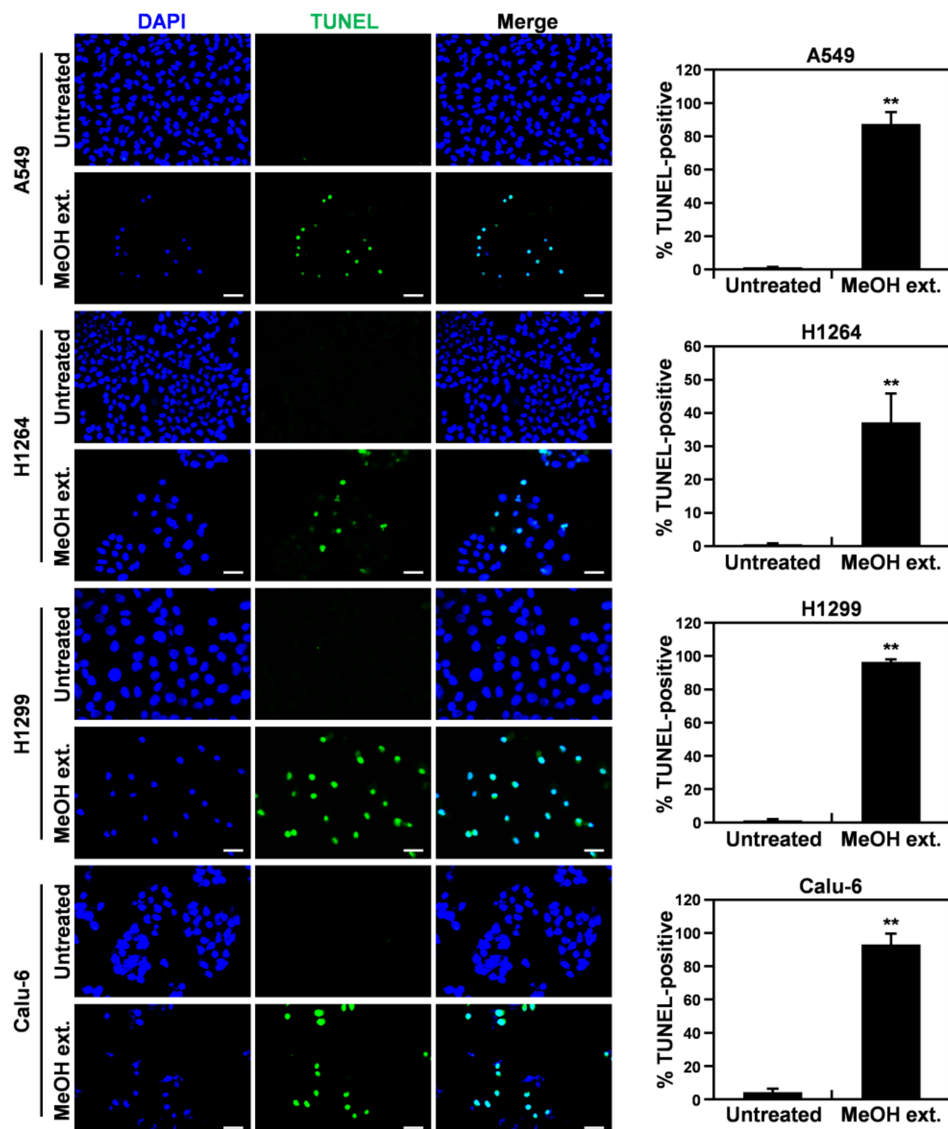

Fig. 2.

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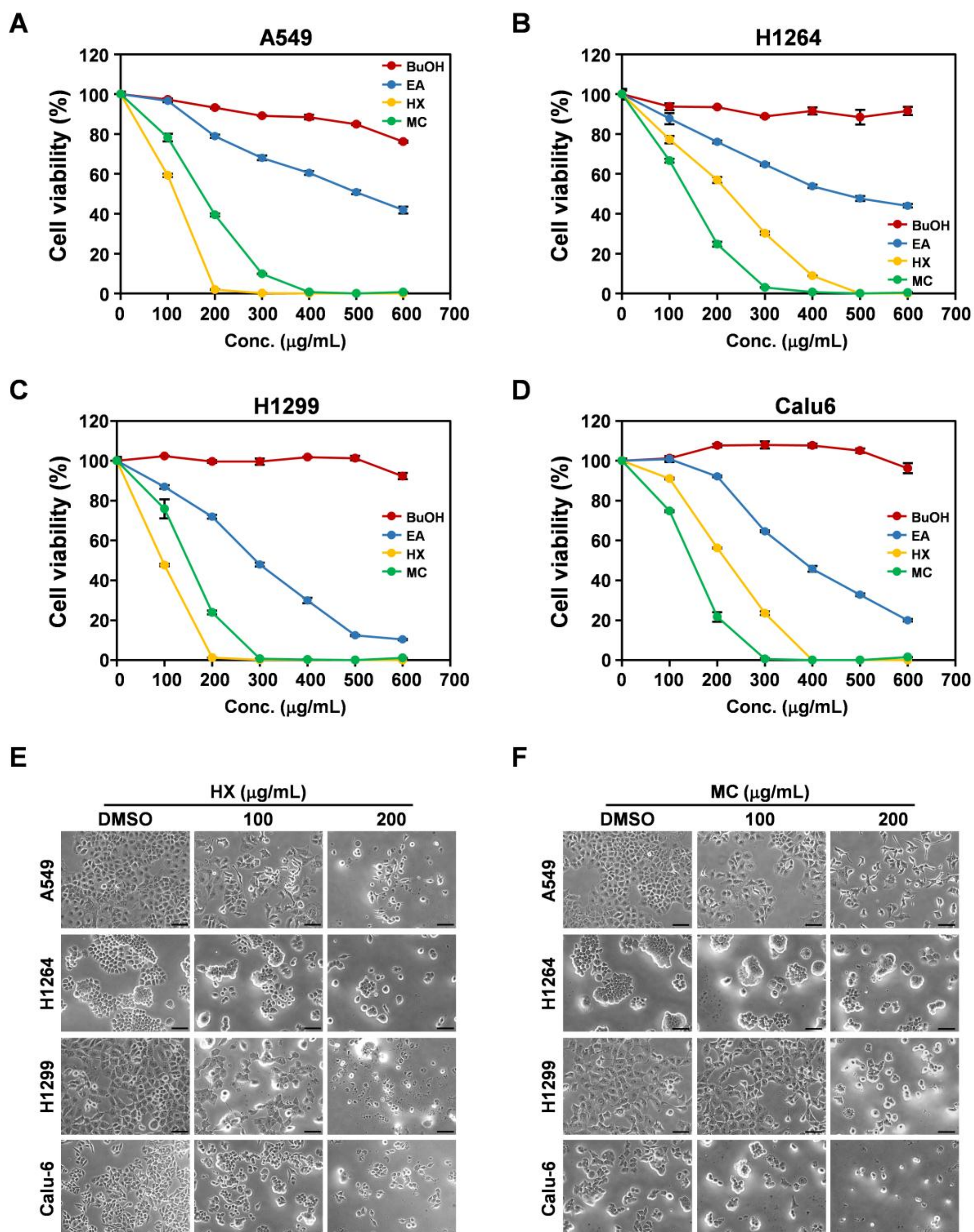

Fig. 3.

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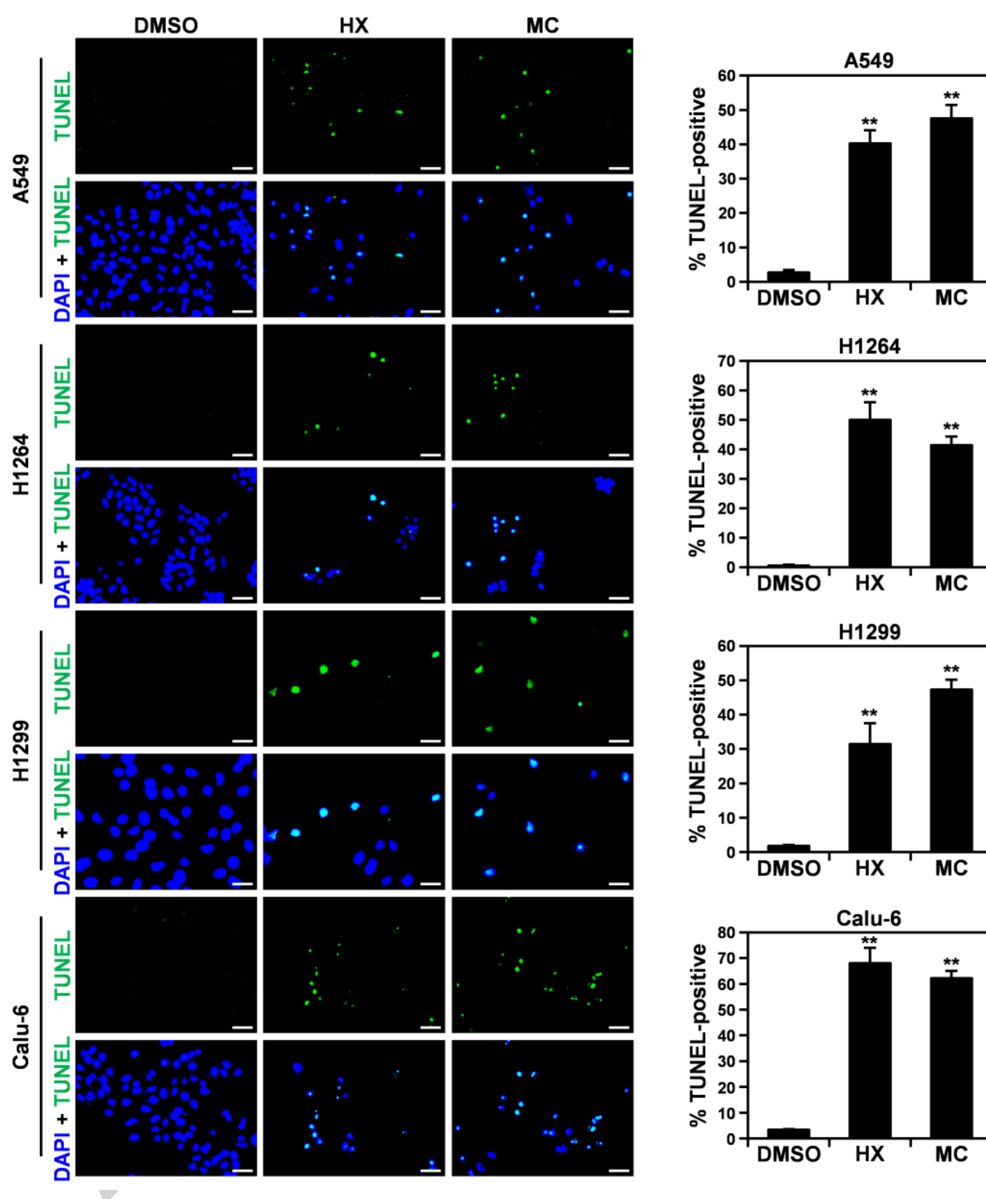

Fig. 4.

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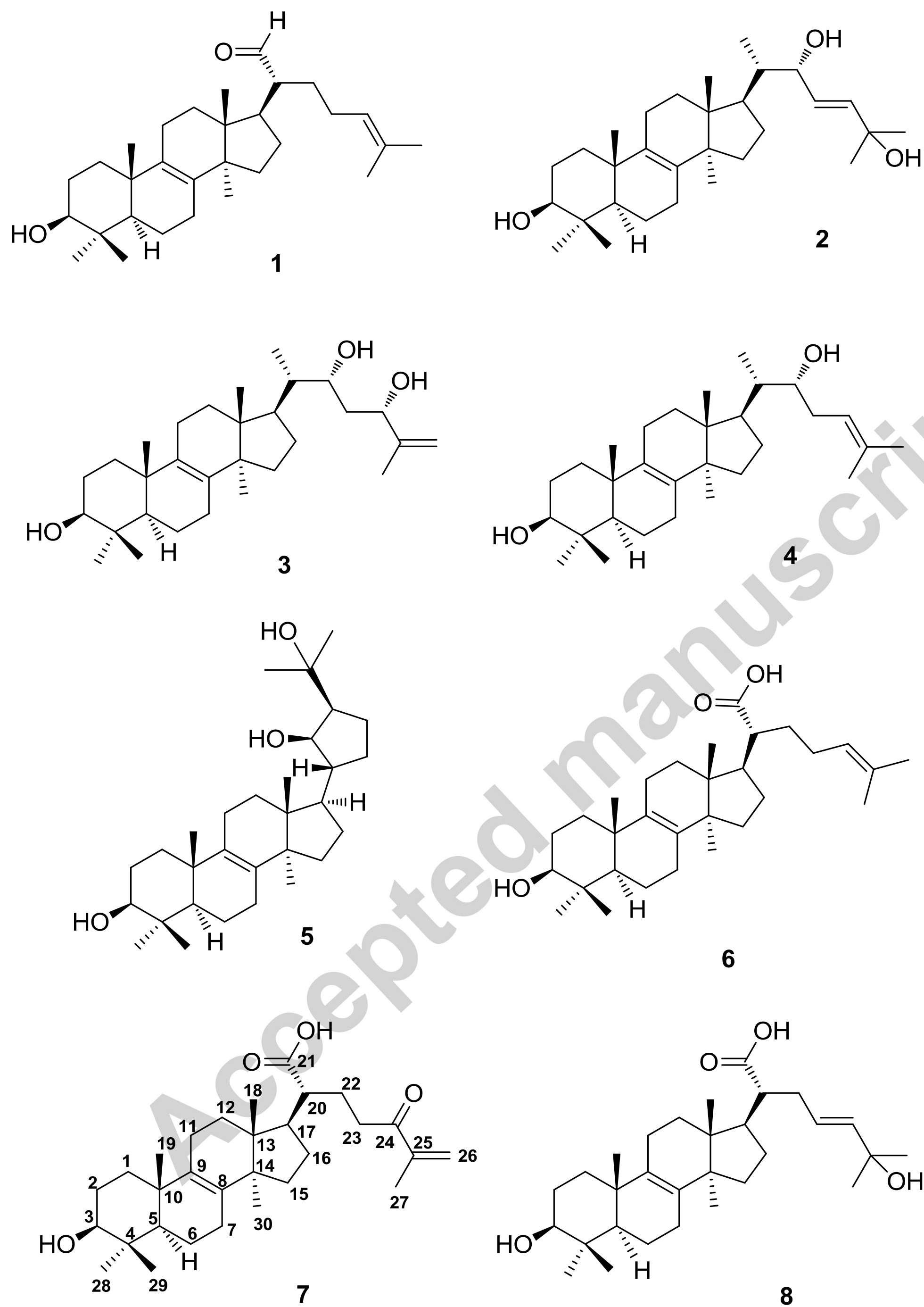

Fig. 5.

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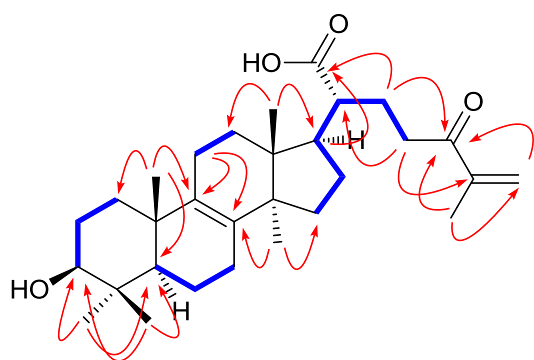

Fig. 6.

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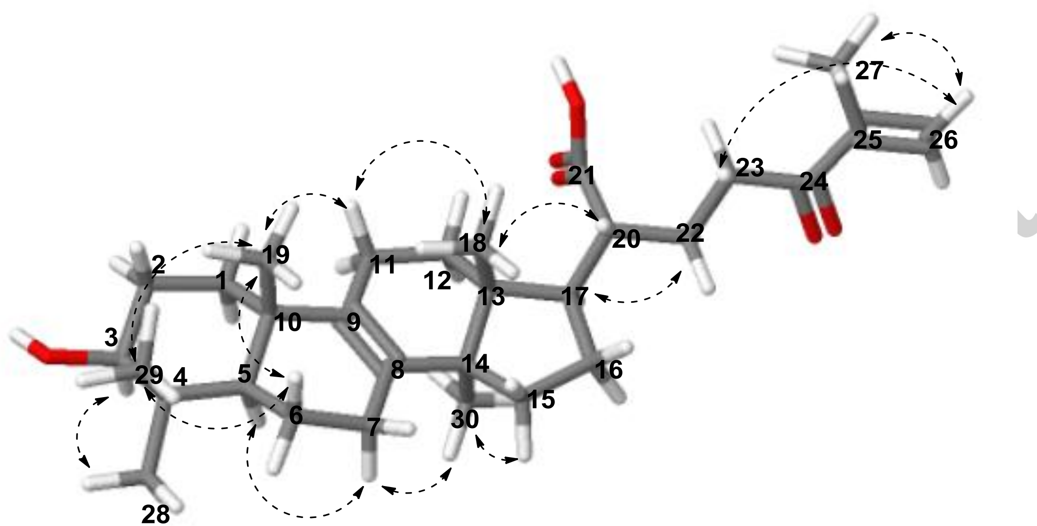

Fig. 7.

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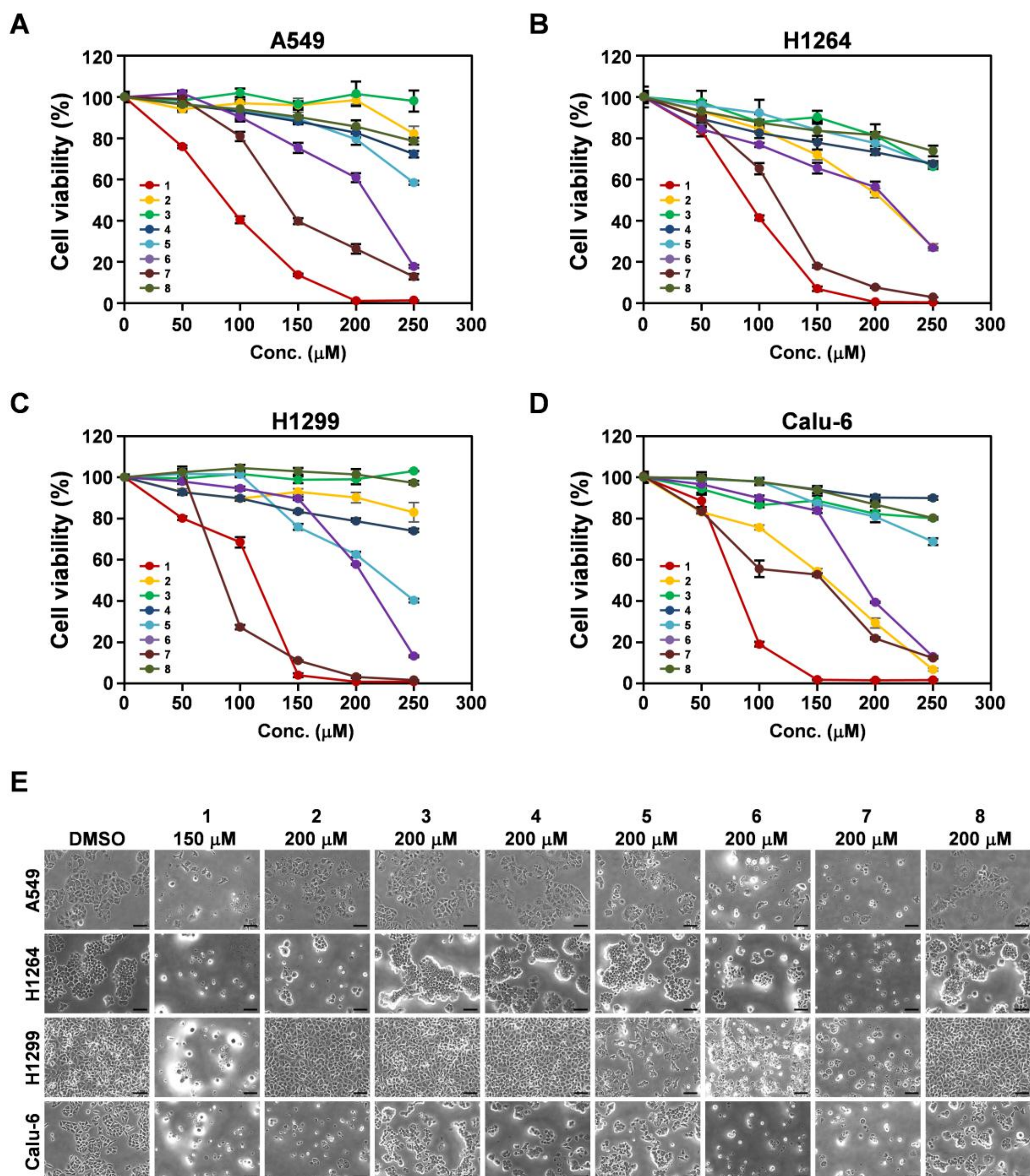

Fig. 8.

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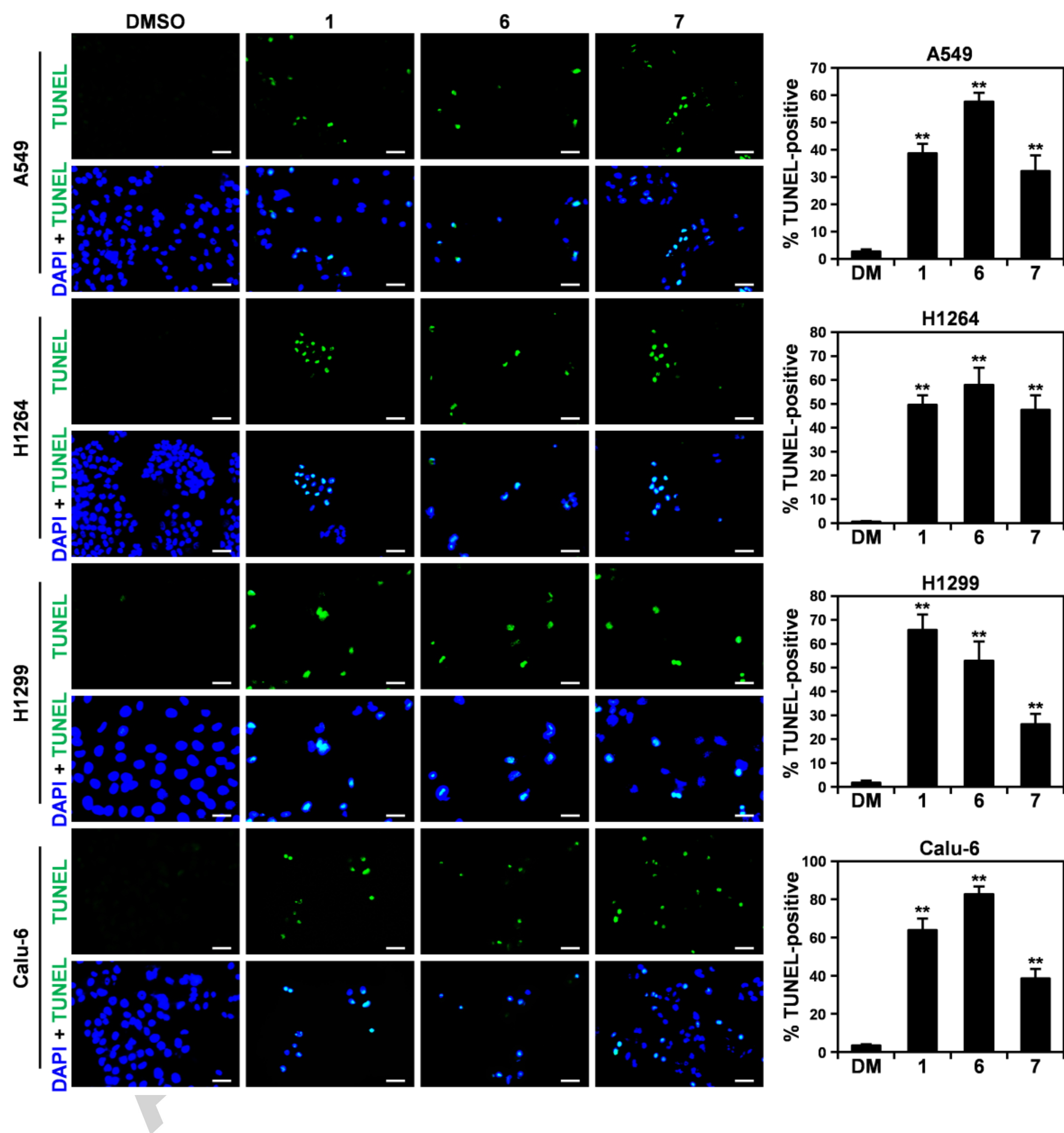

Fig. 9.

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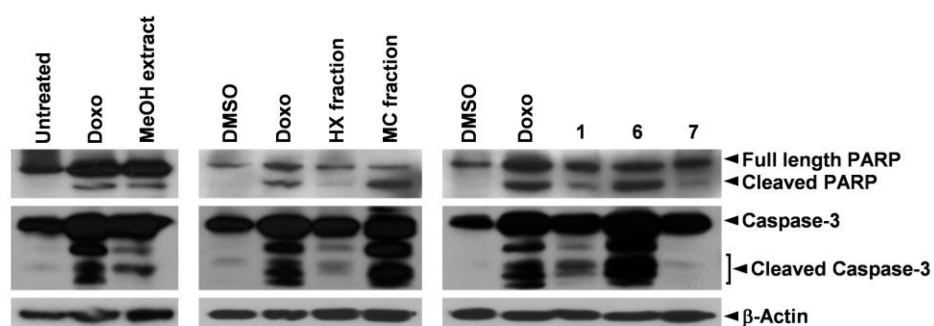

Fig. 10.

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Table 1. IC_{50} values (μg/mL) of four different fractions from the MeOH extract of *I. obliquus* fruiting bodies in human lung adenocarcinoma cell lines.

	Cell lines			
Fraction (s)	A549	H1264	H1299	Calu-6
BuOH	ND[a]	ND	ND	ND
EA	513.5 ± 13.9[b]	440.0 ± 19.5	292.5 ± 4.7	379.4 ± 2.9
HX	119.4 ± 1.7	222.0 ± 5.4	95.3 ± 1.4	225.1 ± 2.0
MC	173.0 ± 2.2	134.1 ± 2.3	147.1 ± 10.3	137.7± 3.6

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[a] ND, not determined.
[b] Values are the means ± SEMs of triplicate determinations.

Table 2. ^{1}H (800 MHz) and ^{13}C NMR (200 MHz) data of chagabusone A in CD_3OD.[a]

Position	7	
	δ_H	δ_C
1	1.23 m, 1.77 m	37.8
2	1.64 m	29.2
3	3.17 dd (11.0, 5.5)	80.4
4		40.7
5	1.07 dd (12.5, 2.0)	52.7
6	1.73 m, 1.56 m	20.2
7	2.10 m	28.4
8		136.3
9		136.8
10		39.0
11	2.05 m, 2.01 m	22.7
12	1.70 m, 1.53 m	30.8
13		46.3
14		51.5
15	1.67 m, 1.26 m	32.3
16	2.00 m, 1.46 m	28.7
17	2.09 m	49.1
18	0.81 s	17.3
19	1.02 s	20.2
20	2.23 m	50.7
21		177.7
22	1.90 m, 1.66 m	29.0
23	2.74 t (7.5)	37.1
24		204.4
25		146.4
26	6.06 s, 5.84 s	126.6
27	1.86 s	18.5
28	1.00 s	29.4
29	0.82 s	16.9
30	0.94 s	25.5

[a]Signal multiplicity and coupling constants (Hz) are in parentheses. The assignments were based on HSQC, HMBC, ^{1}H-^{1}H COSY, and NOESY experiments

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Table 3. IC$_{50}$ values (μM) of the compounds isolated from *I. obliquus* fruiting bodies in human lung cancer cell lines.

Cell lines	Compounds							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
A549	83.5 ± 0.3[a]	ND[b]	ND	ND	ND	227.4 ± 8.6	141.2 ± 3.9	ND
H1264	90.9 ± 1.1	203.7 ± 6.0	ND	ND	ND	218.2 ± 8.7	122.2 ± 6.5	ND
H1299	128.0 ± 6.8	ND	ND	ND	239.3 ± 7.4	210.3 ± 0.3	82.0± 9.4	ND
Calu-6	75.1 ± 14.2	162.1 ± 1.2	ND	ND	ND	184.1 ± 0.7	128.6 ± 2.5	ND

[a] Values are the means ± SEMs of triplicate determinations.

[b] ND, not determined.

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