Bioactivity-based analysis and chemical characterization of cytotoxic compounds from a poisonous mushroom, *Amanita spissacea*, in human lung cancer cells *in vitro*

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Abstract

As part of our systematic study on Korean toxic mushrooms, bioactivity-guided fractionation of the MeOH extract of *Amanita spissacea* (Amanitaceae) fruiting bodies and chemical investigation of its cytotoxic fractions led to the isolation of (9*E*)-8-oxo-9-octadecenoic acid (**1**), (10*E*)-9-oxo-10-octadecenoic acid (**2**), (9*E*)-8-oxo-9-octadecenoate methyl ester (**3**), (9*Z*)-9-octadecenoate-(2'*S*)-2',3'-dihydroxypropyl ester (**4**), (9*Z*)-9-octadecenoic acid (**5**), and palmitic acid (**6**). The structures of the isolates were elucidated by NMR spectroscopic analysis and LC/MS analysis. Among the isolated compounds, compounds **1** and **2** exhibited the most potent cytotoxic activity in all human lung cancer cell lines examined, with IC_{50} values ranging from 255.7 to 321.0 μM and 250.2 to 322.5 μM, respectively. The cytotoxicity of these compounds was also found to be mediated by apoptosis associated with caspase-3 activation. These findings provide experimental evidence suggesting the potential of *A. spissacea* as a promising natural source for the discovery of novel anticancer drug candidates.

Keywords

Amanita spissacea; poisonous mushroom; fatty acids; cytotoxicity; lung cancer; apoptosis

Experimental

General experimental procedures

Optical rotations were calculated using a Jasco P-1020 polarimeter (Jasco, Easton, MD, USA). IR spectra were recorded using a Bruker IFS-66/S FT-IR spectrometer (Bruker, Karlsruhe, Germany). UV spectra were acquired on an Agilent 8453 UV-visible spectrophotometer. LC/MS analysis was performed with an Agilent 1200 Series HPLC system equipped with a diode array detector and a 6130 Series ESI mass spectrometer. Nuclear magnetic resonance (NMR) spectra were documented using a Bruker AVANCE III HD 800 NMR spectrometer with a 5 mm TCI CryoProbe (Bruker, Karlsruhe, Germany) operating at 800 MHz (^{1}H) and 200 MHz (^{13}C). Preparative HPLC was performed with a Waters 1525 binary HPLC pump with a Waters 996 photodiode array detector using Agilent Eclipse C18 columns (21.2 × 250 mm; flow rate: 5 mL/min). Semi-preparative HPLC was performed with a Shimadzu Prominence HPLC System with SPD-20A/20AV Series Prominence HPLC UV-Vis Detectors using Phenomenex Luna HPLC phenyl-hexyl columns (250 × 10 mm; flow rate: 2 mL/min). Column chromatography was performed with silica gel 60 (Merck, 230–400 mesh). Merck pre-coated silica gel F254 plates and reversed-phase (RP)-18 F254s plates were used for TLC. Spots were detected on TLC under UV light or by heating after spraying with anisaldehyde-sulfuric acid.

Mushroom material

Fresh fruiting bodies of *A. spissacea* were collected at Yunggeolleung, Hwaseong of Gyeong Gi-do, Korea, in August 2015. One of the authors (S.J.S.) authenticated a voucher specimen (HCCN26949) of the mushroom, which was then deposited at the Herbarium Conservation Center of National Institute of Agricultural Sciences (HCCN), RDA, Korea.

Extraction and isolation

Dried fruiting bodies of *A. spissacea* (830 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 (19.6 g). The MeOH extract (19.6 g) was then suspended in distilled water (800 mL) and 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, 0.4 g, 0.5 g, and 2.1 g, respectively. To treat human lung cancer cells, the MeOH extract and its fractions were dissolved as stock solutions at concentrations of 100 and 50 mg/mL, respectively, in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) and stored at −80ºC until use.

To isolate the cytotoxic constituents of the fruiting bodies of *A. spissacea*, the HX soluble fraction (1.7 g), which exhibited a significant cytotoxic effect on all the human lung cancer cell lines tested, 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 nine sub-fractions (HA – HI). Fraction HD (193.8 mg) was separated by preparative reversed-phase HPLC using an Agilent Eclipse C18 column (21.2 × 250 mm; flow rate: 5 mL/min) with a gradient solvent system from 60% MeCN to 100% MeCN to give eight subfractions (HD1-HD8). Subfraction HD7 (13.7 mg) was isolated by semi-preparative reversed-phase HPLC using a semi-preparative RP HPLC (250 mm × 10 mm i.d., 10 μm, Phenomenex Luna C18(2) column, flow rate 2.0 mL/min) with 87% MeOH to yield compound **3** (2.2 mg, t_R = 26.0 min). Subfraction HD8 (88.3 mg) was isolated by semi-preparative reversed-phase HPLC using a Phenomenex Luna HPLC phenyl-hexyl column (250 × 10 mm; flow rate: 2 mL/min) with 87% MeOH to yield compound **5** (13.5 mg, t_R = 27.5 min) and compound **6** (13.4 mg, t_R = 23.0 min). Fraction HG (207.0 mg) was separated by preparative reversed-phase HPLC using an Agilent Eclipse C18 column (21.2 × 250 mm; flow rate: 5 mL/min) with a gradient solvent system from 55% MeCN to 100% MeCN to give five subfractions (HG1-HG5). Subfraction HG5 (23.4 mg) was isolated by semi-preparative reversed-phase HPLC using a Phenomenex Luna

HPLC phenyl-hexyl column (250 × 10 mm; flow rate: 2 mL/min) with 87% MeOH to yield compound **4** (3.4 mg, t_R = 24.0 min).

The MC-soluble fraction (0.4 g), which was also found to show cytotoxic activity towards all the human lung cancer cell lines examined, was fractionated by silica gel column chromatography with a gradient solvent system from CH_2Cl_2-MeOH (50:1, v/v) to CH_2Cl_2-MeOH (1:1, v/v) to give nine sub-fractions (CA – CI). Finally, Fraction CG (207.0 mg) was isolated by semi-preparative reversed-phase HPLC using a Phenomenex Luna HPLC phenyl-hexyl column (250 × 10 mm; flow rate: 2 mL/min) with a gradient solvent system from 70% MeOH to 95% MeOH to yield compound **1** (0.7 mg, t_R = 38.5 min) and compound **2** (2.3 mg, t_R = 36.5 min).

All the isolated compounds were then dissolved as stock solutions at a concentration of 50 mM in DMSO and used for treating human lung cancer cells.

Cell culture

The human lung cancer 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 cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA, USA), 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (WelGENE).

Cell viability analysis

A549, H1264, and H1299 (5×10^3 cells/well) and Calu-6 (7.5×10^3 cells/well) were plated in triplicate in 96-well tissue culture plates (Thermo Scientific, Waltham, MA, USA), grown overnight, and incubated in growth medium containing the MeOH extract of *A. spissacea* fruiting bodies, its fractions, or the isolated compounds at various concentrations. Cells were also treated with 0 to 1.5% DMSO-containing growth medium as vehicle controls. At 48 hrs after treatment, the viability of cells was assessed using the WST-1 cell proliferation assay (Daeil Lab Service, Co., Ltd., Seoul, Korea) as previously described [43] and calculated as a

percentage of the corresponding vehicle control. IC_{50} values of the MeOH extract, its fractions, and the isolated compounds were determined by a non-linear regression analysis of the dose-response curve using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

Apoptosis analysis

A549, H1264, and H1299 (7.5×10^3 cells) and Calu-6 (1.0×10^4 cells) were seeded on 12-mm glass coverslips (Paul Marienfeld GmbH & Co, KG, Lauda-Königshofen, Germany) in triplicate, grown overnight, and treated with the MeOH extract, its fractions, and the isolated compounds for 48 hr. Cells were also treated with growth medium containing DMSO as a vehicle control. Apoptotic cells were then detected by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining using the Dead-End labeling kit (Promega, Madison, WI, USA) following the manufacturer's instructions as previously described (Lee et al., 2017). Cells were also counterstained with 0.5 μg/mL 4',6-diamidino-2-phenylindole (DAPI, Sigma) to visualize cell nuclei. The cells were then mounted and examined under a fluorescence microscope (Carl Zeiss Meditec AG, Jena, Germany). The percentage of apoptotic cells was calculated as the ratio of TUNEL-positive to DAPI-stained nuclei counted from six randomly selected high-power fields (400×) on each slide.

Immunoblotting

Calu-6 cells (5×10^5 cells/well) were plated in 60-mm tissue culture dishes (Thermo Scientific), grown overnight, and treated with the MeOH extract, its HX and MC fractions, or the isolated compounds for 48 hrs. Cells treated with 1 μM doxorubicin (Sigma) and DMSO were used as positive and vehicle controls, respectively. The cells were then lysed in RIPA buffer, separated on SDS-PAGE, and probed for cleaved caspase-3, poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Danvers, MA, USA), and β-actin as a loading control, as previously described (Lee et al., 2017).

Statistical analysis

A two-tailed unpaired Student's *t* test was employed for determining the statistical significance of the difference between the vehicle control and the treated groups. All data were presented as the mean ± standard error of the mean (SEM), and *p* values less than 0.05 were considered to be statistically significant differences.

Sample Information

Amanita spissacea is a poisonous mushroom of the genus *Amanita*, widely distributed throughout Korea, Japan, and China. *A. spissacea* has been reported to cause symptoms of toxicity such as nausea and vomiting, hallucinations, and coma in those who mistakenly consume it, from which those individuals recovered 1 or 2 days later (Seok et al., 2011).

Additional Discussion

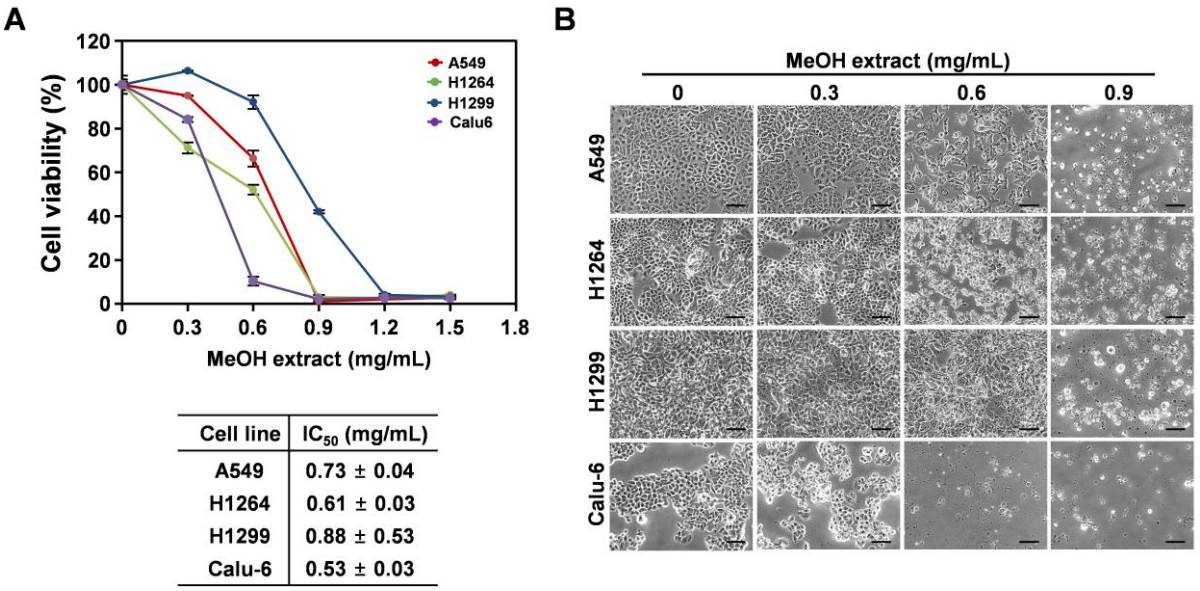
Octadecenoic acids have been shown to induce apoptosis in various types of human cancer cell lines, including gastric cancer, hepatocellular carcinoma, and leukemia cells (Yu et al., 2008). In addition, a recent study reported that an octadecenoic acid-containing extract of *Typhonium giganteum* Engl., an herb used in traditional Chinese medicine for treating cancer, triggers apoptosis mediated by the mitochondrial pathway in human hepatoma cells (Li et al., 2011). Furthermore, the cytotoxic fraction derived from the extract of *Protaetia brevitarsis* larva, which is enriched with octadecenoic acid derivatives, was demonstrated to induce apoptosis mediated by caspase-3 activation in human colon cancer cells (Yoo et al., 2007). Therefore, together with these previously published studies, our data strongly suggest that the cytotoxicity of *A. spissacea* fruiting bodies and their cytotoxic constituents, (9*E*)-8-oxo-9-octadecenoic acid (**1**) and (10*E*)-9-oxo-10-octadecenoic acid (**2**), is mediated through induction of apoptosis accompanied by caspase-3 activation in human lung cancer cells.

Previously, polysaccharides and cyclic peptides, including glucan and amanitin derivatives, derived from several poisonous mushrooms belonging to the genus *Amanita* have been shown to exhibit anticancer activity *in vivo* and *in vitro* (Kiho et al., 1992; Kiho et al.,1994; Tang et al., 2011). However, the biological activity of *A. spissacea*, especially its activity related to cancer, has not yet been investigated. Our study is, to the best of our knowledge, the first study showing the cytotoxicity of *A. spissacea* in human cancer cells and identifying its cytotoxic constituents. In addition, to the best of our knowledge, we, for the first time, showed the cytotoxic activity of mushroom-derived octadecenoic acid derivatives against human cancer cells. Furthermore, we found that *A. spissacea* and its cytotoxic constituents exhibit their cytotoxic effect on human lung cancer cells irrespective of their *p53* status, a tumor suppressor depleted or mutated in more than 50% of human cancers and known to be associated with drug resistance in human cancer cells *in vitro* and in patients with cancer in the clinic (Wang and Sun, 2010). Taken together, although further phytochemical investigation on *A. spissacea* fruiting bodies is required to determine whether their constituents other than the fatty acids (compounds **1** to **6**) isolated in this study are also involved in cytotoxicity against human lung cancer cells, our data suggest the potential use of *A. spissacea* as a natural source for the discovery of novel lead compounds with various pharmacological activities including anticancer potential.

Conclusion

In this study, the fruiting bodies of *A. spissacea*, a poisonous mushroom, were demonstrated to exhibit cytotoxic activity through induction of apoptosis in human lung cancer cells. As part of continuing efforts to discover cytotoxic compounds from Korean natural sources, bioactivity-guided fractionation and chemical investigation of the MeOH extract of *A. spissacea* fruiting bodies revealed the main cytotoxic compounds, (9*E*)-8-oxo-9-octadecenoic acid (**1**) and (10*E*)-9-oxo-10-octadecenoic acid (**2**), in human lung cancer cells with different

p53 status. Our findings provide experimental evidence suggesting a novel biological activity of *A. spissacea* related to cancer, and further support that toxic mushrooms are potential natural sources for the discovery of novel lead compounds for therapeutic intervention in cancer.



Cell line	IC_{50} (mg/mL)
A549	0.73 ± 0.04
H1264	0.61 ± 0.03
H1299	0.88 ± 0.53
Calu-6	0.53 ± 0.03

Figure S1. The MeOH extract of *A. spissacea* fruiting bodies reduces cell viability in human lung cancer cells. (A) Cell viability assessed by WST-1 assay (*upper*) and IC_{50} values of the MeOH extract of *A. spissacea* fruiting bodies in four different human lung cancer cell lines, A549, H1264, H1299 and Calu-6 cells, after 48 hrs of treatment (*lower*). (B) Representative bright-field images (200× magnification) of the human lung cancer cells at 48 hrs after treatment with the MeOH extract. Data are presented as mean ± SEM. Scale bar: 100 μm.

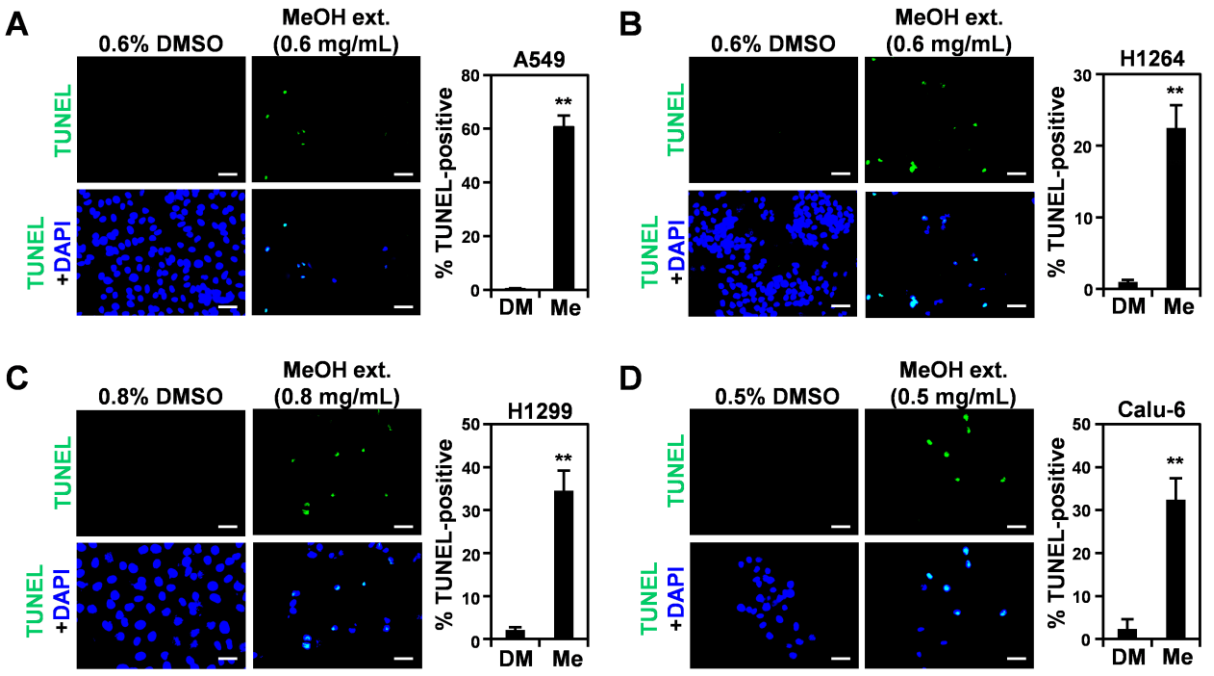


Figure S2. The MeOH extract of *A. spissacea* fruiting bodies induces apoptosis in human lung cancer cells. (A-D) Representative fluorescence images (400× total magnification) of TUNEL (green) and DAPI (blue) staining (*left*) and quantitation of TUNEL-positive cells (*right*) in A549 (A), H1264 (B), H1299 (C), and Calu-6 (D) cells treated with the MeOH (Me) extract or DMSO as vehicle controls at the indicated concentrations for 48 hours. Data are presented as means ± SEMs. Scale bar: 50 μm. ** $p < 0.01$.

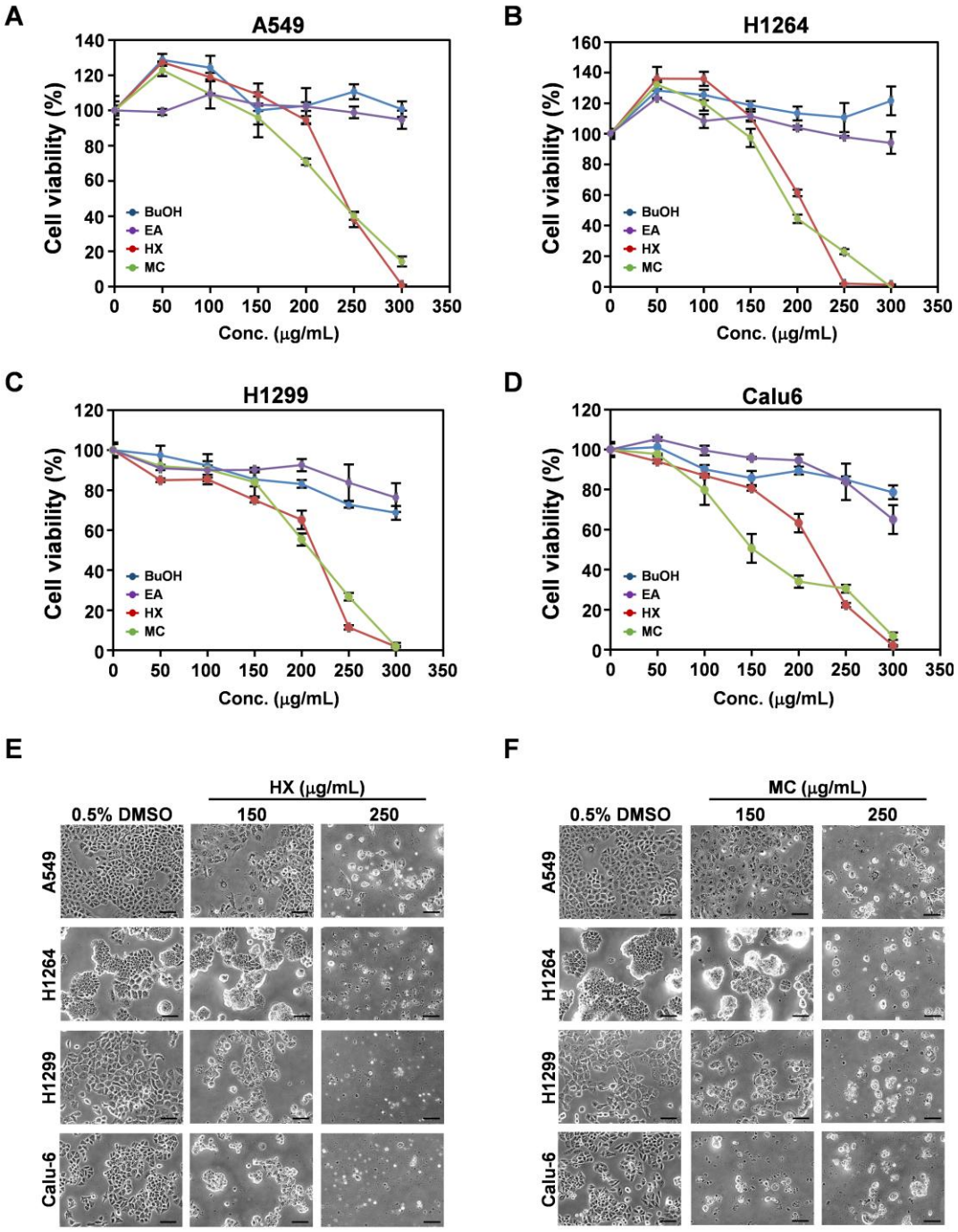


Figure S3. The HX and MC fractions of the MeOH extract of *A. spissacea* fruiting bodies exhibit cytotoxicity in human lung cancer cells. (A-D) The viability of A549 (A), H1264 (B), H1299 (C), and Calu-6 (D) cells assessed by WST-1 assay after treatment with the *n*-BuOH (BuOH), EtOAc (EA), hexane (HX), and CH_2Cl_2 (MC) fractions of the MeOH extract of *A. spissacea* fruiting bodies at the indicated concentrations for 48 hrs. (E-F) Representative bright-field images (200× magnification) of human lung cancer cells at 48 hrs after treatment

with the HX (E) and MC (F) fractions or DMSO as vehicle control at the indicated concentrations. Data are presented as means ± SEMs. Scale bar: 100 μm.

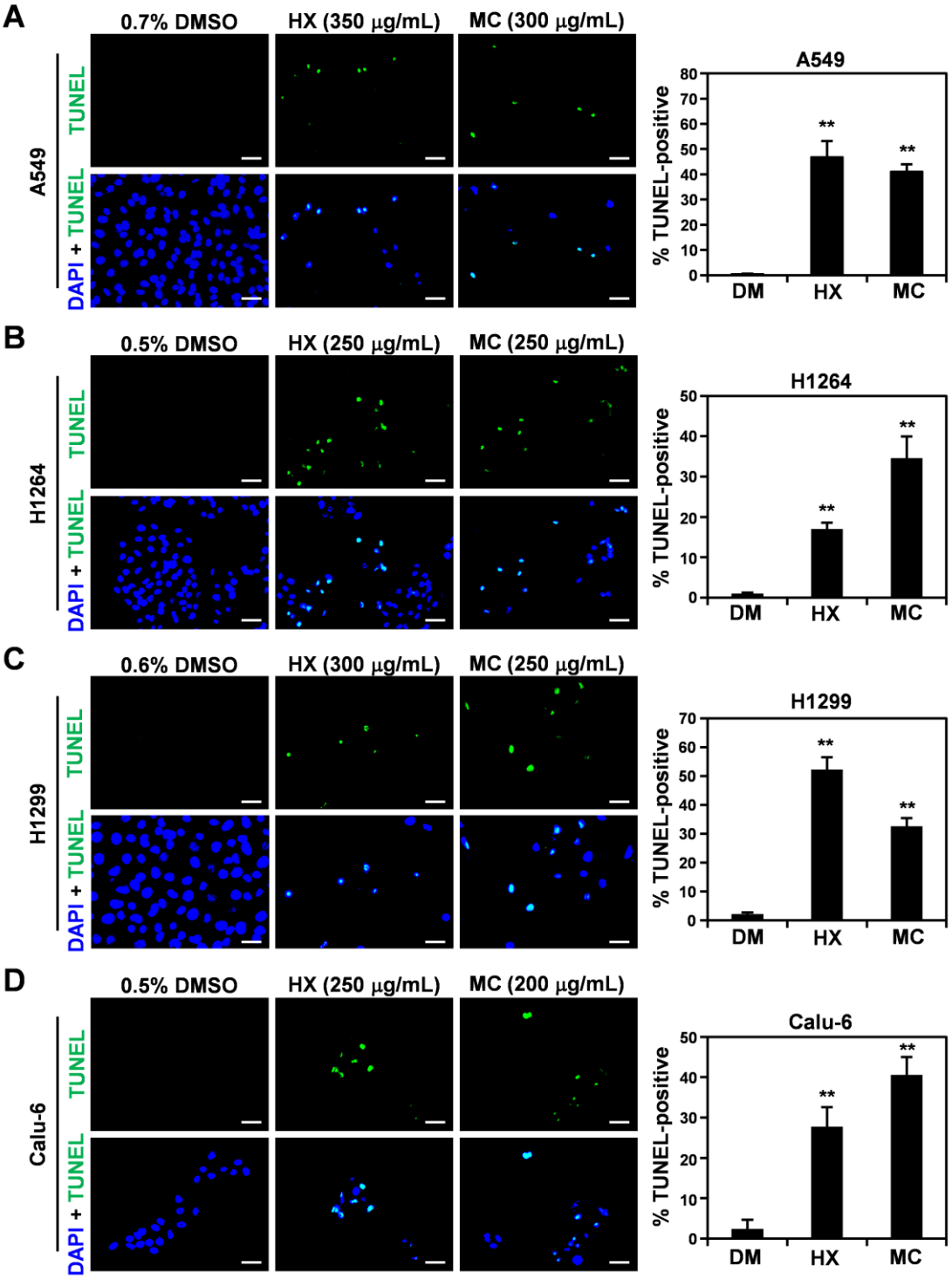


Figure S4. The HX and MC fractions of the MeOH extract of *A. spissacea* fruiting bodies induce apoptosis in human lung cancer cells. (A-D) Representative immunofluorescence images (400× total magnification) of TUNEL staining (*left*) and quantitation of TUNEL-positive cells (*right*) in A548 (A), H1264 (B), H1299 (C), and Calu-6 (D) cells treated with the HX and MC fractions of the MeOH extract of *A. spissacea* fruiting bodies or DMSO

(DM) as vehicle controls at the indicated concentrations for 48 hrs. Data are presented as means ± SEMs. Scale bar: 50 μm. ** $p < 0.01$.

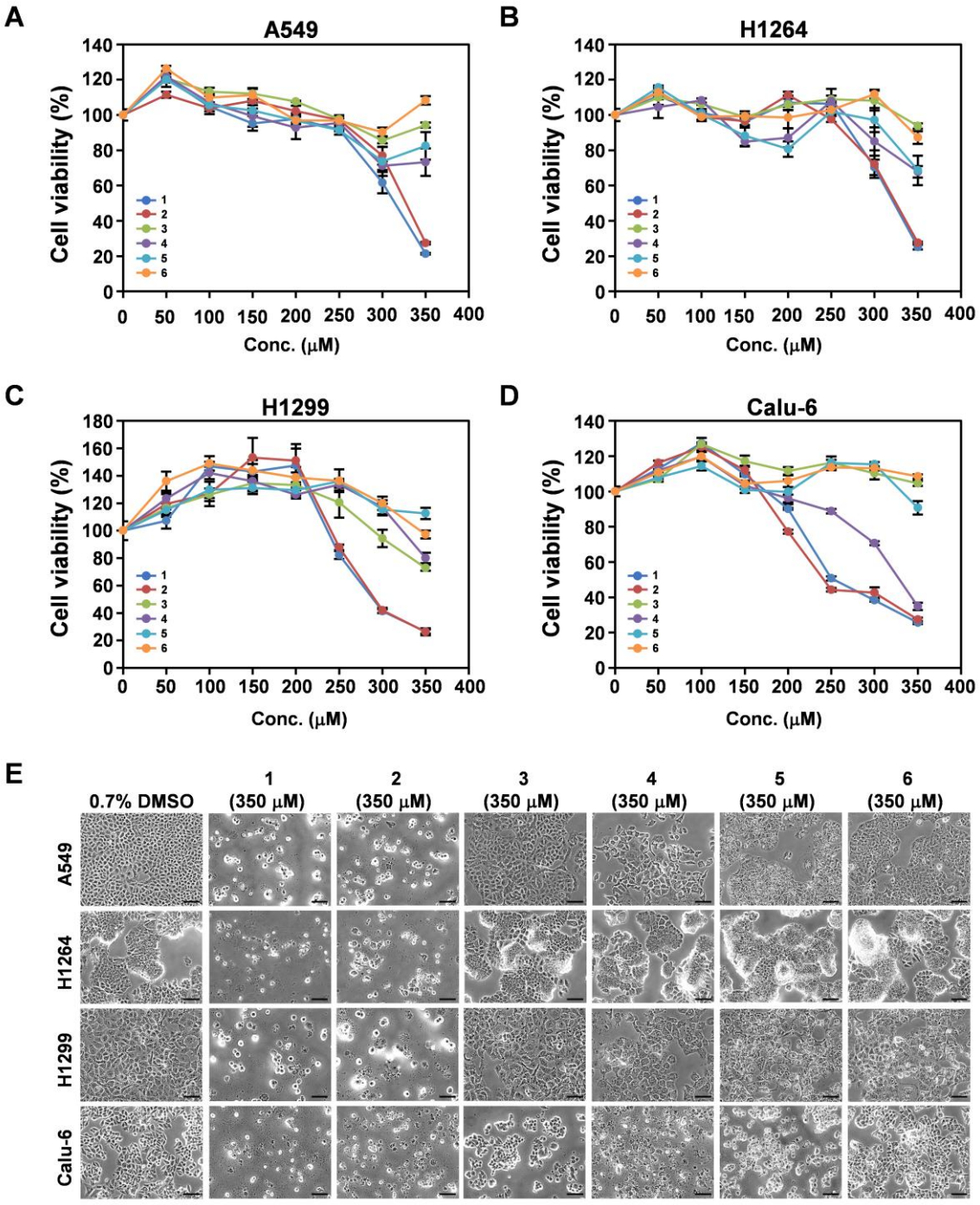


Figure S5. Cytotoxicity of six compounds (**1-6**) isolated from the HX and MC fractions of the MeOH extract of *A. spissacea* fruiting bodies in human lung cancer cells. (A-D) Cell viability assessed by WST-1 assay in A549 (A), H1264 (B), H1299 (C), and Calu-6 (D) cells after treatment with compounds **1-6** at the indicated concentrations for 48 hrs. (E) Representative bright-field images (200× magnification) of the human lung cancer cells treated with the

compounds or DMSO as vehicle controls at the indicated concentrations for 48 hrs. Data are presented as means ± SEMs. Scale bar: 100 μm.

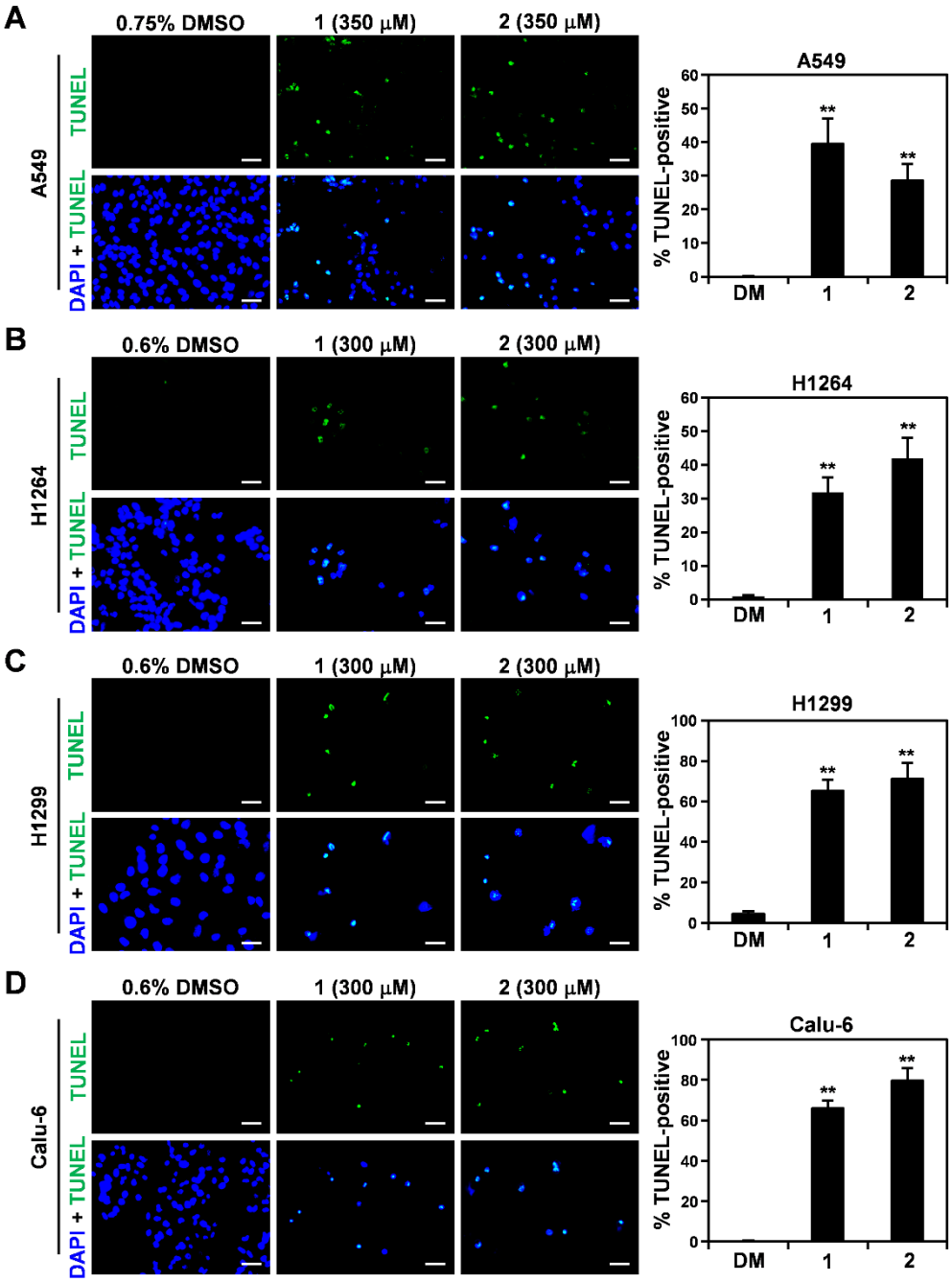


Figure S6. Compounds **1** and **2** isolated from *A. spissacea* fruiting bodies as the main constituents induce apoptosis in human lung cancer cells. (A-D) Representative immunofluorescence images (400× total magnification) of TUNEL staining (*left*) and quantitation of TUNEL-positive cells (*right*) in A548 (A), H1264 (B), H1299 (C), and Calu-6 (D) cells treated with compounds **1** and **2** or DMSO (DM) as vehicle controls at the indicated concentrations for 48 hrs. Data are presented as means ± SEMs. Scale bar: 50 μm. ** $p < 0.01$.

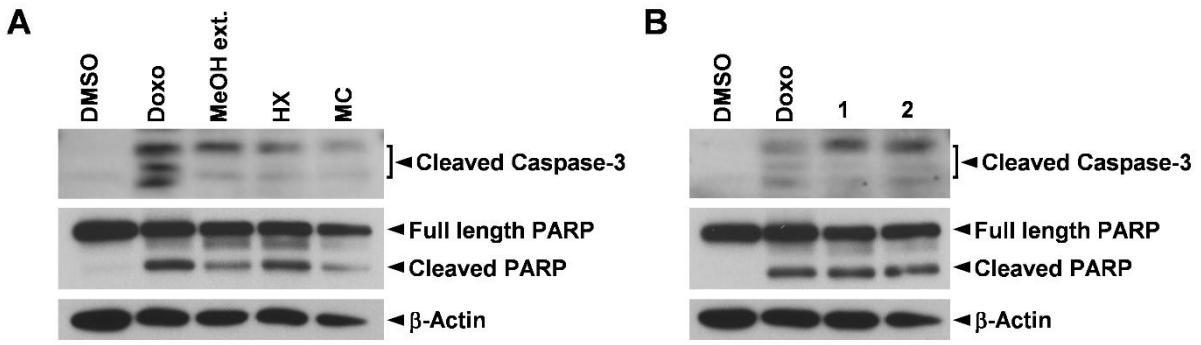


Figure S7. The MeOH extract of *A. spissacea* and its cytotoxic constituents increase cleaved forms of caspase-3 and its substrate, PARP, in human lung cancer cells. (A-B) Calu-6 cells were incubated with 0.5 mg/mL of the MeOH extract, 250 and 200 μg/mL of its HX and MC fractions, respectively (A), or 300 μM of compounds **1** and **2** (B) for 48 hrs. Cells were also treated with 1 μM doxorubicin (Doxo) and 0.5 or 0.6% DMSO as positive and vehicle controls, respectively. Cells were then lysed, separated on SDS-PAGE, and probed for cleaved caspase-3, PARP, and β-actin as a loading control.

Table S1. IC_{50} values (μg/mL) of four different fractions from the MeOH extract of *A. spissacea* fruiting bodies in human lung cancer cell lines.

Fraction (s)	Cell lines			
	A549	H1264	H1299	Calu-6
BuOH	ND[a]	ND	ND	ND
EA	ND	ND	ND	ND
HX	238.4 ± 1.5[b]	218.0 ± 1.6	223.7 ± 2.4	217.3 ± 6.2
MC	234.7 ± 1.7	194.4 ± 2.7	209.4 ± 3.6	162.7 ± 15.1

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

Table S2. IC_{50} values (μM) of the six compounds isolated from the HX and MC fractions of the MeOH extract of *A. spissacea* fruiting bodies in human lung cancer cell lines.

Compounds	Cell lines			
	A549	H1264	H1299	Calu-6
1	311.3 ± 2.0[a]	321.0 ± 2.7	278.3 ± 4.0	255.7 ± 0.4
2	322.5 ± 0.9	321.5 ± 2.9	293.0 ± 6.0	250.2 ± 8.8
3	ND[b]	ND	ND	ND
4	ND	ND	ND	366.7 ± 1.4
5	ND	ND	ND	ND
6	ND	ND	ND	ND

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

References

Kiho T, Katsuragawa M, Nagai K, Ukai S, Haga M. 1992. Structure and antitumor activity of a branched (1→3)-beta-D-glucan from the alkaline extract of *Amanita muscaria*. Carbohydr Res. 224:237-243.

Kiho T, Yoshida I, Katsuragawa M, Sakushima M, Usui S, Ukai S. 1994. Polysaccharides in Fungi. XXXIV. A polysaccharide from the fruiting bodies of *Amanita muscaria* and the antitumor activity of its carboxymethylated product. Biol Pharm Bull. 17:1460-1462.

Lee TK, Roh HS, Yu JS, Baek J, Lee S, Ra M, Kim SY, Baek KH, Kim KH. 2017. Pinecone of *Pinus koraiensis* inducing apoptosis in human lung cancer cells by activating caspase-3 and its chemical constituents. Chem Biodivers. 14:e1600412.

Li Q, Jiang C, Zu Y, Song Z, Zhang B, Meng X, Qiu W, Zhang L. 2011. SFE-CO_2 extract from *Typhonium giganteum* Engl. tubers, induces apoptosis in human hepatoma SMMC-7721 cells involvement of a ROS-mediated mitochondrial pathway. Molecules. 16:8228-8242.

Seok S, Kim YS, Kim WG, Seo JS, Jung MH, Lim KS, Son CH, Lee YS. 2011. Illustrated guide to poisonous mushroom. Pooreun hang bok, Seoul.

Tang YL, Luo Q, Ding W, Ding X, Yang ZR. 2011. Antitumor activity of polysaccharides extracted from two wild amanitas. Sichuan Da Xue Xue Bao Yi Xue Ban. 42:792-796.

Wang Z, Sun Y. 2010. Targeting p53 for Novel Anticancer Therapy. Transl Oncol. 3:1-12.

Yoo YC, Shin BH, Hong JH, Chee HY, Song KS, Lee KB. 2007. Isolation of fatty acids with anticancer activity from *Protaetia brevitarsis* larva. Arch Pharm Res. 30:361-365.

Yu F, Lu S, Yu F, Shi J, McGuire PM, Wang RJ. 2008. Cytotoxic activity of an octadecenoic acid extract from *Euphorbia kansui* (Euphorbiaceae) on human tumour cell strains. Pharm Pharmacol. 60:253-259.