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Chemical Constituents and Anticancer Activity of *Curcuma zedoaria* Roscoe Essential Oil against Non-Small Cell Lung Carcinoma Cells *in Vitro* and *in Vivo*

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ABSTRACT: In this study, we report that the essential oil obtained from *Curcuma zedoaria* Roscoe, known as zedoary, possesses efficient cytotoxic effects on non-small cell lung carcinoma (NSCLC) cells and causes cell apoptosis. Zedoary essential oil increased the sub-G1 population and the level of annexin-V binding and induced cleavage and activation of caspase-3, -8, and -9 and poly(ADP ribose) polymerase. Decreases in the levels of Bcl-2 and Bcl-xL and an increase in the Bax/Bcl-2 ratio were also observed following zedoary essential oil treatment. Notably, zedoary essential oil led to the release of AIF, endonuclease G, and cytochrome *c* into the cytosol and increased levels of p53 in H1299 cells. Our results indicate that zedoary essential oil slightly inhibited the phosphorylation of ERK1/2 and enhanced the phosphorylation of JNK1/2 and p38. Zedoary essential oil also inhibited AKT/NF-κB signaling pathways in H1299 cells. Moreover, intraperitoneal administration of zedoary essential oil significantly suppressed the growth of H1299 cells *in vivo*. In addition, potential active compounds were detected using gas chromatography and mass spectrometry. 8,9-Dehydro-9-formyl-cycloisolongifolene, 6-ethenyl-4,5,6,7-tetrahydro-3,6-dimethyl-5-isopropenyl-*trans*-benzofuran, eucalyptol, and γ-elemene were found in zedoary essential oil. In summary, our findings provide insight into the molecular mechanisms underlying zedoary essential oil-induced apoptosis in NSCLC cells that are worthy of further study.

KEYWORDS: *Curcuma zedoaria Roscoe, apoptosis, p53, MAPK, AKT/NF-κB*

INTRODUCTION

Curcuma zedoaria (Christm.) Roscoe known as zedoary is a common plant found in tropical countries. Zedoary is used as a spice, natural flavor, and herb in India, Indonesia, and China. In China, it is traditionally for the treatment of flatulence, dyspepsia, menstrual disorders, cough, and fever.[1] Zedoary extract has been shown to have anti-allergic,[2] antitumor,[3] analgesic,[4] and antimicrobial activity.[5]

Lung cancer is the leading cause of cancer deaths in both men and women. Non-small cell lung carcinoma (NSCLC) accounts for approximately 80−85% of all lung cancer patients. NSCLC is an aggressive tumor with poor prognosis and a 5 year survival rate of only 16%.[6,7] The high prevalence and mortality rate make the need for functional diagnosis methods and useful medical drug development urgent.

The most efficient strategy for developing anticancer drugs is to induce apoptosis in cancer cells. Characteristics of apoptosis include membrane blebbing, translocation of phosphatidylserine (PS) of the plasma membrane, chromatin condensation, DNA fragmentation, apoptotic bodies, and the caspase cascade of cell death signaling. Apoptosis is triggered through two major pathways, the extrinsic and intrinsic pathways. The extrinsic pathway can induce activation of the intrinsic pathway via caspase-8 activation. Once activated, caspase-8 cleaves Bid to a truncated Bid (tBid); thus, apoptotic signals initiated by death receptors (DRs) can be linked to the mitochondrially mediated intrinsic pathway. The mitochondrially mediated intrinsic apoptosis is initiated by an alteration in mitochondrial permeability, causing the release of apoptogenic factors, such as AIF, cytochrome *c*, and endonuclease G from the intermembranal space. These apoptogenic factors form complexes with apoptotic protease activating factor-1 (Apaf-1) to form the apoptosome that activates caspase-9 and other effector caspases that cleave different substrates.[8,9]

Pharmacological studies and clinical trials demonstrate that zedoary essential oil exhibits many therapeutic activities, such as antioxidant, anticancer hepatoprotection, and antibacterial action.[1,10,12] The main bioactive components in zedoary essential oil include β-elemene, curdione, neocurdione, curzerene, germacrone, and furanodiene.[11] In our study, the extract of zedoary essential oil was analyzed by gas chromatography and

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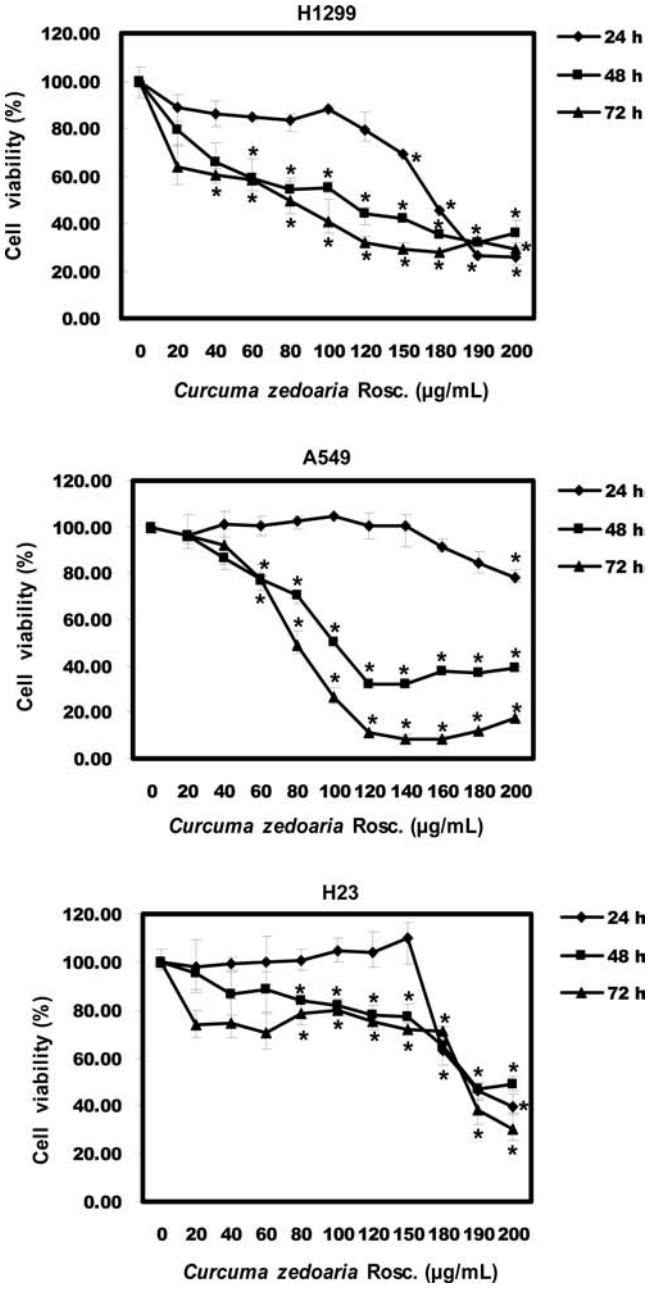

Figure 1. Proliferation inhibitory effect of zedoary essential oil on NSCLC cells. H1299, A549, and H23 cells were treated with various concentrations of zedoary essential oil at 37 °C for 24, 48, and 72 h, respectively. The effect on cell growth was examined by the MTT assay, and the percentage of cell proliferation was calculated by defining the absorption of cells without zedoary essential oil as 100%. This experiment was repeated three times. Bars represent the SEM. Values significantly different from that of the control group are denoted with an asterisk ($P < 0.05$).

mass spectrometry (GC−MS). Via database searching, we identified the components of zedoary essential oil. We also identified the zedoary essential oil antitumor ability by a cell toxicity and animal study. We have shown that the extract of zedoary essential oil has effective antitumor activity.

MATERIALS AND METHODS

Chemicals and Reagents. Annexin-V, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], propidium iodide (PI), and antibodies for caspase-9, caspase-3, Bax, Bcl-2, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), and β-actin were purchased from Sigma (St. Louis, MO). The caspase inhibitor Z-VAD-FMK was obtained from Promega Corp. (Madison, WI). Antibodies for AKT, p-AKT, ERK1/2, p-JNK1/2, JNK1/2, AIF, Endo G, cytochrome *c*, Cox4, and p53 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies for p38, p-ERK1/2, and p-p38 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for rabbit and mouse conjugated with horseradish peroxidase (HRP) were purchased from Chemicon (Temecula, CA). The chemiluminescent HRP substrate was purchased from Millipore Corp. (Billerica, MA).

Plant Material, Isolation, and Fractionation of Zedoary Essential Oil. Zedoary was harvested 5 months after it had been planted in Taichung District Agricultural Research and Extension Station in 2012. The rhizomes of zedoary were powdered and extracted at room temperature; 200 g of the powder was mixed with distilled water and boiled for 3 h. The zedoary essential oil was collected and then dried under sodium sulfate. The yield of zedoary essential oil was 1.6% (milliliters per gram).

GC−MS Analysis of Zedoary Essential Oil. Zedoary essential oil components were conducted on a GC−MS system (Shimadzu GCMS-QP2010) with a capillary column (DB-5, 30 m × 0.250 mm, J&W Scientific, Folsom, CA) using helium as a carrier gas. The flow rate of helium was 1.0 mL/min. The injection temperature was set at 60 °C, increased at a rate of 5 °C/min to 120 °C, held for 13 min, increased at a rate of 25 °C/min to 145 °C, held for 20 min, and finally increased at a rate of 30 °C/min to 280 °C. The MS instrument was set in electron-impact (EI) mode; the ionization energy was 70 eV and the scan rate 0.34 s/scan.

Cell Culture. H1299, A549, and H23 cells were purchased from American Type Culture Collection (Manassas, VA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) in a humidified incubator at 37 °C with 5% CO_2.

Cytotoxicity Assay. The effect of zedoary essential oil on cell cytotoxicity was determined by the MTT assay.[13] Cells were dispersed evenly in culture medium and seeded in a 96-well plate until a density of 1×10^4 cells/well was reached. After the cells had been held at 37 °C and 5% CO_2 for 24 h, the culture medium was replaced with different concentrations of zedoary essential oil. Positive controls were treated with 1% DMSO for the same amount of time. After treatment with zedoary essential oil at various concentrations and incubation for 24, 48, and 72 h, a 40 μL MTT solution was add to each well, and the mixtures were incubated at 37 °C and 5% CO_2 for 1 h. The supernatant was aspirated, and MTT-formazan crystals were dissolved with 110 μL of DMSO. Finally, the absorbance was determined and recorded by a microplate reader at a wavelength of 570 nm.

Analysis for Cell Cycle Distribution. The cell cycle state was determined by using PI staining as reported previously.[14] H1299 cells (5×10^5) were cultured in 6 cm cell culture dishes and treated with 110 μg/mL zedoary essential oil for 12, 24, 48, and 72 h. Then cells from each dish were harvested individually by centrifugation, and the cell cycle distribution was measured by a flow cytometeric assay. Briefly, isolated cells were fixed gently by 2 mL of 100% ethanol at −20 °C overnight and then resuspended in PBS containing 50 μg/mL PI, 0.1 mg/mL RNase A, and 0.1% Triton X-100. The mixture was allowed to stand on ice for 30 min and analyzed via FAC-Scan cytometry (BD Biosciences, San Jose, CA).

Detection of Apoptosis. A cell undergoing apoptosis was detected by PI and annexin-V staining. H1299 cells were treated with 110 μg/mL zedoary essential oil for 24, 48, and 72 h; 5×10^5 H1299 cells were harvested, washed with PBS, and resuspended in 500 μL of binding buffer. PI and annexin-V FITC (5 μL each) were added, and the mixtures were incubated in a dark room for 30 min and analyzed via FAC-Scan cytometry (BD Biosciences).

DNA Fragmentation Assay. Fresh H1299 cell DNA was extracted from 2×10^6 cultured cells using a Sigma genElute Mammalian Genomic DNA Purification Kit following the manufacturer's instructions. The DNA fragmentation is screened by 1% agarose gel

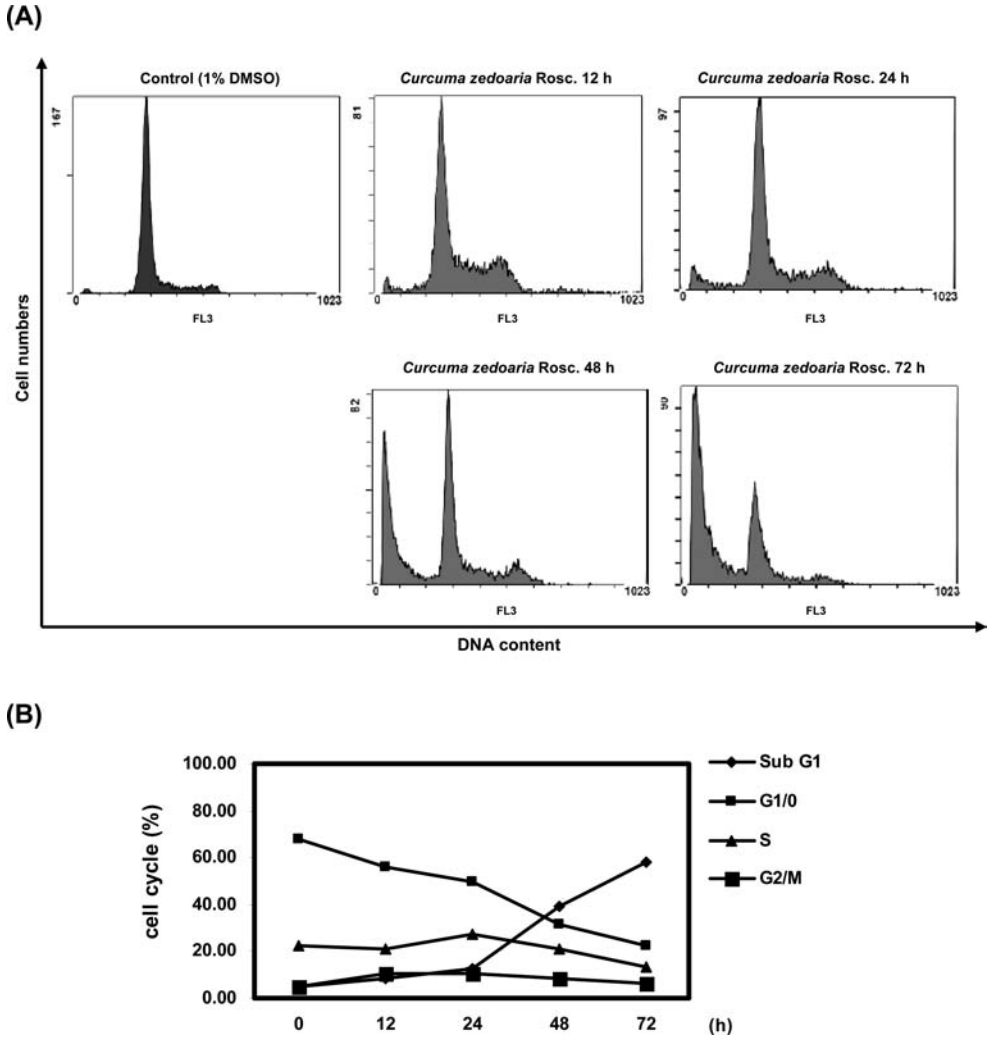

Figure 2. Effect of zedoary essential oil on cell cycle progression in H1299 cells. (A) H1299 cells were treated with 110 μg/mL zedoary essential oil for 12, 24, 48, and 72 h and analyzed for PI-stained DNA content by flow cytometry. (B) The indicated percentages are the means of three independent experiments, each in duplicate. The number of sub-G1 phase H1299 cells increased with time.

electrophoresis. The DNA ladder was visualized with ethidium bromide staining.

Western Blotting Analysis. Cells (1×10^6 per dish) were placed in 10 cm cell culture dishs and treated with various agents as indicated in the figure legends. After being treated with zedoary essential oil, cells were harvested and lysed with ice-cold lysis buffer. Equivalent amounts of proteins in each treatment were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, blocked with 5% (w/v) nonfat milk, and immunoblotted as described previously.[15]

Production of Reactive Oxygen Species (ROS). H1299 cells (2×10^5 per well) placed in 12-well plates were treated with 110 μg/mL zedoary essential oil for 0, 0.5, 1, 2, and 4 h to determine the level of ROS. H1299 cells were harvested and suspended in 500 μL of 10 μM dichloro-dihydro-fluorescein diacetate (DCFH-DA) for ROS measurement. Finally, all samples were incubated at 37 °C for 30 min and analyzed via FAC-Scan cytometry (BD Biosciences).

Cytochrome *c*, AIF, and Endo G Release. The cytosolic and mitochondrial fractions were prepared by resuspending H1299 cells in ice-cold buffer A [1 mM EDTA, 20 mM HEPES, 250 mM sucrose, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 1 mM EGTA, 17 mg/mL PMSF, 2 mg/mL leupeptin, and 8 mg/mL aprotinin (pH 7.4)]. H1299 cells were passed 10 times through a 27 gauge needle. Nuclei and lysed cells were pelleted by centrifugation at 750*g* for 10 min, and the supernatant was then centrifuged at 100000*g* for 15 min. The pellet, representing the mitochondrial fraction, was resuspended in ice-cold buffer A. The supernatant was centrifuged again at 100000*g* for 1 h, and the supernatant from the final centrifugation represents the cytosolic fraction.

***In Vivo* Studies.** *In vivo* tumorigenesis inhibition was assessed by subcutaneous injection of 1×10^5 H1299 cells into the back side of 4–7-week-old BALB/c (nu/nu) nude mice, according to standard protocols of the Institutional Animal Care and Use Committee (IACUC). After 2 weeks, tumor volumes of >100 mm^3 were selected for the next-step inhibition study. Four animals were used for each set of different concentrated zedoary essential oil solutions. Zedoary essential oil was dissolved in a 10% ethanol/PBS solution at different concentrations. The zedoary essential oil solution was injected intraperitoneally (ip) into mice according to standard protocols. Tumor inhibition was assessed by measuring tumor size twice a week, and tumor volume was calculated by the standard formula as (tumor width)^2 × (tumor length)/2.

Statistical Analysis. An independent Student's *t* test was used to compare the continuous variables between two groups, a probability of $P < 0.05$ being considered significant.

RESULTS

Zedoary Essential Oil Inhibited NSCLC Cell Proliferation. The antitumor effect of zedoary essential oil has been successfully reported for a wide range of cancers.[1,16] To evaluate the antitumor effect of zedoary essential oil against

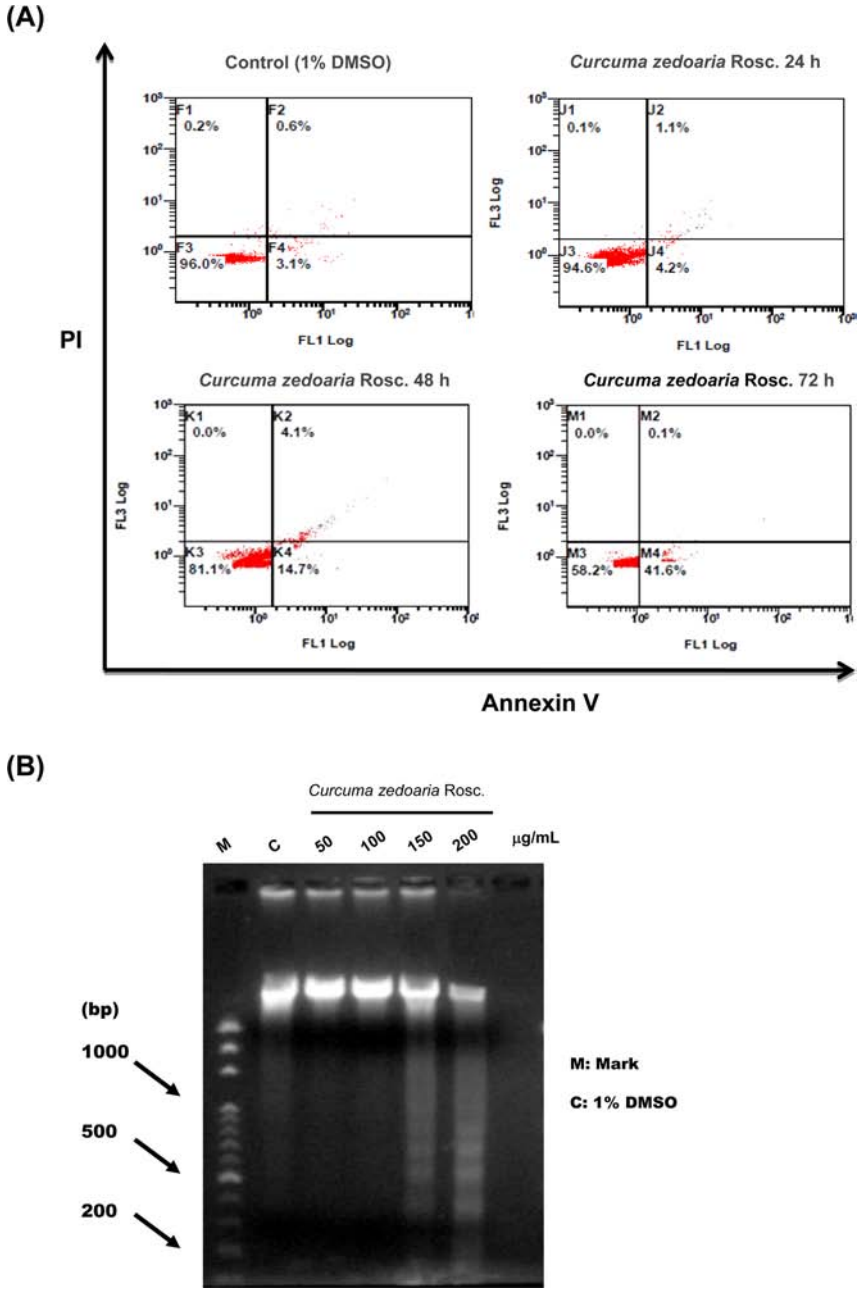

Figure 3. Zedoary essential oil induces apoptosis in H1299 cells. (A) H1299 cells were treated with 110 μg/mL zedoary essential oil for 24, 48, and 72 h. Cell apoptosis percentages were determined by flow cytometry with annexin-V/PI staining. (B) H1299 cells were treated with various concentrations of zedoary essential oil at 37 °C for 48 h. Zedoary essential oil treatment resulted in typical DNA fragmentation as indicated by DNA laddering. M, 100 bp DNA marker. D, 1% DMSO treatment controls.

NSCLC cells, the H1299, A549, and H23 cells were treated with various concentrations of zedoary essential oil (Figure 1) for 24, 48, and 72 h, respectively, and examined for cell viability by the MTT assay. Zedoary essential oil caused a time- and concentration-dependent inhibition of NSCLC cell proliferation. Zedoary essential oil had IC_{50} values ranging from 80 to 170 μg/mL in H1299 cells, 80 to 250 μg/mL in A549 cells, and 180 to 185 μg/mL in A549 cells. Compared to its effects on other NSCLC cells, zedoary essential oil demonstrated the most efficient cytotoxic effects on H1299 cells (Figure 1). These results show that zedoary essential oil exhibits a high potency in inhibiting cell proliferation in NSCLC cells.

Zedoary Essential Oil Induced Cell Death in H1299 Cells. To determine whether zedoary essential oil altered cell cycle progression or induced apoptosis, we performed flow cytometry by using PI staining. H1299 cells were treated with 110 μg/mL zedoary essential oil (the half-maximal inhibitory concentration as assessed by the MTT assay) and then harvested at 12, 24, 48, and 72 h. As shown in Figure 2A, zedoary essential oil induced cell death (sub-G1 phase) in a time-dependent manner. The proportion of sub-G1 phase cells was increased from 11.4 to 53.3% in 72 h (Figure 2B).

Zedoary Essential Oil Induced Apoptosis in H1299 Cells. The increased number of sub-G1 phase cells in flow cytometry is suggestive of apoptosis. To further determine whether zedoary essential oil induced apoptosis in H1299 cells, we performed flow cytometry using PI- and FITC-conjugated annexin-V staining. As shown in Figure 3A, zedoary essential oil

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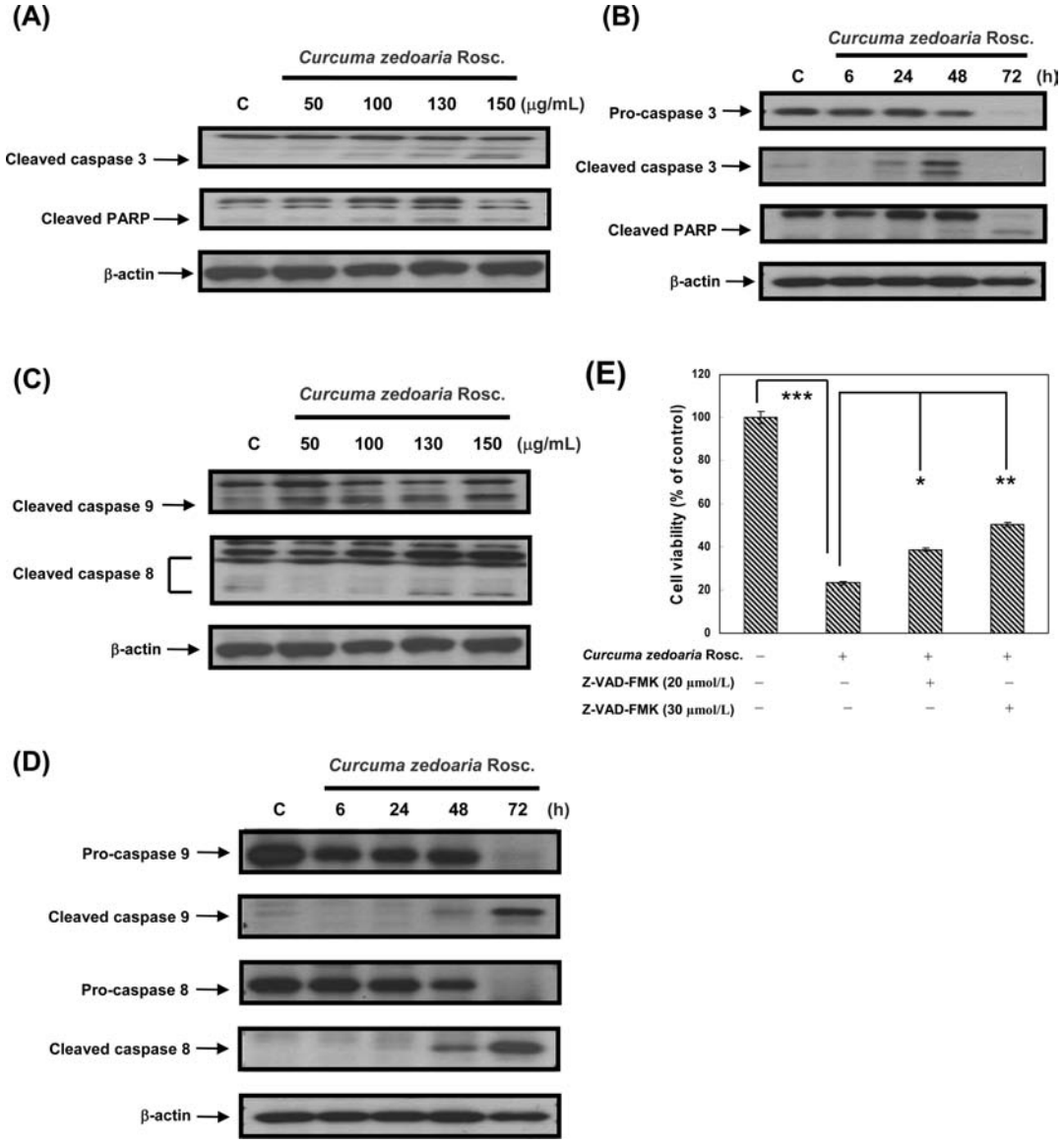

Figure 4. Effect of zedoary essential oil on caspase activity in H1299 cells. (A) H1299 cells were treated with various concentrations of zedoary essential oil for 48 h. Cells were then harvested and lysed for the detection of the expression of cleaved caspase-3, cleaved PARP, and β-actin. (B) H1299 cells were treated with 110 μg/mL zedoary essential oil for 6, 24, 48, and 72 h. Cells were then harvested and lysed for the detection of the expression of pro-caspase-3, cleaved caspase-3, cleaved PARP, and β-actin. (C) H1299 cells were treated with various concentrations of zedoary essential oil for 48 h. Cells were then harvested and lysed for the detection of the expression of cleaved caspase-9, cleaved caspase-8, and β-actin. (D) H1299 cells were treated with 110 μg/mL zedoary essential oil for 6, 24, 48, and 72 h. Cells were then harvested and lysed for the detection of the expression of pro-caspase-9, cleaved caspase-9, pro-caspase-8, cleaved caspase-8, and β-actin. Western blot data are representative of those obtained from at least three separate experiments. (E) Z-VAD-FMK or the vehicle (DMSO) was added to the medium 1 h before the 110 μg/mL zedoary essential oil treatment. After the 72 h incubation, H1299 cell viability was determined using the MTT assay. This experiment was repeated three times. Bars represent the SEM. Values significantly different from that of the control group are denoted with one ($P < 0.05$), two ($P < 0.01$), or three asterisks ($P < 0.001$).

induced cell apoptosis in a time-dependent manner. The highest value was observed after exposure for 72 h, being for 41.6% apoptosis. In comparison with the control, zedoary essential oil treatment resulted in DNA fragmentations, a hallmark of cell apoptosis, in a time-dependent manner (Figure 3B).

Zedoary Essential Oil Induced Cleavage and Activation of Caspase in H1299 Cells. Caspase activation plays an important role in the initiation and success of apoptosis.[17] To monitor the enzymatic activity of caspases during zedoary essential oil-induced apoptosis, we conducted Western blotting. Our result showed that cleavage patterns of caspase-3 were observed in H1299 cells in a concentration-dependent (Figure 4A) and time-dependent (Figure 4B) manner. Cleavage of PARP was also observed in a concentration-dependent (Figure 4A) and time-dependent (Figure 4B) manner. We next determined levels of other caspases associated with apoptosis. Cleavage of caspase-8 and caspase-9 was detected after zedoary essential oil treatment in a concentration-dependent (Figure 4C) and time-dependent (Figure 4D) manner. Consistent with these findings, the general caspase inhibitor Z-VAD-FMK significantly inhibited the zedoary essential oil-induced apoptosis in a dose-dependent manner (Figure 4E).

Zedoary Essential Oil Affected the Production of ROS in H1299 Cells. We next tested whether zedoary essential

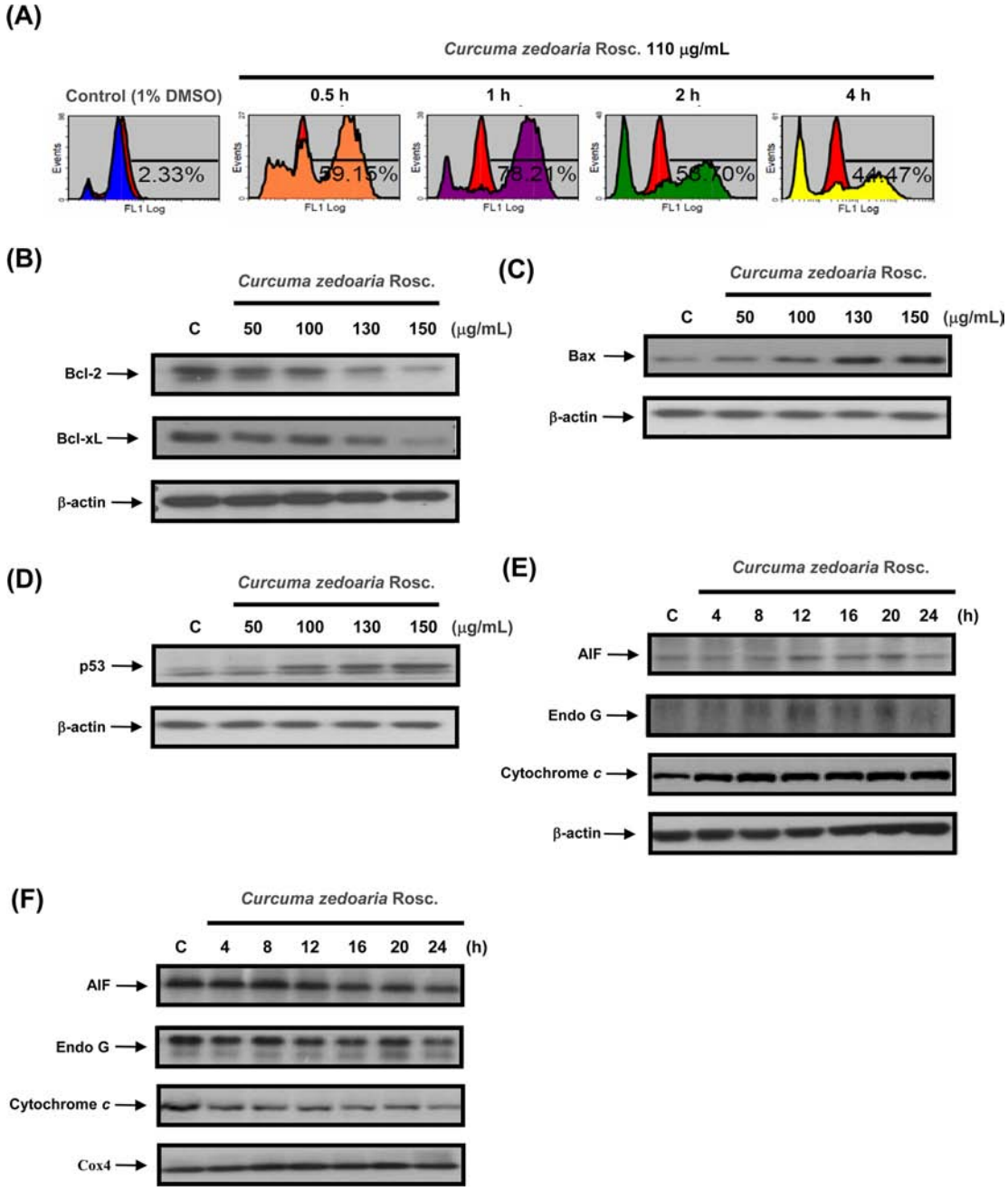

Figure 5. Effect of zedoary essential oil on apoptosis-related proteins in H1299 cells. (A) Cells were treated with 110 μg/mL zedoary essential oil for 0, 0.5, 1, 2, and 4 h for the production of ROS. All samples were analyzed by the flow cytometric assay as described in Materials and Methods. H1299 cells were treated with various concentrations of zedoary essential oil for 48 h. Cells were then harvested and lysed for the detection of expression of (B) Bcl-2, Bcl-xL, and β-actin, (C) Bax and β-actin, and (D) p53 and β-actin. H1299 cells were incubated with 110 μg/mL zedoary essential oil for the indicated periods of time. Levels of AIF, Endo G, and cytochrome *c* in the (E) cytosolic and (F) mitochondrial fractions were analyzed by immunoblotting. Western blot data are representative of those obtained from at least three separate experiments.

oil-induced apoptosis is accompanied by the production of ROS. The results depicted in Figure 5A indicated that zedoary essential oil promoted the production of ROS in a time-dependent manner.

Effect of Zedoary Essential Oil on the Expression of the Bcl-2 Family in H1299 Cells. One of the major mechanisms underlying the ultimate fate of cells in the apoptotic process is the imbalance of expression of anti- and pro-apoptotic proteins.[18] We examined the expression of anti-apoptotic proteins, Bcl-xL and Bcl-2, after treatment with various concentrations of zedoary essential oil. The exposure of H1299 cells to zedoary essential oil resulted in the down-regulation of Bcl-xL and Bcl-2 in a concentration-dependent manner (Figure 5B). However, data in Figure 5C show that zedoary essential oil increased the level of Bax in a concentration-dependent manner. After treatment with zedoary essential oil, optical densitometric analysis revealed an increase in the ratio of Bax to Bcl-2 (data not shown). Moreover, as shown in Figure 5D, zedoary essential oil caused an increase in the level of p53 protein.

Zedoary Essential Oil-Induced Cytochrome *c*, Endo G, and AIF Release. The release of cytochrome *c*, Endo G, and

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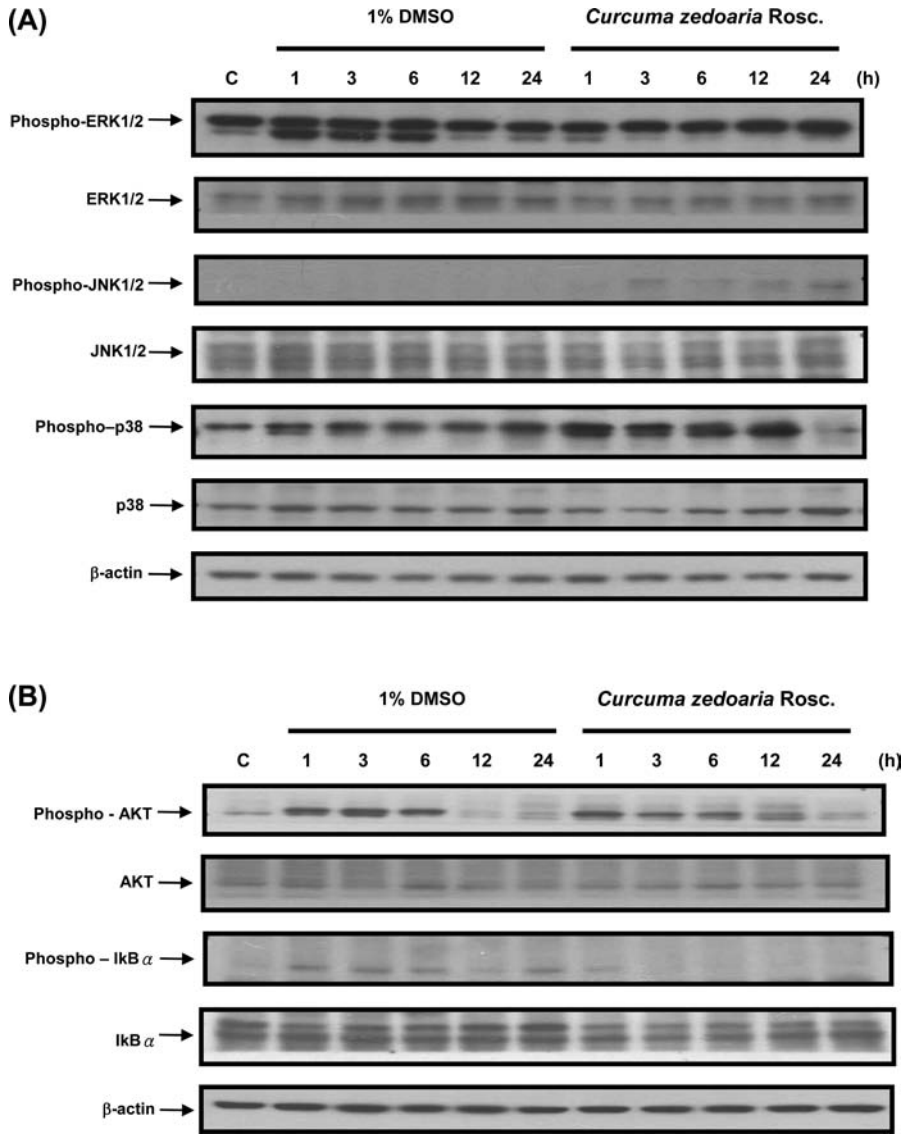

Figure 6. Effect of zedoary essential oil on the MAPK and AKT/NF-κB signaling pathways. H1299 cells were treated with a vehicle (DMSO) or zedoary essential oil (110 μg/mL) for the indicated periods of time. Cells were then harvested and lysed for the detection of the expression of (A) ERK1/2, phospho-ERK1/2, p38, phospho-p38, JNK, phospho-JNK, and β-actin and (B) AKT, phospho-AKT, IκBα, phospho-IκBα, and β-actin. Western blot data are representative of those obtained from at least three separate experiments.

AIF from the mitochondria is the main gate in turning on apoptosis. We next tested whether zedoary essential oil induced the release of AIF, Endo G, and cytochrome *c*. Panels E and F of Figure 5 show that zedoary essential oil led to the release of cytochrome *c*, Endo G, and AIF into the cytosol in a concentration-dependent manner.

Effects of Zedoary Essential Oil on ERK1/2, p38, and JNK1/2 Activation in H1299 Cells. To identify whether zedoary essential oil affects mitogen-activated protein kinase (MAPK) activation, H1299 cells were exposed to zedoary essential oil for various incubation times. Using antibodies specific for phosphorylated ERK1/2, JNK1/2, and p38, Figure 6A indicates that zedoary essential oil slightly inhibited the phosphorylation of ERK1/2 but enhanced the phosphorylation of p38 and JNK1/2 (Figure 6A).

Zedoary Essential Oil Inhibited AKT/NF-kB Signaling Pathways in H1299 Cells. A prominent mechanism linking AKT/NF-κB signaling to NSCLC cancer progression is the abrogation of apoptosis.[19,20] We next identified the effect of zedoary essential oil on the phosphorylation of AKT and IκBα. Figure 6B shows that zedoary essential oil inhibited phosphorylation of AKT and IκBα in a time-dependent manner.

Antitumor Activity of Zedoary Essential Oil in NSCLC Xenografts. To evaluate the *in vivo* antitumor activity of zedoary essential oil, BALB/c (nu/nu) nude mice were implanted subcutaneously with H1299 cells. The control group was treated with a 10% ethanol/PBS mixture, and the treated groups were treated ip five times a week with zedoary essential oil (2.4, 12, 60, and 240 mg/kg). Our results show that ip administration of zedoary essential oil induced a dose-dependent inhibition of H1299 tumor volume (Figure 7A) and a reduction in tumor weight (Figure 7B). For the duration of the experiment, animals did not lose weight (Figure 7B) and no pathologic signs were seen.

Profile of Constituents of Zedoary Essential Oil Extracts. To analyze the involved compositions of the zedoary essential oil, GC−MS was performed. The GC−MS profile of the zedoary essential oil is shown in Figure 8A. Two major

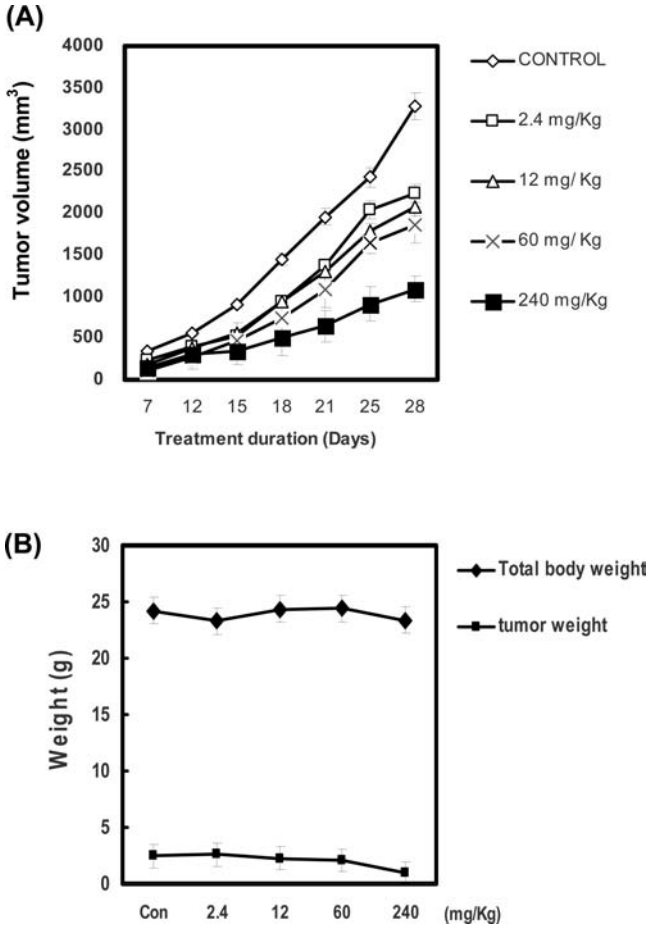

Figure 7. Effect of zedoary essential oil on antitumor activity. H1299 cells were used to establish xenografts in male BALB/c nude mice. Mice (six per group) were given the control or zedoary essential oil (2.4, 12, 60, and 240 mg/kg) by ip injection five times per week: (A) tumor volume and (B) tumor weight (body weight in grams).

compounds identified in zedoary essential oil were 8,9-dehydro-9-formyl-cycloisolongifolene (60%) and 6-ethenyl-4,5,6,7-tetrahydro-3,6-dimethyl-5-isopropenyl-*trans*-benzofuran (12%) (structures shown in panels B and C of Figure 8, respectively). Other compounds such as eucalyptol and γ-elemene were identified as minor compounds.

DISCUSSION

Recent research suggests that zedoary essential possesses anticancer properties.[21,22] Zedoary essential oil could induce apoptosis in hepatic stellate cells.[23] The hexane extract of zedoary essential oil has also been reported to have significant cytotoxicity with respect to HepG2,[24] SNU-1, SiHa, and HL-60 cells.[12] However, its function with respect to NSCLC cells has not been examined. In our study, zedoary essential oil was used to test its cytotoxicity on NSCLC cells. From the MTT assay, the tumor survival rate decreased with an increase in the size of the zedoary essential oil dosage and exposure time. The PI and annexin-V staining result showed that cell apoptosis had occurred and the cell death number increased with zedoary essential oil exposure time. DNA fragmentation confirmed apoptosis.

Cell death plays an important role in the efficacy of chemotherapy. Apoptosis is regulated by programmed cellular signaling pathways that control normal cell homeostasis and are thought to be the principal mechanism by which anticancer drugs kill cancer cells. Importantly, dysregulation of apoptosis is a hallmark of cancer, with both the gain of anti-apoptotic mechanisms and the loss of pro-apoptotic signals contributing to tumorigenesis. The induction of apoptosis in many cancer cells can be divided into the extrinsic and intrinsic pathways. The extrinsic pathway is initiated by the interaction between specific ligands and the death receptor, and their downstream molecules are forms of caspase-8. The intrinsic pathway is initiated by the mitochondrially mediated pathway, and their downstream molecules are forms of caspase-9.[25] However, cross-talk between the extrinsic and intrinsic pathways also occurs.[26] In this study, we observed that zedoary essential oil induced the activition of caspase-8, -9, and -3 in H1299 cells. Interestingly, zedoary essential oil changed the extrinsic and intrinsic pathway-associated proteins, which subsequently promoted caspase-8 and caspase-9 activation and then activated the downstream effector caspase-3 in H1299 cells.

A recent study showed that AKT is constitutively active in >90% of NSCLC cells, contributes to chemotherapeutic resistance, and leads these cancer cells to evade apoptosis.[19] The phosphorylation of Ser473 has been used as an indicator of AKT activity and has been found to correlate with poor prognosis.[20] In our study, zedoary essential oil inhibited Ser473 phosphorylation of AKT, and the inhibition was an earlier event that was followed by a weakened phosphorylation of other downstream members in the AKT signaling pathway. Activated AKT can phosphorylate pro-apoptotic Bcl-2 family member Bad. The Ser136 phosphorylation of Bad promotes its interaction with 14-3-3 proteins in the cytosol, blocking its interaction with Bcl-X_L at the mitochondrial level.[27,28] Our study indicates that zedoary essential oil-mediated inactivation of AKT is associated with weakened Ser136 phosphorylation of Bad, which thus allows Bad to interact with Bax to aggregate on the mitochondrial membrane, resulting in the release of cytochrome *c* to the cytosol. Once cytochrome *c* is released, it binds to APAF1 and leads to the assembly of the apoptosome, which triggers the pathway for the caspase cascade in NSCLC cells. Our study revealed that the pan broad spectrum caspase inhibitor Z-VAD-FMK can only partially alleviate apoptosis. We therefore concluded that zedoary essential oil might induce apoptosis through both caspase-dependent and -independent mechanisms.

Excessive production of mitochondrially derived ROS is an important regulator of apoptosis. Our study showed that zedoary essential oil induced NSCLC cell apoptosis through overproduction of intracellular ROS. ROS have been shown to induce apoptosis by activation of the MAPK pathways.[29] Three classic MAPKs (ERK1/2, p38, and JNK1/2) have been identified in mammalian cells. The involvement of MAPK pathways in the zedoary essential oil-induced apoptosis of NSCLC cells has not been reported. Herein, we attempted to verify whether the MAPK pathways were involved in the process. We found that zedoary essential oil induced JNK1/2 and p38 phosphorylation. Continuous phosphorylation of JNK1/2 plays a crucial role in apoptosis.[30] On the basis of our findings, we hypothesized that zedoary essential oil induces intracellular ROS generation and induces JNK1/2 and p38 phosphorylation, which contribute to the apoptosis of NSCLC cells. However, future studies are necessary to fully understand the mechanism for zedoary essential oil-induced JNK1/2 and p38 phosphorylation.

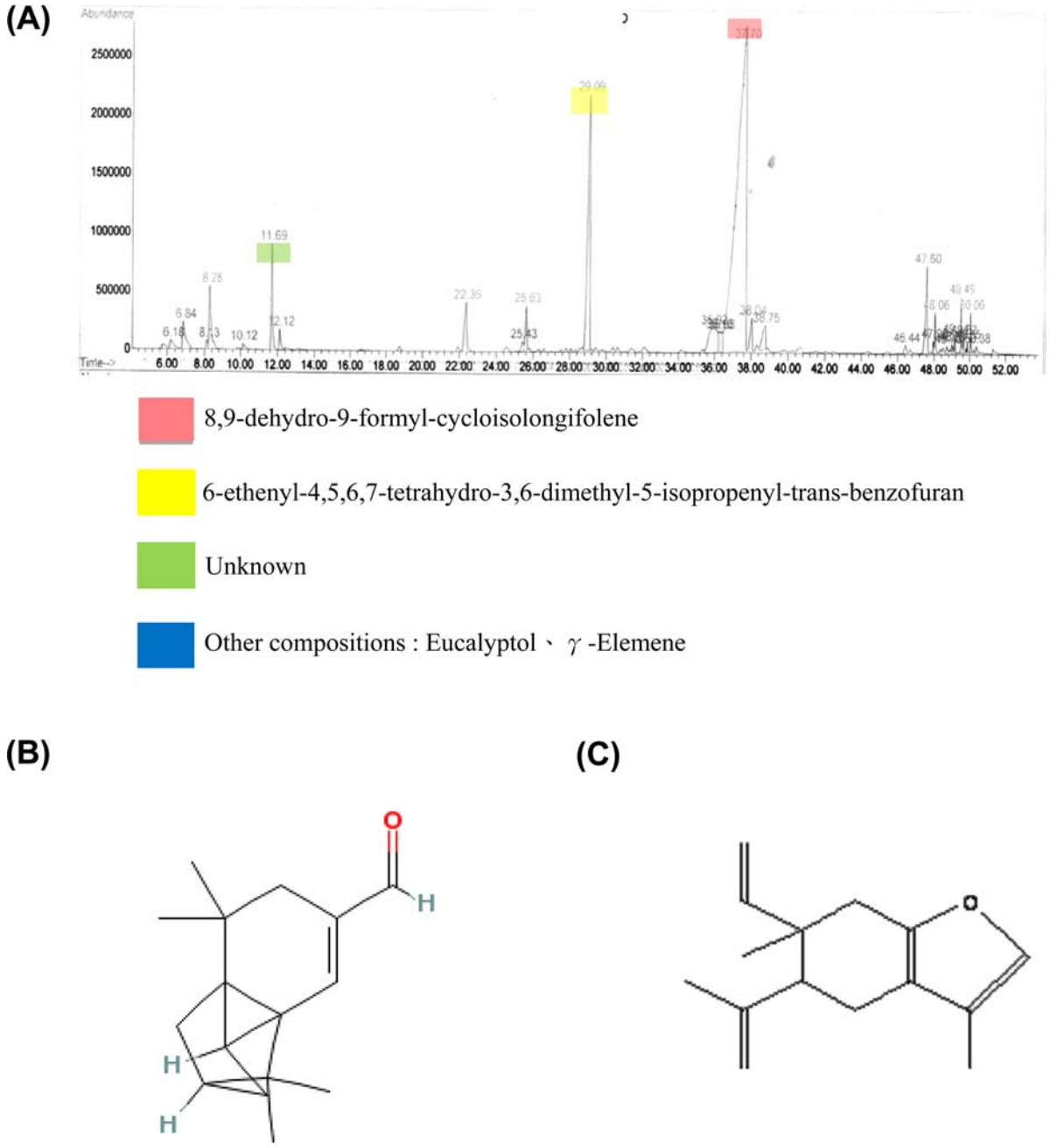

Figure 8. Profile of constituents in zedoary essential oil extracts. (A) GC−MS profile of the zedoary essential oil. (B) Chemical structure of 8,9-dehydro-9-formyl-cycloisolongifolene. (C) Chemical structure of 6-ethenyl-4,5,6,7-tetrahydro-3,6-dimethyl-5-isopropenyl-*trans*-benzofuran.

The *in vivo* tumor inhibition study described here has shown that after being injected for 3 weeks the essential oil as an edible drug can functionally inhibit tumor proliferation. The tumor inhibition rate in mice treated with large doses could reach 40%. This result emphasizes that the zedoary essential oil has great potential to become a new antitumor candidate.

The main components of zedoary essential oil, including neocurdione, curdione, germacrone, curzerene, furanodiene, and γ-elemene, have been reported. However, in our study, we found that the content of an interesting sesquiterpene, 8,9-dehydro-9-formyl-cycloisolongifolene, reached ∼60% in the essential oil. The effective tumor inhibition ability of our results indicates that the essential oil might contain potential compounds for novel antitumor drug discovery. Further study to identify the active compound in this essential oil is needed.

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Notes

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■ ABBREVIATIONS USED

DISC, death-inducing signaling complex; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DRs, death receptors; GC−MS, gas chromatography−mass spectrometry; LPS, lipopolysaccharides; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSCLC, non-small cell lung carcinoma; PARP, poly(ADP ribose) polymerase; PI, propidium iodide; PS, phosphatidylserine; SEM, standard error of the mean.

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