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Ganoderma lucidum Extract Induces G1 Cell Cycle Arrest, and Apoptosis in Human Breast Cancer Cells

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Abstract: *Ganoderma lucidum* (Fr.) Karst is a traditional Chinese herb that has been widely used for centuries to treat various diseases including cancer. Herein, an ethanol-soluble and acidic component (ESAC), which mainly contains triterpenes, was prepared from *G. lucidum* and its anti-tumor effects *in vitro* were tested on human breast cancer cells. Our results showed that ESAC reduced the cell viability of MCF-7 and MDA-MB-231 cells in a concentration-dependent manner with IC_{50} of about 100 μg/mL and 60 μg/mL, respectively. DNA damage was detected by Comet assay and the increased expression of γ-H2AX after ESAC treatment was determined in MCF-7 cells. Moreover, ESAC effectively mediated G1 cell cycle arrest in both concentration- and time-dependent manners and induced apoptosis as determined by Hoechst staining, DNA fragment assay and Western blot analysis in MCF-7 cells. In conclusion, ESAC exerts anti-proliferation effects by inducing DNA damage, G1 cell cycle arrest and apoptosis in human breast cancer cells.

Keywords: *Ganoderma lucidum*; Proliferation; DNA Damage; G1 Cell Cycle Arrest; Apoptosis.

Introduction

Currently, much effort has been made to explore new anti-tumor lead compounds from Traditional Chinese Medicine (TCM). TCM has been playing a key role in the primary health care of China for centuries and is now regarded as an important source for modern drug discovery (Jiang *et al.*, 2003; Zeng *et al.*, 2011). Recently, the pharmacological effects

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of many TCM on tumor cells have been extensively studied and some underlying mechanisms have been revealed, such as inhibition of tumor invasion and induction of cell apoptosis *etc*. (Li-Weber, 2010; Xu *et al.*, 2011).

Ganoderma lucidum (Fr.) Karst, a traditional Chinese herb, has been prescribed to treat various human diseases including hyperglycemia, hypertension, chronic hepatitis, allergy, arthritis, inflammation, and cancer for centuries in East Asian countries (Sliva, 2003). Previous studies have demonstrated that *G. lucidum* extract (GLT) suppressed the motility of highly invasive cancers by inhibiting the constitutively active transcription factors AP-1 and NF-κB (Hu *et al.*, 2002; Sliva *et al.*, 2002). It also suppressed angiogenesis through inhibiting the secretion of VEGF and TGF-β1 in prostate cancer cells (Stanley *et al.*, 2005). Furthermore, combination of GLT with green tea extract has demonstrated a synergistic effect on the invasive behavior of MDA-MB-231 human breast cancer cells (Thyagarajan *et al.*, 2007). Recently, we discovered that two species of *Ganoderma* extracts and one pure compound separated from *G. lucidum* could inhibit tumor cell proliferation by inducing apoptosis and cell cycle arrest (Liu *et al.*, 2009; Wu *et al.*, 2011).

Herein, an ethanol-soluble and acidic component (ESAC) from *G. lucidum* was prepared and its anti-proliferation effect was determined and the potential mechanisms were explored in human breast cancer cells.

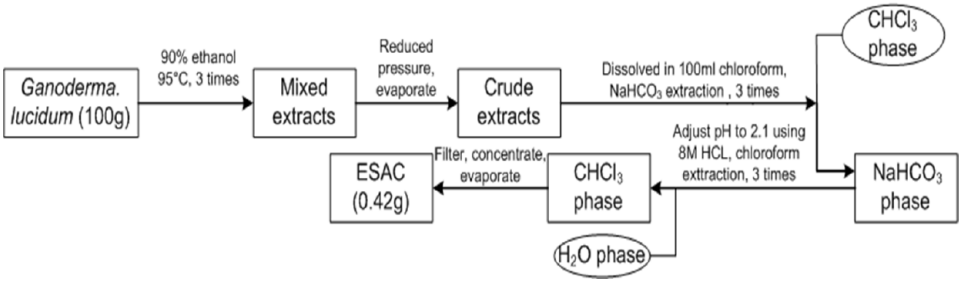
Materials and Methods

Preparation of ESAC from G. lucidum

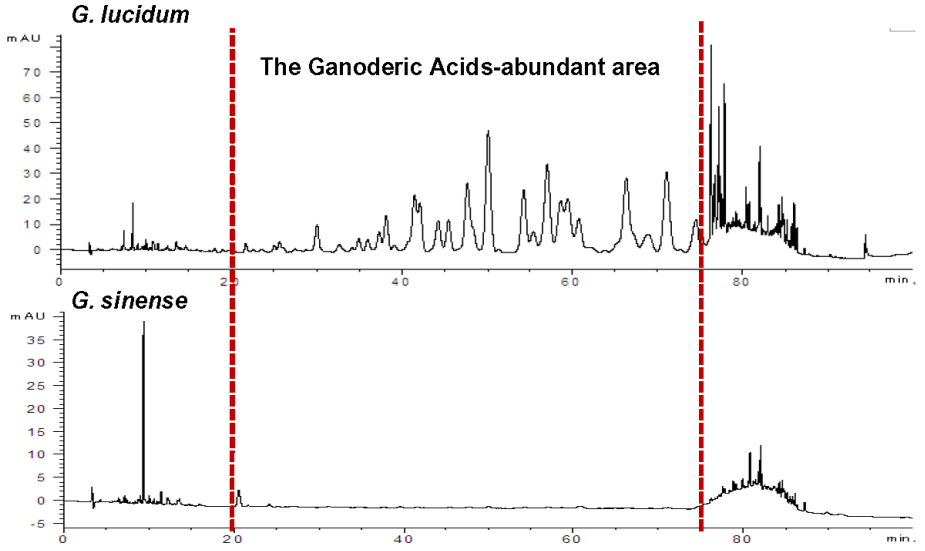
G. lucidum was obtained from Anhui province, China. The voucher specimen of *G. lucidum* was deposited at the Institute of Chinese Medical Sciences, University of Macau, Macao, China. Dried fruiting bodies of *G. lucidum* (100 g) were broken into pieces and ground into powder using a grinder. As shown in Fig. 1A, the powders were extracted with ethanol (90%, 1 L) for 3 h at 95°C. The ethanol extracts were dissolved in chloroform for suspending and extracted with a saturated $NaHCO_3$ solution. The $NaHCO_3$ phase was then collected and adjusted to pH 2.1 with cold HCl solution (8 M). The resulting precipitates were extracted by chloroform. Finally, the ESAC of *G. lucidum* was obtained with a weight of 0.42 g and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL, and stored at −20°C.

Chemical Analysis of Ganoderma Extracts

The samples analysis was performed on an Agilent 1200 HPLC system, which equipped with a vacuum degasser, a binary pump, an autosampler, a diode array detector (DAD). An Agilent ZORBAX Aq C_{18} column (5 μm, 4.6 mm × 250 mm) with a ZORBAX Aq C_{18} column guard column (5 μm, 4.6 mm × 12.5 mm) was used for sample separation. The mobile phase consisted of 0.3% acetic acid solution (A) and acetonitrile (B), which were applied in the gradient elution as follows: 0–5 min, 5–25% B; 5–70 min, 25–30% B; 70–80 min, 30–100% B; 80–100 min, 100% B. The flow rate was set at 1 mL/min.



(A)



(B)

Figure 1. The flow chart of extraction of ESAC and chemical characteristics of ESAC. (A) Grinding fruiting bodies of *G. lucidum* (100 g) were extracted with ethanol, suspended in chloroform, re-extracted with $NaHCO_3$, cold HCL solution and chloroform. (B) The chemical assay of *G. lucidum* (upper) and *G. sinense* (lower). The ganoderic acid abundant area was marked.

The column temperature was maintained at 30°C, and DAD was monitored at 260 nm. An aliquot of 15 μL of sample solution was injected for HPLC analysis.

Cell Culture

The human breast cancer cells MCF-7 and MDA-MB-231 were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO BRL, Carlsbad, CA), 100 U/mL penicillin and 100 mg/mL streptomycin, and grown in a 37°C incubator with 5% CO_2.

Observation of Morphologic Changes

MCF-7 cells were seeded into 6-well plates and treated with indicated concentration of ESAC for 48 h. The cellular morphology was observed with AxioCam HRC CCD camera (Carl Zeiss).

MTT Assay

The effect of ESAC on MCF-7 and MDA-MB-231 cell proliferation was measured using MTT assay, which is based on the ability of live cells to cleave the tetrazolium ring to a molecule that absorbs at 570 nm. Exponentially growing MCF-7 and MDA-MB-231 cells were planted into 96-well plates and after adhesion the cells were treated with ESAC. The cell viability was determined after 48 h incubation by adding 20 μL MTT (5 mg/mL, Molecular Probes, Eugene, OR). Then the MTT-containing medium was aspirated slightly after 4 h and 100 μL DMSO was added to solubilize the formazan followed by shaking 10 min in the dark. The absorbance at 570 nm was recorded using a Multilabel counter (Perkin Elmer, 1420 Multilabel Counter Victor3, Wellesley, MA, USA).

Flow Cytometry Analysis of DNA Content

MCF-7 cells seeded into 6-well plates were treated with ESAC for indicated time. Cells were harvested and fixed in 70% ethanol and then stored at 4°C overnight. Cells were stained in PBS containing 5 μg/mL RNase and 20 μg/mL PI in the dark at room temperature for 30 min and analyzed using a flow cytometry (Becton Dickinson FACS Canto™, Franklin Lakes, NJ). At least 10,000 events were counted for each sample. The DNA content in the G0/G1, S, and G2/M phases was analyzed using ModFit 161 LT version 3.0 software (Verity Software House, Topsham, USA).

Comet Assay

DNA damage was evaluated using the Comet assay as described (Lu *et al.*, 2011) with minor modifications. Briefly, treated cells were harvested after exposed to 100 μg/mL ESAC for 24 h, mixed with 0.75% low melting point agarose, and layered onto microscope slides pre-coated with 0.75% normal melting point agarose. Then, the slides were submerged in pre-chilled lysis solution (1% Triton X-100, 2.5 M NaCl, 1% laurosylsarcosinate and 10 mM EDTA, pH 10.5) for 1 h at 4°C. After soaking with pre-chilled unwinding and electrophoresis buffer (0.3 M NaOH and 1 mM EDTA, pH 13) for 20 min, the slides were subjected to electrophoresis for 20 min at 0.5 V/cm (20 mA), then stained with PI. Individual cells were viewed using an Olympus IX81 fluorescence microscope (Olympus, Japan).

Immunocytochemical Labeling

MCF-7 cells were fixed with 4% formaldehyde (Sigma, Germany) in PBS at 37°C for 30 min, washed with PBS, and then permeabilized with 0.5% Triton X-100 in PBS for

20 min at room temperature. Cells were washed in a blocking solution containing of 5% BSA and 0.2% Triton X-100 and stored in the blocking solution at 4°C until labeling. For labeling, fixed cells were incubated for 2 h at 37°C with the specific antibody against to γ-H2AX in the blocking solution. Following three washes in the blocking solution, cells were incubated with rhodamine-conjugated goat anti-rabbit IgG in the blocking solution at 37°C for 1 h. After three washes, cells were incubated for 10 min at room temperature with Hoechst 33342 (10 μg/mL, Molecular Probes, Eugene, OR). After three washes in PBS, cells were mounted in a 90% glycerol-PBS mixture. Then the cells were visualized and photographed with an IX81 fluorescent microscopy (Olympus, Japan).

DNA Fragmentation Assay

MCF-7 cells were seeded into 6-well plates and treated with 100 μg/mL ESAC for indicated time. The DNAs were extracted and purified with an Apoptotic DNA Ladder Kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's instructions. Equal amounts of purified apoptotic DNA were applied to electrophoresis on a 1% agarose gel at 90 V (1.5 h, RT). Then the DNA fragments were stained and visualized by a UV light and photographed.

Western Blot Analysis

After ESAC treatment cells were collected and lysed in the lysis buffer. The protein contents were quantified with a BCA™ Protein Assay Kit (Pierce, Rockford, IL). Fifty micrograms of total proteins were subjected to 8% SDS-PAGE and transferred onto nitrocellulose membranes. After blocking with 5% nonfat milk in TBST (20 mM Tris, 500 mM NaCl, and 0.1% Tween-20) at room temperature for 2 h, the membranes were incubated with specific primary antibodies against PARP, phospho-H2AX-S139 (γ-H2AX) and p53 (Cell Signaling Technology, Beverly, MA) overnight at 4°C. Then, the membranes were washed with TBST for three times and incubated with horseradish-peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.) in TBST at room temperature for 1 h. After washing three times in TBST for 15 min, the specific protein bands were visualized using an ECL advanced Western blot detection kit. Equal protein loading was verified by re-hybridization of membranes and re-probed with anti-β-actin antibody.

Results

Chemical Characteristics of ESAC

Previous study showed that triterpenes were detected in *G. lucidum* and not found in *G. sinense* (Liu *et al.*, 2009). In order to investigate chemical characteristics of ESAC from *G. lucidum* used in present study, HPLC analysis of ESAC from *G. lucidum* and *G. sinense* were carried out. The chromatogram of samples are shown in Fig. 1B, which indicates that ESAC from *G. lucidum* was abundant with triterpenoids, while ESAC from *G. sinense*

showed no peak emerging in the same marked area. This result was in accordance with our previous report (Liu *et al.*, 2009).

ESAC Decreased the Cell Viability in Breast Cancer Cells

In order to detect the anti-cancer activity of ESAC extracted from *G. lucidum*, we first determined the anti-proliferative effect of ESAC against breast cancer cells. MCF-7 and MDA-MB-231 cells were treated with various concentrations of ESAC for 48 h and the cell viability was detected by MTT assay. As shown in Figs. 2A, 2C, the viability of MCF-7 and MDA-MB-231 cells was notably decreased in a concentration-dependent manner with IC_{50} at about 100 μg/ml and 60 μg/ml, respectively. Compared with previously reported ethanol extracts (Liu *et al.*, 2009), ESAC demonstrated much higher cytotoxicity. Furthermore, the morphological changes of MCF-7 cells after ESAC treatment were also observed. Consistent with the MTT results, the percentage of adherent MCF-7 cells decreased as the ESAC concentration increased (Fig. 2B).

ESAC Mediated G1 Cell Cycle Arrest in Breast Cancer MCF-7 Cells

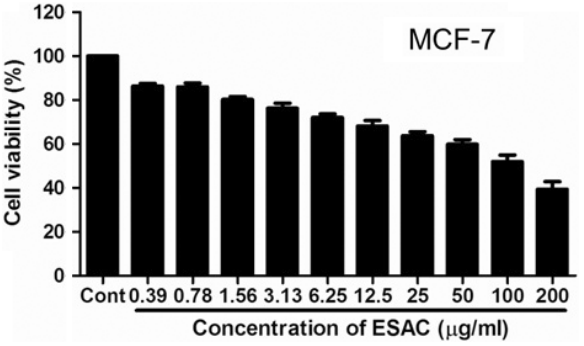
As cell cycle arrest could contribute to cell proliferative inhibition, we therefore inspected the distribution of cells after ESAC treatment in MCF-7 cells. As shown in Figs. 3A and 3C, ESAC effectively mediated G1 cell cycle arrest in a concentration-dependent manner after 48 h treatment. Furthermore, a time-dependent manner was also observed (Figs. 3B and 3D). Consistently, the cells distributed in S and G2/M phases were significantly decreased.

ESAC Induced DNA Damage in MCF-7 Breast Cancer Cells

DNA damage has been regarded as the major cause of genotoxin-induced growth inhibition in various cancer cells (Kaina, 2003; Yang *et al.*, 2009). Herein, to elucidate the possible mechanism of ESAC induced anti-proliferation, we detected the DNA damage after ESAC exposure by Comet assay and examined the expression of γ-H2AX, a specific marker for DNA damage. Comet assay indicated that ESAC elicited comet tails in treated cells (Fig. 4A, right) whereas the control cells showed normal shape (Fig. 4A, left). Elevated protein expressions of γ-H2AX were also detected by Western blot analysis in ESAC treated cells (Fig. 4C). Furthermore, as shown in Fig. 4B, the numbers of γ-H2AX foci were significantly increased after treatment with ESAC for 48 h. Taken together, these results suggested that ESAC induced DNA damage in MCF-7 cells.

ESAC Induced Apoptosis in MCF-7 Breast Cancer Cells

Apoptosis is another factor contributing to cell proliferative inhibition. Therefore, we further performed apoptotic assays to determine whether ESAC induced apoptosis in MCF-7 cells. Hoechst 33342 staining of MCF-7 cells showed that the characteristic



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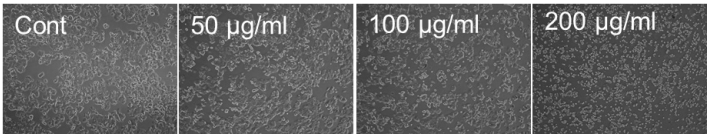

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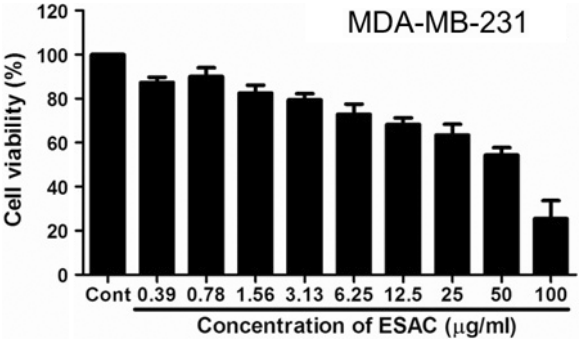

(C)

Figure 2. ESAC decreases the cell viability in breast cancer cells. (A) MCF-7 cells were treated with various concentrations of ESAC for 48 h and the cell viability was tested by MTT assay. (B) Cells were treated with different concentrations of ESAC for 48 h and the morphological changes of MCF-7 cells were taken with an AxioCam HRC CCD phase contrast microscope. Original magnification: ×50. (C) MDA-MB-231 cells were treated with various concentrations of ESAC for 48 h and the cell viability was tested by MTT assay.

apoptotic changes, such as condensation of nuclear chromatin, were observed in ESAC treated (100 μg/mL) cells (Fig. 5A, right, arrow).

We further examined the inter-nucleosomal DNA fragmentation after 100 μg/ml ESAC treatment. After treatment with ESAC for 24 h, ESAC induced little inter-nucleosomal DNA fragmentation while extended incubation time significantly increased the fragmentations (Fig. 5B).

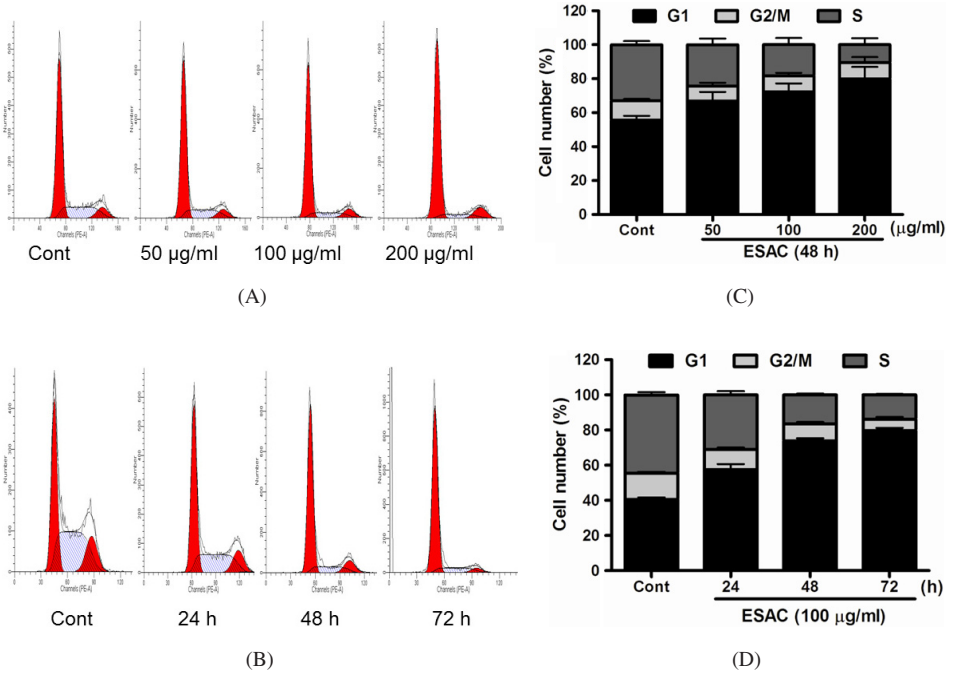

Figure 3. ESAC mediates G1 cell cycle arrest in MCF-7 cells. Cells were exposed to various concentration of ESAC for 48 h (A) or 100 μg/ml ESAC for different time (B). The cell cycle distribution was obtained using flow cytometry. (C and D) Semi-quantitative analyses of the results from (A) and (B).

As MCF-7 cells do not express caspase-3 (Janicke, 2009), we chose poly (ADP-ribose) polymerase (PARP) as the maker for apoptosis (Soldani and Scovassi, 2002). After ESAC treatment, apoptosis was detected as increased expression of a PARP cleavage fragment was observed (Fig. 5C). Moreover, the transcription factor p53 was also up-regulated after ESAC treatment (Fig. 5C).

Discussion

G. lucidum is a widely known Chinese herb used for centuries to treat various diseases in the East Asia area and its extracts were demonstrated to possess numerous therapeutic properties, such as anti-tumor, anti-inflammation, immunomodulation, anti-oxidation *etc.* (Dudhgaonkar *et al.*, 2009; Sudheesh *et al.*, 2010; Yue *et al.*, 2008; Zhu *et al.*, 2007). It has been reported that triterpenes and polysaccharides are the major bioactive components of *G. lucidum* (Xu *et al.*, 2011; Yuen and Gohel, 2005) and the former exhibited the direct anti-tumor effects *via* anti-metastasis (Weng and Yen, 2010), anti-angiogenesis (Stanley *et al.*, 2005), apoptosis-inducing (Liu and Zhong, 2011), cell cycle arrest (Lin *et al.*, 2003) *etc*. Consistent with previous studies, ESAC also displayed potential anti-cancer activities by mediating cell cycle arrest and inducing apoptosis in human breast cancer cells.

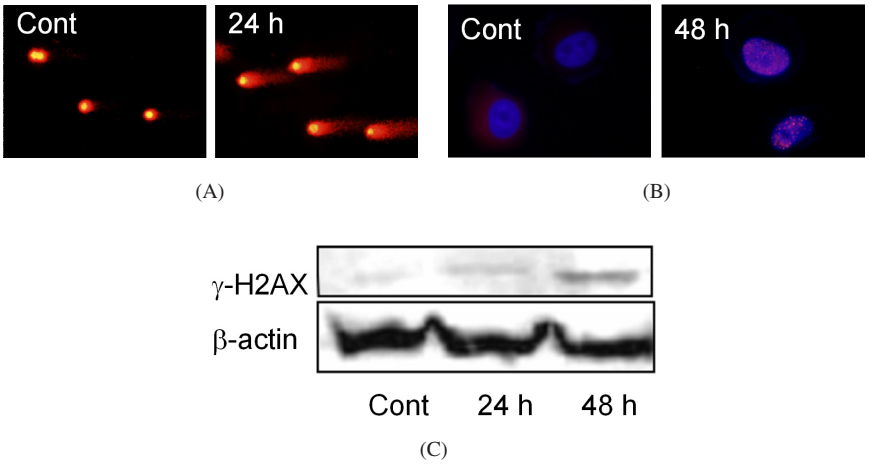

Figure 4. ESAC induces DNA damage in MCF-7 cells. (A) Cells were treated with 100 μg/mL ESAC for 24 h and the DNA damage was detected by Comet assay. Nuclei with damaged DNA have a comet feature with a bright head and a tail, whereas nuclei with undamaged DNA appear round with no tail. (B) The immunocytochemical stains were performed to detect the expression of γ-H2AX in the nuclei. (C) Cells were treated with 100 μg/mL ESAC for indicated time and the level of γ-H2AX was detected by Western blot analysis.

Previous studies showed that ethanol extracts of both *G. lucidum* and *G. sinense* possessed anti-proliferative properties in human cancer cells (Liu *et al.*, 2009). Herein, a revised technology which uses Na_2HCO_3 and $CHCl_3$ extraction to purify the ganoderic acid-abundant component from *G. lucidum* was applied. The HPLC results confirmed that ganoderic acid is abundant in ESAC form *G. lucidum* compared to the counterpart of *G. sinense*. The MTT results also showed that the obtained ESAC with this method exerted higher cytotoxicity towards breast cancer cells than our previous report (Liu *et al.*, 2009).

In MDA-MB-231 breast cancer cells, our previous report showed that an ethanol extract of *G. lucidum* induced G1 cell cycle arrest and inhibited cell proliferation (Liu *et al.*, 2009). The present study showed that ESAC also mediated G1 cell cycle arrest and inhibited cell proliferation in MCF-7 cells suggesting that G1 cell cycle arrest might be contributing to ESAC induced cytotoxicity.

Many types of cancer cells resistance to drug treatment while compounds that restore the normal apoptotic pathways have the potential for effectively treating cancers (Fesik, 2005). In the present study, we showed that ESAC remarkably induced apoptosis in MCF-7 cells as detected by Hoechst staining, DNA fragment assay and Western blot analysis (Fig. 5). PARP is a nuclear protein involved in a number of cellular processes mainly including DNA repair and apoptosis (Underhill *et al.*, 2011) and is cleaved by activated caspase-3 and caspase-7 (Soldani and Scovassi, 2002). As MCF-7 cells do not express caspase-3 (Janicke, 2009), we used cleaved PARP as an indicator of apoptosis. An increase of cleaved PARP resulting from treatment with ESAC was observed (Fig. 5C). p53, a tumor suppressor, plays an important role in apoptotic pathway. Initiating apoptosis by p53 is vital for the proper regulation of cell proliferation in mammal cells (Haupt *et al.*, 2003). In the present study, the

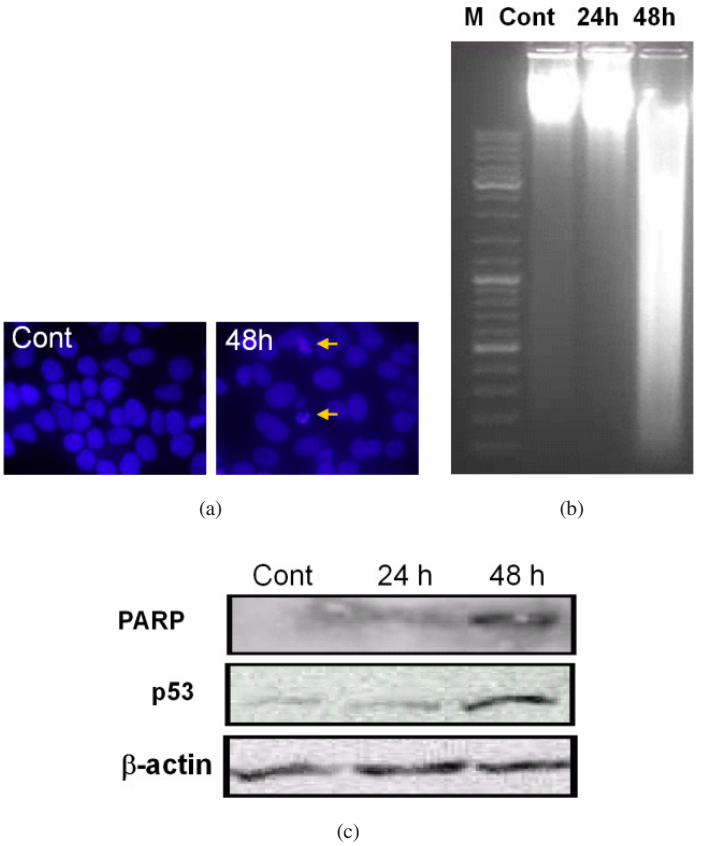

Figure 5. ESAC induces apoptosis in MCF-7 cells. (A) Cells were treated with 100 μg/ml ESAC for 48 h and the apoptotic morphological changes ware detected by Hoechst 33342 staining. The arrow showed the apoptotic cells. (B) Cells were treated with 100 μg/mL ESAC for indicated time and the DNA fragments were separated on 1% agarose gel by electrophoresis. M: marker. (C) Cells were treated with 100 μg/mL ESAC for indicated time and the level of cleavage of PARP and p53 were detected by Western blot analysis.

basal p53 expression level was low in MCF-7 cells. However, elevated expression of p53 after 48 h treatment with ESAC indicated that p53 may be involved in ESAC-trigger apoptosis in MCF-7 cells.

Our study also showed that ESAC elicited DNA damage in MCF-7 cells as determined by Comet assay, Western blot and immunocytochemical labeling. To the best of our knowledge, this is the first report showing that *G. lucidum* extract induced DNA damage. ESAC induced DNA damage after 24 h incubation, which was prior to the presence of apoptosis (Figs. 3A, 5B and 5C), suggesting that DNA damage might be an early step contributing to apoptosis. Phosphorylated H2AX on Ser139 (γ-H2AX) is a marker of DNA damage and is associated with repair of DNA damage (Podhorecka *et al.*, 2010). H2AX phosphorylation is also required for DNA ladder formation and hence lacking of γ-H2AX results in apoptosis (Lu *et al.*, 2006). Consistently, evaluated expression of γ-H2AX was

detected after treatment with ESAC (Figs. 3B and 3C) accompanied with nuclear fragmentation (Fig. 5B).

In conclusion, the current data suggest that the *G. lucidum* extract ESAC induced DNA damage, G1 arrest and apoptosis in MCF-7 breast cancer cells, which provides a new mechanism for the anti-tumor effect of *G. lucidum*.

Acknowledgments

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