Ocimum sanctum Induces Apoptosis in A549 Lung Cancer Cells and Suppresses the In Vivo Growth of Lewis Lung Carcinoma Cells

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Although Ocimum sanctum has been used extensively for its medicinal values in India and China, its antitumor activity against human nonsmall cell lung carcinoma (NSCLC) A549 cells has not been investigated until now. Therefore, the antitumor mechanism of ethanol extracts of Ocimum sanctum (EEOS) was elucidated in A549 cells in vitro and the Lewis lung carcinoma (LLC) animal model. EEOS exerted cytotoxicity against A549 cells, increased the sub-G1 population and exhibited apoptotic bodies in A549 cells. Furthermore, EEOS cleaved poly(ADP-ribose)polymerase (PARP), released cytochrome C into cytosol and simultaneously activated caspase-9 and -3 proteins. Also, EEOS increased the ratio of proapoptotic protein Bax/antiapoptotic protein Bcl-2 and inhibited the phosphorylation of Akt and extracellular signal regulated kinase (ERK) in A549 cancer cells. In addition, it was found that EEOS can suppress the growth of LLC inoculated onto C57BL/6 mice in a dose-dependent manner. Overall, these results demonstrate that EEOS induces apoptosis in A549 cells via a mitochondria caspase dependent pathway and inhibits the in vivo growth of LLC, suggesting that EEOS can be applied to lung carcinoma as a chemopreventive candidate. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: Ocimum sanctum; apoptosis; A549; Lewis lung carcinoma.

INTRODUCTION

Lung cancer is the leading cause of cancer deaths worldwide (Jemal et al., 2006; Parkin, 2001). Approximately 40% of lung cancers are adenocarcinomas, which belong to the subgroup of the nonsmall-cell lung cancers, the most common type of lung cancer in the USA and Asia (Brognard et al., 2001). Although there are various therapies to treat lung cancer including chemotherapy and radiotherapy, these therapies can frequently cause undesirable side effects. Therefore, new drugs without significant adverse side effects are absolutely required. For this aim, using medicinal plants with good efficacy and few side effects is a good strategy in chemoprevention compared with allopathic medicine (Zhu et al., 2005).

Chemoprevention is the use of chemicals in the prevention of cancer, which was first coined by Sporn and Suh (2000). There is accumulating evidence that the overall dietary intake of phytochemicals reduces the risk of cancer (Willett, 1994). Chemopreventive agents such as retinol, beta-carotene, synthetic retinoids and alpha-tocopherol are known to have antitumor potential against lung cancer (Fontham, 1990). Among the medicinal plants, Ocimum sanctum Linne (Labiatae family), commonly known as ‘Holy basil’ has been used extensively in the Ayurvedic system of medicine (Singh et al., 1996). Ocimum sanctum was reported to protect against free radical damage (Balanenu and Nagarajan, 1992) and radiation damage (Devi and Ganasoundari, 1995) and to exert antitumor activity against stomach cancer and hepatoma (Aruna and Sivaramakrishnan, 1992). Nonetheless, the underlying antitumor mechanism of Ocimum sanctum against lung cancer still remains unclear.

Thus, in the present study, the apoptotic mechanism of ethanol extracts of Ocimum sanctum (EEOS) was investigated in human NSCLC A549 cells in vitro and also the effect of EEOS on the in vivo growth of Lewis lung carcinoma (LLC) cells in C57BL6 mice was examined for the first time.

MATERIALS AND METHODS

Chemical reagents. Eagles minimal essential medium (EMEM), RPMI medium and antibiotic antimycotics were purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was from JRH (Lenexa, KS). Bovine serum albumin (BSA), sodium bicarbonate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phenylmethylsulphonyl fluoride (PMSF),
propidium iodide (PI) and ribonuclease A (RNase A) were from Sigma Chemical Co. (St Louis, MO). Protease inhibitor cocktail was from Boehringer Mannheim (Indianapolis, IN) and DC protein assay kit II was from Bio-Rad (Hercules, CA). Antibodies against Bel-2, Bax, procaspase-9, procaspase-3, cytochrome C and PARP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-Akt, Akt, phospho-ERK and ERK were purchased from Cell Signaling Technology (Beverly, MA). Anti-β-actin antibody was obtained from Aldrich-Sigma (St Louis, MO). Goat anti-mouse IgG HRP conjugated secondary antibody and goat anti-rabbit IgG HRP conjugated secondary antibody were from Zymed (San Francisco, CA). 4x NuPAGE LDS sample buffer, 4–12% NuPAGE Bis-Tris gels and sample buffer, 4–12% NuPAGE Bis-Tris gels and NuPAGE MES SDS running buffer were from Invitrogen (Carlsbad, CA). Hybond ECL transfer membrane and the ECL western blotting detection kit were from Amersham Pharmacia (Arlington Heights, IL). X-ray films were from Agfa-Gevaert (CP-BU, N. V., Belgium). The dead end™ fluorometric terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay kit was from Promega (Madison, WI).

Cell culture. The NSCLC A549 cells purchased from ATCC (Manassas, VA, USA) were cultured in RPMI-1640 supplemented with FBS, t-glutamine and penicillin-streptomycin in an atmosphere of 5% CO₂ at 37 °C. Cells were routinely grown in 75 cm² tissue culture flasks (Costar, Cambridge, MA, USA) and harvested with a solution of trypsin-EDTA when they were at the logarithmic phase of growth. LLC cells were kindly provided by Dr Ikuo Saiki (Toyama Medical and Pharmaceutical University, Toyama, Japan) and cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 100 U/mL antibiotic-antimycotics and 2.2 g/L sodium bicarbonate.

Preparation of EEOS. Ocimum sanctum Linne collected in Chennai, India was authenticated by Dr Namin Baek, a pharmacognosist and professor of College of Life Science, Kyunghee University, and deposited at the Cancer Preventive Material Development Research Center (CPMDCRC), Kyunghee University, South Korea, with a voucher specimen (CPMDCRC 07-03-31). Three kg of plant leaves was extracted with ethanol for 3 days at room temperature, concentrated using a rotary evaporator (Eyela, Tokyo, Japan) and freeze-dried to obtain 570 g (yield = 19%) of ethanol extract of Ocimum sanctum (EEOS).

Cytotoxicity assay. The cell viability was examined by MTT assay. A549 cells were seeded onto 96-well plates (1 × 10⁴ cells/well) and treated with various concentrations of EEOS (25, 50, 100, 150 and 200 μg/mL) using paclitaxel as a positive control and DMSO as a negative control for 48 h. MTT was added to each well and incubated for 4 h at 37 °C. Formazan crystals were dissolved by the addition of DMSO solution. The absorbance of each well was determined using a microplate reader (Molecular Devices Co., USA) at 570 nm.

Cell cycle analysis. Using standard methodology (Lee et al., 2006), A549 cells treated with EEOS were harvested, washed twice with cold phosphate-buffered saline (PBS) and fixed in 75% ethanol at –20 °C. After washing twice with cold PBS, the fixed cells were resuspended in 100 μL of PBS containing 10 μL of RNase A (10 mg/mL) and incubated for 1 h at 37 °C. The cells were stained by adding 400 μL of PI (50 μg/mL) for 30 min at room temperature in the dark. After filtering with nylon mesh (40 μm pore), the DNA contents of stained cells were analysed using CellQuest Software with a FACSVantage SE flow cytometry system (Becton Dickinson, Heidelberg, Germany).

Western blot analysis. Total cell lysates were lysed in extraction buffer. The protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). For western blot analysis, aliquots containing 20–50 μg proteins were separated by SDS-polyacrylamide gel electrophoresis and then electro transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). The membranes were subjected to immunoblot analysis and proteins were visualized by the enhanced chemiluminescence (ECL) method (Amersham Corp., Arlington Heights, IL, USA).

Cytochrome C immunoblot. The cytosolic fraction was prepared as described previously (Boularea et al., 2001). A549 cells (6 × 10⁵) treated with EEOS were harvested and washed with cold PBS. Cell pellets were lysed in 40 μL of lysis buffer (20 mM HEPES/NaOH, pH 7.5, 250 mM sucrose, 10 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail) for 20 min on ice. They were homogenized by 10 passages through a 22-gauge needle. The homogenate was centrifuged at 25 000 × g for 30 min at 4 °C and the protein contents in the supernatant were measured using a Bio-Rad DC protein assay kit II. The lysates containing 25 μg of protein were analysed by western blotting for cytochrome C (1:1000 dilution).

TUNEL assay. Individual apoptotic cell death was observed using a dead end fluorometric TUNEL assay kit as described by the manufacturer. A549 cells (2 × 10⁶) treated with 25, 50 and 100 μg/mL of EEOS for 24 h were harvested and washed with cold PBS. The cells were seeded onto a chamber slide, fixed with 4% paraformaldehyde for 15 min and washed with PBS twice for 3 min. Permeabilized cells by immersing the slides in 0.2% Triton X-100 for 5 min were washed with PBS and then equilibrated with 100 μL of equilibrium buffer for 10 min at room temperature. After removing equilibrium buffer, 50 μL of terminal deoxynucleotidyl transferase (TdT) enzyme buffer containing fluorescein-12-dUTP was added to the cells and covered with plastic cover slips, and the slides were incubated for 1 h at 37 °C in the humidified chamber covered with aluminum foil. The plastic cover slips were removed and the reactions were terminated by 2X SSC for 15 min at room temperature. The cells were stained with propidium iodide solution diluted to 1 μg/mL in PBS for 15 min at room temperature in the dark. After washing with PBS, the slides were mounted in 30% glycerol in PBS and analysed under an Axiovert S 100 fluorescence microscope (Carl Zeiss, Inc., USA).

LLC tumor model. Five week old C57BL/6 mice were given food and water ad libitum. The mice were housed...
in a room maintained at 25 ± 1 °C with 55% relative humidity. One week later, the mice were inoculated by s.c. injection of 5 × 10^6 LLC cells in 100 μL PBS into the right flank of the mice. Three days after LLC inoculation, the mice were i.p. injected with EEOS at doses of 50 and 100 mg/kg every other day, while the control mice were administered PBS vehicle. The tumor volumes were measured every other day with a caliper and calculated according to the formula \[(\text{length} \times \text{width}^2)/2\], where length represents the larger tumor diameter and width stands for the smaller tumor diameter. All mice were killed 21 days after LLC inoculation and the tumors were removed and weighed.

**Statistical analysis.** All data were expressed as mean ± SD or SE. The statistically significant differences compared with untreated control were calculated by a paired Student’s *t*-test.

**RESULTS**

**EEOS significantly exhibited cytotoxicity against A549 cells**

The cells were treated with EEOS (25–200 μg/mL) and the cell viability was assessed by the MTT assay. EEOS significantly exerted cytotoxicity against human NSCLC A549 cells in a concentration dependent manner with an IC_{50} value of approximately 176 μg/mL (Fig. 1A) compared with the untreated control. In addition, EEOS at 200 μg/mL showed cytotoxicity against A549 cells, which was almost comparable to 1 μM paclitaxel as a positive control, while 0.1% DMSO solvent solution did not show any significant cytotoxicity as a negative control (Fig. 1B).

**EEOS increased sub-G_1 peaks and TUNEL positive cells of A549 cells**

To confirm the antitumor mechanism of EEOS, cell cycle analysis was performed (Fig. 2A). EEOS increased the apoptotic sub-G_1 peak of A549 cells to 5.9%, 25.4% and 29.2% at concentrations of 25, 50 and 100 μg/mL, respectively (Fig. 2B). In addition, EEOS significantly increased TUNEL positive apoptotic cells in A549 cells in a concentration dependent manner (Fig. 3). These results indicate that the cytotoxicity of EEOS can be exerted via apoptosis induction in A549 cells.

**EEOS regulated apoptosis related proteins in A549 cells**

Cytochrome C binds to Apaf-1 and recruits, activates procaspase-9 in the apoptosome, and induces caspase-3 activation (Hengartner, 2000; Thornberry and Lazebnik, 1998; Wolf and Green, 1999). There is evidence that an early event in apoptosis is the disruption of the mitochondrial membrane potential (Hengartner, 2000; Susin *et al*., 1999). In the current study, EEOS released cytochrome C into the cytosol, implying the disruption of the mitochondrial membrane potential and activated caspase 9 and 3, cleaved caspase-mediated PARP cleavage in A549 cells. In addition, EEOS decreased the expression of antiapoptotic protein Bcl-2 in a concentration-dependent manner without affecting the proapoptotic protein Bax level in A549 cells. These data imply that EEOS increases the ratio of Bax/Bcl-2 protein in A549 cells, suggesting apoptosis induction more than survival or antiapoptosis (Fig. 4). Consistently, EEOS also decreased the phosphorylation of the survival gene Akt and ERK in A549 cells (Fig. 4). These data suggest that EEOS induces apoptosis via activation of caspases and inhibition of Akt and ERK in A549 cells.
**Figure 2.** Effect of EEOS on the sub-G₁ peaks of human A549 cancer cells. (A) Histogram of cell cycle distribution in EEOS treated A549 cells. Cells were cultured in complete medium and treated with the indicated concentrations of EEOS for 24 h and analysed for DNA content by flow cytometry. The sub-G₁ peak was considered as the apoptotic portion. M₁, M₂, M₃ and M₄ represent sub-G₁, G₀/G₁, S and G₂/M, respectively. (B) Bar graph for sub-G₁ DNA contents in EEOS treated A549 cells. All data were expressed as mean ± SD. * p < 0.05, ** p < 0.01, *** p < 0.001 versus control.

**EEOS suppressed the growth of LLC in mice**

To confirm the *in vitro* antitumor activity of EEOS, the LLC tumor model was used in C57BL/6 mice, because LLC cells grow rapidly in mice after inoculation. EEOS did not show any side effects such as weight loss or inactivity during the experiment (data not shown). The animal study revealed that EEOS significantly inhibited the growth of LLC in a time course (Fig. 5A), decreased tumor weight at necropsy (Fig. 5B) and the final tumor size (Fig. 5C) in a dose-dependent manner compared with PBS control.

**DISCUSSION**

Many chemopreventive agents have been developed actively for the prevention and treatment of cancers from medicinal plants, because they usually exert anticarcinogenic activities with fewer side effects than synthesized compounds (Ranga et al., 2005). Among the multibiological activities of *Ocimum sanctum* that have been used traditionally for cancer prevention (Balachandran and Govindarajan, 2005; Ichikawa et al., 2007), there is accumulating evidence regarding the antitumor potential of *Ocimum sanctum*. An ethanol extract of *Ocimum sanctum* leaf inhibited the proliferation and angiogenesis related proteins through the down-modulation of Bcl-2 and VEGF expressions and overexpression of caspase-3 during N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)-induced gastric cancer bearing rats by immunohistochemistry (Manikandan et al., 2007) and also suppressed 7,12-dimethylbenzanthracene (DMBA) and aflatoxin B1 induced skin tumorigenesis in a mouse model (Prakash and Gupta, 2000). Nevertheless, the underlying antitumor mechanism of *Ocimum sanctum* still remains unclear. Therefore, in the current work, the
apoptotic mechanism of ethanol extracts of *Ocimum sanctum* (EEOS) was investigated in A549 cells in vitro and in the LLC tumor model *in vivo*.

To elucidate the cytotoxic mechanism of EEOS against human NSCLC A549 cells with an IC₅₀ of ~176 µg/mL, cell cycle analysis was performed, since the regulation of cell cycle progression is considered to be a potentially powerful strategy for tumor growth control (Grana and Reddy, 1995). EEOS treatment induced a significant accumulation of apoptotic portion sub-G₁ peaks as well as increased green TUNEL positive apoptotic bodies in A549 cells, indicating that EEOS can inhibit the proliferation of A549 cells via apoptosis induction.

Apoptosis plays a crucial role in eliminating the mutated or proliferating neoplastic cells from the biological system (Hickman, 1992). Apoptosis can be triggered in a cell through either the extrinsic pathway or the intrinsic pathway (Broker *et al.*, 2005). In the case of the intrinsic pathway, the cytosolic cytochrome C binds to Apaf-1, leading to activation of procaspase-9 and caspase-3 (Thornberry and Lazebnik, 1998). Activated caspase-3 is the key executioner of apoptosis to induce the cleavage and inactivation of key cellular protein, such as PARP (Thornberry and Lazebnik, 1998; Wolf and Green, 1999). In the present work, western blotting revealed that EEOS activated the caspase 9 and 3 cascade, cleaved PARP, released cytochrome C into the cytosol and increased the ratio of Bax/Bcl-2 protein in A549 cells, strongly suggesting that EEOS induces apoptosis chiefly via the intrinsic mitochondrial dependent pathway.

**Figure 3.** Effect of EEOS on TUNEL positive cells in human A549 cancer cells. Cells were cultured in complete medium and treated with vehicle (0.1% DMSO) or 25, 50 and 100 µg/mL of EEOS. After 24 h treatment, the cells were harvested and washed with cold PBS buffer. A549 cells were stained with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end-labeling (TUNEL) and photographed under an Axiovert S 100 fluorescence microscope.

**Figure 4.** Effect of EEOS on the expression of pro/anti-apoptotic gene products. Cells were incubated with the indicated concentrations of EEOS for 24 h and the whole cell lysates were analysed by western blotting using antibodies for PARP, cytochrome C, procaspase-9, caspase-3, Bcl-2, Bax, phospho-ERK and phospho-Akt as indicated. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. A representative blot was shown from three independent experiments.

**Figure 5.** Effect of EEOS on the growth of LLC inoculated onto the flank of C57BL/6 mice. Three days after tumor inoculation, mice were given i.p. injection of PBS or EEOS every other day for 21 days. n = 7. (A) Tumor growth kinetics, (B) Tumor weight 21 days after LLC inoculation. All data were expressed as mean ± SE. * p < 0.05, ** p < 0.01 versus PBS control, (C) Photographs of tumor sizes of PBS control and EEOS treated groups.
The Akt pathway plays an important role in survival when cancer cells are exposed to different kinds of apoptotic stimuli, such as oxidative and osmotic stresses, irradiation, matrix adhesion, ischemic shock and chemotherapeutic drugs (Chen et al., 1998; Downward, 1998; Franke et al., 1997; Khwaja et al., 1997). In addition, the dysregulation of Akt or ERK is known as a prominent feature of many human cancers including NSCLC (Lee et al., 2002). Thus, the suppression of Akt and ERK phosphorylation can lead to p53 activation, which in turn may lead to the activation of pro-apoptotic signaling pathways (Fraser et al., 2003). The suppression of the phosphorylation of Akt and ERK by EEOS in our study implies that EEOS can regulate the survival of PI3/Akt or the ERK MAPK pathway, an upstream pathway of caspase cascades.

To confirm the antiproliferative and apoptosis-inducing activities observed in cell culture experiments, the in vivo antitumor activity of EEOS was evaluated. EEOS reduced the tumor volume/weight of LLC inoculated onto C57BL/6 mice without any adverse effects including weight loss at doses of 50 mg/kg and 100 mg/kg.

Regarding the effective compounds of Ocimum sanctum, Anandjiwala and his colleagues (Anandjiwala et al., 2006) quantified four major constituents such as eugenol (0.175–0.362%), luteolin (0.019–0.046%), ursolic acid (0.252–0.348%) and oleanolic acid (0.174–0.218%) in this plant by high pressure liquid chromatography (HPLC). These compounds are known to have multidimensional activities. For instance, eugenol possesses anticancer (Yoo et al., 2005) and antiinflammatory (Sharma et al., 1994) activities, and luteolin also has anticancer (Kim et al., 2005) and antiinflammatory (Chowdhury et al., 2002) activities. Similarly, terpenoid compounds such as ursolic acid (Kim et al., 1998) and oleanolic acid (Lazebnik et al., 1994) exhibit hepatoprotective, anti-inflammatory and antihyperlipidemic activities. Moreover, our group (Kim et al., 2007) previously reported that luteolin exerted antitumor activity by caspase activation and ERK/Akt inhibition, which coincides with our current data, suggesting that luteolin can play an important role in Ocimum sanctum induced apoptotic activity via caspase-Rib179 and ERK/Akt inhibition as one of the potent antitumor constituents. Thus, it can be thought that EEOS definitely exhibited antitumor activity through the combination of these antitumor phytochemicals in the present study.

In summary, EEOS increased apoptotic sub-G1, peak and TUNEL apoptotic bodies, released cytchrome C into the cytosol, activated caspase 9 and 3 proteins, cleaved PARP, markedly increased the ratio of Bax/Bcl-2 protein and reduced the phosphorylation of ERK and Akt in human A549 lung cancer cells. In addition, EEOS suppressed the in vivo growth of LLC implanted onto the flank of mice in a dose-dependent manner. Taken together, these findings strongly demonstrate that EEOS can be used effectively for lung cancer treatment as a chemopreventive agent.

Acknowledgement
This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (No. R13-2007-019-00000-0).

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