RESEARCH COMMUNICATION

Oleanolic Acid from *Prunella Vulgaris* L. Induces SPC-A-1 Cell Line Apoptosis Via Regulation of Bax, Bad and Bcl-2 Expression

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Abstract

***Prunella vulgaris* L. (PV) has been used as a herb for chemoprevention of lung cancer. In this study, the main active compound, oleanolic acid (OA) was isolated from an ethanol extract and its chemical structure was identified according to the results of high performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC) and liquid chromatography-mass spectrography (LC-MS). Results for cell viability indictated no notable differences between OA and ethanol extract of PV in lung adenocarcinoma SPC-A-1 cells measured by MTT assay. Consistent concentration-response curves. Fluorescence detection with acridine orange-ethidium bromide was used to evaluate apoptosis of SPC-A-1 cells. OA at 16 and 8 μM group increased significantly the apoptosis rate compared with normal and 1% DMSO groups ($p \leq 0.05$). In addition, immunocytochemistry assays showed increase in Bax and Bad protein expression while Bcl-2 decreased. Moreover, the ratio of Bax/Bcl-2 was heightened by OA treatment. The results suggest OA induced apoptosis of lung adenocarcinoma cells through down-regulating Bcl-2 expression, and up-regulating Bax and Bad expression.**

Keywords: *Prunella vulgaris* L. - oleanolic acid - apoptosis - lung adenocarcinoma cells - Bcl-2 family proteins

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Introduction

Lung cancer is the leading malignancy resulted in death. There are 1.2 million new cases and 1.1 million deaths each year in the world (Parkin et al., 2001). Lung adenocarcinoma is one of the most common types which accounts for about 40% of total cases (Travis, 2002). Currently, there is no effective therapeutic method towards treatment of this disease. Reversing or delaying the occurrence and development of lung adenocarcinoma was considered to be the best measures to decrease the disease morbidity and mortality (Van Zandwijk, 2001). Therefore, exploring new and effective agents which may function to reverse, prevent or delay the occurrence and progression of lung adenocarcinoma is of great significance (Mulshinea et al., 2003).

Promoting tumor cell apoptosis has been recognized as one of most important methods towards tumor treatment. There are a number of cytokines responsible for the induction of apoptosis, in which Bcl-2 family proteins play an important role (Fukazawa et al., 2007). It is recently reported that Bcl-2 family mRNA expression plays a key role in the biological behavior of non-small cell lung carcinomas (NSCLCs) (Grimminger et al., 2010). Bcl-2 over-expression with the imbalance of the ratio Bcl-2/Bax was recognized as the underlying mechanism to induce apoptosis in A549 cell (Liu et al., 2009). Bcl-2 family genes also play an important role in regulation of the apoptosis process in human lung adenocarcinoma cell line SPC-A-1 and its over-expression can also be found in highly metastatic human lung adenocarcinoma cell line SPC-A-1BM (Zou et al., 2008; Yang et al., 2009). Therefore, modulating Bcl-2 family proteins' expression was a promising way towards lung adenocarcinoma treatment.

Prunella vulgaris L (PV), known as self-heal herb, was found that it had strong anti-lung cancer activity on SPC-A-1 cell and C57BL/6 mice model in our previous study (Feng et al., 2010). We first found that the ethanol extract of PV including triterpenes had proliferation inhibition activity on lung adenocarcinoma cell. In our study, we screened the main active compound from the ethanol extract of PV according to cell membrane chromatography as described previously (Dong et al., 2005; data not shown in this paper). Oleanolic acid (OA), an active component isolated from *Prunella vulgaris* L, was screened by cell membrane chromatography and found to inhibit tumor cell growth. Recently, its anti-tumor activity has attracted more attention owing to its low toxicity. Scholars have demonstrated it can inhibit the growth of breast cancer cell (Chu et al., 2010), osteosarcoma cell (Hua et al., 2010), and liver cancer

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cell (Yan et al., 2010), etc. via regulating mitochondrial membrane potential, or intercellular adhesion molecule-1 and vascular endothelial growth factor expressions. Most recently, there is a report demonstrates that OA can inhibit HuH7 cells (human liver cell line) growth via modulating Bcl-2 family expression (Shyu et al., 2010). However, to our knowledge, its effect on lung adenocarcinoma has never been investigated.

The aim of the present study is to isolate OA from *Prunella vulgaris* L. and investigate the effect of OA on lung adenocarcinoma SPC-A-1 cell line. The potential mechanism of OA on inhibiting SPC-A-1 growth was also investigated.

Materials and Methods

Plant material

Prunella vulgaris L (PV) was purchased from medicinal corporation of Bozhou city, Anhui province and authenticated by Professor D.K. Wu, from Nanjing University of Chinese Medicine.

Chemicals

RPMI 1640 medium, Fetal bovine serum (FBS). Bax, Bcl-2 and Bad were offered by Wuhan Boster Biological Technology (Wuhan, China). 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT), poly-L-lysine, ethidium bromide (EB) and acridine orange (AO) were obtained from Sigma. Acetonitrile was HPLC grade (TEDIA, USA). Other reagents were from commercial sources.

Preparation of PV extract

Dry powder of *Prunella vulgaris* L. (58.7 g) was weighed and added into a 1000 mL flask, and then 500 mL 95% (v/v) ethanol was added for reflux at 95°C for 1.5 h. After first extract, the other 500 mL 95% (v/v) ethanol was added for second reflux. The two extracts were merged and concentrated on rotary evaporator under 50°C. The extract was successively washed with distilled water for 2 times and petroleum ether for three times and then obtained 1.71 g final extract.

OA preparation

The extract was dissolved in 100 mL ethanol and added 2 g active carbon for decolorization. The white compounds were obtained after recrystallization. The compounds were disoloved in mixture of $CHCl_3$: MeOH (100:1, v/v) and separated over a silica gel column using a gradient solvent system of $CHCl_3$:MeOH=100:1-1:1 to yield to this compound (300 mg). The final compound was recrystallized under ventilation at room temperature.

HPTLC, HPLC and LC-MS analysis

The ethanol extract of PV (10 mg crude drug/mL), white crystal (0.52 mg/mL) and OA standard substance (0.50 mg/mL) were dotted on the same silica gel G plate with quantitative capillary (2 μL) and developed with cyclohexane:chloroform:ethyl acetate:glacial acetic acid (20:5:8:0.5). Then the silica gel G plate was dried, sprayed with 10% ethanol solution of sulfuric acid and heated at 100 °C to develop the color. Finally, it was observed under sunlight and UV light (365 nm) and photographed with Cannon utilities Zoom Browser EX5.8. Agilent 1200 High Performance Liquid Chromatography (USA), equipped with quaternary pump, an automatic sample injector and Diode Array Detector (DAD) detector, was used. The chromatographic conditions were as follows, chromatographic column was ZORBAX C18 (2.1×100 mm, 3 μm), mobile phase was acetonitrile:distilled water = 75:25 (v/v), with 0.2 mL/min flow rate at 210 nm for 25 min. The column temperature was at 30°C and injection volume was 1 μL. MS analysis was performed on a ThermoQuest mass spectrometer (San Jose, CA, USA) equipped with Finnigan ESI interface. The final ionization conditions were as follows, spray voltag, capillary temperature, sheath gas and auxiliary gas were set at 4.5 kV, 300°C, 40 psi and 20 psi, respectively. In this study, nitrogen was used as both nebulizing gas and auxiliary gas. Argon (Ar) was used as the collision gas (3mTorr) with energy of 30V.

Cell culture and Cell viability by MTT assay

Human lung adenocarcinoma cell line SPC-A-1 was kindly provided by Dr. X.B. Jia from Jiangsu Provincial Academy of Chinese Medicine, China. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS containing 80 units/mL of penicillin and 80 units/mL of streptomycin and maintained at 37°C and 5% CO_2. SPC-A-1 cell suspension (10^4 cell/mL) was inoculated into 96-well plates (100 μL/well) and cultured for 12 h. The cell was starved for12h, then the cells were treated with crude extract of PV (0.0016, 0.008, 0.04, 0.2, 1.0, 5.0, 10.0 mg crude drug/mL) and OA (1, 2, 4, 8, 16, 32, 64 μM) and cultured for 48 h. The cells in positive group were treated with 10 μM fluorouracil (5-Fu, sigma). Finally, the medium was replaced with 0.5 mg/mL MTT and further incubated at 37°C for 4 h, the formazan was resolved by DMSO and its amount was determined by Microplate spectrophotometer (SPECTRAmax190, Moleculor Devices, USA) at 580 nm.

Fluorescence detection of acridine orange

SPC-A-1 cells were seeded on coverslips in 24-well plates and treated with RPMI-1640 medium, 1% DMSO, 16 μM OA, 8 μM OA or 4 μM OA for 24 hours. Then the cells were stained with AO-EB solution and observed under fluorescence microscope (Olympus IX71 inversion microscope, Olympus BX51 fluorescence microscope, Japan). The cell viability was evaluated according to green (normal) or jacinth (apoptosis) color area as calculated by Image-Pro Plus picture analysis software.

Immunocytochemistry assay of Bax, Bad and Bcl-2

The StreptAvidin-Biotin-enzyme Complex (SABC) was used for immuno- cytochemistry staining in this study. Cells were grown on coverslips in 24-well plates and were treated with drug (same with 2.7) for 24 hours. Then the cells were washed with phosphate-buffer solution (PBS, pH = 7.4) and fixed with 4% paraformaldehyde for 90 min. Cells were blocked with 10% primary antibody-origin serum for 20 min at room temperature and further

incubated with Bcl-2, Bax and Bad antibodies (1:200) at 37°C for 120 min. After being incubated with biotin-conjugated secondary antibody for 30 min, SABC was added and was further incubated at 37°C for 20min. Coverslips were exposed to 3,3'-diaminobenzidine (DAB, 0.3 mg/mL) for colorization and were counterstained with hematoxylin. The brown deposit was showed in positive staining. In negative control, PBS was used to replace the primary antibody. The coverslips were observed under microscope.

Statistical analysis

Data were expressed as means ± standard deviation (SD), and one-way ANOVA with SPSS 16.0 software was used to evaluate the statistical significance. The level of difference among means was defined at $p < 0.05$ with the Least Significance Difference Test.

Results

Antiproliferation activity of PV extract on SPC-A-1 cell

As can be seen in Figure 1, the inhibition rates of PV increased from 4.23 ± 4.15% in 0.0016 mg crude drug/mL group to 84.7 ± 0.15% in 10 mg crude drug/mL group. IC 50 of PV extract on SPC-A-1 cells was 2.298 mg crude drug/mL. At 5 and 10 mg crude drug/mL concentration, no significant proliferation inhibition difference was observed on SPC-A-1 cells. The result indicated PV extract has strong antiproliferation activity on lung adenocarcinoma cells.

Analysis and identification of OA

The main activity compound was screened from ethanol extract of PV by cell membrane chromatography. The isolated compounds after recrystallization (0.3 g,) were analyzed by HPTLC, HPLC-DAD and LC-MS. In thin layer chromatography (Figure 2A), the yellow green spot can be seen in the same place (Rf = 0.60) as compared with OA standard substance. As shown in Figure 2B and 2C, the isolated compound was observed at 14.202 min, and thus can be identified as OA on basis of m/z 457, m/z 439, m/z 411, m/z 395 under positive ionization mode.

Antiproliferation activity of OA

In order to evaluate the antiproliferation activity of isolated compound OA, MTT method was used as the evaluation method of PV extract. As shown in Figure 3, OA enhance the inhibition rates from 20.3 ± 2.99% in 1 μM group to 82.1 ± 1.57% in 64 μM group. There is no significant difference between 5-Fu (10 μM) and OA (64 μM). The result indicated OA has also anti-proliferation activity on lung adenocarcinoma cells and was the main active compound in ethanol extract of PV.

OA induced SPC-A-1 apoptosis

To observe the morphological changes of the cells, acridine orange/ethidium bromide (AO/EB) staining assay was performed. As can be seen in Figure 4A, B and C, SPC-A-1 cells treated with medium, 1% DMSO and 4 μM OA group were stained with bright green, and no obvious red-staining was observed. The result in Figure 4B showed

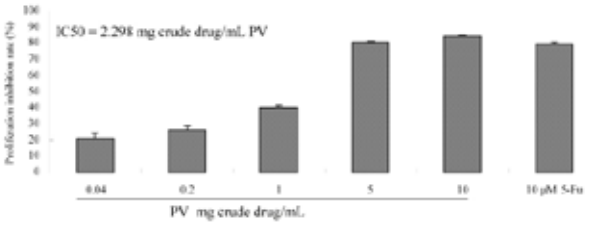

Figure 1. Inhibition of SPC-A-1 Cell Proliferation by the PV Extract. The rate was calculated as following formula: R = (1- OD in sample/OD in normal group)× 100%. Data are means ± SD (n=12)

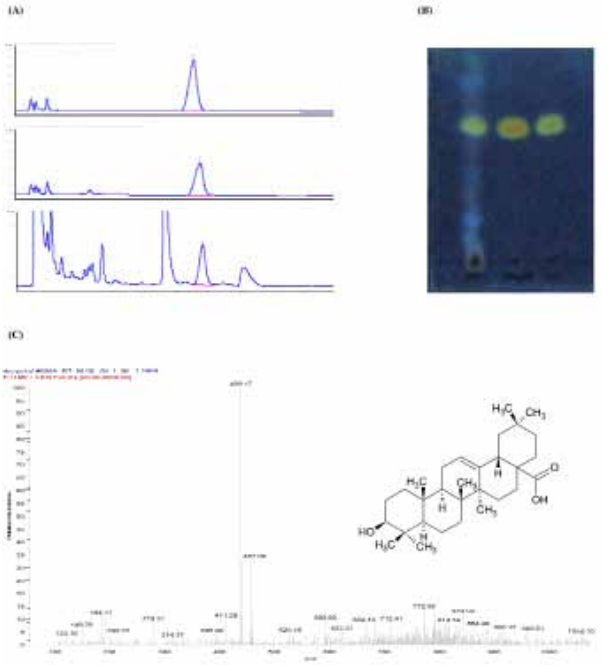

Figure 2. HPLC, HPTLC and LC-MS Chromatography of PV, the Isolated Compound and OA. (A) The HPLC chromatography of PV, the isolated compound and OA. (Aa) the isolated compound; (Ab) standard substance of OA; (Ac) ethanol extract of PV. (B) HPTLC picture, (Ba) ethanol extract of PV; (Bb) isolated compound from PV; (Bc) standard substance of OA. (C) Chemical structure, MS and production spectra of OA

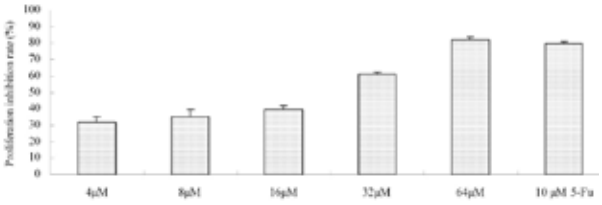

Figure 3. Proliferation Inhibition of OA on SPC-A-1 Cells. Formula is as in Figure 1. Data are means ± SD (n=12)

that there was no significant difference between normal group, 1% DMSO group and 4 μM OA group (0.16 ± 0.13%, 0.52 ± 0.22% vs. 0.61 ± 0.19%). While in Fig. 4B, the cells treated with 16 and 8 μM OA showed significant red-staining represented of apoptosis. It indicated that OA significantly promoted cell apoptosis compared with normal and 1% DMSO group ($p \leq 0.01$).

Effect of OA on expressions of Bad, Bax and Bcl-2

Bad, Bax and Bcl-2 are mainly expressed in cytoplasm. In positive expressions, the proteins were in uneven,

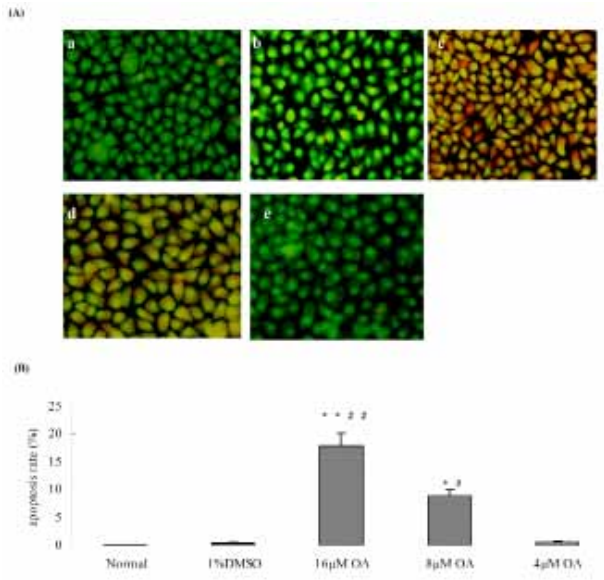

Figure 4. Fluorescence Detection of SPC-A-1 Cells Apoptosis Treated with OA. SPC-A-1 cells treated with drug were incubated with ethidium bromide-acridine orange solution and observed under fluorescence microscopy. (A) Fluorescence pictures, (a) normal group; (b) 1% DMSO group; (c) 16 μM OA group; (d) 8 μM OA group; (e) 4 μM OA group. Magnification: 200×. (B) The score of apoptosis induced by treatment of OA. **P < 0.01, 16 μM OA vs. normal group; *P < 0.05, 8 μM OA vs. normal group; ##P < 0.01, 16 μM OA vs. 1% DMSO; #P < 0.05, 8 μM OA vs. 1% DMSO. Data are expressed as means ± SD (n=3)

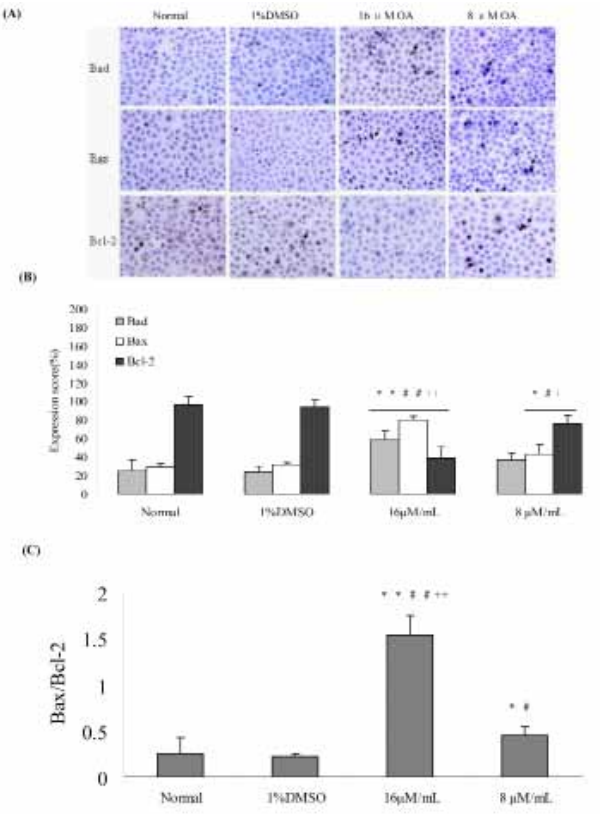

Figure 5. Effects of OA on Expression of Bad, Bax and Bcl-2 in SPC-A-1 Cells. Cells were treated with OA for 48 h. The expression of Bad, Bax and Bcl-2 proteins was detected by immunocytochemistry with SABC method. Black arrows represent the expressions of Bad, Bax and Bcl-2 proteins. Magnification: 200×. **P < 0.01, 16 μM OA vs. normal group; *P < 0.05, 8 μM OA vs. normal group; ##P < 0.01, 16 μM OA vs. 1% DMSO; #P < 0.05, 8 μM OA vs. 1% DMSO. ++ P < 0.01, 16 μM OA vs. 8 μM; + P < 0.05, 16 μM OA vs. 8 μM. Values are expressed as means ± SD (n=3)

scattered or spotty distribution and were dark brown deposits. OA incubation dose-dependently increased Bad and Bax positive staining compared with normal and 1% DMSO group. Furthermore, OA dose-dependently decreased Bcl-2 expression (Figure 5A). In Figure 5B, it could be seen that OA had obvious effects on the level of Bax, Bad and Bcl-2 proteins in SPC-A-1 cells. OA increased the expression of Bad from 25.5 ± 12.0% to 59.0 ± 9.1% and Bax from 29.0 ± 4.5% to 80.5 ± 4.04% while decreased the expression of Bcl-2 from 96.7 ± 8.7% to 39.0 ± 12.5%. There were significant differences in protein expressions between OA group and the control group. It could also be seen in Figure 5C, there is significant difference for the ratio of Bax/Bcl-2 between OA and the control group. The above results indicated that OA promoted the apoptosis of SPC-A-1 cells via up-regulating Bax & Bad and down-regulating Bcl-2 expressions.

Discussion

PV has been applied as a herbal medicine for goiter, scrofula, gall tumors, mammary abscess, etc. in China for centuries and is known as a self-heal herb in Europe (Chinese National Pharmacopoeia Committee, 2010). Recently, its potential anti-tumor activity has attracted much attention. Effects of the aqueous extract of PV on anti-tumor and antimutagenesis have been abundant (Zhang et al., 2006; Collins et al., 2009; Feng et al., 2010). Scholars have demonstrated its aqueous extract role on preventing normal cell from canceration caused by UVB or benzo[a]pyrene (Lee et al., 1988; Horikawa et al., 1994; Vostálováet al., 2010). PV can inhibit the proliferation and induce the apoptosis of human lymphoma cell via JNK, caspase, matrix metalloproteinase-9 and NF-kappaB pathways (Choi et al., 2010; Liu et al., 2010). However, to our knowledge, there is no report on proliferation inhibition of PV ethanol extract on lung adenocarcinoma. In the present study, we demonstrated that ethanol extract of PV can inhibit proliferation of lung adenocarcinoma cells.

Triterpenoic acids mainly included in the ethanol extract of PV have cytotoxic activities and were considered as promising anticancer agents (Lee et al., 2008). In the present study, OA was isolated from PV by silica gel column and identified according to results detected by HPLC, HPTLC and LC-MS. OA was considered to be the main active compound based on the comparison of proliferation inhibition on SPC-A-1 cells. Therefore, its effects were examined in this study.

Inhibiting the abnormal proliferation and promoting the apoptosis of tumor cells are of great significance in tumor therapy (Li et al., 2010). Bcl-2 family proteins, including pro-apoptotic Bax, Bad and anti-apoptotic Bcl-2, play important role in apoptosis of lung adenocarcinoma cell (Ludovini et al., 2008). It is recently reported that Bcl-2 family proteins expression plays a crucial role in the biological behavior of no-small cell lung cancer (Grimminger et al., 2010). Bcl-2 family proteins not

only inhibit the activation and cleavage of caspases, but also block the proteolytic cascade that ultimately dismantles the cell (So et al., 2008; Anagnostou et al., 2010). Many mechanisms responsible for anti-tumor activity of OA, such as antioxidative (Wang et al., 2010), anti-inflammatory (Tsai et al., 2008), anti-virus (Ma et al., 2009), etc., have been reported. In the present study, we demonstrated that OA could decrease Bc1-2 while increase Bax and Bad expressions in SPC-A-1 cells, thus exerted anti-lung adenocarcinoma effects.

In conclusion, in the present study, OA was isolated from ethanol extract of PV and was identified by HPLC, HPTLC and LC-MS. Both OA and PV showed the proliferation inhibition and inducing-apoptosis activity on lung adenocarcinoma SPC-A-1 cells. The apoptosis mechanism of OA on SPC-A-1cell was up-regulating the expressions of Bax and Bad proteins while down-regulating the expression of Bcl-2 protein.

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