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Caffeic acid n-butyl ester-triggered necrosis-like cell death in lung cancer cell line A549 is prompted by ROS mediated alterations in mitochondrial membrane potential

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Abstract. – OBJECTIVE: The aim of the present study was to evaluate the anticancer activity of caffeic acid n-butyl ester against lung cancer cell line A549 and to investigate the underlying mechanism.

MATERIALS AND METHODS: IC50 was determined by MTT assay. Fluorescent probes DCFH-DA, Indo 1/AM, DiOC6 were used to determine ROS, Ca^{2+}, and mitochondrial membrane potential ($\Delta\Psi m$). ATP levels were determined by using ATP liteTM kit. DNA damage was investigated by DAPI and comet assays. Protein expression was investigated by Western blotting.

RESULTS: Caffeic acid n-butyl ester exhibited lowest IC50 of 25 μM against lung A549 cell line. Caffeic acid n-butyl ester reduced the cell viability of A549 cells concentration and time-dependently. It also augmented the discharge of ROS and Ca^{2+} and lessened the mitochondrial membrane potential ($\Delta\Psi m$) and ATP levels in A549 cells. Additionally, caffeic acid n-butyl ester also prompted DNA damage in A549 cell line. Notably, caffeic acid n-butyl ester-stimulated the cytochrome c release only and exhibited no effect on the expression of apoptosis-related protein levels such as caspase-3, caspase-8, and Apaf-1.

DISCUSSION: Caffeic acid n-butyl ester exhibited significant anticancer activity against lung cancer cell line A549. However, the anticancer activity was not due to apoptosis as no significant change was observed in the expression of apoptosis-related proteins. The anticancer activity of caffeic acid n-butyl ester may be attributed to necrosis-like cell death prompted by ROS-mediated alterations in $\Delta\Psi m$.

CONCLUSIONS: Taken together, we conclude that caffeic acid n-butyl ester-induced A549 cells death displayed a cellular pattern characteristic of necrotic cell death and not of apoptosis.

Key Words:
Caffeic acid n-butyl ester, Necrosis, Lung cancer A549 cells, ROS.

Introduction

Lung cancer is considered as one of the main reasons of cancer-associated mortality and it is the major cause of cancer-related deaths in China[1,2]. The sharp increase in the incidence of cancer, the lack of proper cure and the severe side effects associated with the synthetic drugs has made it necessary to search for new and more effective molecules. From the past few decades, across the globe there has been a budding interest in the use of herbal drugs or herb-derived natural products due to their lower side effects. Among the natural products flavonoids form a large group of compounds ubiquitously found across plant kingdom[3]. Flavonoids represent an important part of the human diet and in the United States the estimated regular dietary intake of mixed flavonoids ranges from 500 to 1000 mg. This figure may be even higher for people improving their diets with flavonoid-rich herbal preparations[4,5]. With advancements in medical research, flavonoids are being evaluated for the diversity of bioactivities. So far they have been reported to exhibit a wide range of activities which include, but are not limited to, anti-inflammatory, estrogenic, enzyme inhibition, antimicrobial[3,4] anti-antiallergic, antioxidant[6], and antitumor[7]. Owing to their fairly consistent structure, flavonoids impede the activity of a wide

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range of eukaryotic enzymes and, therefore, exhibit a diversity of activities. The different parts of flavonoid molecules have been considered critical for their bioactivities. Moreover, flavonoids are ubiquitously present in edible plants and beverages and they are therefore expected to have minimal toxicity. Against this backdrop, the current study investigated the antitumor potential of a natural flavonoid caffeic acid n-butyl ester against a panel of cancer cell lines using MTT assay. Our results revealed that caffeic acid n-butyl ester exhibited lowest IC_{50} against lung cancer A549 cancer cell line that was then selected for further study. Flow cytometry and caspase activity, ROS and expression studies, revealed that caffeic acid n-butyl exerts its antitumor activity through scavenging of ROS and alterations in mitochondria membrane potential and then caused cell necrosis.

Materials and Methods

Chemicals and Reagents

Caffeic acid n-butyl ester, propidium iodide (PI), RNase A triton X-100 dimethyl sulfoxide (DMSO), were obtained from Sigma-Aldrich (St. Louis, MO, USA). All primary and secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The fluorescent probes DCFH-DA, Indo 1/AM, $DiOC_6$, 4′-6-diamidino-2-phenylindole (DAPI), fetal bovine serum (FBS), Roswell Park Memorial Institute-1640 (RPMI)-1640 medium, L-glutamine, antibiotics were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA).

Cell Culture

The human cancer cell lines were procured from the Food Industry Research and Development Institute (Hsinchu, Taiwan) and preserved in RPMI-1640 medium tissue culture flasks at 37°C under a humidified 5% CO_2 and 95% air as described previously[8].

Cytotoxicity of Caffeic Acid n-butyl Ester Against Different Cancer Cell Lines

The cytotoxic effect of caffeic acid n-butyl ester was measured against gastric cancer cell line (SNU-5), lung cancer cell line (A-549), prostate (PC-3), breast (MCF-7) and pancreas (MiaPaca-2), using the methyl thiazolyl tetrazolium (MTT) assay. The cytotoxic effect of caffeic acid n-butyl ester against all the cancer cell lines was expressed as IC_{50} values.

Assessment of Morphology and Viability of A549 Cells

Cancer A549 cells were seeded at the density of 2×10^5 cells/well in 6 well plates and treated with caffeic acid n-butyl ester (10, 25, and 50 μM) for 48 h. DMSO (1%) was used as vehicle control. The cells were investigated and photographed under a phase-contrast microscope for caffeic acid n-butyl ester-induced morphological changes. Afterward, cell viability was estimated from each treatment by PI exclusion method and flow cytometry as described previously[9].

Estimation of Cell Cycle Dissemination of A549 Cells

The cells were seeded in 6 well plates at a density of 2×10^5 cells/well and caffeic acid n-butyl ester was administrated to the cells at the concentrations of 0, 10, 25 and 50 μM followed by 24 h of incubation. DMSO (1%) was used as a control. For estimation of DNA content, PBS was used to wash the cells followed by fixation in ethanol at -20°C. This was followed by re-suspension in PBS holding 40 μg/ml PI and, RNase A (0.1 mg/ml) and Triton X-100 (0.1%) for 30 min in a dark room at 37°C. Afterward, the analysis was carried out by flow cytometry as described previously[9].

Determination of ROS, Ca2+ Release and $\Delta\Psi m$

A549 cells were seeded at a density of 2×10^5 cells/well in a 6-well plate and kept for 24 h and then treated with 25 μM caffeic acid n-butyl ester for 6-72 h at 37°C in 5% CO_2 and 95% air. Thereafter cells from all treatment were collected, washed 2 times with PBS and re-suspended in 500 μl of DCFH-DA (10 μM) for ROS estimation, Indo 1/AM (3 μg/ml) for Ca^{2+} generation and $DiOC_6$ (1 μmol/l) for $\Delta\Psi_m$ and incubated at 37°C indark room for 30 min. The samples were then analyzed instantly using flow cytometry as previously described in literature[10].

Evaluation of ATP Level

A549 lung cancer cells (density 1×10^4 cells/well) were plated in 100 μl phenol red-free medium at varied concentrations (0, 10, 25 and 50 μM) of caffeic acid n-butyl ester for 6 h in 96-well microplate and the intracellular ATP content was estimated by Luminescence ATP Detection Assay by ATP liteTM kit (PerkinElmer, Waltham, MA, USA) as described in literature[11].

Comet Assay and DAPI Staining

A549 cells/well at a density of 2×10^5 cells/well were seeded in 6-well plates were admi-

Table I. IC50 of caffeic acid n-butyl ester against different cancer cell lines as determined by MTT assay.

Cell line	IC50 (μM)
Gastric cancer SNU-5	30
Lung cancer A-549	25
Prostate PC-3	50
Breast MCF-7	25
Pancreas MiaPaca-2	50

nistrated with 10 to 50 μM caffeic acid n-butyl ester for 48 h. The cells were then separated into two shares for comet staining by PI and as well as DAPI staining. Afterward, the cell sample was studied and photographs were taken under fluorescence microscopy as previously described[12].

Western Blotting Analysis

The caffeic acid n-butyl ester administrated cells were harvested and lysed. The protein concentrations of the lysates were quantified by BCA assay using specific antibodies. β-actin was used as a loading control. From each sample, equal amounts of protein samples were loaded and separated by electrophoresis on a 12% denaturing SDS gel. Afterward, the proteins were electroblotted onto polyvinylidene difluoride membranes (0.45 m pore size).

Statistical Analysis

The quantitative data was expressed as mean ± SD. The results are representative of three independent biological replicates. The statistical differences between the caffeic acid n-butyl ester-treated and control samples were calculated by Student's *t*-test with a *p*-value of <0.05 was considered statistically significant.

Results

Caffeic Acid n-butyl Ester-induces Changes in Cell Morphological Changes and Viability

Caffeic acid n-butyl ester was evaluated against different cancer cell lines and it was found to exhibit lowest IC_{50} against Lung cancer cell line A549 (Table I). Therefore, it was further evaluated against Lung cancer cell line A549 only.

After administration of several doses of caffeic acid n-butyl ester for varied time intervals, cells were studied for alterations in morphology and viability (Figure 1). Our results revealed that caffeic acid n-butyl ester triggered morphological changes in A549 cells in a concentration-dependent way (Figure 1a). Moreover, it was observed that caffeic acid n-butyl ester also decreased the percent viability of cells in a concentration dependent manner (Figure 1b).

Caffeic Acid n-butyl Ester Caused S Phase Arrest and Inhibition of Related Protein Levels

The cancer cells were administrated with different doses of caffeic acid n-butyl ester for 24 h

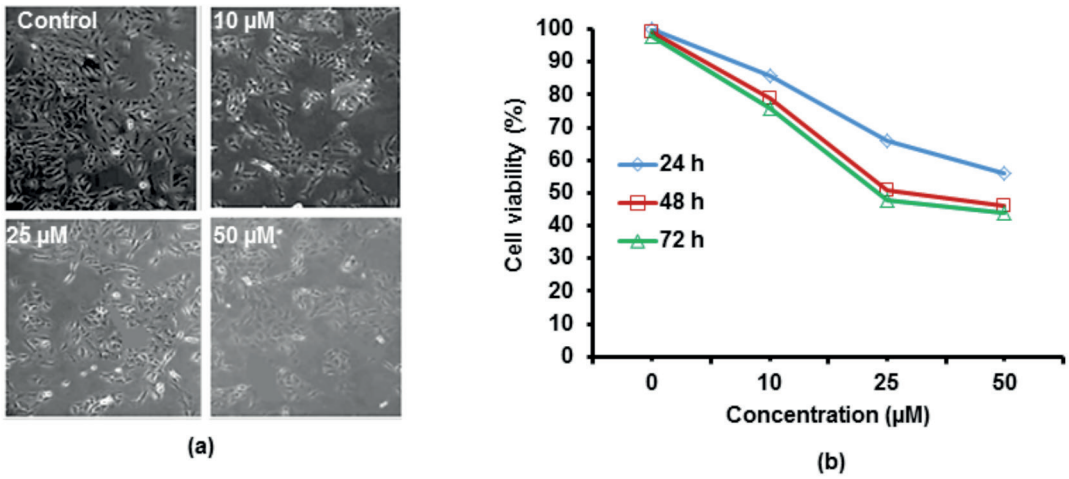

Figure 1. Caffeic acid n-butyl ester-induced alterations in (a) cell morphology and (b) percent cell viablility. A549 cells were administrated with 10, 25 and 50 μM of caffeic acid n-butyl ester for 24, 48 or 72 h. Each value is mean of three replicates ± SD.

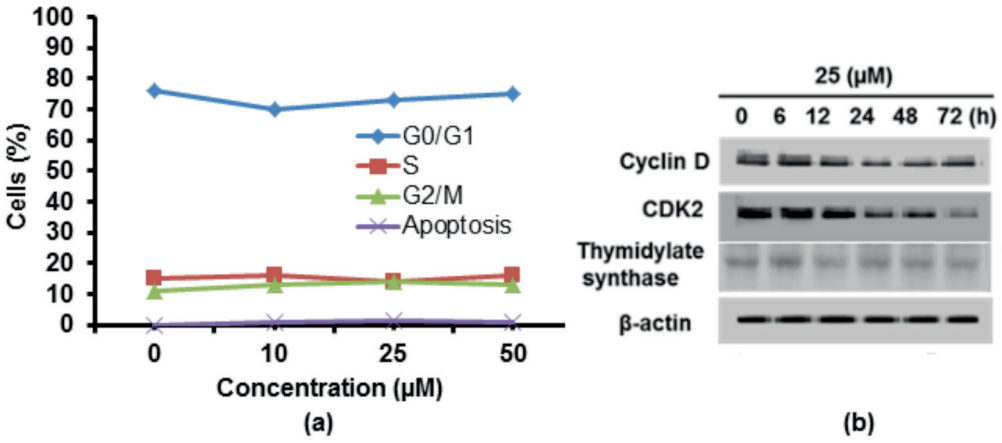

Figure 2. Caffeic acid n-butyl ester triggered S phase arrest and regulated protein expression in A549 cells. Cells were administrated with indicated concentrations of caffeic acid n-butyl for 24 h. ***(a)*** The cells were then collected and examined for cell cycle distribution Each point is mean of three replicates ± SD. ***(b)*** Western blots showing expression of cyclin D, CDK12 and thymidylate synthase in caffeic acid n-butyl ester-treated A549 cells. Each experiment was carried out in at least three times and β-actin was used as an internal control.

and estimation of A549 cell cycle was carried out. Our results indicated that caffeic acid n-butyl ester triggered S phase arrest (Figure 2a). The administration of A549 cells with 10-50 μM caffeic acid n-butyl ester for 24 h caused a greater number of cells in S phase (19%) than control (11%). Also, the decrease in the expression level of cyclin D, CDK12 and thymidylate synthase was observed in caffeic acid n-butyl ester-administrated A549 cells for 6-72 h.

Caffeic Acid n-butyl Ester Augmented the Accretion of ROS and Ca2+, and Reduced the ΔΨm Level

Cells were administrated with 25 μM caffeic acid n-butyl ester for various time periods and the levels of ROS, Ca^{2+} and $\Delta\Psi_m$ were evaluated (Figure 3). A considerable upsurge in intracellular ROS (Figure 3a) and cytosolic Ca^{2+} (Figure 3b), and a significant reduction of $\Delta\Psi_m$ level (Figure 3c) were experienced in the caffeic acid n-butyl ester-treated A549 cells as compared to the control. Moreover, it was detected that caffeic acid n-butyl ester treatment considerably augmented the ROS levels from 12 h to 72 h than the control (Figure 3). Similarly, early caffeic acid n-butyl ester treatment (6 h) increased cytosolic Ca^{2+} level significantly till 72 h exposure (Figure 3b). Furthermore, caffeic acid n-butyl ester considerably reduced $\Delta\Psi_m$ level around 35% in 12 h administration in A549 cells compared to control (Figure 3c).

Caffeic Acid n-butyl Ester-induced Alterations in ATP Levels

A549 cells were administrated with various concentrations of caffeic acid n-butyl ester, and the ATP level was measured. The results indicated that caffeic acid n-butyl ester reduced ATP levels up to 40% at the caffeic acid n-butyl ester 10-50 μM in A549 cells (Figure 3d).

Caffeic Acid N-Butyl Ester Prompted DNA Damage in A549 Cells

The cells were separated from caffeic acid n-butyl ester and DNA damage was evaluated by comet assay as well as DAPI staining. Our results indicated that caffeic acid n-butyl ester caused DNA damage dose-dependently as evident from the comet assay (Figure 4a). DAPI staining further confirmed that caffeic acid n-butyl ester-induced dose-dependent DNA breakage (Figure 4b) as evident from the greater density of white color nuclei.

Caffeic Acid n-butyl Ester Caused Alterations Oxidative Stress and Apoptosis-related Protein Induction

Western blot analysis revealed that caffeic acid n-butyl ester-inhibited the expression of GST, CAT and SOD (Mn) but exhibited no effects on SOD (Cu) expression in A549 cells (Figure 4c). Moreover, caffeic acid n-butyl ester-stimulated the expression of cytochrome in A549 cells. However, no effect was observed in PARP caspa-

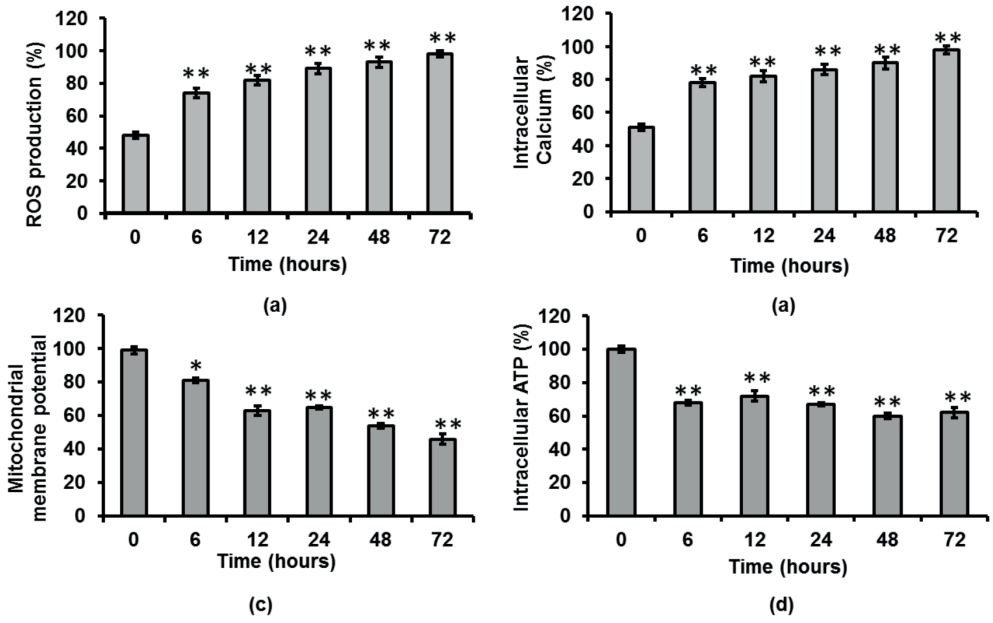

Figure 3. Caffeic acid n-butyl ester caused alterations in the levels of ***(a)*** ROS ***(b)*** $\Delta\Psi_m$ ***(c)*** Ca^{2+} production, and ***(d)*** ATP content in A549 cells. Cells were adminstrated with 50-μM caffeic acid n-butyl for 0, 6, 12, 24, 48 or 24 h. Each experiment was done in triplicates and expressed as mean ± SD (*p<0.05, **p<0.001).

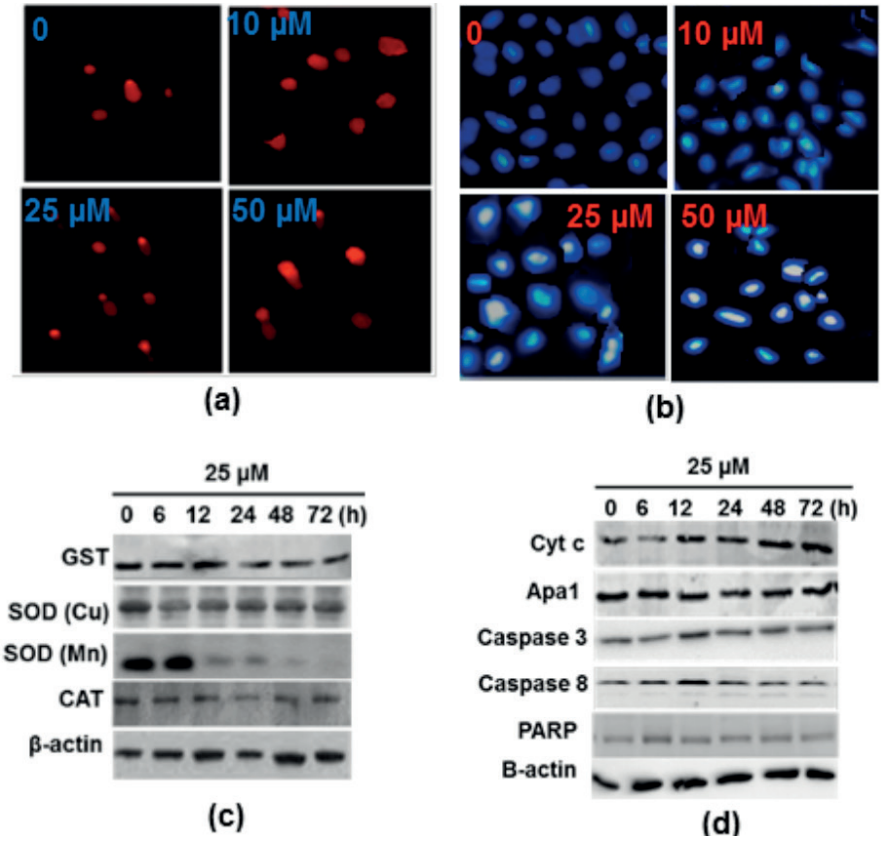

Figure 4. Cells were administrated with various concentrations of caffeic acid n-butyl ester (10, 25 and 50 μM) for 48 h. The cells were then collected and assessed for DNA damage by (a) Comet assay (b) DAPI staining. Western blots showing caffeic acid n-butyl ester-induced the changes in the expression levels of (c) GST, CAT, SOD (Cu), SOD and (Mn) and (d) cytochrome c; PARP, caspase-3, caspase-8, and Apaf-1.

se-3, caspse-8, Apaf-1 protein expression levels in A549 cells (Figure 4d). Taken together, these results indicate that caffeic acid n-butyl ester prompted non-apoptotic cell death in A549 cells.

Discussion

Anti-cancer drugs that induce apoptosis of cancer cells are considered effective in cancer treatment[13-15]. It has been reported that extracts prepared from many plants as well as semi-synthesized compounds can induce apoptotic processes[16,17]. The present study first time reports that caffeic acid n-butyl ester inhibits human lung carcinoma A549 cell line growth through the generation of ROS. Treatment with caffeic acid n-butyl ester lead to accumulation of A549 cells in S phase and underwent necrosis and not apoptosis in a concentration and time-dependent manner. It is well established that several anticancer agents arresting cell cycle progression by downregulating the expression of cyclin D, CDK2 and thymidylate synthase levels, are essential players of DNA replication during S phase. Thus, we propose that caffeic acid n-butyl ester blocked A549 cells in the S phase by augmenting the expression of these proteins.

Our findings indicated that caffeic acid n-butyl ester reduced the $\Delta\Psi_m$ (Figure 3c) and ATP levels (Figure 3d). Moreover, expression analysis by Western blotting revealed that caffeic acid n-butyl ester administration exhibited no significant change in the expression of Apf-1 caspase-3, caspase-8, and PARP proteins. Thus, we suggest that A549 cell death induced by caffeic acid n-butyl ester may not be mediated through apoptotic signaling.

The outcomes of this study also indicated that caffeic acid n-butyl ester augmented the accretion of ROS in A549 cells (Figure 3a). Previous studies[18-20] revealed that reduction in ROS induced by several anticancer drugs is positively associated inhibition of tumor growth and the upsurge of ROS is associated with the apoptotic response. Moreover, it is now well established that elevated ROS generation induces apoptosis by inducing mitochondria permeability transition pore opening, discharge of pro-apoptotic factors and stimulation of cspase-9[21]. Nevertheless, necrosis is accompanied by a loss of $\Delta\Psi_m$ and ATP [22]. Consistent with this, our results revealed that caffeic acid n-butyl ester reduced both the $\Delta\Psi_m$ (Figure 3c) and ATP levels (Figure 3d) in A549 cells. Consequently, our results are in good agreement with other investigations as the generation of ROS, loss of $\Delta\Psi_m$ and ATP exhaustion in mammalian cells causes necrosis due to failure to apoptosome formation[23].

Conclusions

Taken together, our findings indicate that lung cancer A549 cells are subtle to caffeic acid n-butyl ester and their cell death is due to a necrosis-like event triggered by ROS-mediated reduction in $\Delta\Psi_m$ and exhaustion of ATP levels. The present study paves the way for further evaluation of caffeic acid n-butyl ester.

Conflict of interest

The authors declare no conflicts of interest.

References

1) Herbst RS, Heymach JV, Lippman SM. Lung cancer. N Engl J Med 2008; 359: 1367-1380.
2) Shi WY, Liu KD, Xu SG, Zhang JT, Yu LL, Xu KQ, Zhang TF. Gene expression analysis of lung cancer. Eur Rev Med Pharmacol Sci 2014; 18: 217-218.
3) Marder M, Viola H, Bacigaluppo JA, Colombo MI, Wasowski C, Wolfman C, Medina JH, Rúveda EA, Paladini AC. Detection of benzodiazepine receptor ligands in small libraries of flavone derivatives synthesized by solution phase combinatorial chemistry. Biochem Biophys Res Commun 1998: 249; 481-485.
4) Nagaoka T, Banskota AH, Tezuka Y, Saiki I, Kadota S. Selective antiproliferative activity of caffeic acid phenethyl ester analogues on highly liver-metastatic murine colon 26-L5 carcinoma cell line. Bioorg Med Chem 2002; 10: 3351-3359.
5) Alonso DF, Farías EF, Urtreger A, Ladeda V, Vidal MD, Bal De Kier Joffe E. Characterization of F3II, a sarcomatoid mammary carcinoma cell line originated from a clonal subpopulation of a mouse adenocarcinoma. J Surg Oncol 1996; 62: 288-297.
6) Takagaki N, Sowa Y, Oki T, Nakanishi R, Yogosawa S, Sakai T. Apigenin induces cell cycle arrest and p21/WAF1 expression in a p53-independent pathway. Inter J Oncol 2005; 26: 185-190.
7) Urtreger AJ, Ladeda VE, Puricelli LI, Rivelli A, del Carmen Vidal M, Sacerdote de Lustig E, Bal de Kier Joffé ED. Modulation of fibronectin expression and proteolytic activity associated with the invasive and metastatic phenotype in two new murine mammary tumor cell lines. Inter J Oncol 1997; 11: 489-496.
8) Lu HF, Chen YS, Yang JS, Chen JC, Lu KW, Chiu TH, Liu KC, Yeh CC, Chen GW, Lin HJ, Chung JG. Gypenosides induced G0/G1 arrest via inhibition of cyclin E and induction of apoptosis via activation of caspases-3 and -9 in human lung cancer A-549 cells. In Vivo 2008; 22: 215-221.

9) Hsu SC, Kuo CL, Lin JP, Lee JH, Lin CC, Su CC, Lin HJ, Chung JG. Crude extracts of Euchresta formosana radix induce cytotoxicity and apoptosis in human hepatocellular carcinoma cell line (Hep3B). Anticancer Res 2007; 27: 2415-2425.

10) Chiang JH, Yang JS, Ma CY, Yang MD, Huang HY, Hsia TC, Kuo HM, Wu PP, Lee TH, Chung JG. Danthron, an anthraquinone derivative, induces DNA damage and caspase cascades-mediated apoptosis in SNU-1 human gastric cancer cells through mitochondrial permeability transition pores and bax-triggered pathways. Chem Res Toxicol 2011; 24: 20-29.

11) Huan SK, Lee HH, Liu DZ, Wu CC, Wang CC. Cantharidin-induced cytotoxicity and cyclooxygenase 2 expression in human bladder carcinoma cell line. Toxicol 2006; 223: 136-143.

12) Chiang LC, Ng LT, Lin IC, Kuo PL, Lin CC. Anti-proliferative effect of apigenin and its apoptotic induction in human Hep G2 cells. Cancer Lett 2006; 237: 207-214.

13) Wu PP, Liu KC, Huang WW, Ma CY, Lin H, Yang JS, Chung JG. Triptolide induces apoptosis in human adrenal cancer NCI-H295 cells through a mitochondrial-dependent pathway. Oncol Rep 2011; 25: 551-557.

14) Wu SH, Hang LW, Yang JS, Chen HY, Lin HY, Chiang JH, Lu CC, Yang JL, Lai TY, Ko YC, Chung JG. Curcumin induces apoptosis in human non-small cell lung cancer NCI-H460 cells through ER stress and caspase cascade-and mitochondria-dependent pathways. Anticancer Res 2010; 30: 2125-3213.

15) Yu FS, Yang JS, Yu CS, Lu CC, Chiang JH, Lin CW, Chung JG. Safrole induces apoptosis in human oral cancer HSC-3 cells. J Dent Res 2010; 11: 168-174.

16) Calixto JB, Santos AR, Cechinel Filho V, Yunes RA. A review of the plants of the genus Phyllanthus: their chemistry, pharmacology, and therapeutic potential. Med Res Rev 1998; 18: 225-258.

17) Vergote D, Cren-Olive C, Chopin V, Toillon RA, Rolando C, Hondermarck H, Le Bourhis X. (-)-Epigallocatechin (EGC) of green tea induces apoptosis of human breast cancer cells but not of their normal counterparts. Breast Cancer Res Treat 2002; 76: 195-201.

18) Fruehauf JP, Meyskens FL, Jr. Reactive oxygen species: a breath of life or death? Clin Cancer Res 2007; 13: 789-794.

19) Ozturk G, Ginis Z, Akyol S, Erden G, Gurel A, Akyol O. The anticancer mechanism of caffeic acid phenethyl ester (CAPE): review of melanomas, lung and prostate cancers. Eur Rev Med Pharmacol Sci 2012; 16: 2064-2068.

20) Antosiewicz J, Herman-Antosiewicz A, Marynowski SW, Singh SV. c-Jun NH(2)-terminal kinase signaling axis regulates diallyl trisulfide-induced generation of reactive oxygen species and cell cycle arrest in human prostate cancer cells. Cancer Res 2006; 66: 5379-5386.

21) Iwamaru A, Iwado E, Kondo S, Newman RA, Vera B, Rodriguez AD, Kondo Y. Eupalmerin acetate, a novel anticancer agent from Caribbean gorgonian octocorals, induces apoptosis in malignant glioma cells via the c-Jun NH2-terminal kinase pathway. Mol Cancer Ther 2007; 6: 184-192.

22) Halestrap A. Biochemistry: a pore way to die. Nature 2005; 434: 578-579.

23) Green DR, Reed JC. Mitochondria and apoptosis. Science 1998; 281: 1309-1312.