J Nat Med (2010) 64:146–152
DOI 10.1007/s11418-009-0387-8

ORIGINAL PAPER

Free radical scavenging activity and antiproliferative potential of *Polygonum cuspidatum* root extracts

Yun-Wei Lin · Fu-Jung Yang · Chia-Ling Chen · Wei-Ting Lee · Ruey-Shyang Chen

Received: 14 September 2009 / Accepted: 17 December 2009 / Published online: 15 January 2010

Abstract *Polygonum cuspidatum* is widely used as a medicinal herb in Asia. In this study, ethanol and ethyl acetate extracts of *P. cuspidatum* root were assayed for their 1,1-diphenyl-2-hydrazyl (DPPH) and hydroxyl free radical scavenging activities, total phenolics content, protective effect against DNA damage, and antiproliferative activity on human lung cancer cells. The ethanol and ethyl acetate (lipophilic phase) extracts of *P. cuspidatum* had significant scavenging effects on DPPH and hydroxyl radicals. Total phenolics content of ethanol and ethyl acetate (lipophilic phase) extracts of *P. cuspidatum* were 276.78 ± 39.31 and 231.73 ± 5.04 mg/ml, respectively; both extracts protected against hydroxyl radical-induced DNA strand scission. Furthermore, the extracts of *P. cuspidatum* induced apoptosis and inhibited cell growth in A549 and H1650 cell lines, suggesting that *P. cuspidatum* root extracts exhibit an antiproliferative effect on human lung cancer cells.

Keywords *Polygonum cuspidatum* · Free radical · Antioxidant activity · Lung cancer

Introduction

Polygonum cuspidatum Sieb. et Zucc is a medicinally important plant due to the presence of anthraquinone derivatives occurring in the subterranean parts of the plant, and is widely used for treatment of various inflammatory diseases, hepatitis, tumors, and diarrhea [1]. Several active compounds of *P. cuspidatum* have been identified, including resveratrol, emodin, 2-methoxy-6-acetyl-7-methyljuglone, polygonin, emodin monoethyl ether, chrysophanol, reynoutrin, avicularin, and hyperin [2–4]. Previous research showed that resveratrol had antitumor activity [2, 5, 6] and emodin possessed antibacterial activities [7, 8]. *P. cuspidatum* extracts have been reported to inhibit bacterial DNA primase [9], and inhibit acyl-coenzyme A–cholesterol acyltransferase activity [10]. Its ethanol extract also had antiviral activity against hepatitis B virus [7]. In addition, *P. cuspidatum* has been reported to contain a large number of flavonoids, frequently found as glucosides and other constituents, including phenethyl alcohols, sterols, essential oils, and amino acids. The phenolic compounds exhibit a wide range of biological and physiological properties due to their ability to act as antioxidants, free radical scavengers, and chelators of divalent cations [11, 12].

Reactive oxygen species (ROS) are various forms of activated oxygen, which include hydroxyl radical, superoxide radical, and hydrogen peroxide. ROS and free radicals produced by ultraviolet light (UV), ionizing radiation, chemical reactions, and metabolic processes have numerous pathological effects, such as aging, cancer, coronary heart disease, and Alzheimer's disease [13, 14]. ROS have also been shown to play an important role in carcinogenesis by damaging DNA and potentially leading to mutation [15, 16]. Several anti-inflammatory, antiulcerogenic, antinecrotic, neuroprotective, and hepatoprotective drugs have antioxidant activity [17]. The use of traditional medicine is widespread and plants provide a large source of natural antioxidants that might serve as leads for the development of novel drugs. Therefore, investigations of natural

Y.-W. Lin · F.-J. Yang · C.-L. Chen · W.-T. Lee · R.-S. Chen (✉)
Department of Biochemical Science and Technology, National Chiayi University, 300 University Rd, Chiayi 60004, Taiwan, ROC
e-mail: rschen@mail.ncyu.edu.tw

antioxidants and bioactive compounds for preservation of traditional medicines and use in treating certain human diseases have received much attention.

The main objectives of the present study were to determine the free radical scavenging and antioxidant activities of root extracts of *P. cuspidatum*. In addition, we tested the effect of the ethanol extract of *P. cuspidatum* on cell viability and morphology because it was unknown whether *P. cuspidatum* had antiproliferative potential on human lung cancer cells.

Materials and methods

Preparation of *P. cuspidatum* extracts

Root of *P. cuspidatum* was purchased from a traditional Chinese medicines store in Tainan, Taiwan. A voucher specimen (NCYU-RSC-0901) was deposited at the Department of Biochemical Science and Technology, National Chiayi University, Taiwan. As plants were grown for 2 months, fresh roots and rhizomes were gathered for extraction. Commercialized root powder of *P. cuspidatum* was purchased from Yi De Co., Taiwan.

Ethanol (EtOH) extract

Dried, crushed root of *P. cuspidatum* (1 g) was extracted in 150 ml of ethanol for 1 h with shaking. The solution was filtered and concentrated in the rotary vacuum evaporator (Eyela N-1000, Tokyo Rikakikai Co.), and then lyophilized. The extract was stored at −20°C until use.

Ethyl acetate (EtOAc) extract

Root powder of *P. cuspidatum* (1 g) was extracted with 2 ml 50 mM Na-phosphate buffer (pH 7.5) and 5 ml ethyl acetate for 1 h with shaking. The extract was then centrifuged at 6000 rpm for 10 min. The aqueous and organic phases were collected as hydrophilic and lipophilic phase extracts, respectively. The hydrophilic phase was lyophilized and then resuspended in doubly distilled H_2O. The lipophilic extract was evaporated to dryness in the rotary vacuum evaporator, and dissolved in 50% ethanol. The extracts were stored at −20°C until use.

1,1-Diphenyl-2-hydrazyl (DPPH) assay

A 50-μl aliquot of 1 mM solution of DPPH in ethanol was mixed with 150 μl of *P. cuspidatum* extract solution, and incubated at room temperature for 30 min. Reduction of DPPH free radicals was determined by measuring the absorbance at 517 nm. L-Ascorbic acid was used as a positive control. The inhibition ratio (percentage) was calculated from the following equation: % of inhibition = [(absorbance of control − absorbance of test sample)/absorbance of control] × 100% [18].

Hydroxyl radical scavenging activities by electron spin resonance (ESR) spectrometry

Hydroxyl radicals were generated by the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$). The reaction mixture contained H_2O_2 (5 mM), ferrous ammonium sulfate (5 mM), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (100 mM), and different contractions of extracts in phosphate-buffered saline (pH 7.4). Deionized water and 5% ethanol were used for the blank experiment. After incubation of the reaction mixture at 25°C for 8 min, the relative signal intensity was measured by ESR spectrometry (Bruker EMX-10, Germany) using the following conditions: receiver gain, 5.02×10^5; modulation amplitude, 1.6 G; scan time, 167.8 s; filed, 3483 ± 100 G; time constant, 163.8 ms.

Determination of total phenolics content

Total phenolics content was measured according to the Folin–Ciocalteu method with slight modification [19], and gallic acid was used as standard. A 200-μl sample of extract was added to 20 μl of 20% Na_2CO_3. After 2 min, 10 μl of 50% Folin–Ciocalteu reagent was added to the mixture, which was then left for 30 min. Absorbance was measured at 750 nm using a spectrophotometer. Deionized water was used as a blank control and gallic acid as standard. The phenolic compound content was expressed as gallic acid equivalent using the following equation based on the calibration curve: absorbance = 0.019137 gallic acid (mg ml^{-1}) + 0 ($R^2 = 0.99816$).

DNA strand scission assay

The reaction mixture contained ΦX 174 RF1 supercoiled DNA (0.6 μg; New England Biolabs, Beverly, MA, USA), H_2O_2 (0.2 M), 10 mM Tris–HCl, 1 mM EDTA buffer (pH 8.0), and various concentrations of extracts were added prior to H_2O_2 addition. Hydroxyl radicals were generated by irradiation of the reaction mixtures with a 23-W UV lamp (shortwave) at a distance of 10 cm. The mixture was incubated at room temperature for 2 min and analyzed by 0.8% agarose gel electrophoresis and stained with ethidium bromide. The gels were then photographed under UV light.

Cell culture

Human lung carcinoma A549 cells derived from human alveolar type 2 cells (CCL-185; American Type Culture

Collection, Manassas, VA) and human lung adenocarcinoma H1650 cells (CRL-5883; American Type Culture Collection, Manassas, VA) were cultured in RPMI-1640 complete medium. The medium was supplemented with sodium bicarbonate (2.2%, w/v), L-glutamine (0.03%, w/v), penicillin (100 units/ml), streptomycin (100 μg/ml), and fetal calf serum (10%). A549 and H1650 cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Determination of cell growth

The H1650 cells (1×10^5) were treated with *P. cuspidatum* extracts for 24 h. The growth of cells was determined by exclusion of trypan blue staining, and the numbers of cells were counted by a hemocytometer. The stain is excluded from living cells but penetrates dead cells. The proportion of dead cells was determined by counting the cells stained by trypan blue by using a hemocytometer.

Flow cytometric analysis of cell cycle distribution

Human lung cancer A549 cells were plated in 60-mm dishes, cultured overnight, and then treated or not treated with *P. cuspidatum* extract for 24 h. The floating and adherent cells were collected by trypsinization, fixed overnight in 70% ethanol, and resuspended in propidium iodide (25 μg/ml) supplemented with 0.1% RNase A and 0.1% Triton X-100. DNA content was measured with a FACScan flow cytometer (BD Biosciences). These experiments were repeated thrice.

Statistical analysis

For each protocol, three or four independent experiments were performed. Results were expressed as mean ± standard error of the mean (SEM). Statistical calculations were performed using the SigmaPlot 2000 software (Systat Software, San Jose, CA, USA). Differences in measured variables between experimental and control groups were assessed using an unpaired *t* test. $P < 0.05$ was considered statistically significant.

Results

Free radical scavenging activities of *P. cuspidatum* extracts

The DPPH free radical scavenging activities of *P. cuspidatum* extracts are shown in Fig. 1. The ethanol and ethyl acetate (lipophilic phase) extracts from *P. cuspidatum* exhibited significant dose-dependent scavenging effects on DPPH radicals. However, the hydrophilic phase of the EtOAc extract had a relatively lower scavenging effect.

The scavenging ability of *P. cuspidatum* against ·OH was also determined. The ESR spectrum of the spin adduct formed between DMPO and hydroxl radicals generated by the Fenton reaction is a 1:2:2:1 quartet with splitting at $A_N = A_H = 15$ G (Fig. 2). The signal intensities of this DMPO–OH spin system were used to evaluate the scavenging activity of extracts from *P. cuspidatum*. The ethanol extract had a higher inhibition activity than the EtOAc extract; the hydroxyl radical scavenging activities of the ethanol extract (1.5 mg/ml) and EtOAc (lipophilic phase) (1.5 mg/ml) were 72 and 67%, respectively.

Antioxidant activities of *P. cuspidatum* extracts

Polyphenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity [20]. In this study, the total phenolics contents of ethanol and EtOAc extracts of *P. cuspidatum* were measured and expressed as milligrams of gallic acid equivalent. The ethanol extract from *P. cuspidatum* had the highest phenolics contents of 276.78 ± 39.31 mg. Total phenolics contents of EtOAc lipophilic and hydrophilic phases from *P. cuspidatum* extract were 231.73 ± 5.04 and 146.21 ± 7.80 mg/ml, respectively.

ΦX 174 RF1 DNA strand scission induced by UV photolysis of H_2O_2 was also performed to evaluate the protective effect of *P. cuspidatum* extracts on DNA under oxidative stress. The results are shown in Fig. 3. UV illumination alone did not cause DNA strand cleavage, but the treatment of supercoiled DNA with UV plus H_2O_2 led to the conversion of the DNA to open circular form. Interestingly, *P. cuspidatum* extracts can protect UV- and H_2O_2-induced

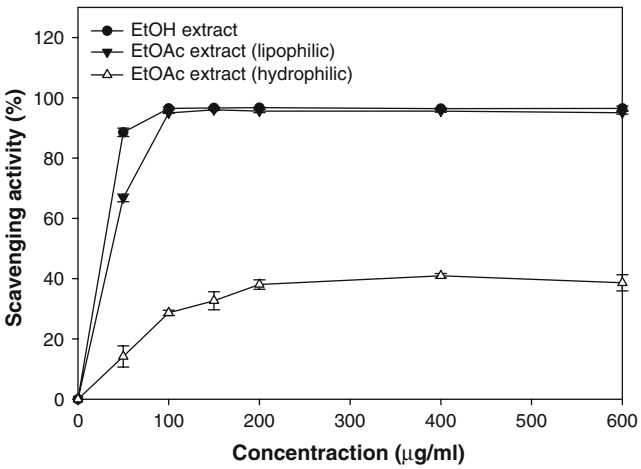

Fig. 1 DPPH free radical scavenging activities of ethanol and ethyl acetate extracts from root powder of extract derived from *Polygonum cuspidatum*

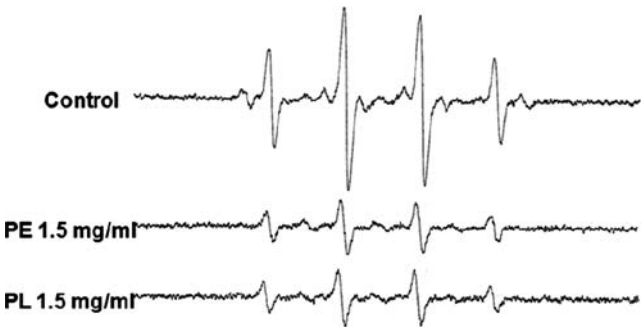

Fig. 2 Hydroxyl radical scavenging activities of ethanol and ethyl acetate extracts from root powder of extract derived from *Polygonum cuspidatum*. The signal intensities of DMPO–OH adduct were determined by electron spin resonance spectrometry and recorded 8 min after reaction initiation in phosphate-buffered solution (pH 7.4) containing 100 mM DMPO and the following reactants: *Control* (4% ethanol), 1.5 mg/ml of ethanol extract (*PE*), ethyl acetate (lipophilic levels) extract (*PL*)

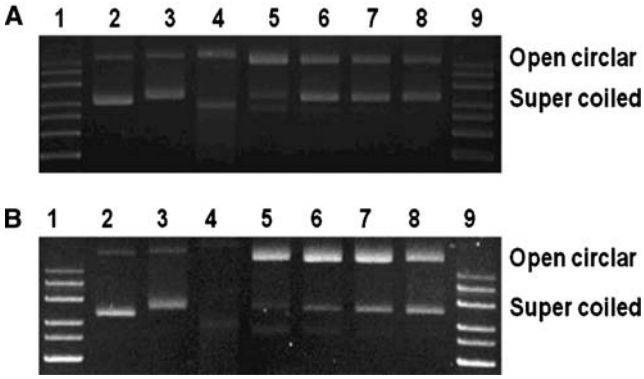

Fig. 3 Protective effect of **a** ethanol and **b** EtOAc (lipophilic phase) extracts of *Polygonum cuspidatum* against DNA strand damage induced by H_2O_2 and UV. *Lane 1* and *9*, marker; *lane 2*, ΦX 174 RF1 supercoiled DNA alone as a control; *lane 3*, ΦX 174 RF1 supercoiled DNA was exposed to UV alone; *lane 4*, UV plus H_2O_2; or plus H_2O_2 and UV in the presence of final concentration of 0.1 mg/ml (*lane 6*), 0.5 mg/ml (*lane 7*), 1 mg/ml (*lane 8*) of *Polygonum cuspidatum* extract. *Lane 5*, UV plus H_2O_2 with 4% ethanol

DNA damage in a dose-dependent manner. Co-treatment with 0.1 mg/ml of ethanol extract or 0.5 mg/ml of EtOAc (lipophilic phase) extract of *P. cuspidatum* can completely reverse the DNA damage. This result shows that *P. cuspidatum* extracts efficiently protect against DNA damage induced by UV and H_2O_2.

Antiproliferative effects of *P. cuspidatum* extract on human lung cancer cells

The cytotoxic effect of the ethanol extract of *P. cuspidatum* on lung cancer H1650 cells was examined by using the trypan blue exclusion assay. A dose-dependent growth inhibition in the H1650 cells by *P. cuspidatum* ethanol extract was observed (Fig. 4a, b). In addition, growth suppression caused by 0.2 mg/ml of the ethanol extract of *P. cuspidatum* in the H1650 cell lines was also demonstrated (Fig. 4c).

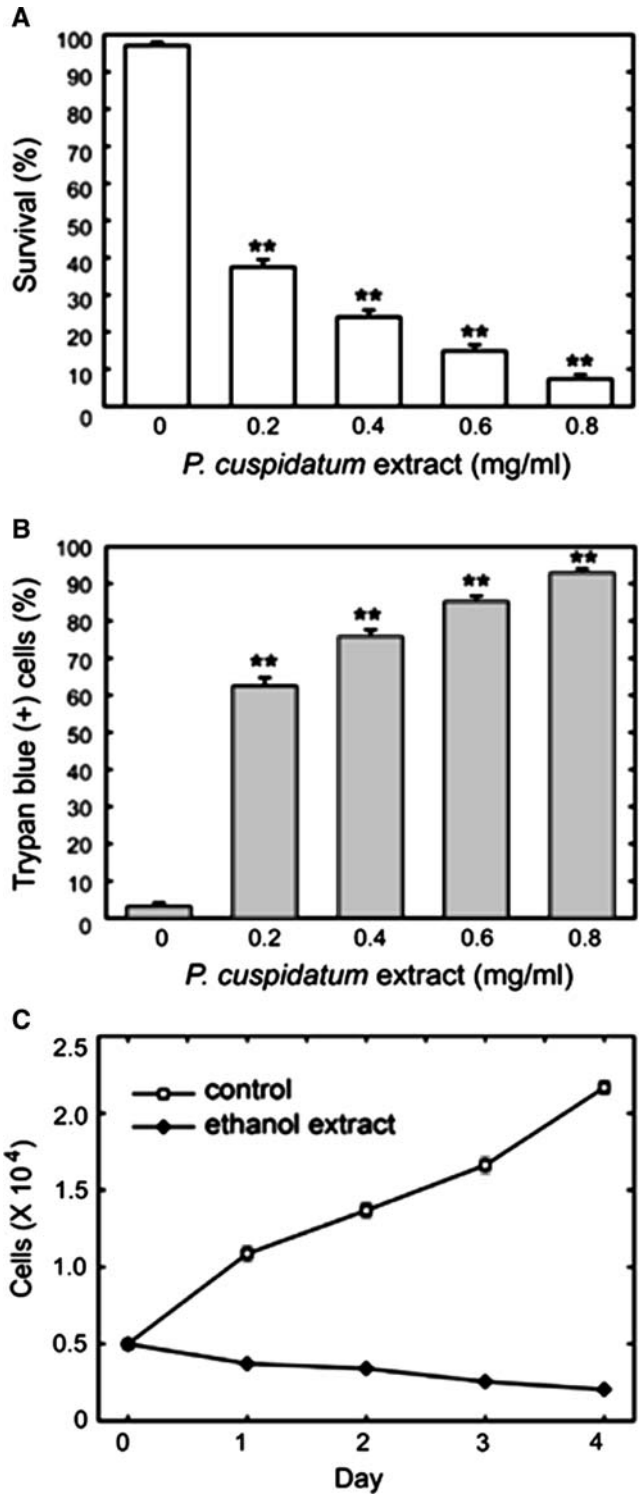

Fig. 4 Effect of ethanol extract of *Polygonum cuspidatum* on the cell viability and growth of lung cancer cell. **a** Cell survival, **b** dead cells, **c** cell growth

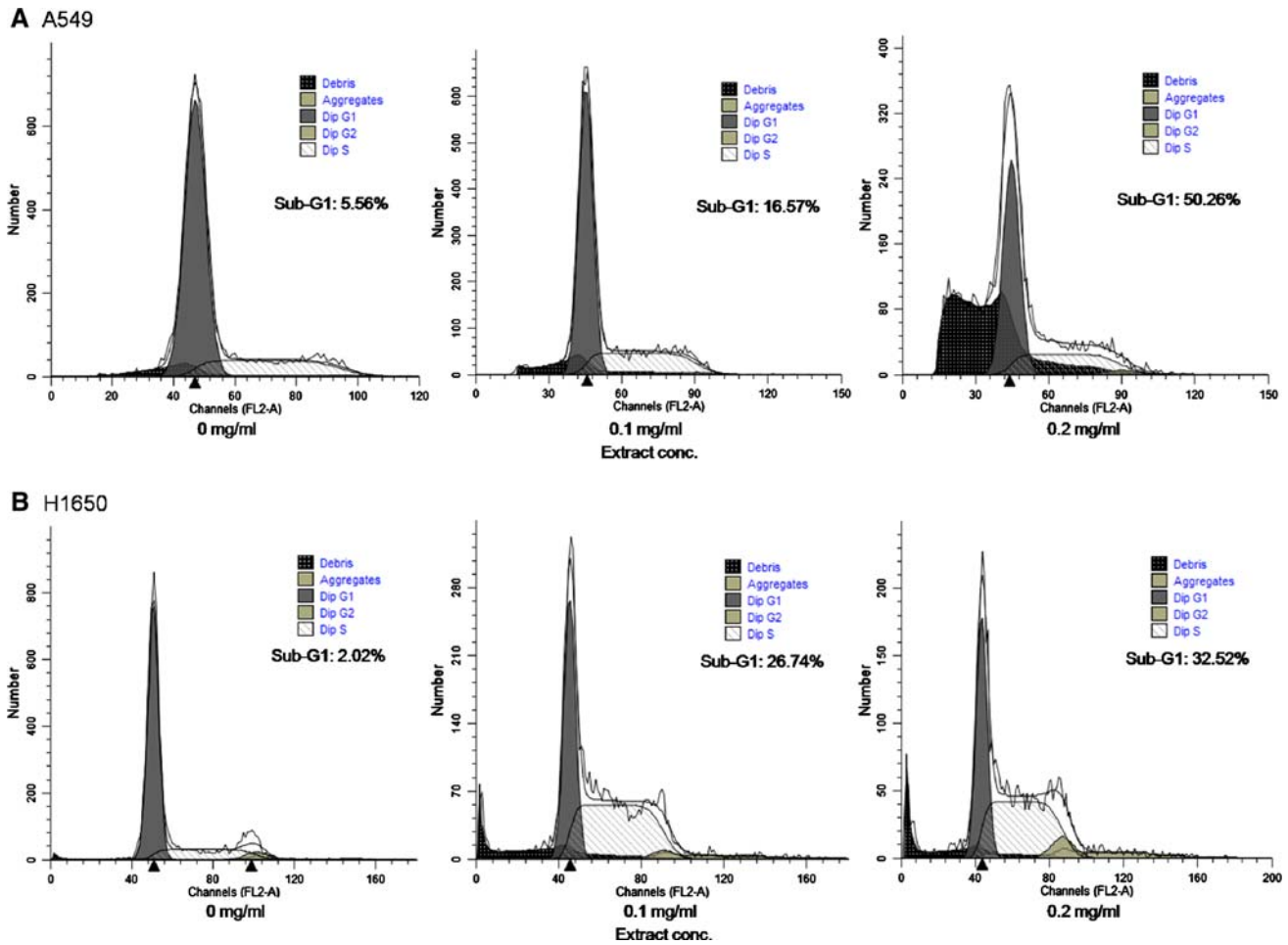

Fig. 5 Flow cytometry analyses of the DNA content of **a** A549 cells and **b** H1650 cells treated with *Polygonum cuspidatum* ethanol extract. The percentages of apoptotic cells (the sub-G1 peak) are shown in each histogram

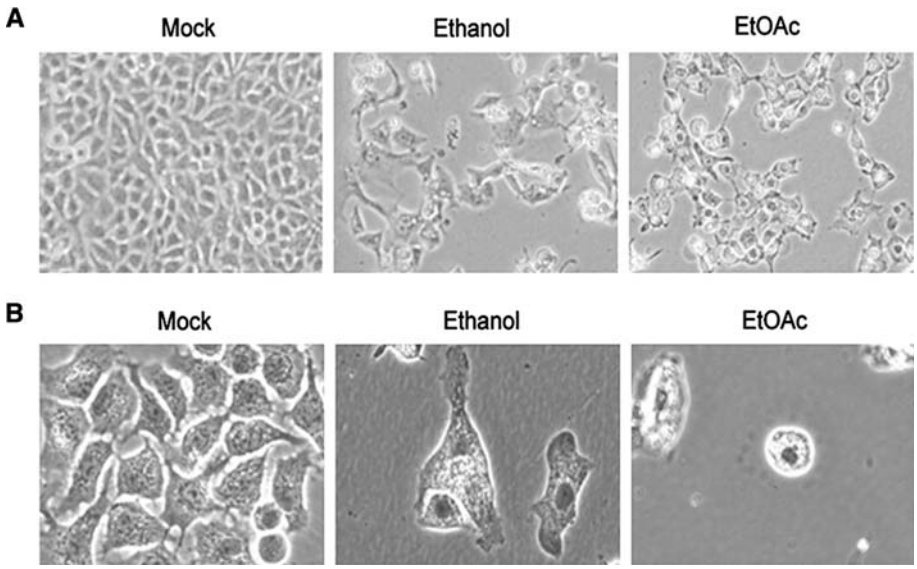

Fig. 6 Effect of ethanol and ethyl acetate (EtOAc) extract of *Polygonum cuspidatum* on the cellular and nuclear apoptotic morphology of human lung cancer A549 cells. **a** ×100, **b** ×200

Cell cycle distribution analysis was performed by flow cytometry, which enabled the identification of the cell distribution during the various phases of the cell cycle. Cells in the sub-G1 region were detected in the carcinoma A549 cells and adenocarcinoma H1650 cell line; 5.56% and 2.02% were found in the untreated cells, respectively.

This proportion increased to 50.26% and 32.52% in the A549 and H1650 cells treated with 0.2 mg/ml ethanol extract, respectively (Fig. 5). The results showed that the proportion of cells in the sub-G1 phase significantly increased on treatment with *P. cuspidatum* ethanol extract.

Effects of ethanol and ethyl acetate (EtOAc) extract of *P. cuspidatum* on the morphological changes in the A549 cell line after incubation for 24 h are shown in Fig. 6. Cellular and nuclear morphology of control (left panel) and A549 cells (right panel) treated with *P. cuspidatum* extracts were examined by light microscopy. Cells treated with *P. cuspidatum* extracts showed chromatin condensation, nuclear disintegration, and vacuolization of cytoplasm as compared with untreated A549 cells.

Discussion

P. cuspidatum has been traditionally used for treatment of various inflammatory diseases, hepatitis, tumors, and diarrhea [1]. In this study, we tested the ethanol and ethyl acetate extracts of *P. cuspidatum* for their free radical scavenging activity, total phenolics content, protective effect against DNA damage, and antiproliferative effect on human lung cancer cells.

The free radical scavenging activities of *P. cuspidatum* extracts were assayed by using the DPPH assay and ESR spectrometry. DPPH is a stable radical that has been used widely to evaluate the antioxidant activity of various natural products. The results indicated that root extracts of *P. cuspidatum* exhibit a significant DPPH radical scavenging effect (Fig. 1). We also tested the hydroxyl free radical scavenging activities of *P. cuspidatum* extracts by using ESR spectrometry. Reactive oxygen species (ROS) are able to damage a wide range of essential biomolecules [21]. Their short lifetimes make these transient free radicals difficult to detect. However, spin-trapping techniques allow in situ detection of reactive species in room temperature aqueous solutions [22]. The results shown in the present study suggest that *P. cuspidatum* is an important natural source for free radical scavengers.

The cellular damage resulting from hydroxyl radicals is strongest among free radicals. Hydroxyl radicals can attack DNA and cause strand scission. In the present study, *P. cuspidatum* extracts decreased the DNA strand scission induced by both H_2O_2 and UV radiation and showed a dose-dependent protection of DNA under oxidative stress that was similar to other plants, such as *P. aviculare* [23], suggesting that *P. cuspidatum* extracts are DNA protectors.

The use of naturally occurring antioxidants has attracted considerable attention and an increasing interest in natural antioxidants, mainly phenolic compounds, that might help prevent oxidative damage has appeared worldwide [24]. Phenolics are widely distributed in the tissue of plants and play an important role as highly effective free radical scavengers and antioxidants. The antioxidant activity of phenolics is principally due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. In addition, phenolics are potential metal chelators [12]. In the present study, the ethanol extract of *P. cuspidatum* had the highest phenolics contents of 276.78 ± 39.31 mg. Thus, the effectiveness of the antioxidant activity of *P. cuspidatum* extract is probably related to the high contents of phenolics.

The results in the present study also clearly demonstrated that *P. cuspidatum* root extracts could exhibit an antiproliferative effect by induction of apoptosis on human lung cancer cells. Apoptosis is a relatively new therapeutic target in cancer research, but these results reveal the initial potential of *P. cuspidatum* as a cytotoxic agent for therapy against human lung cancer. It is therefore worthwhile to isolate the active compounds and identity their structures. Further investigation of the in vivo activity of *P. cuspidatum* (and its constituents) in cancer-bearing mice is also necessary to exploit this nascent promise.

References

1. Choi J, Conrad CC, Malakowsky CA, Talent JM, Yuan CS, Gracy RW (2002) Flavones from *Scutellaria baicalensis* Georgi attenuate apoptosis and protein oxidation in neuronal cell lines. Biochim Biophys Acta 1571:201–210
2. Chu X, Sun A, Liu R (2005) Preparative isolation and purification of five compounds from the Chinese medicinal herb *Polygonum cuspidatum* Sieb. et Zucc by high-speed counter-current chromatography. J Chromatogr A 1097:33–39
3. Jayasuriya H, Koonchanok NM, Geahlen RL, McLaughlin JL, Chang CJ (1992) Emodin, a protein tyrosine kinase inhibitor from *Polygonum cuspidatum*. J Nat Prod 55:696–698
4. Kimura Y, Kozawa M, Baba K, Hata K (1983) New constituents of roots of *Polygonum cuspidatum*. Planta Med 48:164–168
5. Ciolino HP, Daschner PJ, Yeh GC (1998) Resveratrol inhibits transcription of CYP1A1 in vitro by preventing activation of the aryl hydrocarbon receptor. Cancer Res 58:5707–5712
6. Kimura Y, Okuda H (2000) Resveratrol isolated from *Polygonum cuspidatum* root prevents tumor growth and metastasis to lung and tumor-induced neovascularization in lewis lung carcinoma-bearing mice. J Nutr 131:1844–1849
7. Chang JS, Liu HW, Wang KC, Chen MC, Chiang LC, Hua YC, Lin CC (2005) Ethanol extract of *Polygonum cuspidatum* inhibits hepatitis B virus in a stable HBV-producing cell line. Antivir Res 66:29–34
8. Jayatilake GS, Jayasuriya H, Lee ES, Koonchanok NM, Geahlen RL, Ashendel CL, McLaughlin JL, Chang CJ (1993) Kinase inhibitors from *Polygonum cuspidatum*. J Nat Prod 56:1805–1810
9. Hegde VR, Pu H, Patel M, Black T, Soriano A, Zhao W, Gullo VP, Chan TM (2004) Two new bacterial DNA primase inhibitors from the plant *Polygonum cuspidatum*. Bioorg Med Chem Lett 14:2275–2277

10. Park CS, Lee YC, Kim JD, Kim HM, Kim CH (2004) Inhibitory effects of *Polygonum cuspidatum* water extract (PCWE) and its component resveratrol on acyl-coenzyme A-cholesterol acyltransferase activity for cholesteryl ester synthesis in HepG2 cells. Vasc Pharmacol 40:279–284
11. Kahkonen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, Heinonen M (1999) Antioxidant activity of plant extracts containing phenolic compounds. J Agric Food Chem 47:3954–3962
12. Rice-Evans CA, Miller NJ, Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 20:933–956
13. Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408:239–247
14. Halliwell B, Gutteridge JM, Cross CE (1992) Free radicals, antioxidants, and human disease: where are we now? J Lab Clin Med 119:598–620
15. Knight JA (1995) Diseases related to oxygen-derived free radicals. Ann Clin Lab Sci 25:111–121
16. Sawa T, Nakao M, Akaike T, Ono K, Maeda H (1999) Alkylperoxyl radical-scavenging activity of various flavonoids and other phenolic compounds: implications for the anti-tumor-promoter effect of vegetables. J Agric Food Chem 47:397–402
17. Repetto MG, Llesuy SF (2002) Antioxidant properties of natural compounds used in popular medicine for gastric ulcers. Braz J Med Biol Res 35:523–534
18. Hsu CY (2006) Antioxidant activity of extract from *Polygnum aviculare*. Biol Res 39:281–288
19. Duh PD, Yen GC, Yen WJ, Wang BS, Chang LW (2004) Effects of pu-erh tea on oxidative damage and nitric oxide scavenging. J Agric Food Chem 52:8169–8176
20. Bravo L (1998) Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr Rev 56:317–333
21. Halliwell B, Gutteridge JM (1990) Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 186:1–85
22. Quaresima V, Ferrari M (1998) Current status of electron spin resonance (ESR) for in vivo detection of free radicals. Phys Med Biol 43:1937–1947
23. Hsu CY, Chan YP, Chang J (2007) Antioxidant activity of extract from *Polygnum cuspidatum*. Biol Res 40:13–21
24. Stalikas CD (2007) Extraction, separation, and detection methods for phenolic, acids and flavonoids. J Sep Sci 30:3268–3295