



## *Phyllanthus urinaria* triggers the apoptosis and Bcl-2 down-regulation in Lewis lung carcinoma cells

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### Abstract

*Phyllanthus urinaria* (*P. urinaria*), a widely used herb medicine, was tested for the anticancer effect in its water extract for the first time. The water extract of *P. urinaria* significantly decreased the number of Lewis lung carcinoma cells in a dose- and time-dependent manner as determined by MTT assay. However, the water extract of *P. urinaria* did not exert any cytotoxic effect on normal cells such as endothelial cells and liver cells. Result from flow cytometry revealed a dose-dependent increase of dead cells 24 hours after treating Lewis lung carcinoma cells with *P. urinaria* extract. The anticancer activity of *P. urinaria* extract was due to the apoptosis induced in Lewis lung carcinoma cells, which was demonstrated by DNA fragmentation analysis and increased caspase-3 activity. The apoptosis triggered by *P. urinaria* extract in Lewis lung carcinoma cells was associated with the down-regulation of Bcl-2 gene expression, but not with p53, p21 and Bax. Furthermore, the partial inhibition of *P. urinaria*-induced apoptosis in Lewis lung carcinoma cells by pretreatment with cyclosporin A, a mitochondria permeability transition pore inhibitor, suggesting that *P. urinaria* extract induced the apoptosis of Lewis lung carcinoma cells, at least in part, through a mitochondria-associated intrinsic pathway.

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**Keywords:** *Phyllanthus urinaria*; Apoptosis; Bcl-2; Mitochondria

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## Introduction

Apoptosis, also called the programmed cell death, is a specific form of cell death characterized by several morphological and biochemical events [1,2]. It is a general physiological process to remove unwanted cells without damaging the neighboring cells and inducing inflammatory responses. In recent years, many studies have demonstrated that the dysregulation of apoptosis process is involved in the development of neoplastic transformation and tumor growth [3,4]. The induction of apoptosis in tumor cells has been shown to be the most common anticancer mechanism conjoint by many cancer therapies. Thus, to find the potential therapeutic antitumor drugs with potent and selective apoptotic effect would be valuable.

*Phyllanthus urinaria* (*P. urinaria*), one of the herbal plants belonging to the genus *Phyllanthus* (Euphorbiaceae), is widely distributed in China, Southern India and Southern America. It has long been used in folk medicine for the treatment of several diseases such as hepatitis B, nephrolithiasis and in painful disorders [5–7]. The anticancer effect of the genus *Phyllanthus* has only been reported in few papers. *Phyllanthus amarus* could protect the liver from hepatocarcinogenesis induced by N-nitrosodiethylamine in animal model [8,9]. Glycosides isolated from *Phyllanthus acuminatus* have been shown to exert anti-tumor activity on murine P-388 lymphocytic leukemia and B-16 melanoma cell lines [10,11]. Anticancer molecule such as 7'-hydroxy-3',4',5,9,9'-pentamethoxy-3,4-methylene dioxy ligan has also been isolated from the ethyl acetate fraction of *P. urinaria* [12]. However, the mechanism responsible for the anticancer effect is still not clear and needs further investigation. Our initial study has found that the water extract of *P. urinaria* could inhibit the tumor growth of LLC cells transplanted on C57/BL mice. Therefore, we carried out the experimental study on the effect of *P. urinaria* on cultured LLC cells.

In this study, we demonstrated that the water extract of *P. urinaria* could exhibit potent anticancer activity by inducing the apoptosis of Lewis lung carcinoma cells. Many genes such as p53, p21, and genes in Bcl-2 family have been demonstrated to play important roles in deciding the initiation and execution of apoptosis in tumor cells exposed to radiation or anticancer drugs [4,13]. The expression of these genes associated with the apoptosis induced by *P. urinaria* in Lewis lung carcinoma cells were investigated. Cyclosporin A, a mitochondria permeability transition pore inhibitor [14,15], was also used to examine the involvement of mitochondria-related pathway in the *P. urinaria*-induced apoptosis of Lewis lung carcinoma cells.

## Methods

### *Preparation of Phyllanthus urinaria extract*

*P. urinaria* used in this study was identified by Dr. Rong-Chi Yang, the chief of the Chinese Herbal Pharmacy in Chang Gung Memorial Hospital, based on the definition described in Flora of Taiwan [16]. The voucher specimen of *P. urinaria* was deposited and numbered “173156” in the herbarium of National Taiwan University (Taiwan, R.O.C.). The whole plant was minced and extracted with boiling water in the proportion of 1:20 (w/v) for 4 hours and repeated for another 4 hours after adding the same amount of water again. The resulting crude extract was filtered and lyophilized down to dry powder. In average, the yield was 26.4% (w/w) from whole plant of *P. urinaria*. *P. urinaria* extract used in the experiments was prepared by dissolving the dry powder extracted from 100 mg original whole plant in 1 ml sterile water, filtered and used as a 100 mg/ml stock.

### Cell culture

Lewis lung carcinoma cells and WRL 68, human embryonic liver cells were purchased from Culture Collection and Research Center (Taiwan, R.O.C.) and grown in DMEM supplemented with 10% FCS and antibiotics. Human endothelial cells were isolated from umbilical cord vein (HUVECs) and grown in EGM provided by Clonetics (MD, U.S.A.). Cells were maintained in a humidified atmosphere with 5% CO<sub>2</sub>/95% air at 37 °C. To examine the effect of *P. urinaria* extract on cell growth, cells at 70% confluence were treated with 0–3 mg/ml of *P. urinaria* extract for 24 hrs. For longer treatment (0–3 days), cells at 40–50% confluence were used instead.

### MTT assay

Cells with or without *P. urinaria* treatment were washed once with PBS, followed by adding 1 ml DMEM containing 0.05 mg/ml 3-[4, 5]-2, 5-diphenyltetrazolium bromide (MTT). After incubation at 37 °C for 1 hour, the media were removed and formazan crystals in the cells were solubilized in 1 ml DMSO for OD reading at 570 nm using a spectrophotometer.

### Flow cytometry

Lewis lung carcinoma cells with or without *P. urinaria* treatment were washed, and harvested in PBS containing 0.1% bovine serum albumin (BSA). Cells were then fixed in 70% ethanol for at least one hour on ice. Cells were pelleted and resuspended in 0.5 ml PBS containing 0.1% BSA, 0.08 mg/ml propidium iodide and 0.2 mg/ml RNase A to stain the nuclear DNA at 37 °C for 30 min, then analyzed by flow cytometry. The percentage of cells in sub-G1 population was calculated as below, and used to indicate the extent of cell apoptosis [17].

$$\text{Percentage of cells in sub - G1 population} = \frac{\text{Number of cells in sub - G1}}{\text{Total number of cells}} \times 100\%$$

### DNA fragmentation analysis

$1 \times 10^6$  Cells were lysed in 250 µl DNA digestion buffer containing 50 mM Tris.HCl, pH 8.0, 10 mM EDTA, 0.1 M NaCl and 0.5% SDS. The lysate was incubated with 0.5 mg/ml RNase A at 37 °C for 1 hr, and then with 0.2 mg/ml proteinase K at 50 °C overnight. Phenol extraction of this mixture was carried out and DNA in the aqueous phase was precipitated by 25 µl 7.5 M ammonium acetate and 250 µl isopropanol. DNA electrophoresis was performed in 1.5% agarose gel containing 1 µg/ml ethidium bromide at 70 volt, and DNA fragments were visualized by exposing the gel to UV light and photographed.

### Caspase-3 activity

$5 \times 10^6$  cells were lysed in 100 µl lysis buffer containing 10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 350 µg/ml PMSF, 10 µg/ml pepstatin, 10 µg/ml aprotinin and 20 µg/ml leupeptin. Cells were homogenized by three cycles of freeze-thaw method and then centrifuged to

remove cell debris. For each reaction, 40  $\mu$ g of protein sample was incubated in buffer containing 10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT supplemented with 10  $\mu$ M Ac-DEVD-AFC for 3 hours at room temperature and then OD<sub>390</sub> was measured using a spectrophotometer [18].

### RNA isolation and RT-PCR

Total cellular RNA was isolated by lysis in a guanidinium isothiocyanate buffer followed by single step phenol-chloroform-isoamyl alcohol extraction [19]. Briefly, cells were harvested and lysed in solution D containing 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sodium sarkosine and 0.1 M  $\beta$ -mercaptoethanol. Sequentially, 1/10 volume of 2 M sodium acetate (pH 4.0), one volume of phenol and 1/5 volume of chloroform-isoamyl alcohol (49:1, v:v) were added to the homogenate. After vigorous vortexing for 30 seconds, the solution was centrifuged at 10,000  $\times$  g for 15 min at 4 °C. RNA in the aqueous phase was precipitated by the addition of 0.5 ml isopropanol. One  $\mu$ g of total RNA was reverse-transcribed into cDNA by incubating with 200 units of reverse transcriptase in 20  $\mu$ l of reaction buffer containing 0.25  $\mu$ g of random primers and 0.8 mM dNTPs at 42 °C for one hour. Two  $\mu$ l of the cDNA was used for the PCR reaction as templates. The PCR was performed in buffer containing 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 1  $\mu$ M of each primer and 5 units Taq DNA polymerase for 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. The resulting PCR products were analyzed by 1.5% agarose gel electrophoresis. Sequences for the specific primers used in the PCR were summarized in Table 1.

### Statistical analysis

All statistical analyses were performed using SigmaStat statistical software (version 2.0, Jandel Scientific, CA, U.S.A.). Results were represented as means  $\pm$  SEM. ANOVA was carried out when multiple comparisons were evaluated. Values were considered to be significant at *p* less than 0.05.

Table 1  
Oligonucleotides used in RT-PCR

PCNA	sense: 5'-CGCGCAGAGGGTTGGTAGTT-3' antisense: 5'-AAGCCTTCGGAGCGCAGAGT-3'
p53	sense: 5'-AAAACCTACCAAGGCAACTA-3' antisense: 5'-TGAAATATTCTCCATCGAGT-3'
p21	sense: 5'-CATGTCCGATCCTGGTGATG-3' antisense: 5'-AGTGCAAGACAGCGACAAGG
Bcl-2	sense: 5'-TGCAC CTGAC GCCCT TCAC-3' antisense: 5'-AGACAGCCAGGAGAAATCAAACAG-3'
Bax	sense: 5'-ACCAAGAAGCTGAGCGAGTGTC-3' antisense: 5'-ACAAAGATGGTCACGGTCTGCC-3'
GAPDH	sense: 5'-TTCATTGACCTCAACTACAT-3' antisense: 5'-GAGGGGCCATCCACAGTCTT-3'

## Results

### *Effect of P. urinaria on the cell viability of Lewis lung carcinoma cells and normal cells*

To study the potential anticancer effect of *P. urinaria* extract, we first analyzed the cell viability of Lewis lung carcinoma (LLC) cells after *P. urinaria* treatment by MTT assay. As shown in Fig. 1, the number of LLC cells was reduced significantly by *P. urinaria* treatment dose-and time-dependently. For 24 hrs treatment, IC<sub>50</sub> value of *P. urinaria* extract on LLC cells was determined to be 2 mg/ml. Complete loss of cells could be observed with higher dose or longer exposure of *P. urinaria* extract. However, under same treatment, *P. urinaria* extract did not cause any cell loss in normal cells such as HUVECs and WRL 68 cells (Fig. 2).

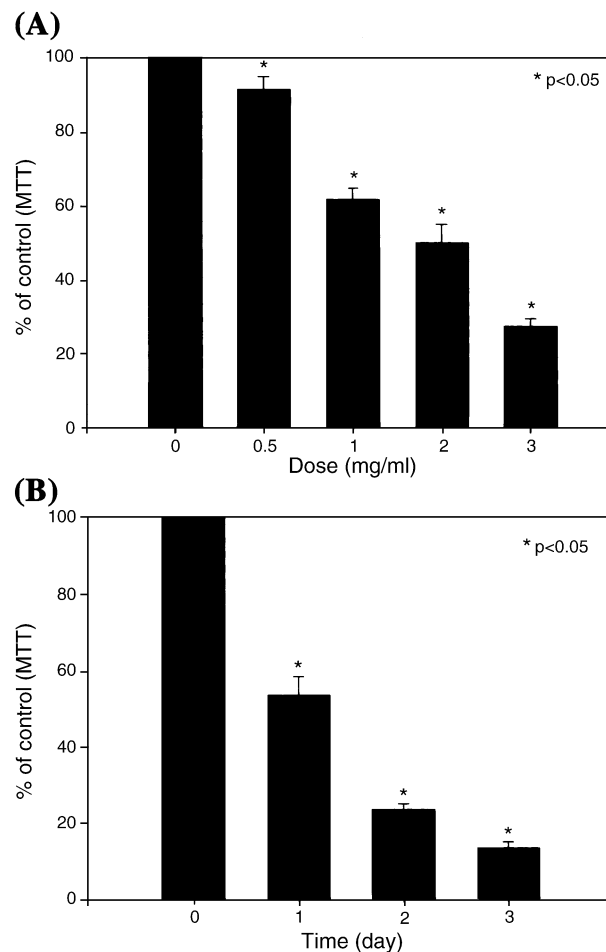


Fig. 1. Anticancer effect of *P. urinaria* extract on LLC cells. (A) Cells were treated with different concentrations of *P. urinaria* extract as indicated for 24 hours. (B) Cells were treated with 2 mg/ml of *P. urinaria* for various time periods. The cell viability was determined by standard MTT assay. The asterisk (\*) represented significantly different value from control,  $p < 0.05$ . Data were means  $\pm$  SEM calculated from three individual experiments.

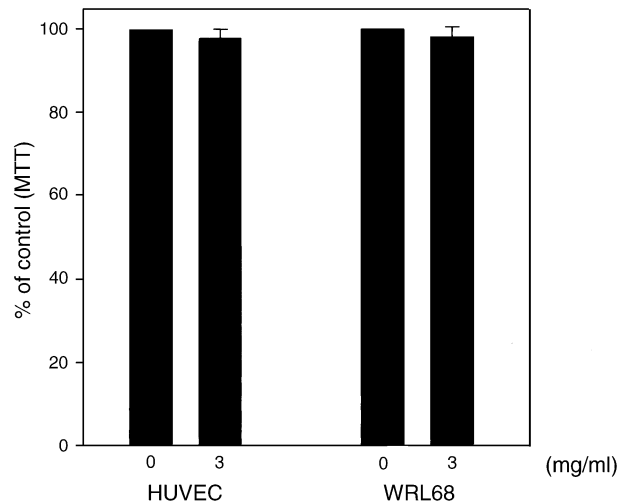


Fig. 2. Effect of *P. urinaria* extract on HUVECs and WRL 68 cells. Cells were treated with 3 mg/ml of *P. urinaria* extract for 24 hours. The cell viability was determined by standard MTT assay. Data were means  $\pm$  SEM calculated from three individual experiments.

### Flow cytometry analysis

Effect of the *P. urinaria* extract on decreasing the total number of LLC cells may be due to the inhibition of cell proliferation or the induction of cell death. To study the acting mechanism of *P. urinaria* extract, flow cytometry was used to analyze the cell cycle distribution of LLC cells after 24 hours treatment with *P. urinaria* extract. The percentage of LLC cells in phase G1, S and G2 + M after the *P. urinaria* treatment was significantly decreased dose-dependently. G1/S ratio was calculated to be  $3.0 \pm 0.03$ ,  $3.13 \pm 0.37$ ,  $2.86 \pm 0.22$ ,  $1.32 \pm 0.11$  and  $1.52 \pm 0.28$  for cells treated with 0, 0.5, 1, 2 and 3 mg/ml *P. urinaria* extract, respectively. *P. urinaria* treatment obviously did not result in the increase of G1/S ratio, the implication of inhibited cell proliferation. In contrast, the dose-dependently increased proportion of cells in sub-G1 phase from 3.71% to 54.25% after treated with *P. urinaria* extract for 24 hrs (Fig. 3), indicating that the anticancer effect of *P. urinaria* extract on LLC cells was mediated by the induction of cell death.

### Induction of apoptosis by *P. urinaria* extract

To confirm that the anticancer effect of *P. urinaria* was due to the induction of cell apoptosis, DNA fragmentation, the hallmark of cell apoptosis, was analyzed in LLC cells after *P. urinaria* treatment. As shown in Fig. 4, *P. urinaria* extract induced the typical pattern of DNA ladder in LLC cells in a time-dependent manner as revealed by DNA gel electrophoresis. Caspase-3 activation is known to play a critical role in executing the apoptosis process as demonstrated by many studies [20–22]. The enzymatic activity of caspase-3, after cells were treated with *P. urinaria* extract, increased dose-and time-dependently (Fig. 5).

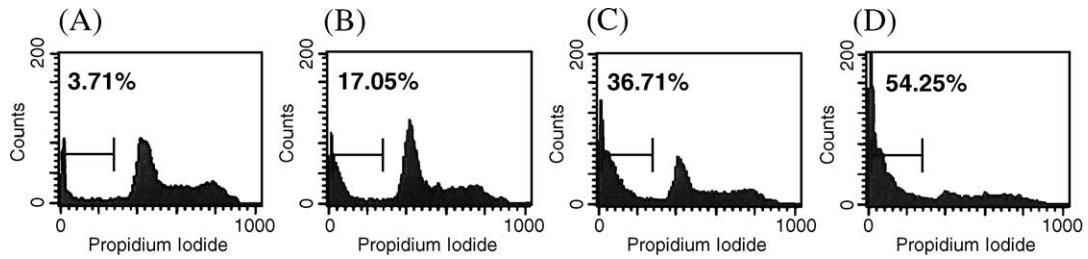


Fig. 3. Cell cycle analysis on LLC cells after *P. urinaria* treatment.  $2 \times 10^5$  cells were treated with different concentrations of *P. urinaria* as indicated for 24 hours. (A) control without *P. urinaria* treatment, (B) 0.5 mg/ml, (C) 1 mg/ml, (D) 2 mg/ml. Representative data from four individual analyses.

#### Down-regulation of *Bcl-2* gene expression in *P. urinaria*-treated LLC cells

To further investigate the molecular mechanism responsible for the *P. urinaria*-induced apoptosis in LLC cells, the gene expression of some apoptosis-related genes such as p53, p21, Bax and Bcl-2 was analyzed by RT-PCR. In Fig. 6, *P. urinaria* treatment caused the down-regulation of Bcl-2 gene expression, while other genes were not affected, which therefore resulted in the relative increase of Bax/Bcl-2 ratio. The unchanged expression of proliferation cell nuclear antigen (PCNA), an universal marker for cell proliferation, further confirmed that the anticancer effect of *P. urinaria* was not acting by interfering the cell proliferation.

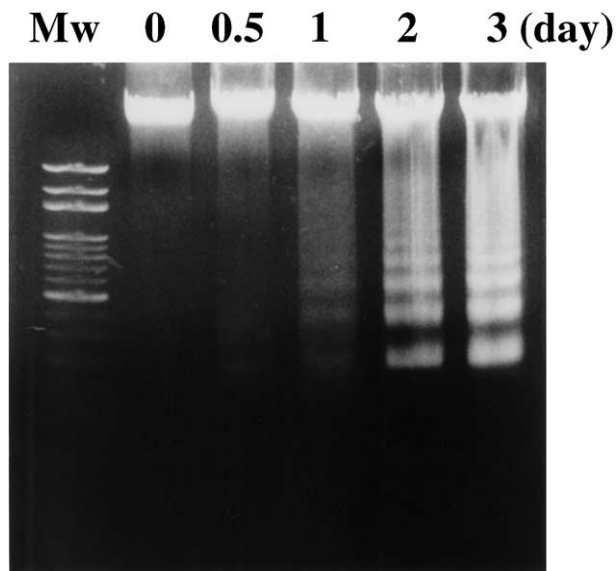


Fig. 4. *P. urinaria* induced the DNA fragmentation in LLC cells time-dependently. Cells were treated with 2 mg/ml of *P. urinaria* for different time intervals. Cells were harvested and isolated DNA was resolved by gel electrophoresis. Representative data from two individual analyses.

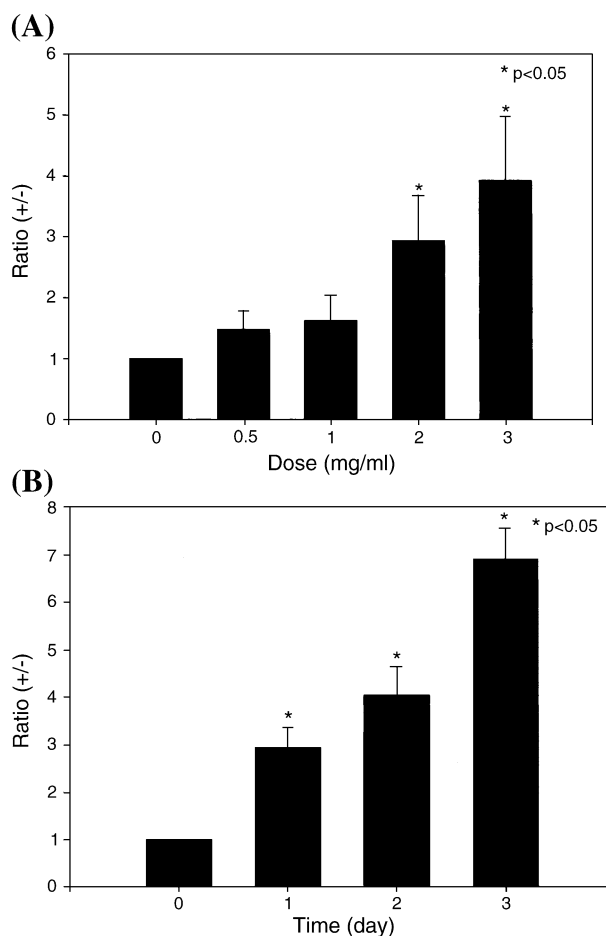


Fig. 5. Increased caspase-3 activity in *P. urinaria*-treated LLC cells. (A) Cells were treated with different concentrations of *P. urinaria* as indicated for 24 hours. (B) Cells were treated with 2 mg/ml of *P. urinaria* for different time intervals. Cells were harvested and processed for caspase-3 activity determination. Value of ratio (+/-) represented the relative intensity of caspase-3 activity in *P. urinaria*-stimulated cells compared to that of control cells. The asterisk (\*) represented significantly different value from control,  $p < 0.05$ . Data were means  $\pm$  SEM calculated from three individual experiments.

#### *Cyclosporin A partially inhibited the P. urinaria-induced apoptosis*

The down-regulation of Bcl-2 expression is known to be involved in the release of cytochrome c from mitochondria. Cyclosporin A, a potent mitochondria membrane transition pore inhibitor, was used in this study to investigate the role of mitochondria in this *P. urinaria*-induced apoptosis pathway. Pretreatment of LLC cells with cyclosporin A at concentrations of 1–7.5  $\mu$ M according to previous similar studies [23–25], dose-dependently increased the cell viability of LLC cells after *P. urinaria* treatment. However, the maximal prevention of LLC cells from apoptosis induced by *P. urinaria* extract was about  $66 \pm 9\%$  (Fig. 7).

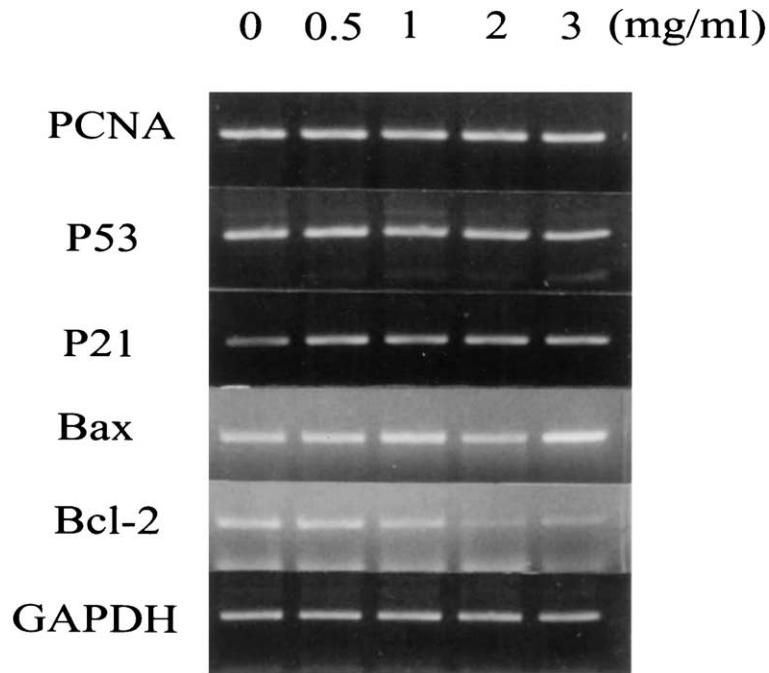


Fig. 6. *P. urinaria* induced the Bcl-2 down-regulation in LLC cells. Cells were treated with different concentrations of *P. urinaria* as indicated for 24 hours and cells were harvested for RNA isolation and RT-PCR analysis. Representative data from three individual analyses.

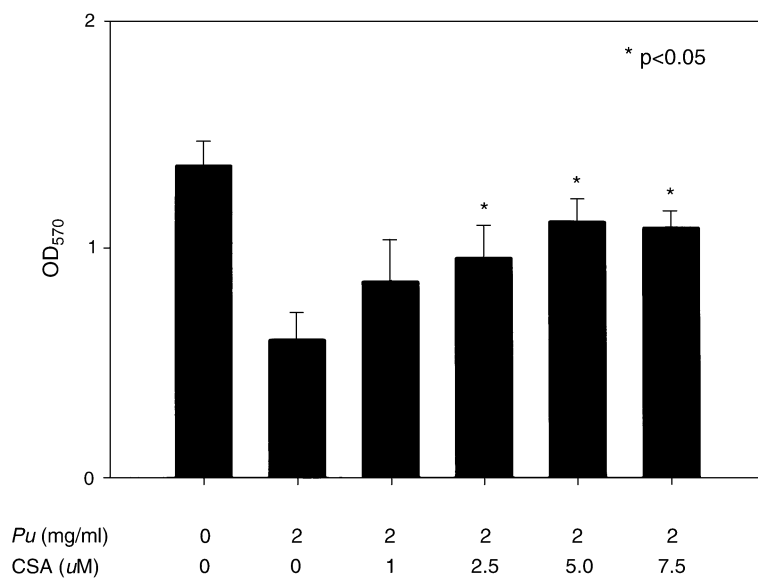


Fig. 7. Cyclosporin A partially reduced the apoptosis in *P. urinaria*-treated LLC cells.  $1 \times 10^6$  cells were pretreated with 1–7.5  $\mu$ M of cyclosporin A for one hour and then treated with 2 mg/ml *P. urinaria* for 24 hours. The cell viability was determined by standard MTT assay. The asterisk (\*) represented significantly different value from control,  $p < 0.05$ . Data were means  $\pm$  SEM calculated from three individual experiments.

## Discussion

The induction of apoptosis is known to be an efficient strategy for cancer therapy. Various stimuli including ionizing radiation, toxins, cytokines and anticancer drugs have been used in many studies to induce the apoptosis in tumor cells [26–30]. Recently, extracts prepared from a variety of plants have been discovered to exhibit ability to trigger the apoptotic process [31–33]. In this study, we have also demonstrated the induction of apoptosis in Lewis Lung carcinoma cells by *P. urinaria* treatment dose- and time-dependently. Our initial study on the in vivo effect of *P. urinaria* also showed inhibited tumor growth of LLC cells transplanted on C57/BL mice and following detailed analysis is currently being carried out in our laboratory.

*P. urinaria* has been used for long time without any clinical side effect reported. Our study also showed that *P. urinaria* extract did not produce cytotoxic effect on normal cells, suggesting that the anticancer effect of *P. urinaria* might be more specific to tumor cells. It also implied that the pH or salt concentration of *P. urinaria* extract was physiologically tolerable by normal cells.

Apoptosis is regulated and executed by different interplay of many genes responsive to various stimuli. Lung carcinoma cells have been induced by a variety of chemical reagents to undergo apoptosis through different pathways such as p53-dependent pathway or Bcl-2 family-related pathway [34,35]. To clarify the molecular mechanism of apoptosis mediated by *P. urinaria*, we examined the expression of genes including p53, p21, Bax and Bcl-2 by RT-PCR. Results indicated that *P. urinaria* induced apoptosis in LLC cells accompanied by the dose-dependent down-regulation of Bcl-2 gene expression, while others were not significantly changed. It has been demonstrated that Bcl-2 family members, such as Bcl-2 itself and Bax, are mediators of apoptosis. The balance of proapoptotic Bax and antiapoptotic Bcl-2 is known to be important in determining whether cells die or survive. Bax/Bcl-2 ratio in a cell acts to regulate its own susceptibility to apoptosis [36]. In the present study, we demonstrated that the relative increase of apoptotic Bax/Bcl-2 ratio was correlated well with *P. urinaria*-induced apoptosis in LLC cells.

Bcl-2 proteins predominantly localize on the outer mitochondrial membrane, and mediate anti-apoptotic effect by stabilizing the mitochondrial membrane, inhibiting permeability transition pore ability and the release of cytochrome c [37]. In contrast, Bax proteins predominantly localize in the cytosol, and upon activation, translocate to the mitochondria and trigger the loss of mitochondria membrane potential and mediate the release of cytochrome c [38]. Release of cytochrome c can be blocked by the mitochondria permeability transition pore inhibitor cyclosporin A. Therefore, the partial prevention of *P. urinaria*-induced apoptosis in LLC cells by cyclosporin A, suggesting *P. urinaria*-induced apoptosis might, at least in part, be correlated with the loss of mitochondrial transmembrane potential. It is likely that both the mitochondria-dependent intrinsic pathway and mitochondria-independent extrinsic pathway are involved in the apoptotic process of LLC cells induced by *P. urinaria* extract.

In this study, we showed that the apoptosis induced by *P. urinaria* extract was possibly mediated through multiple pathways, suggesting multiple ingredients, rather than single component, in *P. urinaria* may act to induce the apoptosis in Lewis lung carcinoma cells. The acting ingredients in *P. urinaria* that exerted the anticancer effect may include polyphenols such as ellagic acid, flavanoids or tanins [39], which are abundant in *P. urinaria*. However, the detailed mechanism responsible for the anticancer effect of *P. urinaria* and the actual functional components still need to be further investigated in the near future.

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