Anti-cancer Effects of *Phyllanthus urinaria* and Relevant Mechanisms

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Phyllanthus urinaria (*P. urinaria*), a widely used herbal medicine, has been reported to possess various biological activities. This report aimed to characterize the whole *P. urinaria* plant, present the anticancer effects of *P. urinaria* both *in vivo* and *in vitro*, and explore relevant mechanisms. The water extract of *P. urinaria* not only significantly reduces the cell viability of various cancer cell lines from different origins but also suppresses tumor development in C57BL/6J mice after implantation of Lewis lung carcinoma (LCC) cells. The anticancer activity of *P. urinaria* extract is mainly due to induced apoptosis of cancer cells as demonstrated by DNA fragmentation and increased caspase-3 activity through both intrinsic and extrinsic pathways. The decrease in viability with *P. urinaria* treatment might be partially associated with down-regulation of telomerase activation and induction of the apoptotic process. In addition, *P. urinaria* also exhibits anti-angiogenic activity that is mediated, at least in part, by suppression of matrix metalloproteinase 2 (MMP-2) secretion and inhibition of MMP-2 activity through zinc chelation. *(Chang Gung Med J 2010;33:477-87)*

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Phyllanthus urinaria (*P. urinaria*), one of the herbal plants belonging to the genus *Phyllanthus* (Euphorbiaceae), is widely distributed in China, South India and Southern America. It has long been used in folk medicine for the treatment of several diseases. The anticancer effect of the genus *Phyllanthus* has been reported in a few papers. *Phyllanthus amarus* protected the liver from hepatocarcinogenesis induced by N-nitrosodiethylamine in animal models.[1] The root of *Phyllanthus acuminatus* has been shown to inhibit the growth of murine P-388 lymphocytic leukemia and B-16 melanoma cell lines.[2,3] 7'-hydroxy-3',4',5,9,9'-pentamethoxy-3,4-methylene dioxy lignan isolated from the ethyl acetate extract of *P. urinaria* was shown to exhibit anticancer activity by inducing apoptosis through the inhibition of telomerase activity and Bcl-2 expression.[4] Our previous study also demonstrated that the water extract prepared from *P. urinaria* has an anticancer effect on Lewis lung carcinoma cells through a similar pathway.[5] In addition, we demonstrated that the anti-tumor and anti-angiogenic effects of *P. urinaria* in mice bearing Lewis lung carcinoma were due to interference with migration of vascular

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endothelial cells but not viability.[6] In clinical application, we often use *P. urinaria* as an anticancer drug for patients, especially in those with hepatocellular carcinoma. Therefore, *P. urinaria* became our study target and we prepared the drug with a standardized protocol under regulation and used it for further investigation. More importantly, no side effect or toxicity was reported in any of the above studies.

Consistency in composition and biological activity are essential requirements for the safe and effective application of medications. It is not easy to meet this standard with botanical preparations because of problems in plant identification, genetic variability, variable growing conditions, and differences in harvesting procedures and processing of extracts. Scientific evaluation of botanical products to elucidate mechanisms of action and clinical efficiency is difficult because of a lack of standardization of botanical products. In addition, lack of standardization and proper quality control puts public safety at great risk. Serious life-threatening toxicities associated with adulteration of herbal products have been reported in the literature.[7,8] Therefore, development of simple and effective methods for the standardization and quality control of botanical products is absolutely critical.

Chinese herbal medicine has many chemicals which could target multiple sites or act synergistically on a single site directly and indirectly. Also, Chinese medicine has multiple medical usages for the treatment of complicated diseases or multiple symptoms as well as disease prevention and improvement of quality of life. Therefore, the first issue is to characterize the compounds in *P. urinaria* as a fingerprint to ascertain batch-to-batch quality control. Second, we want to test the cytotoxicity and explore the relevant mechanisms induced by *P. urinaria in vitro* as well as *in vivo*. Third, we want to determine which compounds of *P. urinaria* are the main chemicals which exert anticancer effects if they go through the same pathways as the water extract of *P. urinaria*. Fourth, we are planning to design a clinical trial of this title plant to further investigate the anticancer effects when combined with other anticancer drugs.

Characterization of preparations of *P. urinaria* with a chemical fingerprint

Analysis of the water extracts of *P. urinaria* by high-performance liquid chromatography-mass spectrometry (HPLC/MS) led to the identification of twelve compounds. The mass structures of these compounds were tentatively assigned based on mass data mining from existing literature. The major compound in *P. urinaria* is corilagin followed by gallic acid and ellagic acid. The identities of the twelve compounds (Fig. 1A) were further confirmed by chemical markers (mass and HPLC retention times) to be gallic and ellagic acid respectively.

Gallic acid, ellagic acid and corilagin can be isolated from different plants.[9-11] Gallic and ellagic acid have demonstrated antioxidant and anti-proliferative activity through inhibition of nuclear factor kappa-B activation both *in vitro* and *in vivo*.[12] Corilagin possesses a potential anti-inflammatory effect through reducing production of the pro-inflammatory cytokines and mediators TNF-alpha, IL-1 beta, IL-6, NO (iNOS) and COX-2 on both the protein and gene levels by blocking NF-kappaB nuclear translocation.[13]

In comparison with the HPLC chromatogram of the original *P. urinara* (Fig. 1A), there were no specific changes (Fig. 1B) after treatment of *P. urinaria* at pH 1-2. This procedure was designed to imitate gastric pH in the chemical profile to mimic oral administration and gastrointestinal handling. Since the intestine and colon have anaerobic bacteria, which have β-glucuronidase activity capable of digesting glucuronides found in herbal extracts, *P. urinaria* aqueous extract was further treated with E. coli β-glucuronidase. There was a substantial change in the chemical fingerprint of *P. urinaria*. The amounts of compounds 3 (brevifolin carboxylic acid) and 12 (ellagic acid) were not altered much (Fig. 1C). Meanwhile, gallic acid was hydrolyzed completely from pH 1-2 treatment after neutralizing to pH 6.8 using NaOH and incubating at a temperature of 37°C (data not shown). This indicates that ellagic acid and brevifolin carboxylic acid could be the major compounds absorbed into the circulation. Further investigation with collection of blood and urine samples from humans or rats is needed for pharmacokinetic study in the future.

Aqueous extract of *P. urinaria* induces apoptosis *in vitro* and *in vivo*

To fully characterize the anticancer potential of water extract prepared from *P. urinaria*, the viability

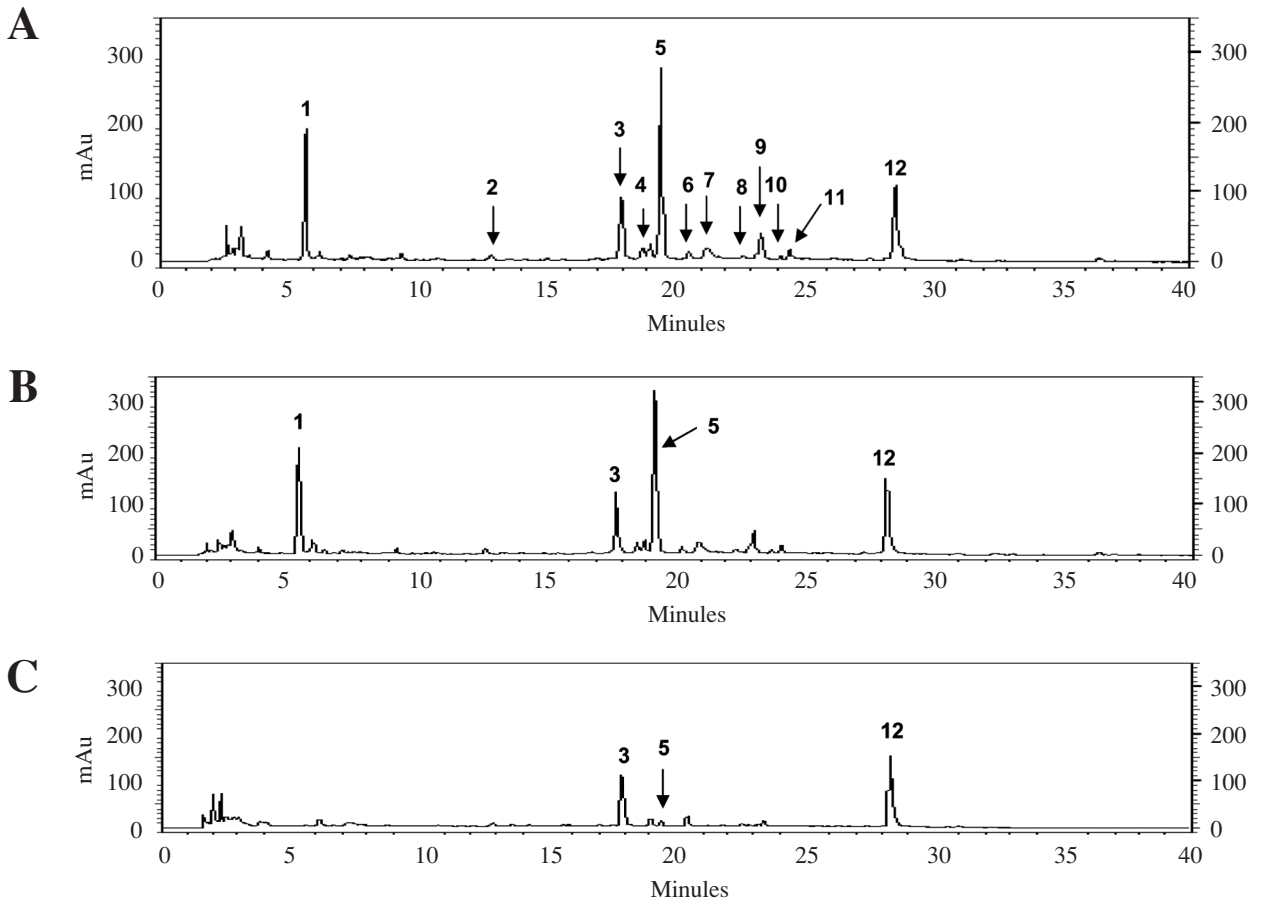

Fig. 1 HPLC chromatograms of (A) *P. urinaria* water extract and (B) *P. urinaria* water extract treated with HCL alone or (C) with HCL followed by β-glucuronidase treatment monitored at 270 nm.

of various cancer cells derived from different human origins including leukemia and solid tumors, and normal cells was investigated. The viability of these cancer cells was significantly decreased by *P. urinaria* treatment in a dose-dependent manner.[14] However, different cancer cells responded to *P. urinaria* with different sensitivities. *P. urinaria* exerted a potent effect in reducing the viability of leukemia cells including HL-60 and Molt-3, within a few hours. K562 cells and cancer cells from solid tumor origins had more tolerance to *P. urinaria* extract. Nevertheless, longer exposures up to 24 hours also resulted in a serious loss of cells. By incubating all these cells with *P. urinaria* extract for 24 hours the half maximal inhibitory concentraion (IC_{50}) was much lower for HL-60 and Molt-3 (0.12 mg/ml and 0.35 mg/ml, respectively). Meanwhile, the highest dose of *P. urinaria* (3 mg/ml) was used to treat normal cells including human endothelial cells and liver cells for 24 hours and no cytotoxic effects were found.[14] This indicated the differential cytotoxic effects of *P. urinaria* extract on different kinds of cancer cells, with no harmful effects on normal cells at higher concentrations.

The morphological changes of cancer cells after *P. urinaria* treatment were further investigated to elucidate the underlying process of reduced viability treatment. The typical morphological characteristics for cell apoptosis, such as cell condensation, plasma membrane blebbing and formation of apoptotic bodies, were confirmed under phase contrast microscopy.[14] The hallmark of cell apoptosis–DNA fragmentation- was also demonstrated in *P. urinaria*-treated cancer cells including NPC, HT-1080,

HepG2, HL-60, Molt-3 and K562. Caspase-3 activity in *P. urinaria*-treated HL-60 and Lewis lung carcinoma (LLC) cells was shown to be increased, which further confirmed that *P. urinaria* could induce cell apoptosis.[5,15]

In vivo study, we showed that the tumor formation in C57BL/6J mice with the implantation of LLC cells could be inhibited by the administration of the aqueous extract of *P. urinaria*. The initial tumor development was delayed or suppressed in the *P. urinaria* group. The average tumor size was significantly reduced in the *P. urinaria* group, clearly demonstrating the *in vivo* anti-tumor effect of *P. urinaria*. There was no obvious toxicity detected in the *P. urinaria* group as shown by the comparable mass and histological examinations of major organs including the liver, lung, spleen, kidney and heart in the *P. urinaria* group and control group.[6] The induction of apoptosis in LLCs could explain, at least in part, the inhibition of tumor growth in the mice with the administration of *P. urinaria*.

The mechanism induced by *P. urinaria* with various cancer cell lines in our previous studies[5,6,14-16] demonstrated the relevant pathways of apoptosis. There is an increase of the Bax/Bcl-2 ratio associated with the apoptosis induced by *P. urinaria* treatment in LLC and HL-60 cells. *P. urinaria* treatment in LCC cells caused the down-regulation of Bcl-2 gene expression, while other genes such as PCNA, P53, P21 and Bax were not affected, which therefore resulted in a relative increase in the Bax/Bcl-2 ratio. The unchanged expression of proliferation cell nuclear antigen, a universal marker for cell proliferation, further confirmed that the anticancer effects of *P. urinaria* was not acting to interfere cells proliferation. The down-regulation of Bcl-2 expression is known to be involved in the release of cytochrome c from mitochondria from the intrinsic pathway.[5] Cyclosporin A, a potent mitochondria membrane transition pore inhibitor, was used to investigate the role of mitochondria in this *P. urinaria*-induced apoptosis pathway. Pretreatment of LLC cells with cyclosporin A[17-19] dose-dependently increased the 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 ± 9% for partial recovery. 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.

Another important mediator of apoptosis in immune cells is the Fas receptor/ligand signaling system.[20,21] The critical elements of the Fas pathway that link receptor-ligand interaction and down-stream activation of caspases, including caspase-3, have been identified. Gene expression of both Fas receptor and Fas ligand were induced in HL-60 cells by treatment with *P. urinaria*. Induction of the Fas receptor/ligand system may result from activation of stress pathways and p53. Since HL-60 cells are p53-null, the *P. urinaria*-induced Fas receptor/ligand system in HL-60 cells was through a p53-independent pathway. The sphingomyelin pathway, a ubiquitous signaling system that links specific cell surface receptors and environmental stress to cellular responses, has been shown to mediate apoptosis in HL-60 cells induced by ionizing radiation and anticancer drugs.[22-25] Ceramide, the hydrolyzed product of sphingomyelin, was found to accumulate before the onset of apoptosis and is often linked to the activation of caspase-3 activity and the Fas receptor/ligand system.[25,26] Fumonisin B1, an inhibitor of ceramide synthase, was associated with *P. urinaria*-induced apoptosis with ceramide synthesis in HL-60 cells. This inhibitory effect of fumonisin B1 on *P. urinaria*-induced apoptosis could be eliminated by the addition of ceramide, and the viability of HL-60 cells returned to a level similar to that in HL-60 cells treated with *P. urinaria* or ceramide alone. This indicates that the activity of ceramide synthase is critical for *P. urinaria*-induced apoptosis in HL-60 cells. *P. urinaria*-induced apoptosis in HL-60 cells is mediated through a ceramide-related pathway.[15]

Aqueous extract of *P. urinaria* inhibits angiogenesis *in vitro* and *in vivo*

P. urinaria not only delayed the onset of tumor development, but also suppressed tumor growth in C57BL/6J mice.[6] It is now known that a decrease in tumor size is often associated with inhibited microvessel formation in the tumor. During the removal of tumor tissues from mice, we noted bleeding was significant in the control group, but rare in the *P. urinaria* group. This prompted us to analyze the effect of *P. urinaria* on the development of *in vivo* neovascularization in the tumor mass.

Immunostaining with anti-CD31 antibody was used to visualize the formation of microvessels in tumors. The microvessel density was markedly reduced in the *P. urinaria* group.[6] Furthermore, chick chorioallantoic membrane (CAM) assay showed *in vivo* effects of *P. urinaria* extract on phorbal-12-myristate-13-acetate (PMA)-induced angiogenesis. As shown in Fig. 2, stereomicroscopic observation showed that the vessels in the control CAM treated with phosphate buffer solution (PBS) alone were small and less branched than the newly sprouting vessels induced by PMA. The angiogenic response was effectively inhibited by the presence of *P. urinaria* extract. Addition of higher concentrations of *P. urinaria* extract led to a decrease in the angiogenic index as determined by counting the number of blood vessel branch points.[27] These results confirmed the inhibitory effect of *P. urinaria* extract on *in vivo* angiogenesis.

Angiogenesis, the formation of new blood vessels to supply enough nutrition and oxygen to a local environment,[28] is known to play an important role in several physiological and pathological processes.[29,30] The primary step in the angiogenic process relies on

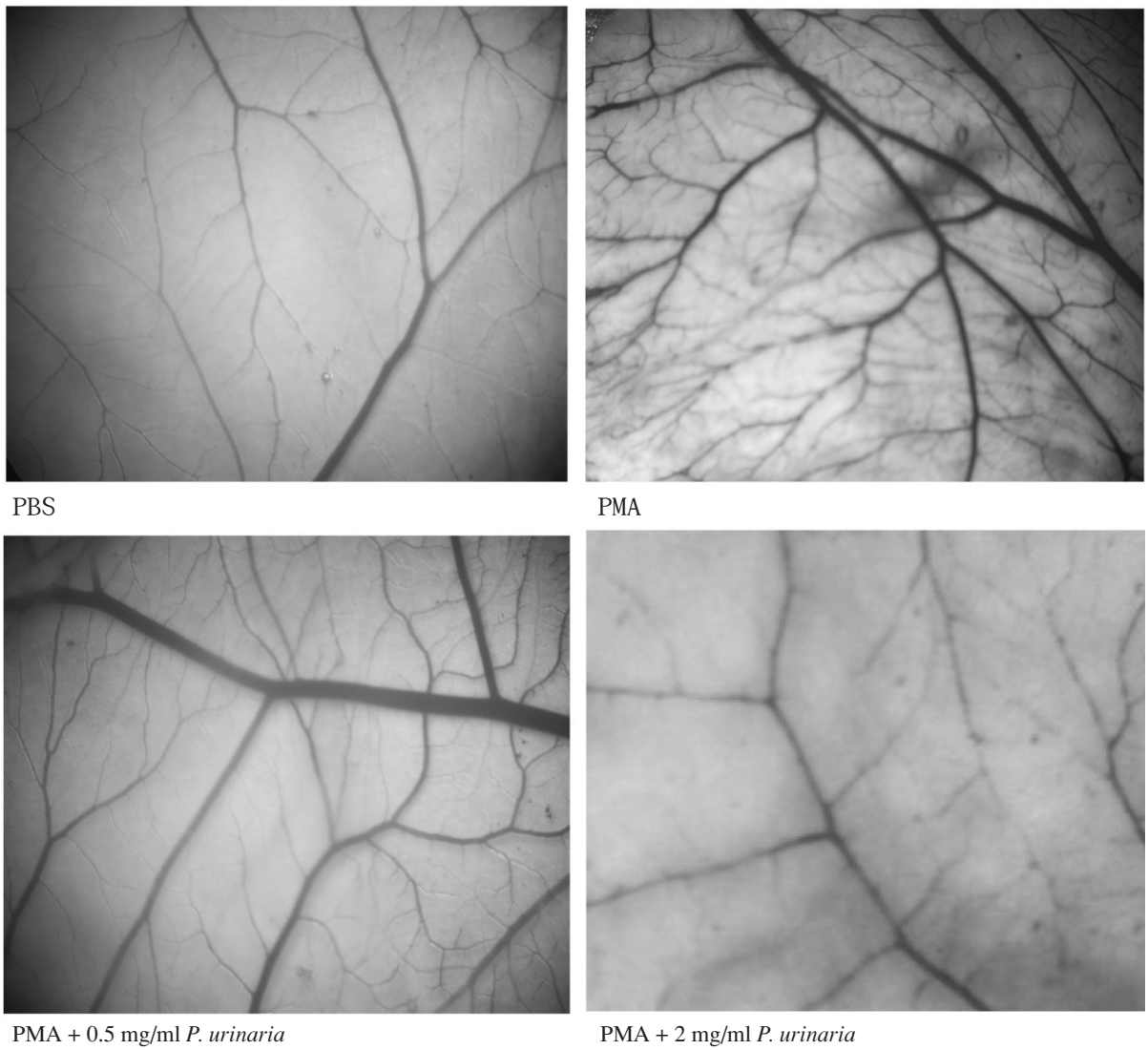

Fig. 2 *P. urinaria* extract inhibited *in vivo* angiogenesis in the chorioallantoic memebranes (CAMs) of chicken embryos.

degradation of the subendothelial basement membrane and surrounding extracellular matrix proteins.[31] Matrix metalloproteinases (MMPs), which degrade extracellular matrix proteins, are involved in angiogenesis both *in vitro* and *in vivo*.[32,33] Therefore, inhibition of the early degradation of extracellular matrix proteins predominantly by MMPs is considered an important strategy to inhibit angiogenesis. The dominant gelatin-type of MMP from CAMs is MMP-2, whereas MMP-9 is minimally present. PMA could induce MMP-2 activity and correlated well with the induction of angiogenesis. In the presence of the aqueous extract of *P. urinaria*, PMA-induced MMP-2 activity was suppressed in a dose-dependent manner (Fig. 3A). *In vitro* study, we showed the inhibitory effect of *P. urinaria* on MMP-2 activity in human umbilical vascular endothelial cells (HUVECs) by gelatin zymography (Fig. 3B).

It is known that multiple regulatory mechanisms are involved in the modulation of MMP-2 expression and activity. Neither the expression of the MMP-2 gene at the transcriptional level nor that of two other mRNAs, MMP-14 and TIMP-2, which have been known to play important roles in regulating MMP-2 activity, was affected by *P. urinaria* treatment (Fig. 4A). The anti-angiogenic effect of *P. urinaria* on vascular endothelial cells was most likely mediated by a mechanism other than MMP transcriptional regulation. However, the process of MMP-2 secretion to the extracellular environment where it actually functions could be another critical mechanism for the regulation of angiogenesis. The protein levels of MMP-2 in conditioned medium after *P. urinaria* treatment were dose-dependently decreased and correlated well with the decrease of MMP-2 activity as analyzed by gelatin zymography. Conversely, the protein levels of MMP-2 in the cytosol were dose-dependently increased after *P. urinaria* treatment (Fig. 4B). Based on the above, a target was involved in the inhibition of secretory pathway of MMP-2 by *P. urinaria*.[27] MMP-2, like other MMPs, is a Zn^{2+}-dependent endopeptidase. Since zinc is essential for

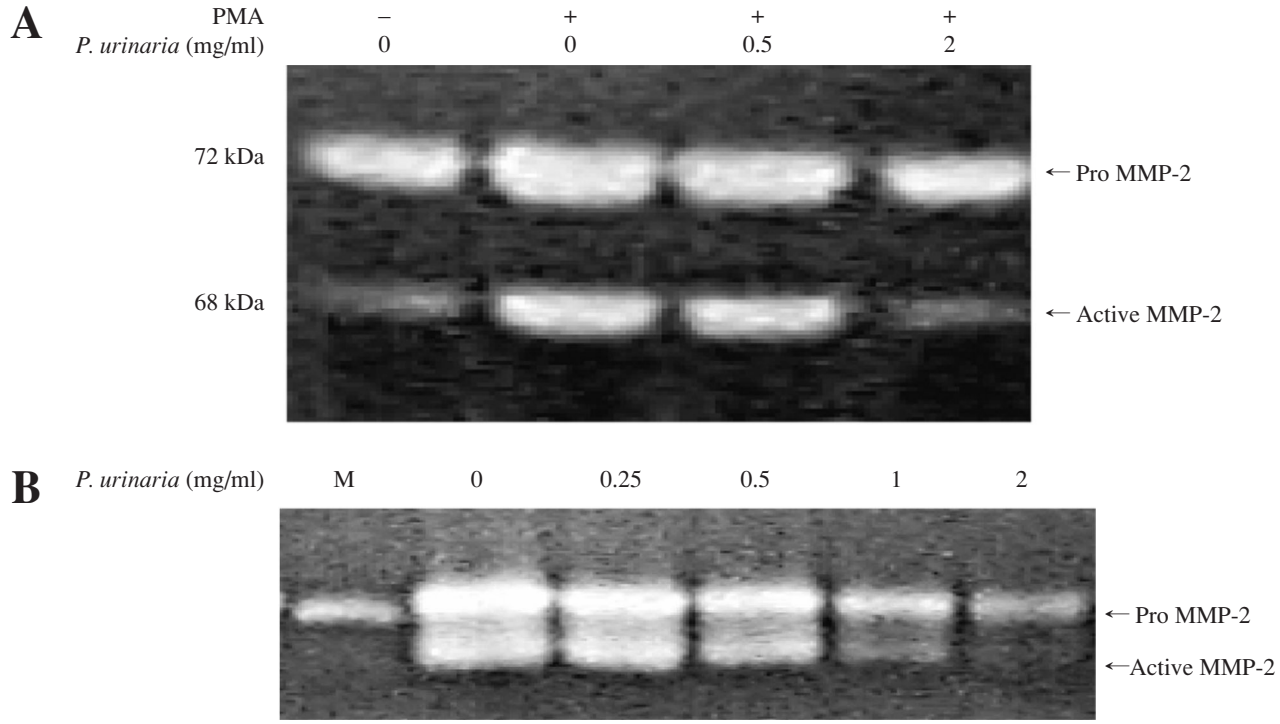

Fig. 3 (A) Inhibition of MMP-2 activity in *P. urinaria* treated chorioallantoic memebranes. The protein extract was prepared by homogenizing the CAMs, centrifuging them, and using the resulting supernatant for gelatin zymography analysis. (B) *P. urinaria* extract dose-dependently inhibited MMP-2 activity in human vascular endothelial cells. Conditioned media taken from human vascular endothelial cells cultured for 24 hours were used for gelatin zymography analysis.

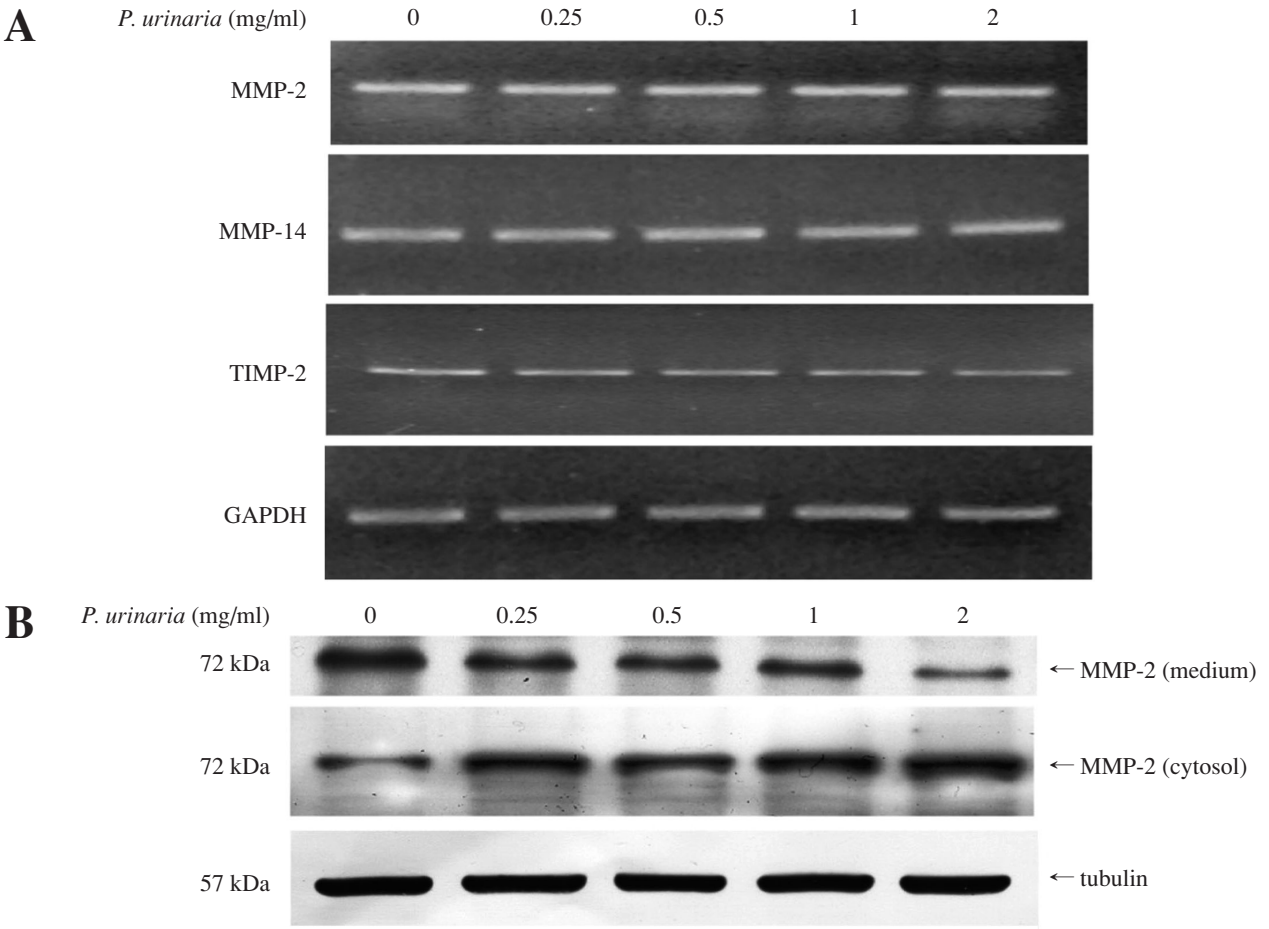

Fig. 4 Cells were treated with *P. urinaria* for 24 hours and the mRNA expression (A) of MMP-2, MMP-14, TIMP-2 and GAPDH were determined by RT-PCR analysis. Cells treated with *P. urinaria* for 24 hours were processed for western blotting (B) to analyze the protein levels of MMP-2 in the conditioned media and cytosol.

the endopeptidase proteolytic capacity to degrade the extracellular membrane (ECM), compounds with zinc-chelating groups, such as thiol or hydroxamate,[34] are often used to inhibit MMP activity. MMP-2 activity was inhibited directly by incubating the conditioned medium of control HUVECs with *P. urinaria*. This inhibition was reversed with the addition of $ZnCl_2$. *P. urinaria*, by acting like a zinc chelator, can exert additional non-cell-mediated inhibitory effects on MMP-2 activity (Fig. 5A). Meanwhile, treatment with *P. urinaria* extract also inhibited the gel-induced tube formation of HUVECs in a dose-dependent manner with unaffected cell viability. $ZnCl_2$ also recovered *in vitro* angiogenesis with gel-induced tube formation of HUVECs previously inhibited by *P. urinaria* (Fig. 5B, C).

Aqueous extract of *P. urinaria* inhibits telomerase activity in NPC-BM1

Resistance to apoptosis and maintenance of telomere integrity, mainly through telomerase reactivation, are features that typically distinguish cancer cells from normal cells. Telomere/telomerase impairment in cancer cells leads to the induction of apoptosis through different mechanisms.[35] First, apoptosis induction by chromosome uncapping results from the inhibition of telomere-binding protein. It has been demonstrated that the presence of damaged DNA foci at dysfunctional telomeres promotes apoptosis in tumor cells. Another mechanism suggests a role for telomerase in the regulation of gene expression. Activation of human telomerase reverse transcriptase (hTERT) gene transcription in cancer cells may be

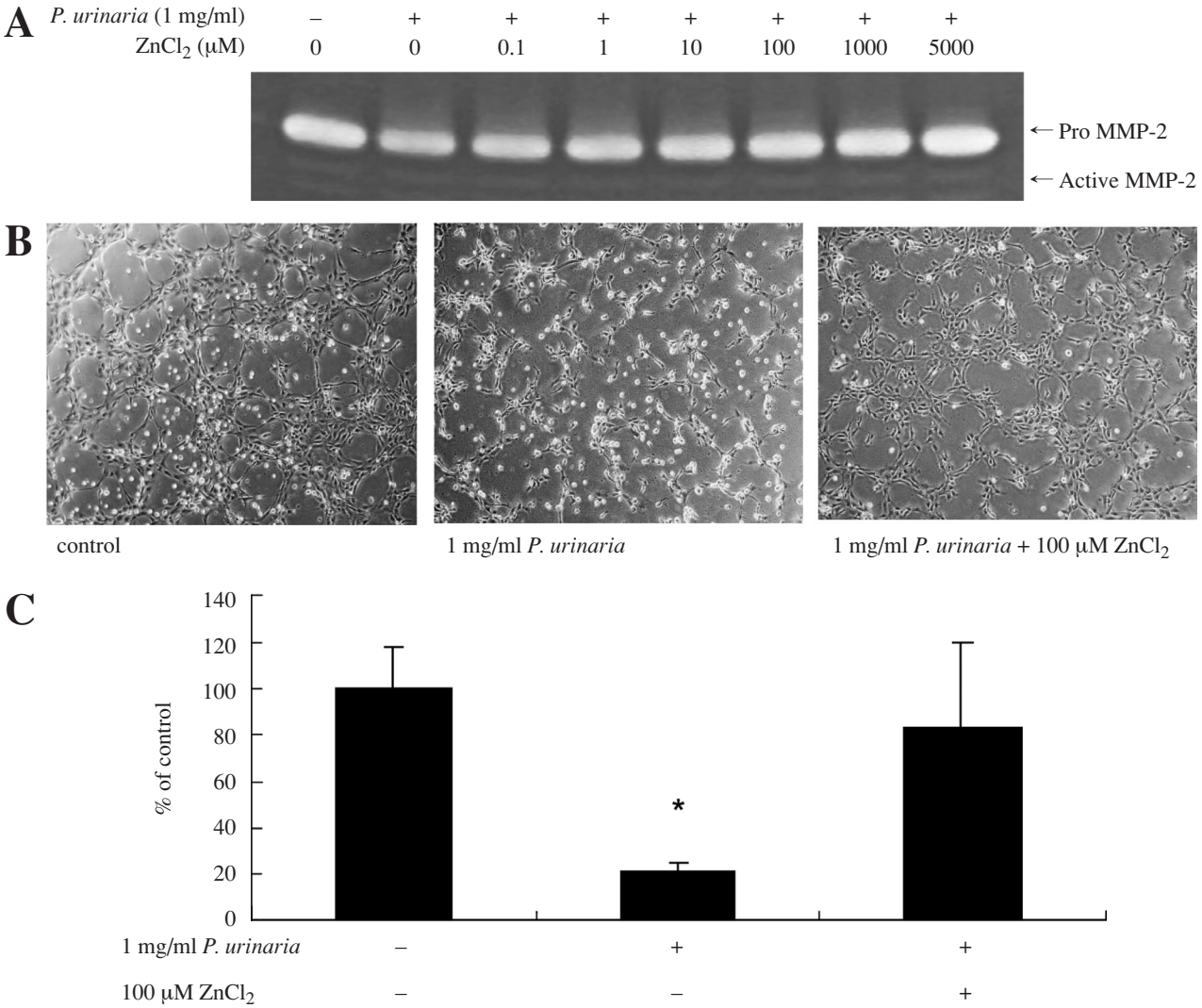

Fig. 5 $ZnCl_2$ reversed the inhibition of MMP-2 activity in conditioned medium directly incubated with (A) *P. urinaria*. Conditioned media taken from cultures of human vascular endothelial cells were incubated with 1 mg/ml *P. urinaria* in the absence or presence of 0 to 5000 μM $ZnCl_2$ for 10 minutes at room temperature and immediately used for zymography analysis. Matrix-induced tube formation of HUVECs inhibited by (B) *P. urinaria* was completely reversed by $ZnCl_2$. Cells were treated with 1 mg/ml *P. urinaria* in the absence or presence of 100 μM $ZnCl_2$. (C) The number of completely formed tubes in each group was determined and compared. The bar value is the mean ± SD of three independent experiments. The asterisk indicates statistical significance in comparison with the vehicle control.

the major, rate-limiting step in telomerase activation. The presence of telomere binding protein 1 (TP1) and human telomerase RNA (hTR) may form an inactive telomerase complex that becomes activated on the incorporation of hTERT during the process of telomerase activation. Because of the important role of telomerase in tumor formation, we investigated the possible effect of *P. urinaria* on telomerase activity in NPC-BM1 cells. Inhibition of telomerase activity with *P. urinaria* treatment was noted in NPC-BM1 cells.[16] As reported,[16] the extract of *P. urinaria* dose-dependently decreased hTERT and TP1 mRNA expression levels, whereas the hTR mRNA level remained the same. In the mean time, we also

demonstrated the down-regulation of c-myc gene expression in *P. urinaria*-treated NPC-BM1 cells. C-myc is a proto-oncogene product with transcriptional activity that can interact with the hTERT promotor to stimulate the expression of hTERT.[36] Therefore, the down-regulation of c-myc gene expression in NPC-BM1 cells could result in a decrease of hTERT expression and telomerase activity.

Summary

P. urinaria is documented worldwide in the treatment of various diseases. We are pioneers in the investigation of anticancer effects of this title plant. We characterized the main compounds of *P. urinaria* which exert anticancer activity and investigated the relevant mechanisms. Our works may potentially contribute to new drug development. Collectively, we have demonstrated the anticancer effect of water extract prepared from *P. urinaria* by exploring its mechanisms related to apoptosis, anti-angiogenesis and suppression of telomerase activity. In addition, *P. urinaria* exerts few cytotoxic effect on normal cells, even at high concentrations and with long exposures. Both the intrinsic and extrinsic pathways are involved in the apoptosis induced by *P. urinaria* extract. Down-regulation of telomerase activity and hTERT expression might accelerate the induction of apoptosis. Meanwhile, the mechanism of *P. urinaria* which interferes with angiogenesis might partially associate with the inhibition of MMP-2 secretion and therefore its extracellular activity, and direct inhibition of MMP-2 activity through chelating zinc. As ellagic acid and gallic acid could be the main components in *P. urinaria*, we further investigated the relevant effects of these two compounds. Primarily, we found that ellagic acid can exert anti-angiogenic effects on the regulation of MMPs similar to that of *P. urinaria*. On the other hand, gallic acid could inhibit cell viability by manipulating the cell cycle and apoptosis. The detailed mechanisms underlying the effects of these two compounds will be further investigated in clinical trials of *P. urinaria* in the near future.

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葉下珠抗腫瘤效應及其相關機轉

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葉下珠 (珍珠草) 是一種臨床上經常使用的中草藥，報告指出葉下珠具有各種不同的生物活性。本文主要分析確認葉下珠全草的可能相關組成，及探討葉下珠體內及體外抗腫瘤的效應及其相關的分子機轉。葉下珠的水抽取物能抑制各種不同腫瘤細胞株的生長，同時對於 C57BL/6J 小鼠所移植上的 Lewis 肺癌細胞也具有抑制效果。而此種抑制腫瘤的效應係經由細胞凋亡，而此係通過內在及外在徑路的分子反應，激活 caspase 家族蛋白酶，切割 DNA 成各種片段。葉下珠的抑制細胞生長，也可能與抑制端粒酶的活性有關，進而加速細胞凋亡的進行。同時，葉下珠亦有抑制血管增生的作用，而此現象乃經由抑制基質金屬蛋白酶的分泌，及葉下珠本身可能爲一種鋅的螯合劑，進而抑制基質金屬蛋白酶的活性。(長庚醫誌 2010;33:477-87)

關鍵詞：葉下珠 (珍珠草)，細胞凋亡，端粒酶，抑制血管增生，鋅

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