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Screening the active constituents of Chinese medicinal herbs as potent inhibitors of Cdc25 tyrosine phosphatase, an activator of the mitosis-inducing p34^{cdc2} kinase

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Abstract: Objective: To screen and evaluate the active constituents of Chinese medicinal herbs as potent inhibitors of Cdc25 phosphatase. Methods: The affinity chromatography purified glutashione-S-transferase/Cdc25A phosphatase fusion protein and Cdc2/cyclin B from the extracts of starfish M phase oocytes are used as the cell cycle-specific targets for screening the antimitotic constituents. We tested 9 extracts isolated from the Chinese medicinal herbs and vegetables including the agents currently used in cancer treatment by measuring the inhibition of Cdc25A phosphatase and Cdc2 kinase activity. The antitumor activity of the extracts was also evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and flow cytometry. Results: Cdc25A inhibitory activity and antitumor activity are detected in the extracts isolated from three Chinese medicinal herbs *Agrimona pilosa*; *Herba solani lyrati*; *Galla chinesis*. Conclusion: We found three extracts isolated from Chinese medicinal herbs have potential inhibitory activity of Cdc25 phosphatase using a highly specific mechanism-based screen assay for antimitotic drug discovery.

Key words: Chinese medicinal herbs, Cdc25 phosphatase inhibition, Cell cycle, Anti-tumor activity
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INTRODUCTION

Cdc25 phosphatases control the activity of cyclin-p34^{cdc2}, a cyclin-dependent kinase (CDK) by regulating the state of tyrosine phosphorylation of p34^{cdc2} kinase. Dephosphorylation by Cdc25 phosphatase allows cyclin-dependent kinase (CDK) activation and the subsequent induction of cell cycle progression (Nilsson and Hoffman, 2000). Three Cdc25 homologs were found in humans: Cdc25A, Cdc25B and Cdc25C (Sadhu *et al*., 1990; Millar *et al*., 1991; Nagata *et al*., 1991). Overexpression of Cdc25A and B, but not C, has been reported in a variety of tumors including breast, ovary, head and neck, and colon cancer with a striking association of tumor aggressiveness and poor prognosis (Galaktionov *et al*., 1995; Cangi *et al*., 2000; Hernandez *et al*., 2001). Cdc25A and B are transcriptional targets of the c-*myc* oncogene (Galaktionov *et al*., 1996). Both Cdc25B and Cdc25C are thought to be regulators of G_2/M transition through their ability to dephosphorylate and activate the CDK1/cyclin B mi-

totic kinase complex, which is required for cell entry into mitosis (Nilsson and Hoffman, 2000). Cdc25A is likely more important for G_1/S phase transition and in preserving genomic integrity (Jinno *et al*., 1994; Hoffman *et al*., 1994; Mailand *et al*., 2000; Molinari *et al*., 2000). As an important cell cycle regulator, the Cdc25A phosphatase is currently considered as potential target for the development of novel therapeutic approaches (Lyon *et al*., 2002). Although several Cdc25A inhibitors have been published in recent years (Baratte *et al*., 1992; Ham *et al*., 1998; Tamura *et al*., 2000; Lazo *et al*., 2001; 2002; Sohn *et al*., 2003), the potential inhibitors isolated from Chinese medicinal herbs are less reported. In the past studies we have found several active constituents from naturally occurring products, especially traditional Chinese medicinal herbs (Yang *et al*., 1996; Zheng *et al*., 1997), which are reported to manifest certain antitumor effects and have been used in clinical practice. The current work was initiated based on the belief that potent Cdc25A phosphatase inhibitors could be obtained by using a highly specific mechanism-based screen assay to evaluate the active constituents isolated from the Chinese medicinal herbs. Here, we report three extracts isolated from Chinese medicinal herbs have potential Cdc25 phosphatase inhibitory activity as well as anti-proliferative activity against human tumor cells. However, it remains to be determined which active constituents contribute to their inhibitory effect on Cdc25A phosphatase.

MATERIALS AND METHODS

Extraction of plant meterial

The material of herbs and vegetables was obtained from the Zhejiang Institution of Traditional Chinese Medicine (TCM) and Beijing Science Academy of Agriculture. The material was ground to a coarse powder and sequentially extracted with water followed by MeOH/CH_2Cl_2 (1:1). The organic extract was evaporated in vacuo and dried to give a maroon solid which was redissolvd in small volume of DMSO for the biological assay.

Cell culture condition

Human leukemia K562 cell line was provide by Dr. Steinmann (Kiel University, Germany). Human KB cell line was from the Shanghai Institute of Cell Biology, Academia. These cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 IU/ml of penicillin-streptomycin in an atmosphere of 5% CO_2 at 37 °C.

Cytotoxicity and MTT assay

IC_{50} value calculations for each cell line were determined by the below described MTT assay (Twentyman *et al*., 1989), where in cells were plated onto 96-well tissue culture dishes at density of 1×10^3 well^{-1} in 200 μl medium. After plating, the cells were allowed to attach for 2 d. The extract was added using DMSO as the vehicles, at maximum concentration of 0.1%. Cells were incubated with various concentrations of the agents for 48 h, at which time 50 μl of 2 mg/ml MTT was added, and the absorbance at 550 nm was determined by a microtiter plate reader. The absorbance at 690 nm was also measured as reference. The same aliquot of drug-free emulsion alone was added to the control cells. Experiments were conducted in triplicate.

Cell cycle analysis

Cell sample preparation and DAPI staining for cytometry analysis were performed according to the method reported previously (Hotz *et al*., 1994). Cell cycle distribution was determined using a CA-II flow cytometer (Partec, Germany). Data analysis was performed by Multicycle software (Phoenix Flow System, San Diego, CA).

Preparation and purification of GST-Cdc25A fusion protein

Bacterial growth and fusion protein induction. An *E. coli* strain, BL 21 (DE3) was transformed by a plasmid encoding the gene fusion constructs of Glutathione-S-Transferase (GST) and human Cdc25A. Bacteria were first grown overnight at 37 °C in the presence of 100 μg/ml ampicilin in LB medium. Four ml of this preculture were inoculated per liter of LB containing 100 μg/ml ampicillin. Incubation was continued at 30 °C until the culture O.D. at 600 nm. reached 0.8–1.0. At this time, 0.4 mmol/L IPTG (isopropyl-BD-thiogalactoside) was added and the culture was incubated at 25 °C for at least 7 h. Cells were then harvested by 3000 g centrifugation for 15 min at 4 °C. Pellets were kept frozen at –80 °C until

extraction. The bacterial pellet was homogenized by sonication at 4 °C in lysis buffer (1% Nonidet-P40, 1 mmol/L EDTA, 1 mmol/L DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 100 μmol/L benzamidine in phosphate buffered saline). The homogenate was centrifuged at 100000 g for 30 min at 4 °C. The supernatant was recentrifuged under similar conditions and the final supernatant was then immediately mixed (6~10 volumes of supernatant/1 volume of packed beads) and rotated with glutathione-agarose beads equilibrated with lysis buffer for 30 min. at 4 °C. The glutathione-agarose beads were washed three times with 10 volumes of lysis buffer followed by four washes with 10 volumes of Tris buffer A (50 mmol/L Tris, pH 8, 50 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT). Elution of the fusion protein was induced by 3~4 successive washes with 10 mmol/L glutathione in Tris buffer A. The efficiency of the elution was monitored by phosphatase assay. Active fractions were pooled and used directly or supplemented with 40% glycerol prior to storage at −80 °C. Glutathione-agarose beads can be recycled by a wash with 1 mol/L NaCl followed by equilibration with lysis buffer.

Assay of GST-Cdc25A phosphatase activity

Assays were performed in microtiter plates as described by Baratte *et al*.(1992). Twenty μl of GST-Cdc25A protein were mixed with 20 μl 100 mmol/L DTT in Tris buffer A and 140 μl of Tris buffer A. The plates were preincubated at 37 °C for 15 min in the incubator. Reactions were initiated by addition of 20 μl of 500 mmol/L *p*-nitrophenylphosphate in Tris buffer A. After a 30 min incubation at 37 °C, absorbance at 405 nm was measured in a Bio-rad microplate reader. Blank values (no GST-Cdc25A protein added) were automatically substracted.

Purification of Cdc2/cyclin B from starfish m phase oocytes extracts

For large scale oocyte extracts preparations, gonads are removed from ripe *Marthasterias glacialis*, and incubated at room temperature with 10 μmol/L 1-methyladenine in Millipore-filtered natural sea water until spawning. By this time all the oocytes have entered the M phase. Oocytes are then recovered from the incubation medium by centrifugation, directly frozen in liquid nitrogen and kept at –80 °C. Oocytes can be stored at –80 °C for several years without substantial loss of Cdc2 kinase activity. M phase oocytes are homogenized in homogenization buffer A (60 mmol/L glycerophosphate, 15 mmol/L *p*-nitrophenylphosphate, 25 mmol/L MOPS (3-(N-morpholino)propanesulfonic acid) (pH 7.2), 15 mmol/L EGTA (ethylene-bis(oxyethylenenitrilo) tetraacetic acid), 15 mmol/L $MgCl_2$, 1 mmol/L DTT, 1 mmol/L sodium vanadate, 1 mmol/L NaF, 1 mmol/L phenylphosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor and 100 μmol/L benzamidine) at a ratio of 2 g of oocytes/ml buffer. After 45 min. centrifugation at 100000 g, the supernatant is recovered and directly used for purification. It is loaded, batch-wise, on p9CKShsl-Sepharose beads at a ratio of 20 ml oocyte extract/1 ml beads. After 30 min. continuous rotation at 4 °C, the beads are centrifuged and washed 5 times with homogenization buffer A. Cdc2/cyclin B is eluted with free p9CKShsl (2 mg/ml). An additional purification step can be added, using Matrex Green A -agarose. Cdc2/cyclin B binds to this resin from which it can be eluted with 0.2 mol/L NaCl. Glycero1 (20% final) is added to the purified enzyme, prior to storage in aliquots at –20 °C or –70 °C. The enzyme is stable for several months under these conditions.

Cdc2 kinase activity inhibition assay

Prior to large scale assays, the activity of the Cdc2/cyclin B kinase preparation is tested according to the following dilution curve of 0.5→1→2.5→5→ 10 μl purified kinase/assay tube (supplement with buffer C:60 mmol/L glycerophosphate, 30 mmol/L nitrophenylphosphate, 25 mmol/L MOPS pH 7.0, 5 mmol/L EGTA, 15 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol, 0.1 mmol/L sodium orthovanadate) to a 10 μl final kinase volume). From the results of this preliminary trial, we recommend dilution of the kinase with buffer C to a final activity of approximatively 12000~20000 counts/min (cpm) incorporated/10 min/10 μl diluted kinase. Assays are run as in the following steps at 30 °C for 10 min in the presence of 15 μmol/L ATP: (1) Mix 10 μl diluted kinase, 6 μl Histone H1 (Sigma, type III-S; 5 mg/ml buffer C) and 6 μl buffer C. These components can be pre-mixed and totally 22 μl mixture was distributed in the each tube just prior to the assay; (2) Add 3 μl of potential

inhibitor to be tested; (3) Start the reaction by adding 5 μl final ATP solution, then mix and incubate for 10 min at 30 °C (final ATP solution: mix 20 μl 32P-ATP (3000 Ci/mmol; 1 mCi/ml) and 90 μl 1 mmol/L cold ATP with 890 μl buffer C. This final ATP solution should display between 300.000 and 600.000 cpm/5 μl; (4) After 10 min incubation at 30 °C, stop the reaction by spotting 25 μl of the reaction mixture on pre-cut P81-phosphocellulose filters (2.5 cm×3 cm). After 20 s, throw the filters in a solution of 10 ml phosphoric acid/liter of distilled water (approximatively 120 filters/400 ml). Wash these filters 5~6 times for at least 5 min with diluted phosphoric acid. Dry the filters and count for 2 min with 3 ml scintillation fluid; (5) Controls were as follows: One filter with 5 μl final ATP solution washed in the diluted phosphoric acid (for filter washing efficiency). One tube with all reagents but histone HI replaced by 6 μl buffer C (for background activity). Negative control: full kinase inhibition in the presence of 100 μl olomoucine18. Count 3 times of 5 μl final ATP solution for final expression of activity in 10^{-12} mol phosphate incorporated/30 μg histone Hl/10 min/10 μl kinase (5 μl final ATP solution contain 450×10^{-12} mol of ATP).

RESULTS

Active constituents of Chinese medicinal herbs inhibit the activity of recombinant Cdc25 phosphatase

The human Cdc25A has been cloned and expressed as a GST fusion protein in *Escherichia coli* and a straightforward colorimetric assay directed towards the search for inhibitors of this key enzyme has been proposed. In our experiment system, a compound/extract is considered as an inhibitor when its IC_{50} (50% inhibitory concentration) is below 10 μmol/L or 10 μg/ml (Table 1) and the values are presented as mean obtained from triplicate samples. During our investigation of Chinese medicinal herbs in the search of biologically active compounds, the crude extracts of *Agrimona pilosa* (E in Fig.1); *Herba solani lyrati* (D in Fig.1); *Galla chinesis* (F in Fig.1) were found to be active on Cdc25 phosphatase in this bioassay. These extracts exhibited also antitumor activity towards K562 and KB cells.

Table 1 Cdc25 phosphatase and Cdc2 kinase inhibitory effect of Chinese medicinal herbs

Extracts	Fig.1	IC_{50} (μg/ml)	
		Cdc25A	Cdc2
Control emulsifier	A	> 1000	> 1000
Lignum sappan	B	20	30
Herba Patriniae	C	11	300
Herba solani lyrati	D	9	700
Agrimona pilosa	E	5	700
Galla chinesis	F	3	220
1628 vegetable	G	40	> 1000
1631 vegetable	H	13	> 1000
β-elemene	I	> 1000	> 1000
Matrine & Oxymatrine	J	> 1000	> 1000

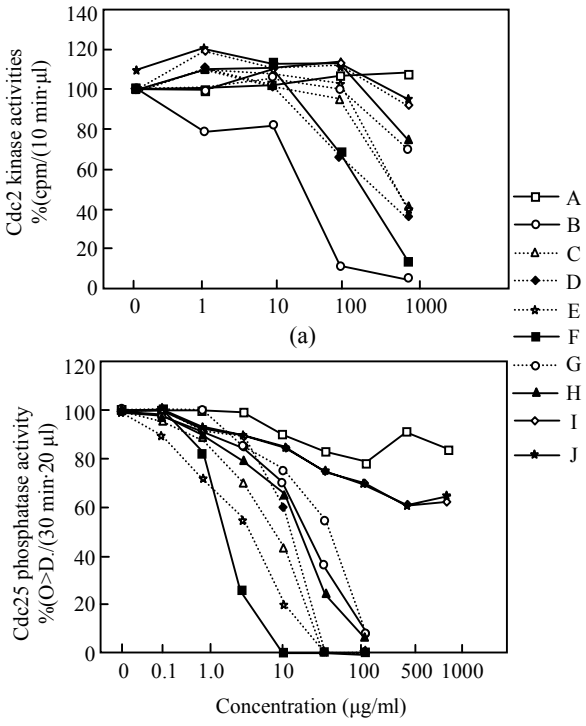

Fig.1 Active constituents of Chinese medicinal herbs inhibit the activity of recombinant Cdc25 phosphatase (a) The Cdc2/cyclin B kinase was purified from oocyte extracts by affinity chromatography on p9CKShs1-Sepharose beads are used as the cell cycle-specific targets for screening the antimitotic constituents. Cdc2 kinase activity inhibition was assayed as described under "MATERIALS AND METHODS." [32P] Phosphate incorporation in histone H1 was measured by direct counting; (b) The Cdc25A phosphatase is inhibited by active constituents of Chinese medicinal herbs. GST-Cdc25A phosphatase was exposed to various concentrations of the extracts and assayed as described under "MATERIALS AND METHODS." Shown is an average of three determinations. The letter of each curve represents same extract as indicated in Table 1

Active constituents of Chinese medicinal herbs inhibit cell proliferation

Proliferation of leukemia cells treated with the potential Cdc25 phosphatase inhibitors was evaluated by MTT assay. When K562 cell line in culture were treated with *Agrimona pilosa* (Fig.2a); *Galla chinesis* (Fig.2b) and *Herba solani lyrati* (Fig.2c) for 48 h, the growth was significantly inhibited. The IC_{50} values of the extracts of *Agrimona pilosa*, *Herba solani lyrati* and *Galla chinesis* for K562 cells are shown in Table 2 and the values are presented as mean±*SD* obtained from triplicate samples. Similar growth inhibitory effect of the three herb extracts was also observed in KB cell lines (data not shown).

Table 2 Growth inhibitory activity of Chinese medicinal herbs

Extracts	IC_{50} (μg/ml)
Agrimona pilosa	77.6±10.3
Galla chinesis	84.0±12.4
Herba solani lyrati	297.3±35.3

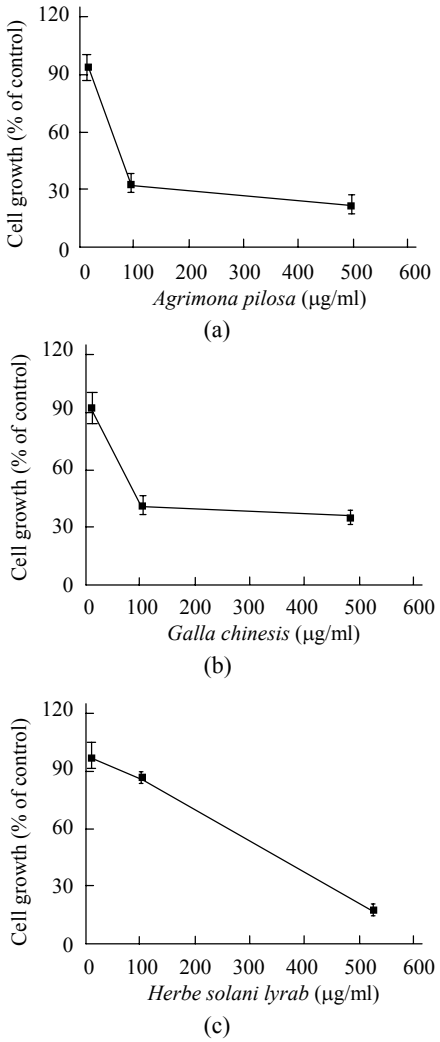

Fig2. Active constituents of Chinese medicinal herbs inhibit tumor cell proliferation

The growth inhibition of K562 cells by the potential Cdc25 phosphatase inhibitors was evaluated by MTT assay as described under "MATERIALS AND METHODS". The treatment of *Agrimona pilosa* (a); *Galla chinesis* (b) and *Herba solani lyrati* (c) at various concentrations for 48 h, the cell growth was significantly inhibited. The values are presented as mean±*SD* obtained from triplicate samples

Active constituents of Chinese medicinal herbs inhibit cell cycle progression

K562 cells, a convenient model for cell cycle analysis, were used to investigate by flow cytometry the effect of the active constituents of *Agrimona pilosa*; *Herba solani lyrati* and *Galla chinesis* on cell cycle progression. As shown in Fig.3, after 24 h of treatment with the extracts at concentrations of 10, 100 and 500 μg/ml, G2M arrest is not detected at treantment of 10 μg/ml, however a significant G2M arrest was observed on the treatment of the three herb extracts at concentrations of 100 and 500 μg/ml. Also, the proportion of S phase cells was markedly decreased in dose-dependent manner. Distinct apoptotic features of the sub-G1 peak were observed during treatment with two extracts. Percentage of apoptotic cells (sub-G1) were respectively 27.8% for 100 μg/ml of *Agrimona pilosa* and 41.2% for 100 μg/ml of *Galla chinesis*. At concentration of 500 μg/ml, the 24 h treatment with *Galla chinesis* caused significant cell

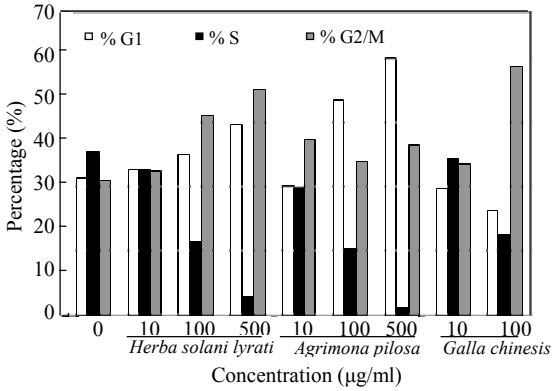

Fig. 3. Active constituents of Chinese medicinal herbs inhibit cell cycle progression

Human leukemia K562 cell line was used for cell cycle analysis by flow cytometry as described under "MATERIALS AND METHODS". After 24 h of treatment with the extracts at concentrations of 10, 100 and 500 μg/ml, the effect of the active constituents of *Agrimona pilosa*; *Herba solani lyrati* and *Galla chinesis* on cell cycle progression is evaluated by detection of G2M arrest

death, so that cell cycle analysis could not be performed. To address the issue whether inhibiting Cdc25A by those activity constituents leading to apoptosis, this phenomenon needs to be further investigated.

DISCUSSION

Traditional and folklore medicine handed on from generation to generation is rich in household remedies and community practice. According to an estimate of World Health Organization (WHO), nearly 80% of the populations of developing countries rely on traditional medicine, mostly plant drugs for their primary health care needs. Traditional medicine has served as a source of alternative medicine, new pharmaceuticals, and healthcare products. Medicinal plants are important for pharmacological research and drug development, not only when plant constituents are used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds (Mukherjee, 2003). For decades, natural products have been a wellspring of drugs and drug leads. Senventy-eight percents of antibacterial and 74% of anticancer compounds are natural products or have been derived from, or inspired by, a natural product (Farnsworth and Loub, 1983; Farnsworth *et al*., 1985; Farnsworth and Soejarto, 1985). It is estimated that nearly three fourths of the plant-derived prescription drugs used worldwide were discovered following leads from local medicine. About 25% of modern medicines are descended from plants first used traditionally according to WHO. Many others are synthetic analogues built on prototype compounds isolated from plants (Choudhary, 2002). Several important modern drugs are extracted directly from plants. It has been estimated that only 6% of all described species have been analyzed chemically and only a small fraction analyzed pharmacologically (Choudhary, 2002). In the USA, the process of synthetic drug discovery and development takes an average of 12 years, and any new drug requires the investment of an average of US $230 million. It is seen that plant based drugs take a comparatively much less time and expenses than synthetic drugs. Hence plant based medicines would be cheaper, unless the market price are inflated by other considerations (Ramakrishnappa, 2003; Wakdikar, 2004). Cdc25 is an excellent prototype target for such an investigation because no potent or selective inhibitors are widely available at present. Cdc25 phosphatases are currently considered as potential targets for the development of novel cancer therapeutic approaches because they have critical roles in controlling cell cycle phase transition, and have been implicated in cancer and Alzheimer's disease (Prevost *et al*., 2003). Screening of Cdc25 phosphatase inhibitors by bioassay led to the successful isolation of several active compounds as selective Cdc25 inhibitors such as Coscinosulfate, a sesterterpene sulfate from sponge (Loukaci *et al*., 2001; Pu *et al*., 2002). We report here the extracts from three Chinese medicinal herbs, *Agrimona pilosa*, *Galla chinesis* and *Herba solani lyrati* as potent Cdc25 inhibitors through bio-screening assay. *Agrimona pilosa* (Xian He Cao) has been used in traditional Chinese remedy against parasites, including taenia, malaria plasmodia, schistosomomas and vaginal trichomonas. Its leaves are rich in vitamin K and are used to promote blood clotting and control bleeding stop bleeding and to prevent infection. *Galla chinensis* is used for chronic cough and diarrhea, spontaneous emission and perspiration as well as night sweat. These three Chinese medicinal herbs were reported effective for treatment of cancer patients. However, the active constituents of them against tumor are largely unknown. Ellagic acid, a phenolic compound, the active ingredient extracted from *Galla chinensis*, is known to be effective against cancer of the rectum and colon, esophagus, liver, lung, tongue and skin. Ellagic acid has been demonstrated to inhibit tumor growth in animal models and clinical studies (Constantinou *et al*., 1995). Studies have shown that treatment of Ellagic acid leads to cell cycle arrest within 48 h and apoptosis within 72 h for cancer cells (Losso *et al*., 2004; Mertens-Talcott and Percival, 2005). Whether Ellagic acid or other active constituents could inhibit Cdc25 phosphatase activity is largely unknown at present. Therefore, it is necessary in future to isolate the active compounds from the extracts of these Chinese medicinal herbs and to determine the Cdc25 inhibitory effect of these active compounds. On the other hand, in our investigation a large amount of apoptosis induced by three extracts has been detected by flow cytometry, of which the

molecular mechanisms also need to clarify (Kim *et al*., 2003).

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