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Chemical and Biological Assessment of a Chinese Herbal Decoction Containing Radix Astragali and Radix Angelicae Sinensis: Determination of Drug Ratio in Having Optimized Properties

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Danggui Buxue Tang (DBT), a Chinese medicinal decoction that is commonly used as a dietary supplement in treating woman with menopausal irregularity, contains two herbs: Radix Astragali (Huangqi) and Radix Angelicae Sinensis (Danggui). The ratio of Radix Astragali and Radix Angelicae Sinensis used in DBT should be 5:1 as described in China in 1247 A.D.; however, the rationale of this formula has not been given. Here, the chemical and biological properties of DBT, prepared from different ratios of the drugs, were determined. Significantly, higher amounts of Radix Astragali-derived astragaloside IV, calycosin, and formononetin and Radix Angelicae Sinensis-derived ferulic acid were found in DBT with Radix Astragali and Radix Angelicae Sinensis in a 5:1 ratio. By using the biological effects of DBT in stimulating osteoblast proliferation, estrogen promoter activation, and anti-platelet aggregation activity, the drug ratio of 5:1 produced the best effects. In addition, the use of ethanol-treated Radix Angelicae Sinensis enhanced the efficacy of DBT, and the treatment further increased the solubilities of chemical constituents. By analyzing the correlation of chemical and biological results, several chemicals showed positive correlation with DBT-induced bioactivities. The current results support the ancient formulation of DBT, and the identified chemicals could serve as markers for quality control of DBT.

KEYWORDS: ***Angelica sinensis*; umbelliferae; *Astragalus membranaceus* var. *mongholicus*; leguminosae; TCM; analytic method**

INTRODUCTION

Traditional Chinese medicine (TCM) is used in medicines as well as in daily dietary supplements in Asia. Historically, TCMs are prepared as decoctions by a unique methodology with a specific combination of different herbs as formulas. However, the functional roles of these requirements are not yet determined. Among thousands of different TCM formulas, Danggui Buxue Tang (DBT; an herbal decoction) is the simplest one. The use of DBT was first recorded in *Neiwaishang Bianhuo Lun* by Li Dongyuan in China in 1247 A.D., which stated that the formula should contain two herbs, 10 qian Radix Astragali and 2 qian Radix Angelicae Sinensis (a qian was a weight unit in ancient China; 1 qian was equal to ∼3 g) and should be boiled in two bowls of water over moderate heat until the final volume is reduced by half (*1*, *2*). DBT is prescribed to women in China to improve menopausal symptoms, and they are recommended to drink DBT decoction daily to raise the "Qi" (the vital energy) and nourish the "Blood" (the body circulation) of the individual (*2*). Although DBT, originally, was prescribed as a medicinal decoction, today it is commonly used as a health food supplement in tea for women's ailments in Asia. Indeed, recent pharmacological results indicated that DBT has the ability to promote hematopoetic function, stimulate cardiovascular circulation, prevent osteoporosis, and act as antioxidatants (*3*, *4*).

Roots of *Astragalus membranaceus* (Fischer) Bunge and *A. membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) P. K. Hsiao are botanical sources of Radix Astragali (*1*), which has been proven to be an immunostimulant, hepatoprotective, antidiabetic, analgesic, expectorant, and sedative drug (*5*, *6*). A good source of Radix Astragali is from cultivated farming in the Shanxi Province of China (*7*). The constituents associated with the activity of Radix Astragali are saponins, isoflavonoids, and polysaccharides. Indeed, the functions of astragaloside IV, calycosin, and formononetin have been described, and they are used as markers for quality control (*5*−*7*). On the other hand, Radix Angelicae Sinensis should be derived from roots of

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Angelica sinensis (Oliv.) Diels (*4*, *8*, *9*). *A. sinensis* root is used to invigorate blood circulation in treating menstrual disorders (*10*, *11*) and to modulate the immune system (*12*). The Gansu Province of China produces over 90% of the total *Angelica* root in China (*9*). The constituents associated with the pharmacological activities of *Angelica* roots are ferulic acid and ligustilide (*9*, *13*).

According to the old theory of herbal formulas in TCM, each decoction (or Fu Fang in Chinese) consists of four elements: Jun (as a king or a master), Chen (as a minister), Zuo (as an assistant), and Shi (as a servant), which work harmoniously together in order to achieve the therapeutic purposes. The action mechanisms of these elements, however, have not been determined. In DBT, Radix Astragali is considered Jun, while Radix Angelicae Sinensis is considered Chen. The rationale of having a specific combination of the two drugs has never been given. As an old tradition, Radix Angelicae Sinensis has to be processed with wine before use. However, it is not commonly used today.

To provide different lines of evidence to support the formulation of DBT, we determined the amounts of Radix Astragali-derived astragaloside IV, calycosin, and formononetin and Radix Angelicae Sinensis-derived ferulic acid and ligustilide in DBT prepared from different ratios of drugs. In parallel, the biological properties of DBT in stimulating osteoblast proliferation, estrogen promoter activation, and anti-platelet aggregation were also determined. Our results showed that the ancient formulation of DBT with a ratio of Radix Astragali to Radix Angelicae Sinensis of 5:1 processed the best chemical and biological properties. Moreover, the role of ancient methods in processing Radix Angelicae Sinensis was elucidated here.

MATERIALS AND METHODS

Plant Materials. Fresh roots were obtained from China from September to October of 2002: 3 year old *A. membranaceus* var. *mongholicus* from Shanxi and 2 year old *A. sinensis* from Minxian of Gansu. These areas were demonstrated to produce good quality Radix Astragali (*8*, *14*) and Radix Angelicae Sinensis (*9*), respectively. The authentication of plant materials was identified morphologically by Dr. Tina Dong at Hong Kong University of Science and Technology during the field collection. Individual samples were prepared from ~1 kg of powder that was ground by a blender from ~40 roots of *A. membranaceus* var. *mongholicus* or *A. sinensis* (*9*, *14*). Their corresponding voucher specimens as forms of whole plants, voucher #02-9-1 for *A. membranaceus* var. *mongholicus* and voucher #02-10-4 for *A. sinensis*, were deposited in the Department of Biology, The Hong Kong University of Science and Technology, China. For the processing of Radix Angelicae Sinensis, the dried roots were sliced and sprayed with rice wine (~15% ethanol at 100 mL for 1 kg of roots). The wetted roots were placed in an oven at 120 °C for 30 min before they were ground for further analysis.

Sample Preparation of DBT. Appropriate amounts of Radix Astragali and Radix Angelicae Sinensis were weighed according to different ratios from 1:1 to 10:1 to total 30 g and then mixed by vortex. The extraction was performed in 240 mL of boiling water for 2 h, and the samples were extracted twice; this extraction followed the ancient preparation and was shown to be the best extracting condition (*2*). For the second extraction of DBT, the residue from the first extraction was filtered, and the same extracting condition was applied. The extract was dried under vacuum and stored at −80 °C. For the assay of calycosin, formononetin, ferulic acid, and ligustilide, about 2 g of each extract was weighed into a 50 mL centrifugal tube, and 20 mL of methanol was added for sonication at 40 min. The supernatant was collected after centrifugation after two extractions and was dried, and the residue was dissolved in 2 mL of methanol for high-performance liquid chromatography (HPLC) analysis. This methanol extraction removed polysaccharides from DBT extracts. For the assay of astragaloside IV, the residues obtained as the aforementioned method were dissolved in 20 mL of water. The water phase was extracted three times by 60 mL of water-saturated butanol. The butanol phase was collected, combined, and then washed two times by 40 mL of ammonia solution (40 mL of concentrated ammonia in 60 mL of water). The ammonia phase was discarded, and the butanol phase was then evaporated to dryness. The residue was accurately dissolved in 5 mL of methanol for HPLC analysis.

Quantitative Analysis of Active Constituents. Astragaloside IV was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ferulic acid was from Sigma (St. Louis, MO). Calycosin, formononetin, and ligustilide (*z*-isoform) were provided by Prof. Pengfei Tu of Peking University; their purities, confirmed by HPLC, were higher than 99.0%. Analytic grade reagents were from Merck (Darmstadt, Germany). For the calibration of astragaloside IV, calycosin, formononetin, ferulic acid, and ligustilide, the standards were weighed and dissolved in methanol to give serial concentrations from 0.01 to 25 μg/mL, and three injections onto HPLC were performed for each dilution (*2*). The concentrations of these compounds in the samples were calculated according to the regression parameters derived from the standard curve. A Shimadzu SCL-10Avp System equipped with Solvent Delivery Module (Shimadzu Corporation, Kyoto, Japan), LC-10ATvp pump, and UV/vis Photodiode Array Detector SPD-M10Avp was used for all analysis. Chromatographic separations were carried out on a NOVA-PAK C_{18} column (particle size 4 μm, 3.9 mm × 150 mm) with acetonitrile (as solvent A):water (as solvent B) as the mobile phase at a flow rate of 1.0 mL/min at room temperature. For the astragaloside assay, a linear gradient elution was applied from 8 to 30% A starting from 0 to 15 min and from 30 to 75% A starting from 15 to 45 min. For the calycosin, formononetin, ferulic acid, and ligustilide assay, a linear gradient elution was applied from 0 to 6% A starting from 0 to 20 min, from 6 to 20% A starting from 20 to 30 min, and from 20 to 98% A starting from 30 to 50 min. Samples were filtered through a Millipore syringe filter unit. A twenty microliter sample was injected into HPLC analysis, and signals were detected at 203 nm for astragaloside IV, 254 nm for formononetin, 265 nm for calycosin, and 313 nm for ferulic acid and ligustilide with a photodiode array detector.

For the calibration of total polysaccharide, the anthrone−sulfuric acid method was used (*15*). The polysaccharide was precipitated by 70% ethanol; the precipitate was dried by air and dissolved in hot water at a desired concentration of about mg/mL. Dextran from *Leuconostoc mesenteroides* was weighed and dissolved in 100 mL of water to give about μg to mg/mL. A standard solution (0.6 mL), or prepared sample, was adjusted to 2.0 mL final volume. Then, 4.0 mL of freshly prepared 0.2% anthrone−sulfuric acid was added. Absorbance at 625 nm was measured after 30 min of color reaction. For flavonoid calibration, rutin was weighed and dissolved in 50 mL of 70% methanol to give serial concentrations from 0.1 to 20 μg/mL. The absorbances of samples were detected at 249 nm by UV spectrophotometry (Beckman DU 650). For the determination of total saponins, 0.5 mL of sample was added with 0.5 mL of 8% vanillin and 5 mL of 72% sulfuric acid. The mixture was placed in a 62 °C water bath. Twenty minutes later, the mixture was cooled to room temperature and the absorbance was measured at 540 nm.

MG-63 Cell Growth and Alkaline Phosphatase Assays. Human osteosarcoma cell line MG-63 was obtained from the American Type Culture Collection and was grown in modified Eagle's medium (MEM), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified CO_2 (5%) incubator at 37 °C. Culture reagents were from Invitrogen Technologies (Carlsbad, CA). Before 2−3 days of the treatment, the medium was changed to MEM-α without phenol red containing 2% charcoal−dextran-treated fetal bovine serum. MG-63 cells were seeded onto a 96 well plate at 2000 cells/well in 0.1 mL of estrogen-free medium. The next day, the media was replaced by 0.1 mL of media containing the appropriate DBT extracts for 3 days. 17β-Estradiol (Sigma), dissolved in dimethyl sulfoxide, was used as a control. The cell growth was determined by 3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT;

Table 1. Chemical Composition of DBT in Different Ratios of Drugs

	Radix Astragali:Radix Angelicae Sinensis							Radix	Radix Angelicae
marker[a]	1:1	2:1	3:1	4:1	5:1	7:1	10:1	Astragali	Sinensis
astragaloside IV	0.277	0.218	0.254	0.376	0.508[c]	0.293	0.210	0.365	*b*
calycosin	0.153	0.118	0.110	0.129	0.186[c]	0.110	0.177	0.088	
formononetin	0.082	0.095	0.116	0.135	0.155[c]	0.142	0.120	0.142	
ferulic acid	0.248	0.274	0.277	0.253	0.351[c]	0.291	0.320		0.149
ligustilide	0.438	0.470	0.340	0.266	0.204[d]	0.340	0.370		0.131
total flavonoids	19.4	17.8	20.4	20.1	26.5[c]	20.7	23.9	15.5	1.7
total saponins	50.3	51.0	57.6	56.9	62.3[c]	55.0	51.0	56.3	3.4
total polysaccharides	103.9	117.7	166.6	129.4	227.7[c]	217.7	188.3	123.8	92.2

[a] The amounts of astragaloside IV, calycosin, formononetin, ligustilide, ferulic acid, total flavonoids, total saponins, and total polysaccharides in Radix Astragali, Radix Angelicae Sinensis, and DBT were determined as described in the Materials and Methods. Values are expressed in mg/g of dry material and are in means ± SEM, $n = 5$. The SEM values are not shown for clarity, which is less than 5% of the mean. [b] Below the detection limited. [c] The difference by comparing to the lowest group is significant ($p < 0.01$). [d] The difference by comparing to the highest group is significant ($p < 0.01$).

Sigma) assay. The absorbance at 570 nm was measured using an enzyme-linked immunosorbent assay plate reader (Dynatech MR 5000) (*16*).

The alkaline phosphatase activity in MG-63 cells was measured by the hydrolysis of *p*-nitrophenyl phosphate (Sigma) as described previously (*17*). Briefly, cells were plated in 33 mm dishes and incubated with phenol red-free medium plus 10% charcoal-stripped fetal bovine serum. After 48 h, cells were washed with ice-cold phosphate-buffered saline (PBS; pH7.4), scraped into 0.2 mL of 0.1% Triton X-100, and rapidly frozen and thawed three times to complete the lysis. One hundred microliters of the homogenate was added to the substrate solution, which contained 10 mM *p*-nitrophenyl phosphate in 100 mM diethanolamine buffer (pH 10.5) supplemented with 0.5 mM $MgCl_2$. After 30 min of incubation at 37 °C, the reaction was terminated by the addition of 0.1 mL of 2 M NaOH, and the alkaline phosphatase activity was determined spectrophotometrically (405 nm) by measuring *p*-nitrophenyl released from the substrate. The enzyme activity was expressed as μmol of substrate cleaved per mg of cell protein.

Estrogen Promoter Assay in MCF-7 Cells. The responsive elements of estrogen receptor 5′-GGT CAC AGT GAC C-3′ were multiplied three times (*18*) and subcloned into pTAL-Luc mammalian expression vector (Clontech, Mountain View, CA) with a downstream reporter of firefly luciferase gene; this DNA construct was named as pERE-Luc. MCF-7 cells were stably transfected with pERE-Luc; the selection of stable cell was reported previously (*19*). The stable transfected MCF-7 cells were maintained in MEM containing nonessential amino acid, sodium pyruvate, and 10% fetal bovine serum. The medium was then changed to MEM-α without phenol red containing 2% charcoal−dextran-treated fetal bovine serum for 2−3 days. MCF-7 cells were seeded in 24 well plates at 25000 cells/cm^2 and incubated in a 5% CO_2 water-saturated growth chamber at 37 °C. DBT was applied on day 2 of the culture for 48 h. The medium was aspirated, and MCF-7 cells were washed with cold PBS. The cells were lysed with 100 μL/well of lysis buffer at 4 °C and subsequently frozen for 15 min at −80 °C. The lysates were collected after thawing and centrifugation. Fifty microliters of the supernatant was used to determine the luciferase activity; the activity was normalized by protein.

Anti-Platelet Aggregation Assay. Blood was collected from adult New Zealand white rabbits (fed regular diet of rabbit chow) by a 10 mL syringe through a polyethylene cannula placed in the common carotid artery. The first few milliliters of blood were discarded, and the rest was diluted to 10-fold with 3.8% trisodium citrate. The platelet-rich plasma was achieved by centrifugation at 800 rpm for 10 min. The platelet-poor plasma was achieved by centrifugation at 4000 rpm for 10 min; this plasma was used as a background reading in the assay. Extracts were added 5 min before ADP (inducer; 10 μM final). The aggregations at 5 (maximum; Amax) and at 1 min (A1′) were recorded by a Sanda-196 platelet aggregator (Shanghai, China). Ticlopidine was used as a positive control. The inhibition activity of platelet aggregation was calculated by the following formula: (ADP-induced A_{max} − sample-induced A_{max})/(ADP-induced A_{max}) × 100%.

Other Assays and Statistical Analysis. In statistical analysis of different samples, SPSS software (version 11.0.0 from Statistical Product and Service Solutions, Chicago, IL) was used. The total protein was measured with a Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). Data are expressed as means ± standard errors of the mean (SEM) of triplicate determinations; where *n* was normally over 5. The evaluation of statistical significance was determined by one-way analysis of variance.

RESULTS

Chemical Optimization of the Drug Ratio. Radix Astragali-derived astragaloside IV, calycosin, and formononetin, Radix Angelicae Sinensis-derived ferulic acid and ligustilide, and total saponins, total flavonoids, and total polysaccharides were determined in DBT extracts that were prepared from drugs of different ratios from 1:1 to 10:1. The rationale to use these chemicals for analyses is that they have been demonstrated to have known biological functions as described previously (*2*, *5*, *6*). The identification, calibration conditions, and their recoveries of these chemicals in HPLC analyses were fully described in ref *2*. To find the best combination of drugs in DBT, the chemical compositions of DBT with different ratios of Radix Astragali and Radix Angelicae Sinensis were determined. The amounts of chemical markers within DBT showed significant variation under seven groups of drug ratios, which are summarized in **Table 1**. The amount of astragaloside IV at the ratio of 5:1 was higher than the other groups, which showed ∼2-fold difference as compared to the lowest group, i.e., a 10:1 drug ratio. The ratio at 5:1 also contained higher amounts of calycosin, formononetin, and ferulic acid; the difference could be at ∼2-fold in comparison to the lowest groups. In contrast, the amount of ligustilide was found to be the lowest at the drug ratio of 5:1, while the group at 2:1 showed the highest amount. Besides the five chemical markers, the amounts of total saponins, total flavonoids, and total polysaccharides were determined, and in line with the previous chemical analyses, it was found that DBT preparation from a 5:1 drug ratio had higher amounts (**Table 1**).

Biological Optimization of the Drug Ratio. Stimulation of blood circulation and improvement of osteoporosis problems are the major functions of DBT in treating women's aliments during menopause (*2*, *4*). Thus, the effect of DBT with different ratios of Radix Astragali and Radix Angelicae Sinensis on the proliferation and differentiation of cultured ostoblast MG-63 cells was determined. Cultured MG-63 cells are a common cell line used in analyzing bone formation (*20*). The herbal extract induced both the proliferation and the differentiation of ostoblast MG-63 cells in a dose-dependent manner; 1 mg/mL of extract showed submaximal induction effects and therefore was used in all assays (see ref *2*). In cultures treated with DBT for 72 h,

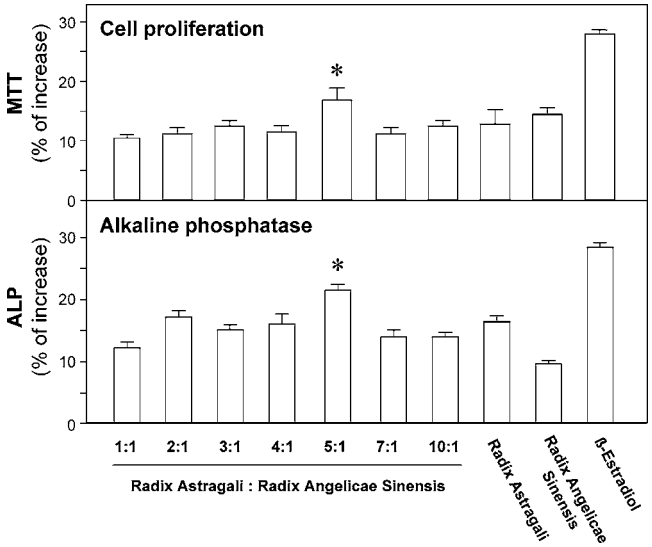

Figure 1. Extracts of Radix Astragali, Radix Angelicae Sinensis, and DBT increased the proliferation and the expression of alkaline phosphatase (ALP) in cultured MG-63 cells. β-Estradiol (0.01 μM) was used as a positive control. DBT (1 mg/mL) at different ratios of Radix Astragali and Radix Angelicae Sinensis were tested. Values of cell proliferation (by MTT assay) and expression of ALP are expressed in percentage of increase as compared to control cultures (without herbal extract) and in means ± SEM, where $n = 5$, each with triplicate samples. *The difference by comparing to the lowest group is significant ($p < 0.01$).

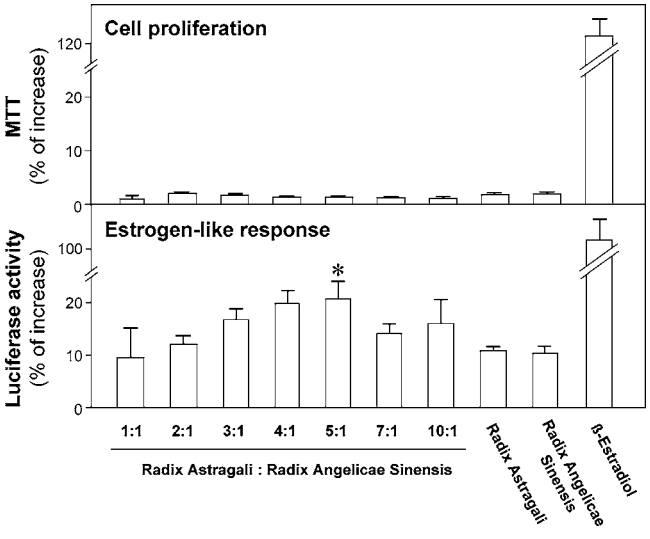

Figure 2. Extracts of Radix Astragali, Radix Angelicae Sinensis, and DBT increased the activity of estrogen-responsive element in cultured MCF-7 cells. Extracts (1 mg/mL) from different group as in **Figure 1** were tested. β-Estradiol (0.01 μM) was used as a positive control. DBT at different ratios of Radix Astragali and Radix Angelicae Sinensis were tested. Values of cell proliferation (by MTT assay) and promoter-driven luciferase (pERE-Luc) are expressed in percentage of increase as compared to control cultures (without herbal extract) and in means ± SEM, where $n = 5$, each with triplicate samples. *The difference by comparing to the lowest group is significant ($p < 0.01$).

a significant increase (~20%) of MG-63 cells' proliferation and differentiation was observed when compared with the untreated cells. In both of the bioassays, the activities derived from a single drug, either Radix Astragali or Radix Angelicae Sinensis, were lower than that of DBT, at least when compared to several groups here (**Figure 1**). By using the MTT assay, the drug ratio at 5:1 processed a much stronger activity in stimulating MG-63 cell proliferation, which was 10−20% higher than the other groups (**Figure 1**, upper panel). The level of alkaline phosphatase expressed by cultured MG-63 cells, serving as an indicative marker for bone cell differentiation, was also increased by the herbal extracts. As with the cell proliferation, the 5:1 drug ratio induced enzymatic activity to a higher extent when compared to other groups (**Figure 1**, lower panel).

The estrogenic effect of DBT was also tested in cultured MCF-7 cells. MCF-7 is a breast cancer cell line that is commonly used for testing estrogenic effects (*21*). A promoter−reporter DNA construct (pERE-Luc) corresponding to the responsive elements of estrogen receptor was stably transfected into MCF-7 cells. Different extracts of DBT were applied onto the cultures for 2 days. Two parameters were determined as follows: cell number and luciferase activity. As shown in **Figure 2**, upper panel, DBT was not able to alter the proliferation of MCF-7 cells, even at a higher concentration of DBT (data not shown). In contrast, the estrogen-driven promoter activity was markedly induced by DBT; the best group in inducing the promoter activity was the 5:1 group (**Figure 2**, lower panel). The difference could reach ~2-fold by comparing the highest (5:1) and the lowest (1:1) groups.

The activity of DBT in preventing ADP-induced platelet aggregation was determined. The drug ratios at 5:1 and 7:1 showed that the two groups had a higher activity in preventing the platelet aggregation (**Figure 3**). Treatment of single herbal extract, either Radix Astragali or Radix Angelicae Sinensis, showed a much lower (~50% reduction) anti-platelet aggregation activity as compared to DBT. Ticlopidine serving as a

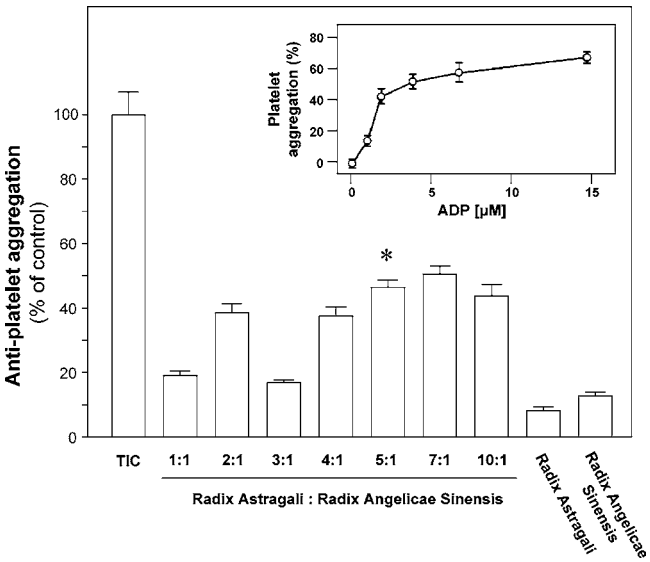

Figure 3. Extracts (1 mg/mL) from different groups as in **Figure 1** were tested for anti-platelet aggregation activity. ADP (10 μM) was used as an inducer. Values in anti-platelet aggregation activity are expressed in percentage of positive control (0.2 mM ticlopidine; TIC). The dose response of ticlopidine is shown in the insert, where 50% inhibition is at 0.2 mM. The values are in means ± SEM, where $n = 5$, each with triplicate samples. *The difference by comparing to the lowest group is significant ($p < 0.01$).

control prevented the aggregation of platelets (*22*) in a dose-dependent manner (**Figure 3**, insert).

Role of Processed Radix Angelicae Sinensis in DBT. In the original description of DBT, Radix Angelicae Sinensis had to be processed with wine. This unique preparation of the roots was used in the past but is not used today. However, whether this ethanol treatment is important in DBT function has not been determined. The chemical and biological properties of DBT were

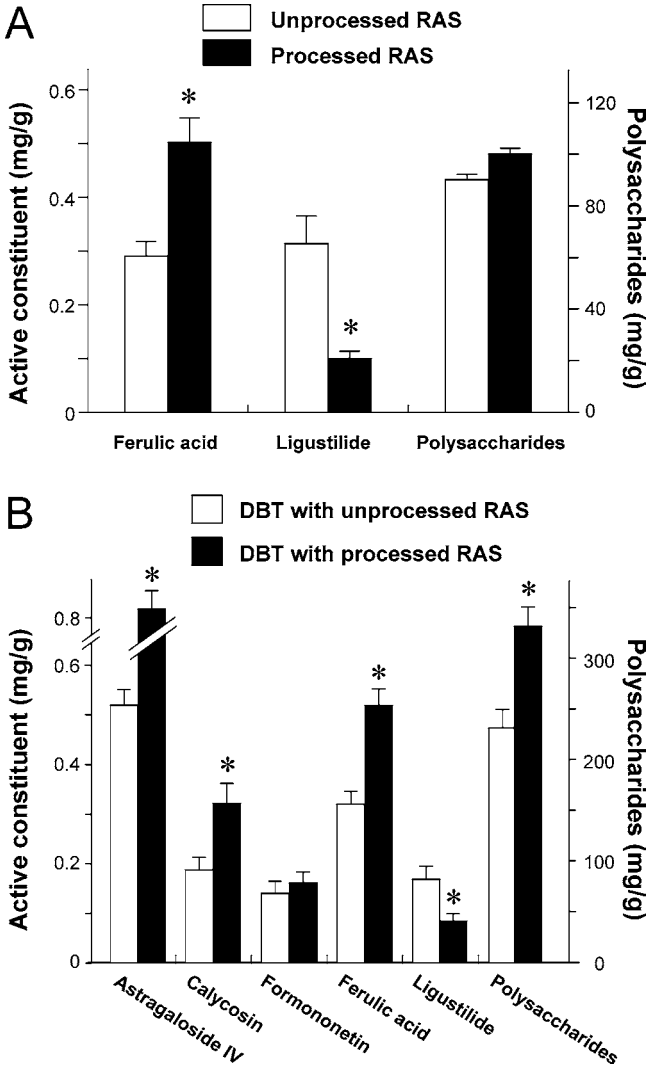

Figure 4. Effect of processed Radix Angelicae Sinensis in chemical composition of DBT. Ethanol-treated Radix Angelicae Sinensis was used in DBT preparation. The amounts of astragaloside IV, calycosin, formononetin, ligustilide, and ferulic acid were calibrated in Radix Angelicae Sinensis (RAS) and DBT as in **Table 1**. (**A**) Amount of ferulic acid, ligustilide, and total polysaccharides in ethanol-treated Radix Angelicae Sinensis. (**B**) Amounts of astragaloside IV, calycosin, formononetin, ferulic acid, ligustilide, and total polysaccharides in DBT prepared from an herb ratio of 5:1 of Radix Astragali and Radix Angelicae Sinensis. Values are expressed in mg/g of dry material and in means ± SEM, where $n = 5$. *The difference by comparing to unprocessed Radix Angelicae Sinensis is significant ($p < 0.01$).

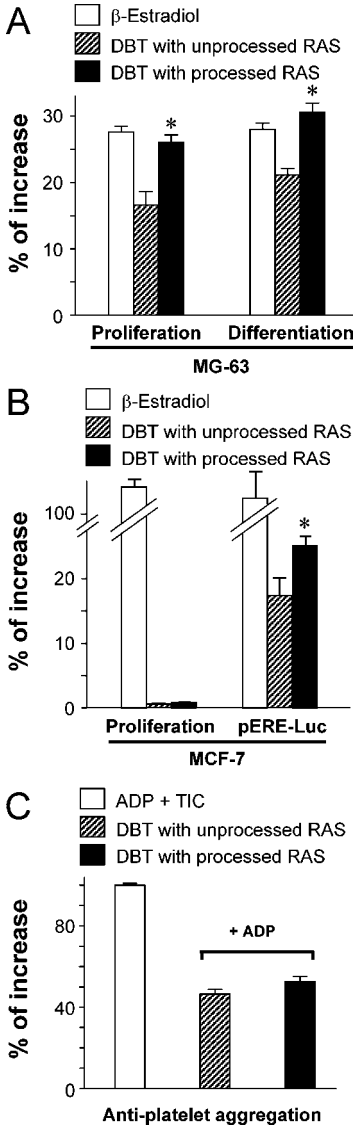

Figure 5. Effect of processed Radix Angelicae Sinensis in biological properties of DBT. Extracts (1 mg/mL) from DBT of a drug ratio of 5:1 from either processed or unprocessed Radix Angelicae Sinensis (RAS) were used. Different bioassays are (**A**) proliferation and differentiation of MG-63 cell, (**B**) proliferation and estrogenic effect in MCF-7 cell, and (**C**) anti-platelet aggregation. β-Estradiol (0.01 μM) and ticlopidine (TIC; 0.2 mM) were used as positive controls. Values are expressed in percentage of increase as compared to control cultures (without herbal extract) and in means ± SEM, where $n = 5$, each with triplicate samples. *The difference by comparing to unprocessed Radix Angelicae Sinensis is significant ($p < 0.01$).

compared by using unprocessed and processed Radix Angelicae Sinensis. By extracting Radix Angelicae Sinensis alone, the ethanol-treated roots showed a ~1.5-fold increase of ferulic acid in the extract, but this treatment decreased the amount of ligustilide by over 60% (**Figure 4A**). In addition, DBT from Radix Astragali and Radix Angelicae Sinensis (either processed or unprocessed) at a 5:1 ratio were prepared. In the chemical analyses, DBT prepared from ethanol-treated Radix Angelicae Sinensis contained significantly higher amounts of astragaloside IV, calycosin, formononetin, and total polysaccharides; the difference could range from ~10 to ~2-fold. In contrast, the treatment caused a reduction of ligustilide by ~30% in the DBT (**Figure 4B**). Thus, ethanol treatment on Radix Angelicae Sinensis increased the amount of ferulic acid but decreased ligustilide in DBT extracts.

The biological property of DBT prepared from processed Radix Angelicae Sinensis was determined. In all bioassays, DBT prepared from processed Radix Angelicae Sinensis showed higher biological responses as compared to that of DBT prepared from unprocessed Radix Angelicae Sinensis. The increase of activity was more robust in the induction of MG-63 cell proliferation and differentiation; DBT prepared from processed Radix Angelicae Sinensis increased the two parameters by ~10% when compared with that from unprocessed Radix Angelicae Sinensis (**Figure 5A**). A small but significant increase of estrogenic effect of processed DBT in MCF-7 was also revealed (**Figure 5B**). However, the effect of the processed Radix Angelicae Sinensis on anti-platelet aggregation was insignificant (**Figure 5C**). In view of the chemical and biological

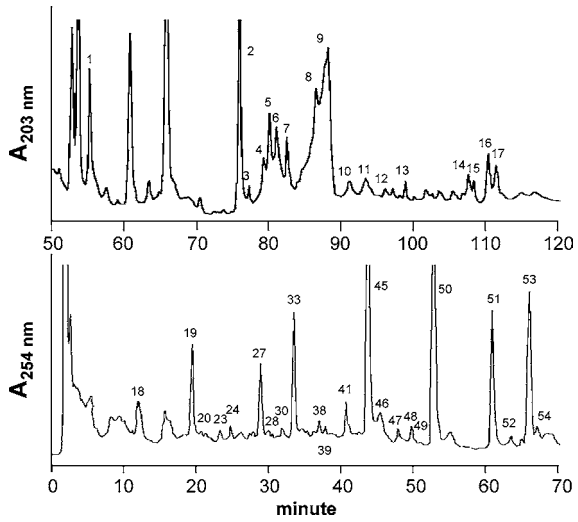

Figure 6. Fifty-four peaks in typical HPLC fingerprints of DBT are shown here. In the HPLC fingerprint of 203 nm, astragaloside IV and another 16 peaks had retention times between 70 and 120 min. In the HPLC fingerprint of 254 nm, ferulic acid, calycosin, formononetin, ligustilide, and another 32 peaks had retention times between 0 and 70 min. The 54 peaks are numbered, where astragaloside IV (1), ferulic acid (19), calycosin (33), formononetin (50), and ligustilide (53) are identified and serve as standards.

results, the ancient wisdom in the processing of Radix Angelicae Sinensis seemed to have a unique function during the preparation of DBT.

Correlation between Chemical Fingerprints and Bioactivities of DBT. By using different wavelengths of the HPLC detector, 54 chemical peaks were detected in DBT extracts (**Figure 6**), and this analysis was applied to all extracts being tested; that is, in total, over 100 different DBT extracts from varied preparations were analyzed. Among these 54 peaks, the corresponding markers of Radix Astragali-derived astragaloside IV, calycosin, and formononetin and Radix Angelicae Sinensis-derived ferulic acid and ligustilide were identified. In analysis of correlation, the identified 54 peak areas together with the contents of total saponins, total flavonoids, and total polysaccharides were considered as independent variables. The results of the four bioactivities, which included proliferation and differentiation of MG-63 cells, estrogenic property in MCF-7 cells, and anti-platelet aggregation activity, were considered as dependent variables. By analyzing the correlation of the two variables, coefficients of correlation between the 57 chemical data and the bioassay data of DBT were obtained. The coefficient shows the possible actions of these chemical peaks in different bioassays; that is, the positive value suggests an active role while the negative value suggests an opposite role.

In the analysis of MG-63 cell proliferation, the content of astragaloside IV, formononetin, total saponins, and total flavonoids affected the bioactivity very significantly (**Figure 7**). For the differentiation of MG-63 cells, the content of formononetin, total saponins, and total flavonoids would affect the bioactivity very significantly. In the analysis of estrogen promoter in MCF-7 cell, the contents of ferulic acid would affect the bioactivity very significantly. Last, calycosin and total polysaccharides were two very significant factors in the assay of anti-platelet aggregation. In contrast, the amount of ligustilide shows an opposite effect in all of the bioassays, which suggests the negative role of this chemical. Among the 57 chemical data, there are other components, such as the peaks 5−15, which have a higher correlation coefficient with the biological activities, but the identities of which have not be revealed.

DISCUSSION

In our previous study, we have demonstrated that the yields of astragaloside IV, calycosin, formononetin, and ferulic acid in DBT extracts were the highest when the extraction was done in 8 volumes of solvent, extracted for 2 h, and extracted twice (*2*). This extraction resembles the original method of having the drugs boiled in two bowls of water over moderate heat until the final volume was reduced by half. Here, we further demonstrated that the drug ratio of Radix Astragali and Radix Angelicae Sinensis should be maintained at 5:1 to achieve the best chemical composition and biological responses. Moreover, the ethanol-treated Radix Angelicae Sinensis should be used in DBT. In summary, our results strongly support the efficacy of this 800 year old formulation of DBT, and the original formulation has been optimized chemically and biologically. Although we do not know how Chinese in the past could make up such an optimized formula, we should not change the

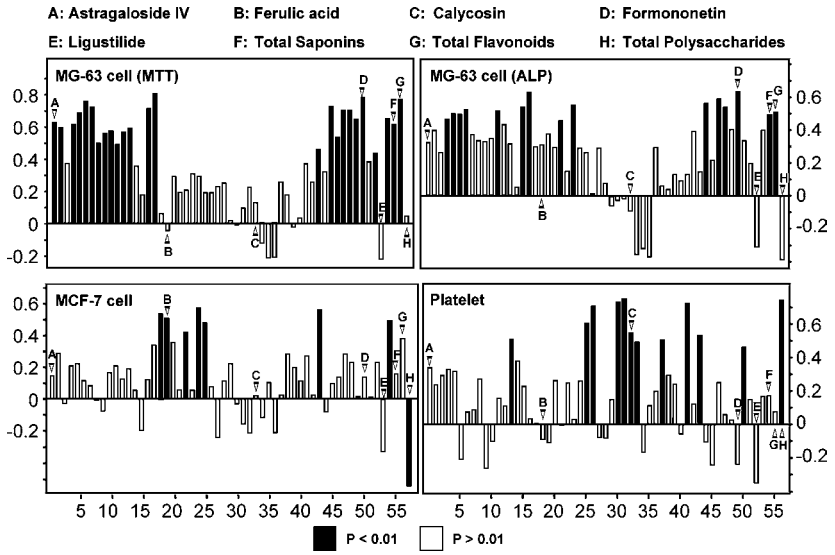

Figure 7. Correlation coefficients between 57 chemical data with four bioassays. The correlation coefficient is at the *Y*-axis, and the peak number is at the *X*-axis. Individual chemical markers are indicated by arrowheads and denoted by astragaloside IV (**A**), ferulic acid (**B**), calycosin (**C**), formononetin (**D**), ligustilide (**E**), total saponins (**F**), total flavonoids (**G**), and total polysaccharides (**H**).

formulation easily unless we completely understand the basic biology of this decoction.

In addition to the aforementioned in vitro assays, the requirement of a 5:1 ratio of Radix Astragali and Radix Angelicae Sinensis in DBT has been tested and proven in animal studies. In DBT-administrated mice, the formulation with Radix Astragali and Radix Angelicae Sinensis at a 5:1 ratio was the most effective decoction in triggering the immune responses (*23*, *24*). In angiogenesis, DBT increased the formation of capillaries and the count of blood vessels, and again, the ratio of Radix Astragali−Radix Angelicae Sinensis at 5:1 showed the best effect (*25*). DBT is known to enhance myocardial mitochondrial as well as red blood cell glutathione status, thereby increasing their resistance to oxidative stress-induced injury in animal. In DBT-administrated rats, the herbal extracts protected against myocardial ischaemia−reperfusion injury in a dose-dependent manner (*26*). A more potent cardioprotection was revealed in DBT treatment than that of extracts from Radix Astragali, or from Radix Angelicae Sinensis, or from a mixture of two root extracts (not boiled together).

An optimized condition in extracting active constituents from DBT could be a good explanation for having the best biological properties. However, under what mechanism could DBT having two drugs provide better solubility of active constituents? The explanations for higher yields of active constituents in DBT could be due to several possibilities. First, compounds such as saponins, over 2% in total dry weight, in Radix Astragali (*15*) help the solubility of other components that are being extracted from Radix Angelicae Sinensis; that is, astragalosides increase the solubility of Radix Angelicae Sinensis-derived ferulic acid. Second, ferulic acid is readily oxidized under heat, which therefore could be degraded due to the boiling (*9*). However, when Radix Angelicae Sinensis is boiled together with Radix Astragali, components derived from Radix Astragali may prevent this oxidization process and thus produce a higher yield of ferulic acid in DBT (*9*). Third, the stability of those active constituents could be improved by having a cocktail of different chemicals with DBT decoction. Nevertheless, the solubilities of the chemical markers, as well as the biological properties within DBT, agree very well with the ancient formulation of the two drugs in a decoction.

Chemically and biologically, ethanol-treated Radix Angelicae Sinensis could provide a better DBT. According to the medicinal theory of TCM, nonprocessed Radix Angelicae Sinensis is considered to be extremely irritating to our body. The irritation reduces the biological functions of Radix Angelicae Sinensis; these functions include activating blood circulation, enriching blood, and stimulating menstrual discharge (*27*). Traditionally, this irritant is believed to be eliminated by treatment of ethanol. Ligustilide is considered to be the main irritant in Radix Angelicae Sinensis. Having an excess amount of ligustilide could result in nausea, xeransis, and anaesthesia of the oral cavity and tongue (*28*). As shown in this study (**Figure 4**), the ethanol treatment markedly reduced the amount of ligustilide in both Radix Angelicae Sinensis alone and in DBT, which subsequently increased the bioactivities of DBT. The ethanol treatment of Radix Angelicae Sinensis is known to cause the evaporation of volatile oils, including ligustilide. In contrast, specific processing of Radix Astragali was not described in the ancient formulation. Historically, the dried roots of *Astragalus* are recommended, which is still commonly used today.

Our result on the correlation analysis (**Figure 7**) suggests that the amounts of astragaloside IV, formononetin, ferulic acid, total saponins, total flavonoids, and total polysaccharide are in accord with the results of bioassays. In contrast, the amount of ligustilide could serve as an opposite indicator for DBT functions. From our analysis, other chemical peaks from the HPLC fingerprints also show a positive correlation; however, the identities of these chemicals are not revealed. Whether these chemicals are crucial to the functions of DBT have not been determined. With the positive indicative markers for DBT, one can develop a mean in the quality control of DBT.

Although we have revealed the chemical properties of DBT, the action mechanisms of DBT in cells or in our body are still not yet known. Following this line of direction, proteomic analysis of DBT in culture cells has been used in our laboratory to reveal DBT-specific activated proteins. The protein profiles of cultured cells after the treatments of extracts from Radix Astragali alone, Radix Angelicae Sinensis alone, or DBT will be resolved. These profiles will provide hints in answering the role of DBT in cells. Again, this study could be done more easily in DBT, which only contains two drugs.

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LITERATURE CITED

(1) Zheng, X. Y. *Pharmacopoeia of the People's Republic of China*; Chemistry Industry Press: Beijing, China, 2005.

(2) Song, Z. H.; Ji, Z. H.; Lo, C. K.; Dong, T. T. X.; Zhao, K. J.; Li, O. T. W.; Haines, C. J.; Kung, S. D.; Tsim, K. W. K. Chemical and biological assessment of a traditional Chinese herbal decoction prepared from Radix Astragali and Radix Angelicae Sinensis: Orthogonal array design to optimize the extraction of chemical constituents. *Planta Med.* **2004**, *70*, 1222−1227.

(3) Wu, D. Z.; Song, C. Q.; Hen, Z. F.; Kong, J. L.; Fan, Y.; Hu, Z. B. Effects of Angelicae Sinensis decoction for supplementing blood on the cardiac function in myocardial ischemia reperfusion injury of rats. *Pharmacol. Clin. Chin. Mater. Med.* **1999**, *15*, 3−6.

(4) Ning, L.; Chen, C. X.; Jin, R. M.; Wu, Y. P.; Zhang, H. G.; Sun, C. L.; Song, C. Q.; Hu, Z. B. Effect of components of Dang-Gui-Bu-Xue decoction on hematopenia. *Chin. J. Chin. Mater. Med.* **2002**, *27*, 50−52.

(5) Sinclair, S. Chinese herbs: A clinical review of Astragalus, Ligusticum, and Schizandrae. *Altern. Med. Rev.* **1998**, *3*, 338−344.

(6) Wagner, H.; Bauer, R.; Xiao, P. G.; Chen, J. M.; Michler, G. *Chinese Drug Monographs and Analysis: Radix Astragali (Huangqi)*; Verlag: Wald, Germany, 1997; pp 1−17.

(7) Ma, X. Q.; Shi, Q.; Duan, J. A.; Dong, T. T. X.; Tsim, K. W. K. Chemical analysis of Radix Astragali (Huangqi) in China: A comparison with its adulterants and seasonal variations. *J. Agric. Food Chem.* **2002**, *50*, 4861−4866.

(8) Wagner, H.; Bauer, R.; Xiao, P. G.; Chen, J. M.; Michler, G. *Chinese Monographs and Analysis: Angelica Sinensis*; Verlag: Wald, Germany, 2001; Vol. 3.

(9) Zhao, K. J.; Dong, T. T. X.; Tu, P. F.; Song, Z. H.; Lo, C. K.; Tsim, K. W. K. Molecular genetic and chemical assessment of Radix Angelica (Danggui) in China. *J. Agric. Food Chem.* **2003**, *51*, 2576−2583.

(10) Mineo, S.; Takayasu, M.; Syoichi, S.; Masao, Y.; Naokata, M. Evaluation of Angelicae Radix (Touki) by the inhibitory effect on platelet aggregation. *Chem. Pharm. Bull.* **1991**, *39*, 2046−2048.

(11) Liu, J.; Burdette, J. E.; Xu, H.; Gu, C.; Breemen, R. B.; Bhat, K. P.; Booth, N.; Constantinou, A. I.; Pezzuto, J. M.; Fong, H. H.; Farnsworth, N. R.; Bolton, J. L. Evaluation of estrogenic activity of plant extracts for the potential treatment of menopausal symptoms. *J. Agric. Food. Chem*. **2001**, *49*, 2472−2479.

(12) Wilasrusmee, C.; Kittur, S.; Siddiqui, J.; Bruch, D.; Wilasrusmee, S.; Kittur, D. S. *In vitro* immunomodulatory effects of 10 commonly used herbs on murine lymphocytes. *J. Altern. Complement. Med.* **2002**, *8*, 467−475.

(13) Ou, S.; Kwok, K. C. Ferulic acid: Pharmaceutical functions, preparation and application in foods. *J. Sci. Food Agric*. **2004**, *84*, 1261−1269.

(14) Ma, X. Q.; Duan, J. A.; Zhu, D. Y.; Dong T. X.; Tsim, K. W. K. Chemical comparison of Astragali Radix (Huangqi) from different ragions of China. *Nat. Med.* **2000**, *54*, 213−218.

(15) Dong, T. T. X.; Cui, X. M.; Song, Z. H.; Zhao, K. J.; Ji, Z. N.; Lo, C. K.; Tsim, K. W. K. Chemical assessment of roots of *Panax notoginseng* in China: Regional and seasonal variations in its active constituents. *J. Agric. Food. Chem*. **2003**, *51*, 4617−4623.

(16) Li, S. P.; Zhao, K. J.; Ji, Z. N.; Song, Z. H.; Dong, T. T. X.; Lo, C. K.; Cheung, J. K. H.; Zhu, S. Q.; Tsim, K. W. K. A polysaccharide isolated from *Cordyceps sinensis*, a traditional Chinese medicine, protects PC12 cells against hydrogen peroxide-induced injury. *Life Sci.* **2003**, *73*, 2503−2513.

(17) Lbbotson, K. J.; Harrod, J.; Gowen, M.; D'Souza, S.; Smith, D. D.; Winkler, M. E.; Derynck, R.; Mundy, G. R. Human recombinant transforming growth factor alpha stimulates bone resorption and inhibits formation in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 2228−2232.

(18) Klinge, C. M. Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res.* **2001**, *29* (14), 2905−2919.

(19) Tung, E. K. K.; Choi, R. C. Y.; Siow, N. L.; Jiang, J. X. S.; Ling, K. K. Y.; Simon, J.; Barnard, E. A.; Tsim, K. W. K. P2Y2 receptor activation regulates the expression of acetylcholinesterase and acetylcholine receptor genes at vertebrate neuromuscular junctions. *Mol. Pharmacol.* **2004**, *66*, 794−806.

(20) Ilbbotson, K. J.; Harrod, J.; Gowen, M.; D'Souza, S.; Smith, D. D.; Winkler, M. E.; Derynck, R.; Mundy, G. R. Human recombinant transforming growth factor alpha stimulates bone resorption and inhibits formation in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 2228−2232.

(21) Boue, S. M.; Wiese, T. E.; Nehls, S.; Burow, M. E.; Elliott, S.; Carter-Wientjes, C. H.; Shih, B. Y.; McLachlan, J. A.; Cleveland, T. E. Evaluation of the estrogenic effects of legume extracts containing phytoestrogens. *J. Agric. Food Chem*. **2003**, *51*, 2193−2199.

(22) Ashida, S. I.; Abiko, Y. Inhibition of platelet aggregation by a new agent, *Ticlopidine Thromb. Haemost.* **1979**, *40*, 542−550.

(23) Wu, B. C.; Sun, X. F.; Yang, G. Y. Studies on different combination of the Dang Gui Buxue decoction. *Chin. Med. Mater. Hei Long Jiang* **1989**, *4*, 41−45.

(24) Li, Y. K.; Xu, J.; Zhang, X. C. Pharmacology study on Dang Gui Buxue decoction. *Chin. J. Herbal Pharmacol. Ther*. **1992**, *8*, 16−20.

(25) Lei, Y.; Chen, K. J. Study on angiogenesis effect of Radix Astragala, Radix Angelicae Sinensis & their combination. Seminar on Chinese Medical Sciences (Macau), 2003; pp 110−117.

(26) Mak, D. H. F.; Chiu, P. Y.; Dong, T. X. X.; Tsim, K. W. K.; Ko, K. M. Dang-Gui Buxue Tang produces a more potent cardioprotective effects than its component herb extracts and enhances glutathione status in rat heart mitochondria and erythrocytes. *Phytother. Res*. In press.

(27) Ye, Y. N.; Liu, E. S.; Li, Y.; So, H. L.; Cho, C. C.; Sheng, H. P.; Lee, S. S.; Cho, C. H. Protective effect of polysaccharides-enriched fraction from *Angelica sinensis* on hepatic injury. *Life Sci.* **2001**, *69*, 637−646.

(28) Xie, M. *Modern Study of the Medical Formulae in Traditional Chinese Medicine*; Xueyuan Press: Beijing, China, 1997; Vol. 1, pp 603−604.

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