JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Chemical and Biological Assessment of Angelicae Sinensis Radix after Processing with Wine: An Orthogonal Array Design To Reveal the Optimized Conditions

Janis Y. X. Zhan, Ken Y. Z. Zheng, Kevin Y. Zhu, Cathy W. C. Bi, Wendy L. Zhang, Crystal Y. Q. Du, Qiang Fu, Tina T. X. Dong, Roy C. Y. Choi, Karl W. K. Tsim,* and David T. W. Lau*

Section of Marine Ecology and Biotechnology, Division of Life Science, and Center for Chinese Medicine, The Hong Kong University of Science and Technology, Clear Water Bay Road, Hong Kong, China

Supporting Information

ABSTRACT: The roots of *Angelica sinensis* [Angelica Sinensis Radix (ASR)] have been used as a common health food supplement for women's care for thousands of years in China. According to Asian tradition, ASR could be processed with the treatment of wine, which subsequently promoted the biological functions of ASR. By chemical and biological assessments, an orthogonal array design was employed here to determine the roles of three variable parameters in the processing of ASR, including oven temperature, baking time, and flipping frequency. The results suggested that oven temperature and baking time were two significant factors, while flipping frequency was a subordinate factor. The optimized condition of processing with wine therefore was considered to be heating in an oven at 80 °C for 90 min with flipping twice per hour. Under the optimized processing conditions, the solubilities of ferulic acid and *Z*-ligustilide from ASR were markedly increased and decreased, respectively. In parallel, the biological functions of processed ASR were enhanced in both anti-platelet aggregation and estrogenic activation; these increased functions could be a result of the altered levels of ferulic acid and *Z*-ligustilide in wine-processed ASR. Thus, the chemical and biological assessment of the processed ASR was in full accordance with the Chinese old tradition.

KEYWORDS: *Angelicae sinensis* (Oliv.) Diels, traditional Chinese medicine, orthogonal array design, quality control, processing with wine

INTRODUCTION

Angelica Sinensis Radix [roots of *Angelica sinensis* (Oliv.) Diels; family Umbellaceae; Danggui in Chinese; ASR] is well-known as "female ginseng" and has been used for more than 2000 years in China as a health food supplement for women's health. The use of ASR was first cited in *Shennong Bencao Jing* (200−300 A.D., Han Dynasty in China); the described functions were to replenish blood, to invigorate blood, and to relieve pain. Nowadays, ASR is being used commonly to promote blood circulation[1] and to modify the immune system[2] in the treatment of menstrual disorders, as well as being a laxative for chronic constipation of aged and debilitated patients.[3] The Chinese Pharmacopoeia (2010) recorded that *Angelica* root in China is derived from the root of *A. sinensis*; however, *Angelica acutiloba* (Sieb. et Zucc.) Kitag. is mainly found in Japan and *Angelica gigas* Nakai is mainly found in Korea. Besides the common usage in Asia, *Angelica* root is also being used as a health food product in Europe and the U.S.A. Therefore, the demand for *Angelica* root is enormous throughout the world.

The processing of Chinese crude herbs is well-known to have its unique function, e.g., to reduce toxicity, to enhance efficacy, and to stabilize active ingredients. To obtain these effects, different processing methods have been developed for Chinese herbs, which could be classified into the following categories: parching, stir-baking with liquid, calcining, roasting in fresh cinders, steaming, boiling, etc. Among different types of processing, the treatment with wine is one of the most commonly used methods. According to an old tradition, ASR should be prepared with the treatment of yellow wine. The wine-processed ASR is believed to have better functions in endorsing the "blood" circulation.[4] Indeed, the wine-processed ASR is recommended to be used in many herbal decoctions, e.g., *Danggui Buxue Tang*, *Siwu Tang*, and *Jiuzhuan Huangjing Dan*.[5] In functional analysis of *Danggui Buxue Tang*, a common herbal decoction for women's aliments, the wine-processed ASR could increase the bioactivity of the decoction in inducing bone cell proliferation and/or differentiation, as well as its estrogenic properties in breast cancer cells.[6] Although the roles of processing with wine (wine processing) have been proposed in ASR functions, an optimized method has not been determined chemically and biologically. More important, the consequence of wine processing in ASR has not been revealed. Here, we attempted to optimize the wine-processing method via an orthogonal array experimental design and to reveal the biological distinctions of this processed ASR.

Over 70 compounds have been isolated and identified from ASR; most of them are phthalides, terpenes, and aromatic compounds. Among these chemicals, ferulic acid, senkyunolide I,

Received: February 10, 2011
Revised: April 25, 2011
Accepted: April 26, 2011
Published: April 26, 2011

Table 1. Orthogonal Array Design in Establishing the Conditions of Processing with Wine

	processing parameters		
group[a]	oven temperature (°C)	time (min)[b]	flipping frequency[c]
1	60	60	2
2	60	90	8
3	60	120	4
4	80	60	4
5	80	90	2
6	80	120	8
7	100	60	8
8	100	90	4
9	100	120	2

[a] Nine groups having different combinations of parameters during the wine processing. Each group contained 50 g of ASR as the starting material, and five batches of preparations were performed (n = 5). [b] Baking time in the oven. [c] Flipping frequency per hour.

senkyunolide H, and *Z*-ligustilide are the most abundant and are considered as biologically active components within ASR.[7−9] Indeed, ferulic acid and volatile oils are being used as markers.[10] Here, these four components are chosen as marker chemicals to ensure the quality of wine-processed ASR. Three levels of parameters of processing were considered, and nine different combinations of variables were established for calibration. Having the solubilities of aforementioned four chemical markers, the optimized conditions of processing with wine were determined. Moreover, cell-based assays were developed here to compare the biological activities of different wine-processed ASR.

MATERIALS AND METHODS

Plant Materials. Fresh roots of *A. sinensis* were obtained from Minxian of Gansu in China in October of 2009, which was demonstrated to produce the best quality ASR.[11] The authentication of the herbs was confirmed morphologically by one of the authors, Dr. Tina Dong, at Hong Kong University of Science and Technology during the field collection. Individual samples were prepared from ∼1 kg of dried sliced ASR roots.

Preparation of Wine-Processed ASR. Three different parameters of extraction and three levels for each were studied by orthogonal array design of L_9 (3^4), and nine combinations of the wine-processing method having different parameters were established. For the processing of ASR, about 50 g of the dried roots were sliced and sprayed with 5 mL of yellow wine (Best Spirits, Zhejiang, China; 15−16% alcohol) or 15% (v/v) ethanol and then processed in an oven according to different variations of the parameters (see Table 1), and in total, nine groups of processed ASR were obtained, each group with triplicate samples. In the extraction of ASR, about 15 g of processed ASR was weighed, ground into powder by a blender, then boiled in 120 mL of water for 2 h, and extracted twice. For the second extraction of processed ASR, the residue from the first extraction was filtered and the same extracting conditions were applied. The extract was dried under vacuum and stored at −80 °C, which was used for chemical and biological determination.

Chemical Analysis. Ferulic acid was from Sigma (St. Louis, MO). *Z*-Ligustilide was kindly provided by Prof. Pengfei Tu, Medical College of Peking University. Senkyunolide I and senkyunolide H were from Shanghai Sunny Biotech (Shanghai, China); their purities, confirmed by high-performance liquid chromatography (HPLC), were higher than 98.0%. Analytical- and HPLC-grade reagents were from Merck (Darmstadt, Germany). For the assay of ferulic acid, senkyunolide I, senkyunolide H, and *Z*-ligustilide, about 0.4 g of ASR extract was weighed into a 50 mL centrifugal tube and 20 mL of methanol was added for sonication at 60 min. After centrifugation (2500*g* for 10 min), the supernatant was collected and filtered through a 0.45 μm Millipore syringe filter unit for HPLC analysis.

A Waters HPLC system consisting of a 600 pump, a 717 autosampler, and an ultraviolet−visible (UV−vis) photodiode array 2996 detector was used for all analyses. Chromatographic separations were carried out on a Prevail 18 column (particle size, 4 μm; 4.6 × 250 mm) with acetonitrile, with 1% acetate acid in water as the mobile phase at a flow rate of 1.0 mL/min at room temperature. The running condition was developed as below: an isostatic elution was applied at 22% acetonitrile starting from 0 to 18 min, and a linear gradient elution was applied from 22 to 100% acetonitrile starting from 18 to 60 min. A total of 10 μL of sample was injected for HPLC analysis, and the signals were detected at 325 nm for ferulic acid and *Z*-ligustilide and at 280 nm for senkyunolide I and senkyunolide H with a photodiode array detector. For the calibrations of four markers, the standards were weighed and dissolved in methanol to give serial concentrations from 0.01 to 100 μg/mL. Three injections onto HPLC were performed for each dilution. The concentrations of these compounds in the extracts were calculated according to the regression parameters derived from the standard curves.

Anti-platelet Aggregation Assay. Blood was collected from adult New Zealand white rabbits through a polyethylene cannula placed in the common carotid artery by a 10 mL syringe. 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 150*g* for 10 min. The platelet-poor plasma was achieved by centrifugation at 2000*g* for 10 min; this plasma was used as a background reading in the assay.[12] The ASR extracts were added 5 min before adenosine 5′-diphosphate (ADP, inducer; 10 μM final). The aggregations at 5 min (maximum; A_{max}) and at 1 min (A1′) were recorded using a Sanda-196 platelet aggregator (Shanghai, China). Ticlopidine (TIC) was used as a positive control. The inhibition activity of platelet aggregation was calculated by the formula: (ADP-induced A_{max} − sample-induced A_{max})/(ADP-induced A_{max}) × 100%.

MCF-7 Cell Proliferation and Estrogenic Assay. Human mammary epithelial carcinoma MCF-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were grown in modified Eagle's medium (MEM), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM pyruvate, 100 units/mL penicillin, and 100 units/mL streptomycinin in a humidified CO_2 (5%) incubator at 37 °C. All culture reagents were purchased from Invitrogen Technologies (Carlsbad, CA). The cell proliferation was measured by the 3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[13] In brief, the cells were cultured in 96-well plates and were treated with ASR extract for 24 h. MTT (Sigma) was added to the cultures, and then the cells were extracted by dimethyl sulfoxide (DMSO) solvent. The absorbance at 570 nm was measured. β-Estradiol (Sigma; 100 nM) served as a positive control for the proliferation of the MCF-7 cell.

The responsive elements of estrogen receptor 5′-GGT CAC AGT GAC C-3′ were multiplied 3 times[14] and subcloned into pTAL-Luc mammalian expression vector (Clontech, Mountain View, CA), having a downstream reporter of firefly luciferase gene; this DNA construct was named as pERE-Luc. Cultured MCF-7 cells were transfected with pERE-Luc to generate stable cells according to a previous report; the selection of the stable cell was reported previously.[15] The stable transfected MCF-7 cells were maintained in MEM containing non-essential amino acids, 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 30 000 cells/cm^2 and incubated in a 5% CO_2

water-saturated growth chamber at 37 °C. Different groups of ASR extracts were applied to 1-day-old cultures for 24 h. The medium was aspirated, and MCF-7 cells were washed with ice-cold phosphate-buffered saline (PBS). The cells were lysed with 100 μL/well lysis buffer at 4 °C and, subsequently, frozen for 15 min at −80 °C. The lysates were collected after thawing and centrifugation. A total of 50 μL of the supernatant was used to determine the luciferase activity; the activity was normalized by the amount of protein. The amount of protein was determined by Bradford's method (Bio-Rad, Hercules, CA).

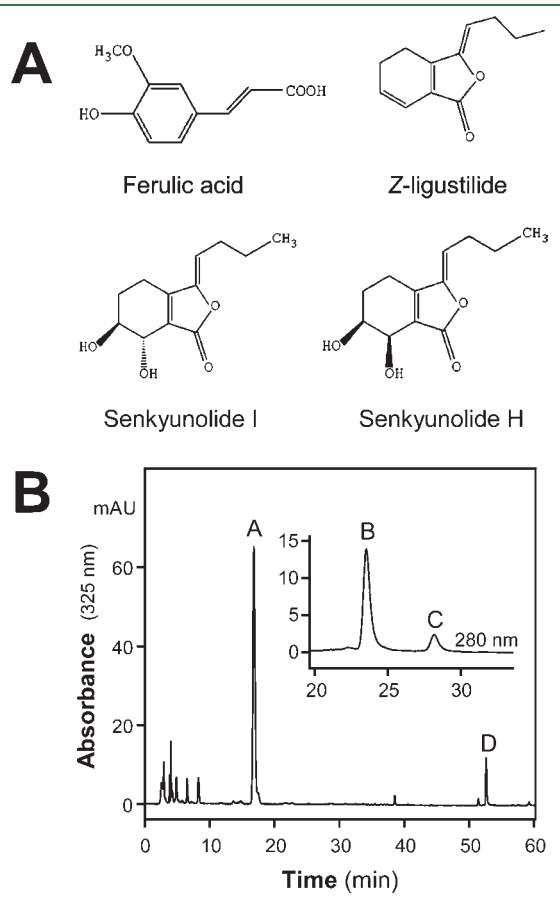

Figure 1. Determinations of ferulic acid, senkyunolide I, senkyunolide H, and Z-ligustilide in the processed ASR using HPLC. (A) Chemical structures of ferulic acid, senkyunolide I, senkyunolide H, and Z-ligustilide were shown. (B) Signals were detected at an absorbance of 280 nm [(B) senkyunolide I and (C) senkyunolide H] and 325 nm [(A) ferulic acid and (D) Z-ligustilide] by using a Prevail C_{18} column. The running conditions were described in the Materials and Methods. A typical chromatogram is shown; $n = 4$.

Statistical Analysis. In hierarchical clustering analysis of different samples, conjoint analysis of SPSS software (version 11.0 from Statistical Product and Service Solutions, Chicago, IL) was used. Statistical tests were performed using one-way analysis of variation (ANOVA). The significant differences between treatments were analyzed by an independent t test of SPSS. Statistically significant changes were classified as (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$.

RESULTS

Chemical Analysis of ASR after Processing. In an orthogonal array design analysis of wine processing of ASR, the parameters of the tested variations were shown in Table 1. These parameters were chosen according to possible critical factors in the old tradition of processing with wine, which included oven temperature, incubation time, and flipping frequency. In total, nine different groups were established. The major chemical components in ASR, including ferulic acid, senkyunolide I, senkyunolide H, and Z-ligustilide, were determined by HPLC (Figure 1A). The quantitation of marker chemicals was carried out by measuring the peak area according to the regression equation (Figure 1B and Table 2). Using the established HPLC method, the calibration curves of ferulic acid, senkyunolide I, senkyunolide H, and Z-ligustilide exhibited good linearity in a specific range of concentrations. The correlation coefficients (r^2) of those chemical markers were from 0.9993 to 0.9998. The precision and repeatability of the chemical measurement were excellent, having a relative standard deviation (RSD) <5% (Table 2). The recovery experiment was carried out to evaluate the method accuracy. Known amounts of the chemical markers were included in various groups of the processed ASR and extracted accordingly; their recoveries were from 97 to 102% (Table 2). Thus, the employed HPLC method was validated in performing the quantitative analysis.

The amount of ferulic acid, senkyunolide I, senkyunolide H, and Z-ligustilide in the processed ASR showed significant variation under different groups of wine treatment (Figure 2). The amount of ferulic acid at group 5 was higher than the others, which showed ~50% increase compared to the lowest ferulic-acid-containing group (e.g., group 1). Among the nine groups, the amount of senkyunolide I and senkyunolide H was not significantly altered. In contrast, the yield of Z-ligustilide in group 5 was significantly lower than that from other groups, i.e., ~30% lower compared to group 1 (Figure 2). Different parameters were analyzed statistically, and the results were summarized in Table 3. The oven temperature and baking time were two distinct factors, and flipping frequency was a subordinate factor. The oven temperature ($p < 0.05$) was a crucial factor in the yield of ferulic acid. The yield of senkyunolide I as well as senkyunolide H could be affected by the oven temperature ($p < 0.1$). For the yield

Table 2. Method Validation of Ferulic Acid, Senkyunolide I, Senkyunolide H, and Z-Ligustilide by HPLC Analysis[a]

chemical	calibration curve	correlation factor (r^2)	linearity (μg/mL)	precision RSD (%)	repeatability RSD (%)	recovery (%)
ferulic acid	$y = 59458x - 71138$	0.9994	0.01−100	1.70	2.85	97.21
senkyunolide I	$y = 23494x + 1217$	0.9996	0.01−100	1.45	3.91	98.47
senkyunolide H	$y = 14642x - 257.2$	0.9993	0.01−100	2.03	4.46	101.12
Z-ligustilide	$y = 16214x - 32478$	0.9998	0.01−100	1.14	3.52	102.45

[a] These samples were subjected to HPLC analysis. The calibration curve was used to calibrate the concentration of various chemical constituents. The mean values were expressed here, and the standard error of the mean (SEM) values of the five tested chemicals were less than 5% of the mean. The calibration was repeated 5 times ($n = 5$).

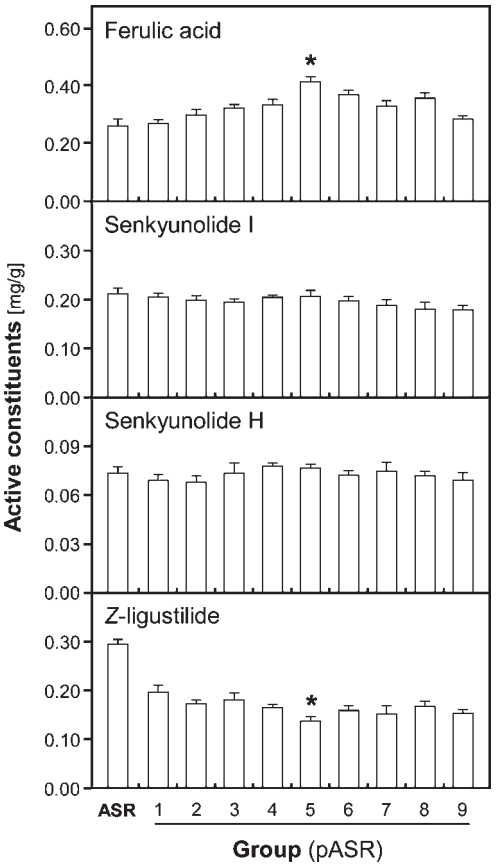

Figure 2. Comparison of four chemical markers in the extracts of processed ASR. The wine-processed ASR (pASR) was generated in different groups as described in Table 1. The unprocessed ASR (ASR) served as a control. Values are expressed in milligrams per gram of dry material and in mean ± SEM, where $n = 5$. The difference by comparing to the lowest group for ferulic acid is significant (∗, $p < 0.05$), and the difference by comparing to the highest group for *Z*-ligustilide is significant (∗, $p < 0.05$).

Table 3. Analysis of Variances in Chemical Constituents in Different Groups

index	source of variation[a]	F^b	p^c
ferulic acid	*A*	22.76	<0.05
	B	4.99	>0.1
	C	0.74	>0.1
senkyunolide I	*A*	9.98	<0.1
	B	1.67	>0.1
	C	0.38	>0.1
senkyunolide H	*A*	9.61	<0.1
	B	4.35	>0.1
	C	1.02	>0.1
Z-ligustilide	*A*	111.48	<0.01
	B	26.93	<0.05
	C	18.06	<0.1

[a] The variables are *A*, oven temperature; *B*, baking time; and *C*, flipping frequency per hour. The calibration was repeated 5 times ($n = 5$). [b] Calibrated using SPSS statistics, where $F_{0.01}(2,2) = 99.00$; $F_{0.05}(2,2) = 19.00$; $F_{0.1}(2,2) = 9.00$. The degree of freedom is 2. [c] Level of significance.

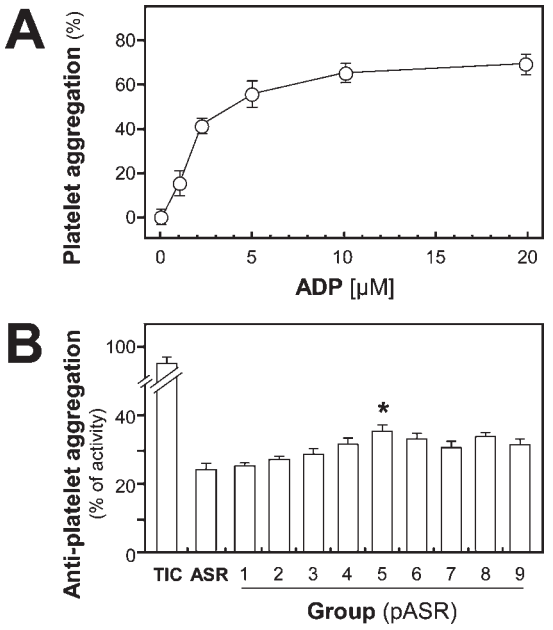

Figure 3. Anti-platelet aggregation activities of wine-processed ASR. (A) ADP induced platelet aggregation in a dose-dependent manner. The percentage of platelet aggregation at 5 min (A_{max}; sub-maximal) was presented. (B) Extracts derived from ASR after wine proceeding (pASR) prevented ADP-induced platelet aggregation. The extracts at 1 mg/mL were used in all cases. ADP (10 μM) was used as an inducer. Values in anti-platelet aggregation are expressed as the percentage of activity, as described in the Materials and Methods. TIC (0.2 mM) served as a control. Values are in mean ± SEM ($n = 5$), each with triplicate samples. The difference by comparing to the lowest group is significant (∗, $p < 0.05$).

of *Z*-ligustilide, the oven temperature ($p < 0.01$) and the baking time ($p < 0.05$) showed more significant roles than that of the flipping frequency ($p < 0.1$). Thus, group 5 here seemed to be rather distinct, as compared to others; i.e., the processed ASR from group 5 contained the highest amount of ferulic acid but the lowest amount of *Z*-ligustilide (Figure 2).

Biological Activity of Processed ASR. Besides the chemical analysis, the bioactivities of those processed ASR from different groups were also tested here. The stimulation of blood circulation is considered as one of the major functions of wine-processed ASR in treating women's aliments during menopause.[16] Thus, the activity of ASR extracts deriving from different processing methods in preventing platelet aggregation was determined. Application of ADP induced platelet aggregation in a dose-dependent manner (Figure 3A). The extracts of wine-processed ASR prevented the ADP-induced platelet aggregation. Group 5 had the highest activity in preventing the aggregation of platelets by over 35%, and the processed ASR of groups 4, 6, and 8 also showed significantly higher anti-platelet aggregation activity (Figure 3B). Interestingly, these groups were being processed at 80 or 100 °C. These results suggested that the oven temperature during ASR processing could be an important parameter for this aggregating activity. Additionally, groups 4, 5, and 6 and groups 7, 8, and 9, classified by oven temperature, could be compared under different baking times (see Table 1). The inhibitory activities of platelet aggregation of processed ASR from groups 5 and 8 (both baked for 90 min) showed much stronger activity than those of other groups at different baking

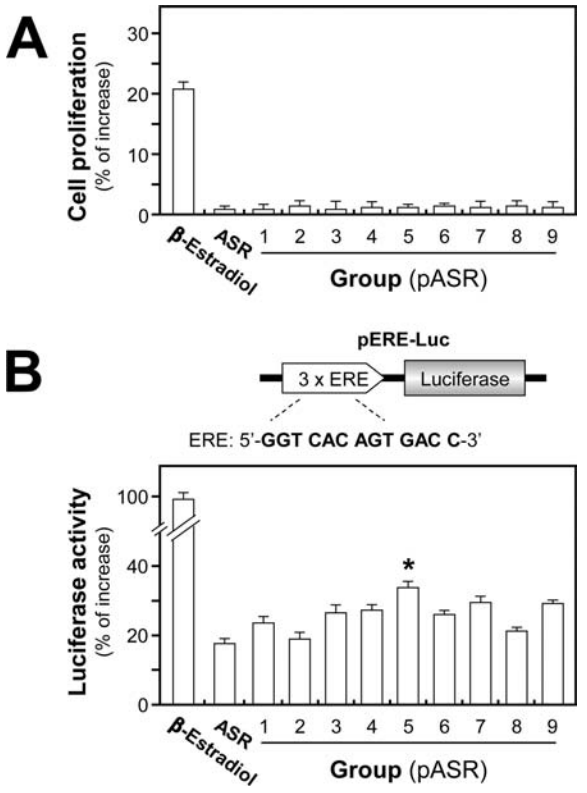

Figure 4. Estrogenic effects of wine-processed ASR in cultured MCF-7 cells. (A) Cultured MCF-7 cells were treated with extracts from ASR or from wine-processed ASR (pASR) at 1 mg/mL. β-Estradiol (100 nM) served as a positive control. (B) pERE-Luc was constructed by 3 repeats of estrogen response element (ERE) in a luciferase promoter−reporter vector (upper panel). Cultured MCF-7 cells were treated with the extracts from pASR. Values of cell proliferation and promoter-driven luciferase (pERE-Luc) are expressed as a percentage of increase compared to control cultures (without herbal extract) and in mean ± SEM, where n = 5, each with triplicate samples. (*) Difference by comparing to the lowest group is significant ($p < 0.05$).

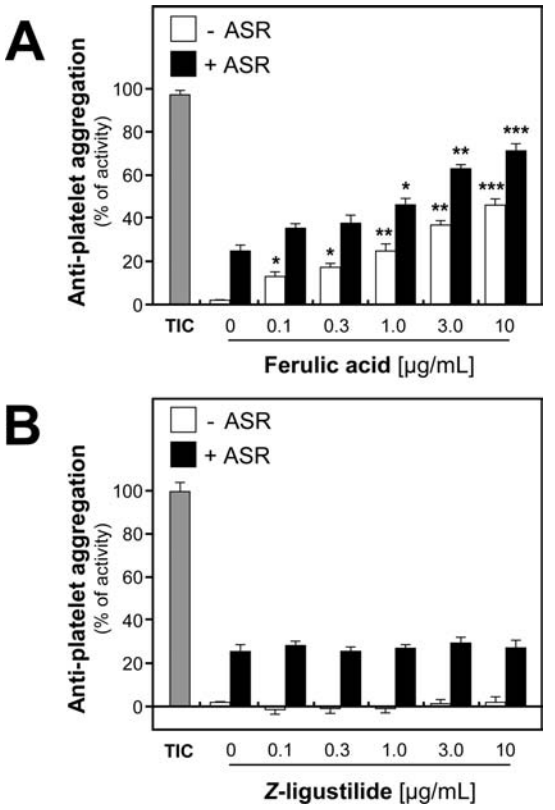

Figure 5. Ferulic acid and Z-ligustilide affect ASR function in anti-platelet aggregation. Different amounts of (A) ferulic acid and (B) Z-ligustilide at 0−10 μg/mL were co-applied with ASR extract (1 mg/mL) in platelets, and subsequently, the activities were determined as in Figure 3B. ADP (10 μM) was used as an inducer. Values in anti-platelet aggregation are expressed as the percentage of activity, as described in the Materials and Methods. TIC (0.2 mM) served as a control. Values are in mean ± SEM, where n = 5, each with triplicate samples. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$.

times. Again, this result suggested that the baking time of processed ASR affected the bioactivity.

The estrogenic property of ASR was tested in cultured MCF-7 cells. A promoter−reporter DNA construct (pERE-Luc) containing three repeats of estrogen-responsive element was stably transfected into cultured MCF-7 cells (Figure 4). The extract from different wine-processed ASR was applied onto the cultures for 1 day. Two biological effects, cell viability and promoter-driven luciferase activity, were subsequently determined. The marked proliferation of MCF-7 cells was considered as the side effect in stimulating breast cancer cell growth. As shown in Figure 4A, the processed ASR in all groups was not able to alter the proliferation of MCF-7 cells, even at high concentrations of the herbal extract (data not shown). In contrast, the pERE-Luc activity was induced by the processed ASR. The best group in inducing the promoter activity was group 5, and groups 7 and 9 also showed significantly higher estrogenic activity (Figure 4B); these groups were processed with 80 or 100 °C baking temperature. The results showed that the oven temperature during the processing was an important parameter for the estrogenic activity.

Chemical and Biological Correlation in ASR. To give further explanation of the relationship between the chemical composition and biological function of processed ASR, we compared the contents and bioactivities of the four tested chemicals in the nine groups. From the analysis of variances, the extraction efficiency of ferulic acid, senkyunolide I, and senkyunolide H was dependent upon oven temperature, while Z-ligustilide was dependent upon oven temperature and baking time (Table 3). Because of the obvious differences of ferulic acid and Z-ligustilide in the processed ASR, their stimulatory activities on blood circulation were evaluated. Application of ferulic acid demonstrated a dose-dependent response in anti-platelet aggregation activity, while Z-ligustilide did not have any effect on platelet aggregation (Figure 5). A similar situation was observed when the chemicals were applied together with ASR extracts (Figure 5). On the other hand, application of ferulic acid decreased the proliferation of cultured MCF-7 cells in a dose-dependent manner (upper panel in Figure 6A) and showed very little effect on the estrogenic activity (lower panel in Figure 6A).

In contrast, Z-ligustilide showed the opposite effect of ferulic acid. The proliferation of MCF-7 cells was stimulated, dose-dependently, by application of Z-ligustilide (upper panel of Figure 6B); the stimulation reached over a 20% increase under the effect of 10 μg/mL Z-ligustilide. In the estrogenic assay, Z-ligustilide did not possess any effect up to 10 μg/mL (lower panel of Figure 6B). Different amounts of Z-ligustilide together with ASR were added to the culture, and the effects were similar to

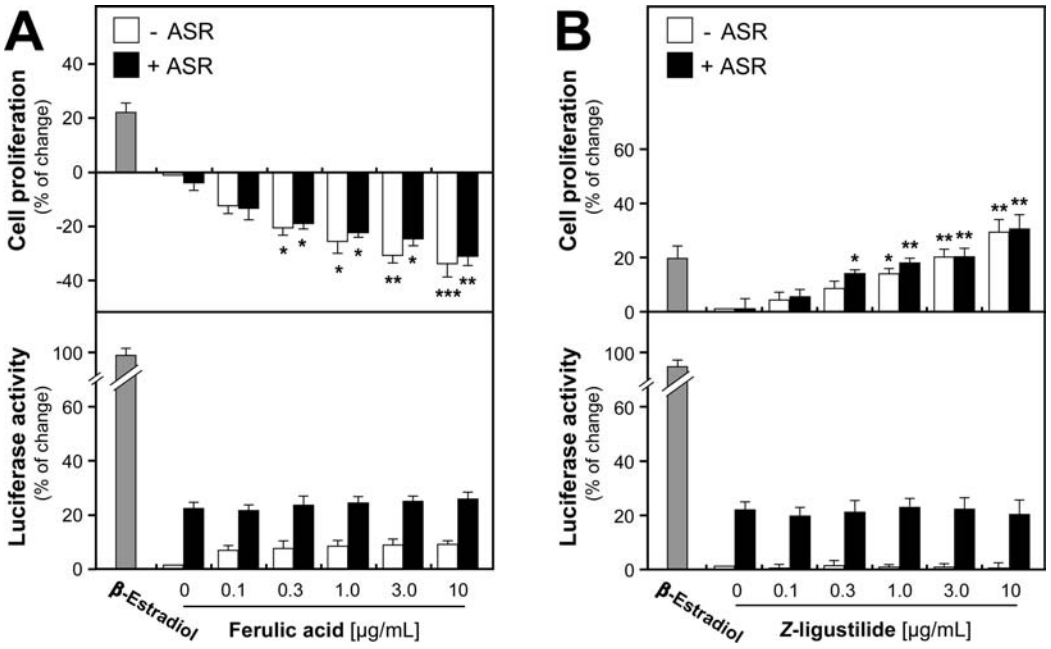

Figure 6. Ferulic acid and *Z*-ligustilide affect ASR function in cultured MCF-7 cells. Different amounts of (A) ferulic acid or (B) *Z*-ligustilide were co-applied with or without ASR extract (1 mg/mL) onto cultured pERE-Luc stable transfected MCF-7 cells. The assays were performed as in Figure 4. β-Estradiol (100 nM) was used as a control. Values are expressed as the percentage of change compared to the control culture (without any drug treatment) and in mean ± SEM, where $n = 5$, each with triplicate samples. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$.

that of the single application of *Z*-ligustilide (Figure 6). These results suggested that ferulic acid and *Z*-ligustilide would affect the activities of ASR in anti-platelet aggregation and MCF-7 cell proliferation. Under this scenario, the highest amount of ferulic acid and the lowest amount of *Z*-ligustilide in group 5 could provide a good explanation for its maximal biological properties and/or its minimal side effect.

DISCUSSION

In China, the herbal processing has been developed for thousands of years, and now this is an important part of the herbal usages. After a long history of practicing, the Chinese have demonstrated the requirement of herb processing according to different properties of herbs. For example, the toxicity of *Achyranthes* root, bitter almond kernel (Semen Armeniacae Amarum), and kansui root could be reduced through the processing.[17] On the other hand, the functions of *Aster* root and coltsfoot (*Tussilago farfara*) flower in arresting cough could be strengthened after baking with honey.[18]

According to Chinese medicinal theory, the "wine processing" has three reasons: (i) promoting the drug absorption into the blood, (ii) enhancing the blood circulation and accelerating drug delivery, and (iii) reducing the harmful ingredients. This method of processing is commonly used today. For example, the side effects of *Dichroa* root in treating malaria could be reduced by baking with wine.[19] The stir baking of Mulberry Twig with yellow wine has an effect in eliminating pathogenic dampness and relieving arthralgia.[20] In Chinese ancient usage, ASR had to be processed with yellow wine; i.e., a heated pan was used to fry ASR with yellow wine. However, this traditional method could not control the temperature, which is not conducive for quality assurance. In recent years, oven baking rather than the traditional stir baking has been proposed,[21] and therefore, this method has been employed. Here, an optimized condition of ASR processing with wine, via an orthogonal array design assay, was established, which should be processed under heating in an oven at 80 °C for 90 min with flipping twice per hour. The current results provide a stable and consistent wine-processing methodology on the basis of the traditional one. This standardized processing method is suitable for large-scale production of processed ASR. Besides ASR, the traditional processing with wine is commonly applied in other crude herbs of traditional Chinese medicine (TCM). Thus, the current orthogonal array experimental design could pave a direction in optimizing the processing of other herbs with wine.

The reasons of the wine processing in ASR are still unknown. Here, we attempted to understand the rationale by revealing the change of chemical content and biological efficacy. The results showed that ferulic acid and *Z*-ligustilide had changed inversely; i.e., the content of ferulic acid increased, while that of *Z*-ligustilide decreased, in the extract from processed ASR. Ferulic acid, serving as a chemical marker of ASR in Chinese Pharmacopoeia,[10] possesses the functions in reducing the level of nitrite and oxygen free radicals, lowering blood lipids, resisting bacteria, diminishing inflammation, suppressing tumor, and enhancing immune function.[22−24] In our study, ferulic acid showed the anti-platelet aggregation activity, as well as the inhibitory effect on proliferation of MCF-7 cells. This evidence suggested that ferulic acid was one of the active compounds in ASR. Therefore, the enhancement of ferulic acid in ASR after wine processing could increase its biological effects. For *Z*-ligustilide, it is effective in treating vasodilatation, improving microcirculation effects, and regulating the contractility of vascular and uterine smooth muscle.[25−27] On the other hand, the side effects of *Z*-ligustilide have also been reported.[28] In *Danggui Buxue Tang*, a high level of *Z*-ligustilide could reduce the solubility of chemicals in Astragali Radix after water boiling. In addition, a high level of *Z*-ligustilide could decrease the osteogenic activity of *Danggui Buxue*, as well

as induce the proliferation of MCF-7 cells.[28] These results suggested that the functions of *Z*-ligustilide could depend upon its concentration, as well as its applied environment, e.g., the targeted biological responses. Traditionally, the unprocessed ASR contains a high concentration of volatile oil, which is considered to be extremely irritating to our body.[29] This irritant within unprocessed ASR may be too high for its pharmacological efficacy; therefore, ASR should be processed by wine to reduce the level of *Z*-ligustilide. In gas chromatography−mass spectrometry (GC−MS) analysis, over 40 compounds of ASR were detected. In comparison to unprocessed ASR, about 30 volatile compounds declined obviously in wine-processed ASR (see the Supplementary Table in the Supporting Information). Among these volatile oils, *Z*-ligustilide is the main irritant in ASR. An excess amount of *Z*-ligustilide results in xeransis, nausea, and anesthesia of the oral cavity and tongue.[29] On the basis of this evidence, *Z*-ligustilide may be considered as a negative regulator in ASR, which should be reduced by the wine processing.

Yellow wine, a common Chinese alcoholic beverage containing 14−20% alcohol, has been used for a long history in processing of Chinese herbs. Here, the yellow wine from Shaoxing was chosen for analysis. This yellow wine is the oldest and most representative yellow wine. Besides alcohol, the wine contains other ingredients, and indeed, this uncertainty is hard to be standardized. Therefore, we studied whether 15% ethanol could be used to substitute the wine. The result showed that 15% ethanol-processed ASR and wine-processed ASR were rather similar chemically; however, these two methods have resulted in distinct bioactivities. The biological activities of wine-processed ASR were better than those of 15% ethanol-processed ASR, especially significantly in the cell proliferation of MCF-7 cells (see the Supplementary Figure in the Supporting Information). Thus, other ingredients within yellow wine might result in these differences. The unknown reason should be revealed further.

■ ASSOCIATED CONTENT

Supporting Information. Volatile components in ASR after wine processing (Supplementary Table) and comparison of chemical and biological assessment of wine- and ethanol-processed ASR (Supplementary Figure). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: (852) 2358-7332 (K.W.K.T.); (852) 2358-5818 (D.T.W.L.). Fax: (852) 2358-1559 (K.W.K.T.); (852) 2358-1559 (D.T.W.L.). E-mail: botsim@ust.hk (K.W.K.T.); davidlau@ust.hk (D.T.W.L.).

Funding Sources

This research was supported by grants from the Research Grants Council of Hong Kong (6419/06M, N_HKUST629/07, and 662608 to K.W.K.T. and 661110 to D.T.W.L.) and the Croucher Foundation (CAS-CF07/08.SC03 to K.W.K.T.).

■ ABBREVIATIONS USED

ASR, Angelica Sinensis Radix; TCM, traditional Chinese medicine; ERE, estrogen response element

■ REFERENCES

(1) Yim, T. K.; Wu, W. K. Myocardial protection against ischaemia-reperfusior injury by a *Polygonum multiflorum* extract supplemented "Dang-Gui decoction for enriching blood", a compound formulation, *ex vivo*. *Phytother. Res.* **2000**, *14*, 195–199.

(2) Wilasrusmee, C.; Kittur, S.; Siddiqui, J.; Bruch, D.; Wilasrusmee, S.; Kittur, D. S. *In vitro* immunomodulatory effects of ten commonly used herbs on murine lymphocytes. *J. Altern. Complementary Med.* **2002**, *8*, 467–475.

(3) Liu, C. X.; Xiao, P. G. *An Introduction to Chinese Meteria Medica*; Peking Union Medical College and Beijing Medical University Press: Beijing, China, 1993; p 168.

(4) Ye, D. J.; Yuan, S. T. *Dictionary of Chinese Medicinal Herbs Preparation*; Shanghai Scientific and Technical Publishers: Shanghai, China, 2005; pp 257−259.

(5) Wang, X. T.; Cao, H.; Liu, Y. P. *Plucking and Processing Technology of Chinese Medicine*; Huaxia Press: Beijing, China, 2000; pp 132−138.

(6) Dong, T. T. X.; Zhao, K. J.; Gao, Q. T.; Ji, Z. N.; Zhu, T. T.; Li, J.; Duan, R.; Cheung, A. W. H.; Tsim, K. W. K. Chemical and biological assessment of a Chinese herbal decoction containing Radix Astragali and Radix Angelicae Sinensis: Determination of drug ratio in having optimized properties. *J. Agric. Food Chem.* **2006**, *54*, 2767–2774.

(7) Liu, C. X.; Xiao, P. G.; Li, D. P. *Modern Research and Application of Chinese Medicinal Plants*; Hong Kong Medical Publisher: Hong Kong, China, 2000; pp 166−169.

(8) Yu, Y.; Lin, B. Q.; Yu, L.; Hua, Y. Q.; Duan, J. A.; Li, S. P. Inhibitory effects of two ferulates from *Angelica sinensis* on platelet aggregation and oxytocin-induced uterine contraction. *Open Bioact. Compd. J.* **2009**, *2*, 43–46.

(9) Zhu, M.; Tang, Y. P.; Duan, J. A.; Guo, J. M.; Guo, S.; Su., S. L.; Shang, E. X.; Qian, D. W.; Ding, A. W. Roles of paeoniflorin and senkyunolide I in Si Wu decoction on anti-platelet and anti-coagulation activities. *J. Sep. Sci.* **2010**, *33*, 3335–3340.

(10) Shao, M. L. *Pharmacopoeia of the People's Republic of China (Part I)*; Chemical Industry Press: Beijing, China, 2010; pp 124−125.

(11) 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.

(12) Ashida, S. I.; Abiko, Y. Inhibition of platelet aggregation by a new agent, ticlopidine. *Thromb. Haemostasis* **1979**, *40*, 542–550.

(13) Gao, Q. T.; Choi, R. C. Y.; Cheung, A. W. H.; Zhu, J. T. T.; Li, J.; Chu, G. K. Y.; Duan, R.; Cheung, J. K. H.; Jiang, Z. Y.; Dong, X. B.; Zhao, K. J.; Dong, T. T. X.; Tsim, K. W. K. Danggui Buxue Tang—A Chinese herbal decoction activates the phosphorylations of extracellular signal-regulated kinase and estrogen receptor α in cultured MCF-7 cells. *FEBS Lett.* **2007**, *581*, 233–240.

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

(15) 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.

(16) Dong, W. G.; Liu, S. P.; Zhu, H. H.; Luo, H. S.; Yu, J. P. Abnormal function of platelets and role of *Angelica sinensis* in patients with ulcerative colitis. *World J. Gastroenterol.* **2004**, *10* (4), 606–609.

(17) Nie, S. Q.; Li, Z. L. The effect of processing on the toxicity and efficacy of *Achyranthes* root, bitter almond kernel and kansui root. *China J. Chin. Mater. Med.* **1996**, *21*, 153–156.

(18) Wang, Z. Y. *Aster* root and coltsfoot flower in orange pills should be honey-baked. *China J. Chin. Mater. Med.* **1990**, *09*, 25–27.

(19) Ye, D. J.; Ding, A. W.; Cai, B. C.; Ni, Z. Study on processing method of *Dichroa* root. *Chin. Tradit. Pat. Med.* **1981**, *2*, 19–21.

(20) Chen, J. M.; Chen, L.; Chen, J. Z. Orthogonal array design to optimize wine-processing method of Mulberry Twig. *Chin. Tradit. Pat. Med.* **2010**, *12*, 28–30.

(21) Lai, Y. X.; Fan, S. G. Orthogonal array design to optimize production processing of Rhizoma Chuanxiong. *West China J. Pharm. Sci.* **2005**, *20*, 446–449.

(22) Suzuki, A.; Yamamoto, M.; Jokura, H.; Fujii, A.; Tokimitsu, I.; Hase, T.; Saito., I. Ferulic acid restores endothelium-dependent vasodilation in aortas of spontaneously hypertensive rats. *Am. J. Hypertens.* **2007**, *20*, 508–513.

(23) Cheng, C. Y.; Ho, T. Y.; Lee, E. J.; Su, S. Y.; Tang, N. Y.; Hsieh, C. L. Ferulic acid reduces cerebral infarct through its anti-oxidative and anti-inflammatory effects following transient focal cerebral ischemia in rats. *Am. J. Chin. Med.* **2008**, *36*, 1105–1119.

(24) Cheng, C. Y.; Su, S. Y.; Tang, N. Y.; Ho, T. Y.; Lo, W. Y.; Hsieh, C. L. Ferulic acid inhibits nitric oxide-induced apoptosis by enhancing $GABA_{B1}$ receptor expression in transient focal cerebral ischemia in rats. *Am. J. Chin. Med.* **2010**, *31*, 889–899.

(25) Du, J.; Bai, B.; Kuang, X.; Yu, Y.; Wang, C.; Ke, Y.; Xu, Y.; Tzang, A. H. C.; Qian, Z. M. Ligustilide inhibits spontaneous and agonists- or K+ depolarization-induced contraction of rat uterus. *J Ethnopharmacol.* **2006**, *108*, 54–58.

(26) Shi, L. F.; Zheng, X. M; Cai, Z.; Wu, B. S. Comparison of influence of essential oil from *Ligusticum chuanxiong* Hort. on microcirculation in rabbit conjunctiva bulbar before and after decomposition of ligustilide. *Chin. J. Pharmacol. Toxicol.* **1995**, *9*, 157–158.

(27) Yan, S.; Qiao, G. F. Effect of the oil of *Angelica sinensis* on contractial function of isolated uterine smooth muscle of mice. *Chin. Tradit. Herb. Drugs* **2000**, *31*, 604–606.

(28) Zheng, Y. Z.; Choi, R. C. Y.; Li, J.; Xie, H. Q. H.; Cheung, A. W. H.; Duan, R.; Guo, A. J. Y.; Zhu, J. T. T.; Chen, V. P.; Bi, C. W. C.; Zhu, Y.; Lau, D. D. W.; Dong, T. T. X.; Lau, B. W. C.; Tsim, K. W. K. Ligustilide suppresses the biological properties of Danggui Buxue Tang: A Chinese herbal decoction composed of Radix Astragali and Radix Angelica Sinensis. *Planta Med.* **2009**, *76*, 439–443.

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