



Anti-platelet aggregation activity and chemical analysis of raw and wine stir-fried Chuanxiong Rhizoma (Kot-Hua-Bua)

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Abstract

Chuanxiong Rhizoma (CR) is the dried rhizome of *Ligusticum chuanxiong* Hort. It is known as Kot-Hua-Bua in Thai traditional medicine. Two types of CR, raw CR and wine stir-fried CR, were commercially available in traditional drugstores in Thailand. Raw CR was found to have more inhibitory effect on ADP-induced platelet aggregation than wine stir-fried CR (34.36 ± 5.80 vs. $9.31 \pm 3.14\%$, respectively; $p = 0.009$). Their chemical profiles using HPLC-DAD and TLC-UV-densitometric methods were similar, but the amount of several bioactive compounds found in wine stir-fried CR, e.g., senkyunolide H, senkyunolide I, ferulic acid, 3-butyridenephthalide, and levistolide A, were lesser. 5-Hydroxymethylfurfural was the artifact compound detected only in wine stir-fried CR. These results indicated that even both raw CR and wine stir-fried CR were the rhizome of the same herb, but their bioactivity and chemical quality were clearly different and should be concerned.

Key Words: Chuanxiong Rhizoma, *Ligusticum chuanxiong*, Anti-platelet Aggregation, HPLC-DAD, TLC

Introduction

Chuanxiong Rhizoma (CR) is the dried rhizome of *Ligusticum chuanxiong* Hort., family Apiaceae (Umbelliferae). It is used as a traditional Chinese medicine for activating blood and moving *qi*, dispelling wind to relieve pain [1]. In Thailand, CR is imported from the People's Republic of China and used under the name "Kot-Hua-Bua". It has been used as an ingredient in many Thai traditional formulae. Our previous study found that more than one type of CR was commercially available in traditional drugstore in Thailand. They were raw CR and wine stir-fried CR [2]. Raw CR is the general dried rhizome, whereas wine stir-fried CR is prepared by soaking raw CR in yellow wine and stir-frying with mild heat until dry [3].

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Wine stir-frying is considered to be one of the special processing methods of the Chinese Materia Medica to transform the medicinal property to a unique function [4]. Based on Chinese traditional knowledge, wine stir-frying could potentiate the circulation property of *qi* and blood of CR [5]. Alteration of chemical compositions of CR after treatment with wine under high temperature was studied. It was found that the content of ferulic acid was increased, but those of butylphthalide and senkyunolide A were decreased; whereas Z-butylidene-phthalide and Z-ligustilide were relatively stable [6]. The other study suggested that coniferyl ferulate was hydrolysed to ferulic acid, whereas Z-ligustilide was hydroxylated to senkyunolide I and senkyunolide H, or dimerized to riligustilide and levistolide [7]. Some of these degraded products have been proved to have anti-platelet aggregation activity [8-11], supporting the increased blood circulation property of wine stir-fried CR. However, activities of raw CR and wine stir-fried CR have never been directly compared. Therefore, the aim of this study was to investigate the anti-platelet aggregation activity of both CR. Their chemical profiles were also described herein.

Materials and Methods

Plant materials and extraction: Raw CR and wine stir-fried CR were purchased from a traditional drugstore in Bangkok, Thailand, in April 2011. Authentication was carried out by Prof. Li Min, School of Chinese Pharmacy, Chengdu University of Traditional Medicine. The voucher specimens (KHB and KHB-F) were deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Silpakorn University, Thailand. The ground samples (5 g) were extracted with 50 ml of 80 % methanol at 100°C, twice, to obtain 1.18 and 1.94 g of the extracts of raw CR and wine stir-fried CR, respectively. Standard ferulic acid and adenosine diphosphate (ADP) were purchased from Sigma Chemical (St Louis, U.S.A).

Anti-platelet aggregation assay: Platelet aggregation was measured by turbidimetric method using aggregometer (Aggram™, Helena laboratory, USA) according to the previous study [12]. Blood samples were obtained from healthy volunteers who did not take any medicines and vitamin supplements at least 14 days prior to blood collection. The study protocol was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand, and every volunteer signed informed consent. Blood was collected into plastic tube containing 3.2 % sodium citrate (1:9 v/v). Platelet-rich plasma (PRP) was prepared by centrifugation of the blood at 200 g for 10 min at 21°C. Remaining blood was re-centrifuged at 1500 g for 15 min at 21°C to obtain platelet-poor plasma (PPP). A platelet count was performed on the PRP to ensure that platelet concentration was in the range of 200 - 600 × 10³ platelet/μl. PPP was set as blank and it was assumed that PPP represented 100 % light transmission and PRP represented 0 % light transmission. The extract was dissolved in DMSO, diluted with normal saline (0.91 % NaCl) and 25 μl of the extract solution was added to the PRP (200 μl) to give the final

concentration of 1 mg/ml. The mixture was incubated at 37°C under stirring condition (600 rpm) for 3 min. Then, 10 μM of ADP was used to initiate platelet aggregation. The changes in light transmittance were recorded for 6 min. The results are presented as a percentage of maximum platelet aggregation and calculated as % inhibition compared with the vehicle control. Acetylsalicylic acid (ASA) in 0.5 % DMSO at the concentration of 500 μM was used as a positive control. The experiments were independently done in triplicate. Data were statistically analyzed by one way-ANOVA with Tukey test for a significance level of $p < 0.05$.

Isolation of 5-hydroxymethylfurfural (5-HMF): Dried ground wine stir-fried CR (100 g) was sequentially percolated with *n*-hexane and dichloromethane to give 0.84 and 1.77 g of the extracts, respectively. The dichloromethane extract was subjected to a silica gel (E. Merck, Germany) column chromatography eluted with *n*-hexane : ethyl acetate (3:1, v/v). Pure 5-HMF (330 mg) was obtained. Its structure was elucidated based on NMR (Bruker 300 Ultrashield) and GC-MS (Hewlett Packard 6890 GC coupled to a 5975 quadrupole MS) experiments.

5-hydroxymethylfurfural (5-HMF): Reddish brown oil. ¹H NMR (300 MHz, CDCl₃): 9.56 (s, 2-CHO), 7.23 (d, J=3.6Hz, H-3), 6.52 (d, J=3.6 Hz, H-4), 4.71 (s, 5-CH₂). ¹³C NMR (75 MHz, CDCl₃): 177.8 (2-CHO), 160.9 (C-5), 152.3 (C-2), 123.2 (C-3), 110.0 (C-4), 57.5 (5-CH₂). EIMS 70 eV, *m/z* (rel. int.): 126 (73), 109 (10), 97 (100), 69 (27), 53 (14), 41 (49).

High-performance liquid chromatography: The extracts were dissolved in methanol to give a solution containing 100 mg/ml. The standard solutions of ferulic acid (1 mg/ml) and 5-HMF (0.5 mg/ml) were prepared in methanol. An injection volume was 10 μl. The chromatographic separation was performed on a Zorbax Eclipse XDB-C18 column (4.6 × 250 mm, 5 micron) (Agilent Technologies, USA). An HPLC system consisted of Agilent 1100 series pump, an on-line solvent degasser, an autosampler, a photodiode-array detector (DAD), and a Chemstation software version A.08.01 operated at 27°C. The mobile phase consisted of methanol (A) and 1 % v/v acetic acid in water, pH 2.7 (B). A gradient method was used in which mobile phase B was set to 100 % at time zero until 5 min, subsequently decreased linearly to 60 % at 45 min, 20 % at 55 min, held at 20 % until 60 min. The flow rate was 1.0 ml/min, and detection wavelength was 270 nm.

Thin-layer chromatography: The extracts were dissolved in 80 % methanol to the concentration of 100 mg/ml. The standard solutions of ferulic acid and 5-HMF were prepared at 2 mg/ml in methanol. Twenty μl of each extract and 5 μl of each standard were applied as a 10 mm band by Nanomat4 (Camag, Switzerland) on the silica gel 60F₂₅₄ plate (E. Merck, Darmstadt, Germany). The mobile phase consisted of toluene : ethyl acetate : formic acid (8.5:1.5:0.5, v/v/v) and the developing distance was 10 cm. After air-drying, TLC plates were visualized under UV at 254 nm. Densitometric scanning was performed on

a Camag TLC scanner II (Muttentz, Switzerland) in the absorbance mode at 270 nm in conjunction with Camag CAT3.1 software. The detected bands were also scanned for UV spectra in the range of 200-400 nm.

Results and Discussion

Chuanxiong Rhizoma crude drugs commercially available in Thailand were raw CR and wine stir-fried CR [2]. Their inhibitory activities on the ADP-mediated platelet aggregation were investigated and the results are shown in **Figure 1**. At the concentration of 1 mg/ml, platelet aggregation after treated with 80 % methanol extracts of raw CR and wine stir-fried CR were 50.25 ± 4.67 and 69.53 ± 2.90 %, respectively. As compared with the vehicle control (0.5 % DMSO), percent inhibition of raw CR was significantly stronger than that of wine stir-fried CR (34.36 ± 5.80 vs. 9.31 ± 3.14 %, respectively; $p = 0.009$). Activity of raw CR was not significantly different from the positive control, ASA at 500 μ M (26.07 ± 5.26 %, $p = 0.548$). These results clearly indicated that wine stir-fried CR was less active than raw CR.

Chemical compositions of raw CR and wine stir-fried CR were investigated by HPLC and TLC chromatograms, as shown in **Figures 2** and **3**, respectively. Identification of each HPLC peak and TLC band was based on its retention time (R_t), retardation factor (R_f) and its UV spectrum (**Table 1**) by comparing with the published data [7, 13-18]. Their overall chemical profiles were similar. However, the sizes of several peaks found in a HPLC chromatogram of wine stir-fried CR were much smaller than those of raw CR (**Table 1**). Peak areas of senkyunolide I (peak no. 3) and senkyunolide H (peak no. 4) were drastically smaller. Peak areas of ferulic acid (peak no. 2), 3-butylidenephthalide (peak no. 7), and levistolide A (peak no. 9) were also reduced for more than 50 %. This indicated that the contents of these compounds were decreased by the wine treatment under high temperature. Also, from TLC analysis, the weaker band intensities of senkyunolide I, ferulic acid, and levistolide A (bands no. 3, 2 and 9, respectively) were clearly observed in wine stir-fried CR (**Figure 3**). Among all compounds, senkyunolide I, ferulic acid, Z-ligustilide and 3-butylideneph-

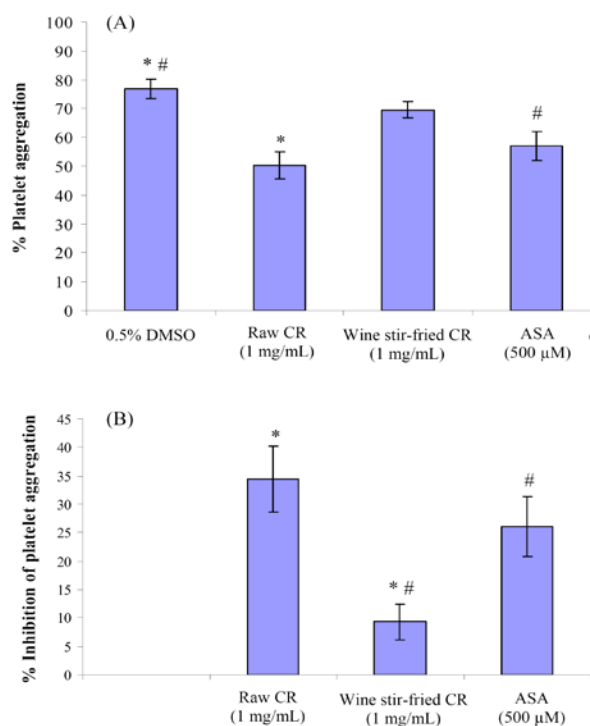


Figure 1 Anti-platelet aggregation of 80 % methanol extract of raw Chuanxiong Rhizoma and wine stir-fried Chuanxiong Rhizoma; (A) % platelet aggregation after added the extracts and (B) % inhibition of platelet aggregation compared with 0.5 % DMSO vehicle control. Data are expressed as mean $\pm$ SEM (n=5). * # Differences are statistically significant ($p < 0.05$).

thalide have been reported for antiplatelet aggregation activity [8-11, 19]. This was the undoubted reason why wine stir-fried CR had less inhibitory effect on platelet aggregation than raw CR.

On the contrary, the previous publications reported that the contents of active compounds, e.g. ferulic acid and senkyunolide I, were found to be increased after the treatment of CR with wine under high temperature [6, 7]. This might be due to the difference that the previous

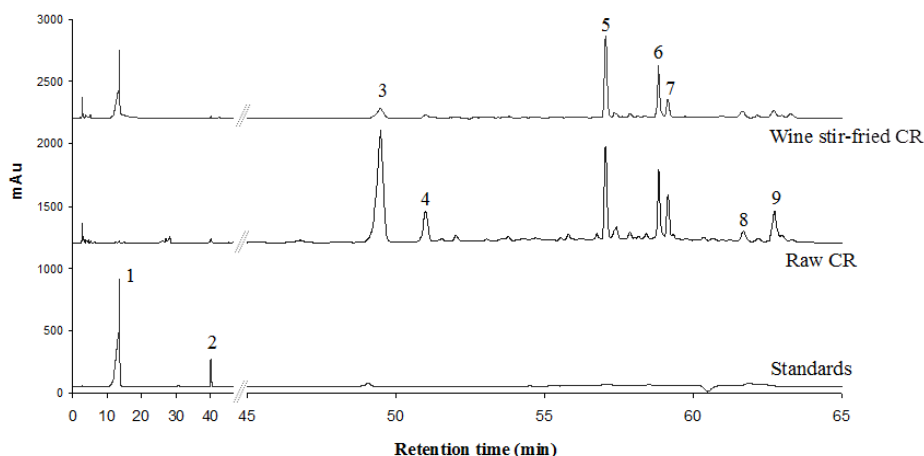


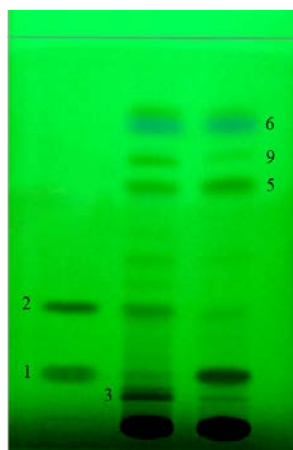
Figure 2 HPLC chromatograms of 80 % methanol extracts of raw Chuanxiong Rhizoma and wine stir-fried Chuanxiong Rhizoma detected at 270 nm. Refer to Table 1 for the identification of the label peaks.

Table 1 Identification of peaks and bands in HPLC-DAD and TLC of 80 % methanol extracts of Chuanxiong Rhizoma

Peak/ Band no.	Compound	R _t (min)	R _f	λ _{max} (nm)	Difference of HPLC peak area (%) ^a
1	5-hydroxymethylfurfural	13.6	0.14	284	- ^b
2	Ferulic acid	40.2	0.28	232, 296, 324	-59
3	Senkyunolide I	49.5	0.08	276	-91
4	Senkyunolide H	51.0	-	276	-90
5	Senkyunolide A	57.0	0.64	280	-16
6	Z-Ligustilide	58.8	0.83	284, 328	-33
7	3-Butylidenephthalide	59.1	-	244, 260, 312	-61
8	Riligustilide	61.7	-	280	-33
9	Levistolide A	62.7	0.71	232, 276	-78

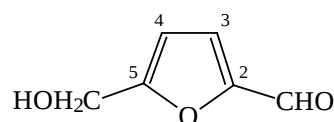
^a Difference of HPLC peak area of wine stir-fried Chuanxiong Rhizoma compared with raw Chuanxiong Rhizoma^b Detected only in wine stir-fried Chuanxiong Rhizoma

studies freshly processed the crude drug in laboratory, but this study used commercially available wine stir-fried CR imported from China. Under the condition of wine stir-frying, chemical compositions were extracted from plant cells by wine and reabsorbed into cell skeleton possibly causing them ready to be released during decoction the crude drug. However, they also might be easily decomposed by exposure to air during storage. Therefore, long duration of transportation and unsuitable stocking condition might cause the alteration of chemical compositions from the freshly prepared wine stir-fried CR. Moreover, post-harvesting and processing methods in each municipality of China could be lacked of uniform standards, which might also cause uncertainty in the quality of the prepared crude drugs [4].

**Figure 3** TLC chromatogram of 80 % methanol extracts of raw CR and wine stir-fried Chuanxiong Rhizoma detected under UV light at 254 nm. Lane 1 = standards, lane 2 = raw CR, lane 3 = wine stir-fried Chuanxiong Rhizoma. Refer to Table 1 for the identification of the label bands.

One point of the interest of this study was that the HPLC peak at R_t 13.60 min and the TLC band at R_f 0.14 were observed only in wine stir-fried CR. The compound was isolated and identified as 5-hydroxymethylfurfural (5-HMF) (**Figure 4**) by spectroscopic study and comparing

data with reference [19]. 5-HMF is a common product often found in carbohydrate-rich foods after processing at high temperature [20]. It is the decomposition product of hexoses, e.g. fructose, glucose and sucrose [21-22]. This compound has been reported in some Chinese crude drugs after processing [23-28]. In previous studies on wine stir-fried CR, the presence of 5-HMF has never been mentioned [6, 7]. However, there was a report on sugar-treated under high temperature of CR showing an additionally remarkable peak in HPLC chromatogram [13], but the peak was unidentified and could possibly be 5-HMF. In this study, raw CR sample treated by heating in an oven at 100°C for 3 hours was investigated, but 5-HMF was not detected. 5-HMF in wine stir-fried CR might come from the adjuvant or wine added during the processing procedure, since the detection of 5-HMF in Chinese rice wine produced in some areas was reported [29] and its content increased after heating [30]. From the result of this study, this compound could be used as a marker to distinguish between raw CR and wine stir-fried CR.

**Figure 4** Chemical structure of 5-hydroxymethylfurfural.

Conclusion

This study investigated anti-platelet aggregation activity of Chuanxiong Rhizoma available in Thailand. Comparing with raw CR, wine stir-fried CR was less active. Contrary to the previous report [6, 7], the contents of most bioactive compounds of wine stir-fried CR were obviously decreased. The variation of quality of crude drugs used in each study possibly resulted in the disagreeable results. It was also clearly suggested that variation in quality of CR could affect the efficacy of the medicine. Moreover, use of Chuanxiong Rhizoma or Kot-Hua-Bua in Thai traditional medicine should be much concerned, since wine stir-frying is not the processing method recorded in Thai traditional knowledge.

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