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ORIGINAL ARTICLE

Combination of Platelet Bio-Specific Extraction and High-performance Liquid Chromatography with Diode-Array Detection/Liquid Chromatography-Mass Spectrometry Method for Analyzing Platelet-targeted Compounds in Rhizoma Chuanxiong

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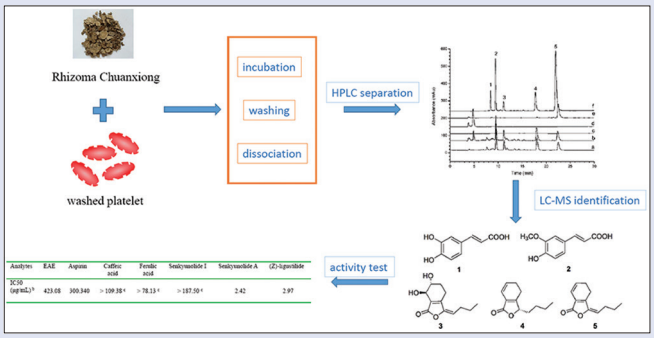
ABSTRACT

Context: Platelet is an important pharmacological target as it participates in complex processes of coagulation and hemostasis. Excessive platelet aggregation is responsible for the formation of pathogenic thrombi in patients with atherothrombotic disease. **Objectives:** To expedite the search for platelet-targeted active candidates in Rhizoma Chuanxiong (root of *Ligusticum chuanxiong* Hort [*Umbelliferae*]). **Materials and Methods:** Rhizoma Chuanxiong ethyl acetate extract (EAE) was obtained by refluxing extraction method. A platelet bio-specific extraction combined with high-performance liquid chromatography with diode-array detection/liquid chromatography-mass spectrometry (HPLC-DAD/LC-MS) approach was employed to screen and identify the platelet-targeted compounds in Chuanxiong EAE, and the results were confirmed by *in vitro* antiplatelet aggregation test using turbidimetry method. Finally, HPLC-DAD analysis was employed for the quantitative determination of these hit compounds in Chuanxiong EAE. **Results:** A total of five hit components were tentatively detected and identified by HPLC-DAD/LC-MS, two of which, senkyunolide A and (Z)-ligustilide, were confirmed their antiplatelet activity by *in vitro* platelet aggregation experiment (both with IC_{50} <3 μg/mL, 2.42 μg/mL [12.60 μM] and 2.97 μg/mL [15.63 μM], respectively). Meanwhile, the quantitative results indicated that those two strongest inhibitors are the main components in Chuanxiong EAE (87.891 ± 1.668 mg/g and 109.058 ± 1.383 mg/g, respectively). **Conclusion:** Senkyunolide A and (Z)-ligustilide are the main active antiplatelet compounds in Rhizoma Chuanxiong EAE. The proposed platelet bio-specific extraction combined with HPLC-DAD/LC-MS method is of value for the discovering potential bioactive components in natural products.

Key words: High-performance liquid chromatography with diode-array detection/liquid chromatography-mass spectrometry, platelet bio-specific extraction, potential antiplatelet active candidates, Rhizoma Chuanxiong

SUMMARY

- Five specific compounds were screened out as platelet targeted compounds in Rhizoma Chuanxiong EAE through platelet extraction combined with HPLC analysis. Among them, senkyunolide A and (Z)-ligustilide showed strong inhibition effect on THR induced platelet aggregation in vitro. Meanwhile, the quantitative determination proved the highest content of these two compounds in Rhizoma Chuanxiong EAE.



Abbreviations used: Rhizoma Chuanxiong EAE: Ethyl acetate extract of Rhizoma Chuanxiong; THR: Thrombin; ESI-MS: Electrospray ionization-mass spectrometer; WP: Washed platelet; ACD: Acid citrate dextrose; LOD: Limit of detection; LOQ: Limit of quantification

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INTRODUCTION

Natural product is a rich source of lead components in drug discovery and continued to be served as the basis for many pharmaceuticals.[1-3] Due to the complicated chemical system of natural products, even for well-documented formulations, bioactive constituent screening and analysis are extremely difficult. Although extraction and separation of chemical components from medicinal herbs followed by pharmacological evaluation are the conventional procedure for screening bioactive components in natural products, the method is time- and sample-consuming in addition to inefficient for direct

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screening of bioactive constituents.[4-6] Usually, drugs should interact with cells or other bio-targets before exert their functions. Based on this principle, bio-extraction directly using living cells or cell membranes to extract active candidate from natural products has been reported widely in recent years.[7] This method takes the integrative characteristic of the pharmaceutical activity of natural products into account, allows for analysis of various natural products with very low concentration in a very short time.[8]

As a pivotal role in the thrombotic process that follows rupture, fissure, or erosion of an atherosclerotic plaque,[9] there are many receptors and proteins on the surface of platelet membrane, which are related to signaling net of platelet aggregation procedure;[10] this promotes the possibility of using platelet as a biomaterial to screen potential platelet-targeted or active candidate components in natural products. There are many natural products showed good anti-platelet effect, including inhibition of platelet membrane receptors, impacting on nucleotide system, inhibition of platelet granules secretion, and impacting on arachidonic acid system.[11,12] In this context, the screening, identification, and analysis of potential anti-platelet active compounds from natural products are of considerable interest. For example, Nie *et al.* using chicken thrombocyte extract to screen platelet ligand bio-target chemicals from Rhizoma Zingiberis found four typical compounds with antiplatelet aggregation activity being isolated.[13] Wang *et al.* studied antiplatelet aggregation candidates from *Panax notoginseng* (Burkill) F. H. Chen (*Araliaceae*) using the platelet extraction method, and five characteristic active compounds were found to bind to the platelets membrane.[14] Yu *et al.* found five compounds in the Mailuoning injection through the platelet-binding process and the results indicated that one of the compounds, caffeic acid, could significantly inhibit platelet activation.[15]

On the basis of our previous study, some natural products showed good antiplatelet aggregation effect,[16] and a simultaneous screening and analysis of antiplatelet active alkaloids in Rhizoma Corydalis were achieved successfully.[17] This study aimed to establish a rapid and efficient platelet bio-specific extraction combined with high-performance liquid chromatography with diode-array detection/liquid chromatography-mass spectrometry (HPLC-DAD/LC-MS) analysis and *in vitro* antiplatelet aggregation test to screen and identify the potential platelet-targeted bioactive candidates from one kind of traditional "HuoXueHuaYu" Chinese medicine Rhizoma Chuanxiong (root of *Ligusticum chuanxiong* Hort [*Umbelliferae*]). The structures of the screened potential chemicals were elucidated, their antiplatelet activity was checked *in vitro*, and quantitative analysis was also conducted with HPLC-DAD method.

SUBJECTS AND METHODS

Chemicals and reagents

The reference compounds caffeic acid, ferulic acid, senkyunolide I, senkyunolide A, and (Z)-ligustilide (≥98% purity, determined by HPLC) were purchased from PUSH Bio-technology Co., Ltd. (Chengdu, China). Platelet agonist thrombin (THR) was a product of Sigma (St, Louis, MO, USA). Methanol (chromatographic grade) was purchased from Beijing InnoChem Science and Technology Co., Ltd. (Beijing, China). All of the experimental water was purified by water purification system (DZG-303A, Ai-ke, Chengdu, China). Other chemicals not mentioned were of analytical grade. Rhizoma Chuanxiong was purchased from Chongqing Xhoo Medicine Co., Ltd. (Chongqing, China) in March 2016. The voucher specimens of Rhizoma Chuanxiong were deposited at the School of Chemistry and Chemical Engineering, Chongqing University, Chongqing, China.

Sample preparation and platelet bio-specific extraction

Preparation of Rhizoma Chuanxiong ethyl acetate extract

Transferred 100 g of dried Rhizoma Chuanxiong fine powder to a glass-stoppered conical flask, accurately added 400 mL of 80% ethanol, and sonicated for 20 min at room temperature. Then, the mixture was refluxing extracted in 80°C water bath for 1 h and repeated for two times. After filtration, the extracts were combined and evaporated at 45°C under reduced pressure to remove ethanol. Finally, the extracts were further liquid–liquid extracted with ethyl acetate for twice. The ethyl acetate extract (EAE) was then collected and rotary evaporated (ZFQ 85A, Shanghai Medical Instrument Special Factory, Shanghai, China) at 30°C under reducing pressure to obtain the EAE of Rhizoma Chuanxiong. The dried extract was dissolved in methanol and used for antiplatelet aggregation assay directly. Alternatively, the Rhizoma Chuanxiong EAE dissolved in methanol was filtered through a 0.22 μm membrane filter (Shanghai Xinyao Purification Equipment Factory, Shanghai, China) for further HPLC analysis.

Experimental animals

Experimental schemes involving conscious animals were approved by the Institutional Animal Ethical Committee of Chongqing University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animal of the National Institute of Health (Publication No. 80-23, revised 1996). Efforts were made to minimize the number of animals used and their suffering. Healthy white rabbits (2.2 ± 0.6 kg) were obtained from Animal Farm in Chongqing, China. Rabbit platelets were used for extraction process and antiplatelet aggregation assay.

Preparation of rabbit's washed platelets

To prepare washed platelet (WP),[18] the blood from the rabbit carotid artery was collected in plastic tubes with acid citrate dextrose (1.32% trisodium citrate, 0.48% citric acid, and 1.47% glucose) as anticoagulant (6:1, v/v). After centrifugation at 196 ×g for 12 min at room temperature, the platelet-rich plasma was isolated and further centrifuged at 2259 ×g for 10 min. The bottom platelet pellet was washed twice by washing buffer (113 mM NaCl, 4 mM KCl, 24 mM $NaH_2PO_4 \cdot 2H_2O$, 24 mM $Na_2HPO_4 \cdot 12H_2O$, 0.2 mM EGTA, and 0.14 mM glucose [250 mL, pH 7.4]). Finally, platelets were suspended in Tyrode's solution (134 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 0.34 mM $NaH_2PO_4 \cdot 2H_2O$, 12 mM $NaHCO_3$, 5 mM glucose, and 5 mM HEPES [250 mL, pH 7.4]) to obtain a platelet count of 2×10^8 platelets/mL. This platelet suspension was used for platelet extraction and antiplatelet aggregation tests directly.

Procedure of platelet bio-specific extraction

Referred to the previous reported method,[14] the Chuanxiong EAE (25 mg/mL, 130 μL) and the WP suspension (3.9 mL) were incubated at 37°C for 30 min with shaking. After incubation, the mixture was centrifuged at 1465 ×g for 8 min with a Centriprep® 50 K device (Merck Millipore Ltd. Tullagreen, Carrigtwohill, Co. Cork, Ireland) to obtain filtrate, and then the suspension was subjected to HPLC analysis. To remove the unbound component, the platelet precipitate collected in the bottom of the filter was washed with phosphate-buffered saline (PBS) (pH 7.4) and subsequently centrifuged for 8 min at 1465 ×g and repeated five times till there was no relative peak shown in HPLC chromatograms. After that, the last washing eluent was collected and subjected to HPLC analysis. Next, denatured the receptor on platelet membrane by addition of 2 mL of 80% methanol solution to the filter and sonicated at room temperature for 20 min, the potential active components were liberated. Centrifuged this denatured platelet suspension at 10,000 ×g for 10 min, combined the suspension, and

dried the solvent under N_2 circumstance. Finally, the dried substance was dissolved with methanol and then subjected to HPLC analysis. To get the blank desorption eluent, the Chuanxiong EAE was replaced with methanol solvent to incubate with WP suspension and did the same operations described above.

High-performance liquid chromatography with diode-array detection analysis and liquid chromatography-mass spectrometry identification

Conditions of high-performance liquid chromatography with diode-array detection analysis

HPLC analysis was performed on an Agilent 1260 Series Liquid Chromatography System (Agilent Technologies, Palo Alto, California, USA), which equipped with a vacuum degasser, a binary pump, an auto-sampler, and a DAD, controlled by an Agilent ChemStation software. An Agilent ZORBAX SB-C18 column (250 × 4.6 mm i.d., 5 μm) along with a precolumn (ZORBAX SB-C18 guard column, 12.5 × 4.6 mm i.d., 5 μm) was employed. The separation was achieved by a gradient solvent system consisted of solvent A (0.3% formic acid solution) and solvent B (methanol) as mobile phases. The gradient elution profile was as follows: 0–3 min, 25%–60% B; 3–8 min, 60%–70% B; 8–26 min, 70%–80% B; 26–28 min, 80%–25% B; 28–30 min, keep 25% B for 2 min. The solvent flow rate was 0.6 mL/min, DAD detection wavelength was set at 294 nm, the column temperature was kept at 35°C, and the injection volume for all samples was 10 μL.

Liquid chromatography-electrospray ionization-mass spectrometry identification

Electrospray ionization-MS (ESI-MS), consisting of a single quadrupole detector as the mass detector (Waters, Milford, MA, U. S. A.), coupled with HPLC was used for LC-MS analysis. The ESI-MS conditions were as follows: the ESI was used in a positive mode. Nitrogen gas was used for desolvation at a flow rate of 550 L/h at 350°C. The capillary voltage was 3000 V, ionization source temperature was 100°C, and the cone voltage was 30 V. MS data were recorded in the full scan mode (*m*/z 100–600).

In vitro antiplatelet aggregation assay

The antiplatelet aggregation study was performed on a SC-2000 Platelet Aggregometer (Beijing Success Technology Development Co., Ltd., Beijing, China) by turbidimetric method as described in previous report.[16] Platelet aggregation agonist THR (0.25 U/mL) was prepared with 0.9% saline solution. WP (300 μL) was preincubated with Chuanxiong EAE solution (10 μL) or reference standard solution (10 μL) at 37°C for 3 min, before being stimulated with THR (10 μL). Platelet aggregation was recorded for 5 min with stirring. Methanol was used as blank control. All tests were performed within 3 h after blood collection. Aggregations were measured and expressed as percent changes in light transmission with respect to Tyrode's solution. The percentage of aggregation inhibition is estimated by the formula: inhibitory (%) = (maximal aggregation of the blank control − maximal aggregation of drug-treated WP) ×100%/maximal aggregation of the blank control, and data are expressed as means ± standard deviation (SD).

Validation of the quantitative analysis

A stock solution containing five standards was prepared by dissolving accurately weighed reference compounds in methanol and then diluted to appropriate concentrations with methanol for the construction of calibration curves. Twelve concentrations of the stock solution were analyzed in duplicates, and calibration curves were obtained by plotting the peak area versus the concentrations of the analyte. The limit of detection (LOD) and limit of quantification (LOQ) for each analyte was calculated on the basis of signal-to-noise ratio (S/N) of about 3 and 10, respectively.

To assess the precision of the development method, intra-day variations were determined. The certain concentration of standards solutions were tested. For intra-day variability test, the mixed standards solutions were analyzed for six times within 1 day (n = 6). Variations of peak areas of compounds were expressed by relative SD.

Statistical analysis

All data are presented as mean ± SDs of at least three different experiments. The statistical analysis was performed with SPSS (version 18, SPSS, Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

High-performance liquid chromatography analysis of Chuanxiong ethyl acetate extract

Studies have found that phenolic acids (such as ferulic acid and coniferyl ferulate) and phthalides (such as senkyunolide A and [Z]-ligustilide) are two kinds of main components in Rhizoma Chuanxiong crude drug.[19] There are many studies for chemical profiling of Rhizoma Chuanxiong, and GC-MS,[20] HPCE-UV,[21] HPLC-UV,[22] and HPLC-DAD-MS[23] methods had been used to investigate its ingredients. However, some of the volatile phthalides such as (Z)-ligustilide is unstable at high temperature, and the dimeric phthalides are also thermos-labile and retro-Diels-Alder reactions can easily take place below 100°C. Therefore, GC-MS is not suitable to analyze the chemical compounds of Rhizoma Chuanxiong, which leads to the development of LC-based method. Referred to previous study,[22] HPLC-DAD was employed in the present study for separating main compounds in Rhizoma Chuanxiong EAE.

After optimizing the mobile phase system (acetonitrile-water and methanol-water, the ratio of formic acid in the water), gradient elution program, and detection wavelength, the optimum HPLC conditions were achieved. Under the optimized conditions, the compounds in Chuanxiong EAE realized baseline separation [Figure 1].

Platelet bio-specific extraction of compounds from Chuanxiong ethyl acetate extract

Optimization of operative conditions for platelet-based screening

To obtain the best screening performance of platelet extraction, several parameters, including the different ways of incubation (incubated under constant temperature, oscillated under constant temperature), washing (gently pipetting with pipette, gently shaken with shaker), and dissociation (incubated with PBS [pH 3.9] at 37°C for 30 min with shaking, sonicated with 80% methanol at room temperature for 20 min), and the ionic strength of the washing buffer PBS (1 mM and 10 mM) was compared and investigated. The results were shown in Figure 2.

As shown in Figure 2A, since the oscillation increased the chances of potential compounds targeting platelets and platelet binding, more related peaks appeared in the denatured desorption of platelet incubated with Chuanxiong EAE sample which employed oscillated under constant temperature. The results suggested that compounds in Chuanxiong EAE would bind with platelet more easily and the process of platelet extraction was more completed in such condition. The washing steps after incubation play important roles in ligand fishing because unbound or non-specific compounds may lead to false positive results. The excess ions in the washing buffer solution could neutralize the surface charge of platelet and hence led to significant decrease in the electrostatic interaction between the potential compound in Rhizoma Chuanxiong

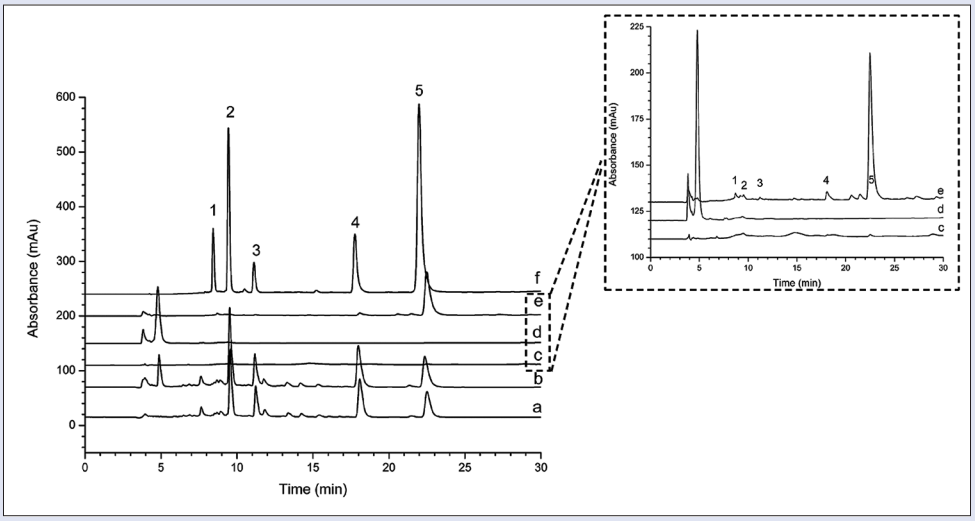

Figure 1: High-performance liquid chromatography chromatograms of (a) Chuanxiong ethyl acetate extract sample solution, (b) supernatant solution after Chuanxiong ethyl acetate extract treated with washing platelet, (c) the final washing elute of platelet incubated with Chuanxiong ethyl acetate extract sample, (d) the denatured desorption elute of platelet incubated in blank solvent without Chuanxiong ethyl acetate extract sample, (e) the denatured desorption elute of platelet incubated with Chuanxiong ethyl acetate extract sample, (f) mixed standards solution of caffeic acid (1), ferulic acid (2), senkyunolide I (3), senkyunolide A (4), and (z)-ligustilide (5)

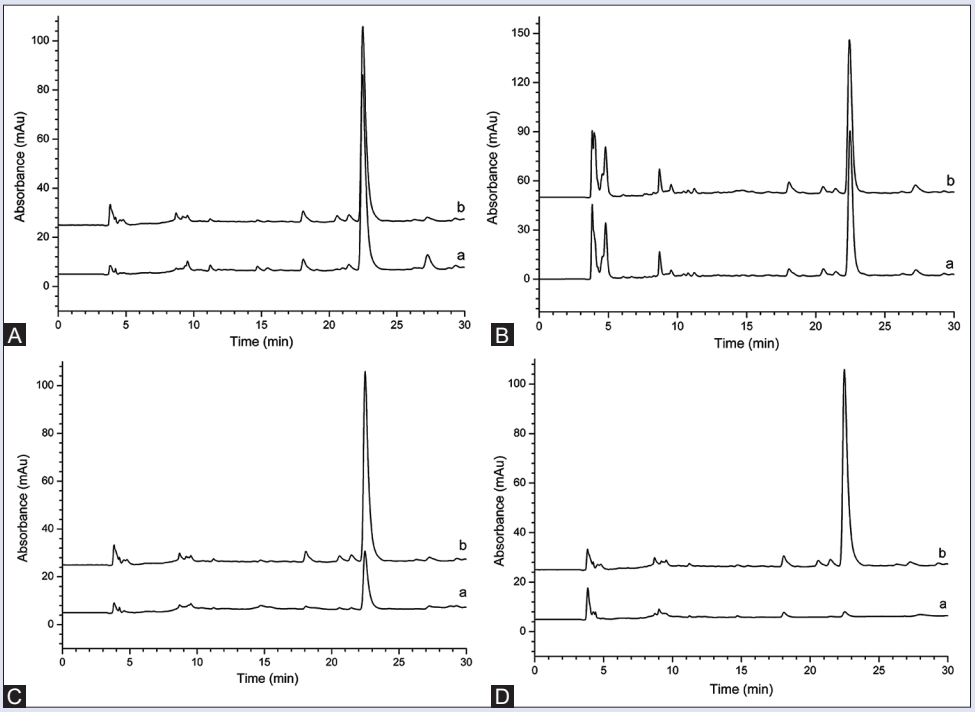

Figure 2: Effects of (A) incubation way (a) incubated under constant temperature (b) oscillated under constant temperature, (B) ionic strength of phosphate-buffered saline buffer (a) 1 mM (b) 10 mM, (C) washing way (a) gently pipetting with pipette (b) gently shaken with shaker, and (D) desorption way on the platelet extraction efficiency of Rhizoma Chuanxiong ethyl acetate extract (a) incubated with phosphate-buffered saline (pH 3.9) at 37°C for 30 min with shaking (b) sonicated with 80% methanol at room temperature for 20 min

EAE and platelet membrane protein or receptor.[19] Figure 2B shows that an increase in the ionic strength of the PBS buffer medium from 1 mM to 10 mM had little impact on the extraction result; this results indicated that such a low degree of ionic strength did not affect the binding efficiency. Investigation on different washing way was also carried out. The results indicated that directly pipetting with pipette repeatedly was too strong for non-specific compound washing in the extraction process [Figure 2C]. It was found that sonication with 80% methanol was the optimal degeneration way [Figure 2D]. Thus, this desorption way was adopted in the screening steps.

Application to Rhizoma Chuanxiong ethyl acetate extract

Figure 1a and b shows chromatograms of the Rhizoma Chuanxiong EAE before and after incubation with WP under the experimental conditions

described above. The denatured solution of platelet was dried with N_2 and redissolved in 500 μL methanol before injecting to HPLC system. Blank cells with vehicle solution were also evaluated to verify whether the absorbed ingredients were from the platelet itself or not [Figure 1d]. After completely washing and eluting from platelet pellets, no relative peaks were appeared in the fifth washing solution [Figure 1c], and five peaks were detected in the chromatogram of denatured solution [Figure 1e].

Identification of the potential platelet-targeted compound

For MS analysis, the positive ion mode of ESI was selected as it easily provided extensive information of the target compounds via collision-induced dissociation (CID) fragmentations. The specific MS fragmentations of compounds are summarized in Table 1. Most of the compounds gave $[M + H]^+$ ions, and this ion was significant in the MS spectra. The neutral loss of H_2O was also observed; the loss of H_2O could be adapted to identify the presence of a carbonyl group or hydroxy in ortho-position. Furthermore, compounds 3 and 5 also gave a $[M + Na]^+$ fragment. By comparing the retention time [Table 1], online UV spectra [Figure 3b], and MS data [Figure 3a and Table 1] of the analyzed sample with those of available standards under the same analysis condition and previous reported results,[24,25] peak 1–5 were identified as caffeic acid (1), ferulic acid (2), senkyunolide I (3), senkyunolide A (4), and (Z)-ligustilide (5), respectively. Their HPLC retention times and UV and MS data are presented in Table 1. Moreover, the chemical structures of the identified platelet-targeted compounds are shown in Figure 4.

In vitro antiplatelet aggregation effect

Based on the result of platelet bio-specific extraction, the anti-aggregation effect of Chuanxiong EAE and the pure reference standard compounds (potential platelet-targeted compounds) were examined *in vitro*. There are many kinds of methods for evaluating the platelet aggregation effect, such as method based on turbidimetry, shear-induced platelet aggregation method, scattering particle detection method, microplate reaction method, platelet counting method, and flow cytometry.[26] Compared with other methods, method based on turbidimetry owned simpler operation steps and much lower experimental cost. In this study, the maximum platelet aggregation rates under certain conditions were tested, and the method based on turbidimetry was chosen. As one of the strongest platelet agonists, THR was employed to induce platelet aggregation, and the results of aggregation suppression rate (IC_{50}) for Chuanxiong EAE and the pure reference compounds are shown in Table 2. Chuanxiong EAE had significant inhibitory activity on platelet aggregation induced by THR (IC_{50} = 423.08 μg/mL), which was an equivalent effect with positive control drug aspirin (IC_{50} = 300.340 μg/mL), this result proved that Chuanxiong EAE could be served as one valuable target for potential targeted platelet compounds screening. Among the five compounds bind with platelet specifically, senkyunolide A and (Z)-ligustilide showed strong antiplatelet aggregation effect *in vitro* (both with IC_{50} below 3 μg/mL, 2.42 μg/mL [12.60 μM] and 2.97 μg/mL [15.63 μM], respectively), while other three hit compounds, caffeic acid, ferulic acid, and senkyunolide I, did not show significant activity at a relatively high concentration (over 75 μg/mL, with IC_{50} >109.38 μg/mL [607.13 μM], >78.13 μg/mL [402.34 μM] and >187.50 μg/mL [836.61 μM], respectively).

It was obvious that not all components which were capable of binding to the platelet surface possessed *in vitro* antiplatelet (THR-induced) aggregation activity. This phenomenon might be explained by the affinity-intrinsic activity theory, namely, except for the affinity to receptor or protein; compound should also own intrinsic activity to generate excitement or inhibition effect.[23] All of the hit five compounds exhibited the affinity to platelet membrane so that could be screened out with the platelet bio-specific extraction; however, only senkyunolide A and (Z)-ligustilide did have the intrinsic inhibition activity to THR-induced platelet aggregation. Although ferulic acid is one of the most commonly reported antiplatelet agents through the arachidonic acid pathway,[27,28] it did not show significant platelet inhibition activity to the THR-induced aggregation in this study.

Determination of the screened five candidates in Chuanxiong ethyl acetate extract by high-performance liquid chromatography with diode-array detection

Method validation

The stock solution of five reference compounds was diluted to appropriate concentrations for the construction of calibration curves. Each solution was determined in duplicates. A satisfactory linear relationship (*r*: 0.9991–0.9996) was obtained between the concentration of the analyte and corresponding peak area. LODs and LOQs of the five compounds were determined as lower than 21.53 ng/mL and 71.77 ng/mL per injection, respectively. The precision results indicated that the intra-day variations were lower than 0.63% for all analytes. Table 3 shows the detailed information regarding calibration curves, correlation coefficient (*r*), linear range, LOD, LOQ and precision for the developed HPLC method, which indicated that the method was precise and sensitive for the quantitative evaluation of compounds in Rhizoma Chuanxiong.

Quantitative determination

The developed HPLC method was applied to determine the concentrations of five platelet-targeted components in Rhizoma Chuanxiong EAE simultaneously. Moreover, the content determination was examined three times within 1 day; the result in Table 3 was exhibited with mean ± SD value (n = 3). According to the established calibration curves, the specific content of each compound in Chuanxiong EAE solution (99.5 μg/mL) was calculated. It was found that the primary component in Chuanxiong EAE was (Z)-ligustilide (109.058 ± 1.383

Table 1: Retention time, mass spectrometer data, and ultraviolet λ_{max} values of the hit platelet-targeted compounds in Rhizoma Chuanxiong ethyl acetate extract

Peak number	t_R (min)	λ_{max} (nm)	Main positive ion	Molecular formula (neutral form)	Identification
1	8.786	325	181 ($[M+H]^+$)	$C_9H_8O_4$	Caffeic acid
2	9.652	325	195 ($[M+H]^+$), 177 ($[M+H-H_2O]^+$)	$C_{10}H_{10}O_4$	Ferulic acid
3	10.324	280	247 ($[M+Na]^+$), 207 ($[M+H-H_2O]^+$)	$C_{12}H_{16}O_4$	Senkyunolide I
4	18.287	280	193 ($[M+H]^+$), 175 ($[M+H-H_2O]^+$)	$C_{12}H_{16}O_2$	Senkyunolide A
5	22.715	330	213 ($[M+Na]^+$), 191 ($[M+H]^+$), 173 ($[M+H-H_2O]^+$)	$C_{12}H_{14}O_2$	(Z)-ligustilide

t_R: Retention time

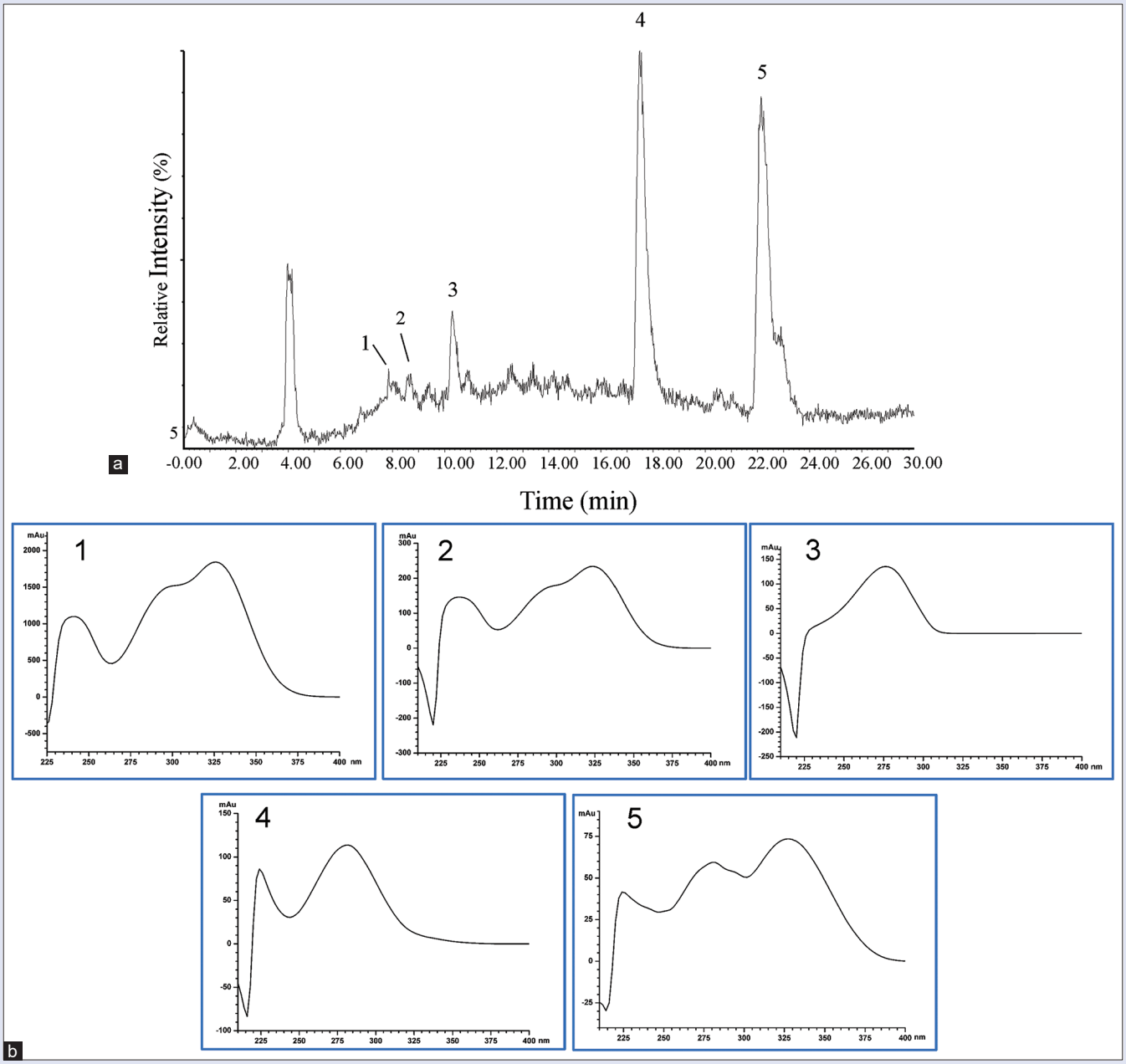

Figure 3: Total ion chromatogram in positive ion mode (a) of Chuanxiong ethyl acetate extract and ultraviolet spectra (b) of the hit five compounds in Chuanxiong ethyl acetate extract

mg/g), then was senkyunolide A (87.891 ± 1.668 mg/g), while caffeic acid showed relatively low abundance (0.113 ± 0.013 mg/g) in Rhizoma Chuanxiong EAE.

Combined the content determination data and *in vitro* antiplatelet aggregation result together, it could be noticed that the two highest content components in Chuanxiong EAE showed the best anti-aggregation effect. However, some trace constituents in Chuanxiong EAE may also have such activity, but not be detected and identified by platelet bio-specific extraction. The possible reasons are as follows: (1) platelet bio-specific extraction at limited sensitivity might not effectively recognize the trace active constituents in complex matrix; (2) the trace active compounds might be easily replaced competitively by higher-content constituent; and (3) HPLC/LC-MS conditions might not be suitable for the detection of trace active compound in the complicated matrix of natural product. For example, it is reported that tetramethylpyrazine is a strong inhibitor for platelet, but the concentration of it in Rhizoma Chuanxiong is too low to be detected.[29]

CONCLUSION

Based on the affinity of small-molecule compounds and biomaterials, platelet extraction combined with HPLC analysis was used to screen potential platelet-targeted components in Rhizoma Chuanxiong EAE. The screening result was confirmed by *in vitro* antiplatelet assay. Among the five hit compounds, only senkyunolide A and (Z)-ligustilide showed strong inhibition effect on THR-induced platelet aggregation, while the other three components (caffeic acid, ferulic acid, and senkyunolide I) had no or very weak inhibit

Table 2: Antiplatelet aggregation effect of ethyl acetate extract and single compounds from Rhizoma Chuanxiong (*n*=3)[a]

Analytes	EAE	Aspirin	Caffeic acid	Ferulic acid	Senkyunolide I	Senkyunolide A	(Z)-ligustilide
IC_{50} (μg/mL)[b]	423.08	300.34	>109.38[c]	>78.13[c]	>187.50[c]	2.42	2.97

[a]Platelet aggregation was induced by thrombin (0.25 U/mL); [b]The IC_{50} values are expressed as the sample concentration to produce 50% inhibition of platelet aggregation in comparison to vehicle group; [c]<50% inhibition at this concentration. EAE: Ethyl acetate extract

Table 3: Method optimization and the content determination result

Analytes	Liner regression					Intra-day precision (*n*=6), RSD (%)	Concentration (*n*=3)	
	Calibration curve	Correlation coefficient (r^2)	Liner range (μg/mL)	LOD (ng/mL)	LOQ (ng/mL)		μg/mL	mg/g
Caffeic acid	Y=1183.60x+13.60	0.9995	0.010-2.644	9.76	32.53	0.46	0.012±0.013	0.113±0.013
Ferulic acid	Y=632.19x+60.69	0.9993	0.056-14.423	21.53	71.77	0.53	3.594±0.135	36.129±1.354
Senkyunolide I	Y=548.06x+44.44	0.9994	0.053-13.462	2.57	8.55	0.41	1.582±0.056	15.896±0.565
Senkyunolide A	Y=155.52x+38.12	0.9996	0.150-38.460	12.76	42.56	0.63	8.745±0.166	87.891±1.668
(Z)-ligustilide	Y=354.92x+242.83	0.9991	0.156-80.000	5.59	18.65	0.54	10.851±0.138	109.058±1.383

LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation

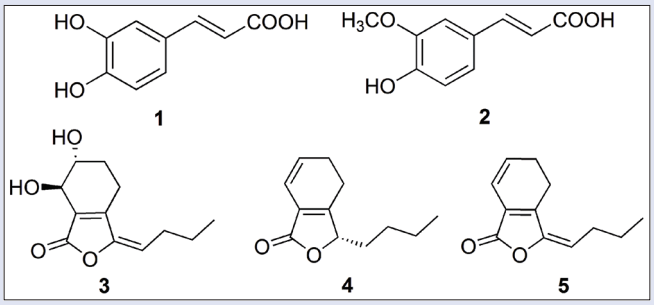

Figure 4: Chemical structures of the five potential bioactive constituents identified from Rhizoma Chuanxiong ethyl acetate extract, including caffeic acid (1), ferulic acid (2), senkyunolide I (3), senkyunolide A (4), and (Z)-ligustilide (5)

efficacy. Furthermore, results of content determination demonstrated that the two strongest inhibitors showed highest content in Chuanxiong EAE. Although the proposed platelet bio-specific extraction combined with HPLC analysis method has some limitations with restrict to the affinity-intrinsic activity and some competitive binding effect for identifying the specific active compound in complex natural product matrix, this method could be a very simple, rapid, straightforward approach in discovering potential bioactive components in natural products. Meanwhile, by combining the results of platelet bio-specific extraction, LC-MS structure identification and antiplatelet activity assay, two compounds, senkyunolide A and (Z)-ligustilide, as promising drug candidates targeting platelet membrane receptors were discovered.

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Conflicts of interest

There are no conflicts of interest.

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