Differential proteomic analysis of platelets suggested target-related proteins in rabbit platelets treated with Rhizoma Corydalis

Chun-Hong Li, Cen Chen, Qian Zhang, Chen-Ning Tan, Yuan-Jia Hu, Peng Li, Jian-Bo Wan, Gang Feng, Zhi-Ning Xia and Feng-Qing Yang

School of Chemistry and Chemical Engineering, Chongqing University, Chongqing, China; Division of Imaging Science & Biomedical Engineering, King’s College, London, UK; State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macao, China

ABSTRACT
Context: Corydalis yanhusuo W.T. Wang (Papaveraceae) (Rhizoma Corydalis) showed inhibitory effects on rabbit platelet aggregation induced by ADP, thrombin (THR) or arachidonic acid (AA).
Objective: This study separates and identifies the possible target-related platelet proteins and suggests possible signal cascades of RC antiplatelet aggregation.
Materials and methods: Based on comparative proteomics, the differentially expressed platelet proteins treated before and after with 50 mg/mL RC 90% ethanol extract (for 15 min at 37°C) were analyzed and identified by two dimensional gel electrophoresis (2-DE) and MALDI-TOF-MS/MS. To further verify the possible signalling pathways of RC antiplatelet aggregation function, the concentration of calcium (Ca\(^{2+}\)) was measured by Fura-2/AM fluorescence (Ex 340/380 nm, Em 500 nm) (RC final concentrations of 0.0156–0.1563 mg/mL), the levels of P-selectin and cyclic guanosine monophosphate (cGMP) were quantified by ELISA (OD. 450 nm) (RC final concentrations of 0.0156–1.5625 mg/mL), and the 5-hydroxytryptamine (5-HT) level was measured using ortho-phthalaldehyde (OPT) fluorescence (Ex 340 nm, Em 470 nm) (RC final concentrations of 0.3125–1.5625 mg/mL).
Results: The expression of 52 proteins were altered in rabbit platelets after the treatment and the MALDI-TOF-MS analysis indicated that those proteins include 12 cytoskeleton proteins, 7 cell signalling proteins, 3 molecular chaperone proteins, 6 proteins related to platelet function, 16 enzymes and 7 other related proteins. Furthermore, RC extract could decrease the levels of 5-HT [inhibition rate of 96.80% (\(p<0.05\), vs. THR-activated group) treated with 0.7813 mg/mL of RC], Ca\(^{2+}\) [172.73 ± 5.07 to 113.56 ± 5.46 nM (\(p<0.001\), vs. THR-activated group) treated with 0.0313 mg/mL of RC] and P-selectin [13.48 ± 0.96 ng/3 × 10\(^8\) to 11.64 ± 0.17 ng/3 × 10\(^8\) (\(p<0.05\), vs. THR-activated group) treated with 0.0156 mg/mL of RC], and increase in cGMP level [38.93 ± 0.57 to 50.26 ± 4.05 ng/3 (\(p<0.05\), vs. THR-activated group) treated with 1.5165 mg/mL of RC].
Discussion and conclusion: The present study indicated that P2Y12 receptor might be one of the direct target proteins of RC in platelets. The signal cascades network of RC after binding with P2Y12 receptor is illustrated in the figure. The mechanism of RC antiplatelet effect is still unclear.

Introduction
Rhizoma Corydalis (RC), the dried tubers of perennial herb Corydalis yanhusuo Y. H. Chou & Chun C. Hsu) W. T. Wang ex Z. Y. Su & C. Y. Wu (Papaveraceae), has a wide range of pharmacological activities such as promotion of blood circulation, antioxidant, alleviating pain, inhibition proliferation of cancer cell, antiulcer, antihypertension and vasorelaxant (Leung et al. 2003; Wu et al. 2007; Gao et al. 2009; Wang et al. 2010; Qu et al. 2015). The function of improving the blood circulation to treat thrombosis associated cardiovascular diseases of RC became increasingly attractive. And among hundreds of chemical components such as organic acids, amino acids, sugars, steroids and volatiles in RC (Zhang et al. 2008; Shi et al. 2011; Su & Guo 2011; Wang et al. 2012; Yang et al. 2014), alkaloids are revealed to be its main chemical compounds and major pharmacologically active ingredients (Cheng et al. 2006; Wang et al. 2010; Yang et al. 2014). For example, the previous research has reported the antiplatelet aggregation effect of the dehydrocorydaline by increasing the platelet cyclic adenosine monophosphate (cAMP) and decreasing cGMP level, as well as inhibiting the expression of the fourth factor (PF4) induced by ADP (Yang et al. 1989; Ding et al. 2007). In addition, the work by our group has demonstrated that RC extracts showed strong antiplatelet activity against AA, THR and ADP-induced platelets aggregation (Chen et al. 2016). In reality, as a therapeutic natural drug for arterial and venous thrombosis, the mechanism of RC on antiplatelet effect is still unclear.

Platelets, small subcellular fragments, have crucial roles in haemostasis and thrombosis after tissue trauma and vascular...
injury (Coller & Shattil 2008; Smyth et al. 2009). Platelet aggregation in blood vessels causes thrombosis and results in various clinical disorders. Therefore, the underlying mechanisms of platelet activation and aggregation play important roles in preventing and treating cardiovascular and thrombosis-related diseases. Platelet proteomics is an effective strategy to identify a wide range of signal cascades involved in platelet activation (Marcone et al. 2015). Among various techniques used in platelet proteomics, gel-free and iTRAQ together with MS or LC-MS/MS, 2-DE coupled to kinds of MS were frequently applied to characterize platelets in cardiovascular diseases and in exploring effects of antiplatelet drugs (Coppinger et al. 2007; Arias-Salgado et al. 2008; Parguina et al. 2010, 2011; López-Farré et al. 2011). One of the application of 2-DE coupled to MS is to identify the expression of platelet proteins and suggest the possible signalling pathways after treated with traditional Chinese medicines (TCMs) and active compounds, such as Panax notoginseng (Burkill) F. H. Chen (Araliaceae) (Yao et al. 2008b), salvianolic acids (Yao et al. 2008a) and salvianolic acid B (Ma et al. 2011) from Salvia miltiorrhiza Bunge (Labiatae).

In the present study, a method combining two-dimensional electrophoresis (2-DE) and MALDI-TOF-MS/MS analysis was applied to separate and explore target-regulated platelet proteins, and then based on the results to suggest possible signalling pathways of antiplatelet aggregation of RC. Furthermore, the Ca\(^{2+}\), P-selectin, 5-HT and cGMP levels in platelets treated with RC were also measured to further confirm its antiplatelet mechanism.

### Materials and methods

#### Regents and chemicals

Pentobarbital sodium, HEPES, THR, ADP, arachidonic acid (AA), ortho-phthalaldehyde, Fura-2/AM and 5-HT were from Sigma (St, Louis, MO). NH\(_4\)HCO\(_3\), K\(_2\)Fe(CN)\(_6\), Na\(_2\)S\(_2\)O\(_3\), acetonitrile, trifluoroacetic acid (TFA), trypsin and the protease inhibitor cocktail (P-9599) were from Sigma (Promega, Madison, WI). Immobiline DryStrips (IPG strips) (pH 4–7, 13 cm), Drystrip cover fluid, 2-D clean-up kit and IPG buffer (pH 3–10 and pH 4–7) were obtained from GE Healthcare (GE Healthcare Bio-Science, Uppsala, Sweden). N, N-methylene-bis acrylamide, acrylamide, dithiothreitol (DTT), iodoacetamide, low melting-temperature agarose, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), glycine, urea, thiourea and coomassie brilliant blue G-250 were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Protein marker was obtained from Microgain-Tiantai (Chengdu, China). The ampholytes (pH 3–10 and pH 4–7) were obtained from Beijing Bio-De Biotechnology Co., Ltd. (Beijing, China). Analytical reagent grade such as ethyl alcohol absolute, sodium carbonate anhydrous, ethylenediaminetetraacetic acid disodium salt (EDTA-Na\(_2\)), Triton X-100 and acetic acid sodium salt trihydrate were obtained from Chengdu Kelong Chemical Works (Chengdu, China). Analytical reagent grade glyciner and acetone were purchased from Chongqing Chuandong Chemical Co., Ltd. (Chongqing, China). Silver nitrate was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The ultrapure water used for the whole experiment was produced by Milli-Q water purification system (DZG-303A, Ai-ke, China). P-selectin kit and cGMP kit were from Beijing Branch of the Science and Technology Co., Ltd. (Beijing, China). *Rhizoma Corydalis* was purchased from Chongqing Xhoo Medicine Co., Ltd. (Chongqing, China) in autumn of 2014, and the identification of RC was confirmed by the corresponding author. The voucher specimens of *Rhizoma Corydalis* were deposited at the School of Chemistry and Chemical Engineering, Chongqing University, Chongqing, China.

#### Instruments

Electrophoresis power supply-EPS 601, ImageScanner III and Etan IPGpator 3 systems were purchased from GE Healthcare (GE Healthcare Bio-Science, Uppsala, Sweden). High speed refrigerated centrifuge was from Huanan Cence Instrument Companies (TGL-20M, Huanan, China). Spectrafuge mini centrifuge was from Haimeen Kylin-Bell Lab Instrument Co., Ltd. (LX-100, Jiangsu, China). The decolourization shaking table was from Shanghai Yangong Biochemical Works (Shanghai, China). Shimadzu RF-5301PC fluorescence spectrophotometer and UV-2450 spectrophotometer (Tokyo, Japan) were used for measuring calcium and protein concentrations, respectively. Platelet aggregation test was performed on a SC-2000 Platelet Aggregometer (Beijing Success Technology Development Co., Ltd., Beijing, China). Sunrise microplate reader was purchased from Tecan Company (Salzburg, Austria). ABI 4800 MALDI-TOF/TOF Proteomics Analyzer was from Applied Biosystems (Wilmington, DE).

#### Preparation of RC sample

The dried RC powder (50 g) was extracted with 150 mL 90% ethanol in an ultrasonic cleanser tank for 20 min, along with reflux extraction for 1 h at 80 °C, filtered and another fresh 150 mL 90% ethanol was added into the residue, repeated the reflux extraction and filtered. The filtrates were combined and dried at 45 °C using a rotary evaporator and further dried in an oven at 50 °C. The obtained RC 90% ethanol extract was dissolved in natural saline before incubated with washed platelets.

#### Preparation of rabbit washed platelet

Healthy rabbits (2–3 kg) were from animal farm in Chongqing, China. Rabbit washed platelets were used for proteomic analysis and identifying target-related proteins. All experimental procedures were approved by the Institutional Animal ethical Committee of Chongqing University and were conducted according to the Guide for Care and Use of Laboratory Animal of the National Institute of Health (Publication No. 80-23, revised 1996). Preparation of rabbit washed platelet was processed according to the previous report with some modifications (Xia et al. 2012). Blood from rabbit carotid artery was obtained in plastic tubes with ACD (0.8% aitric acid, 2.2% sodium citrate, 2.45% glucose) anticoagulant (ACD: blood =1:6, v/v). After centrifugation at 196 × g for 12 min at room temperature, platelet rich plasma (PRP) was isolated and then centrifuged again at 2325 × g for 12 min. The suspension solution was obtained as platelet poor plasma (PPP). Washed platelets were obtained by washing the bottom platelet pellet twice with washing buffer (138 mM NaCl, 12 mM NaHCO\(_3\), 2.7 mM KCl, 0.36 mM Na\(_2\)PO\(_4\)-2H\(_2\)O, 5.5 mM glucose and 0.2 mM EGTA). Finally, platelets were re-suspended in Tyrode buffer (134 mM NaCl, 12 mM NaHCO\(_3\), 2.9 mM KCl, 0.34 mM Na\(_2\)PO\(_4\)-2H\(_2\)O, 0.8 mM MgCl\(_2\)-6H\(_2\)O, 5 mM HEPES, 5 mM glucose) with a cell density of 3 × 10\(^8\) cells/mL.

#### Protein extraction

Washed platelets were incubated with natural saline (blank control) or 50 mg/mL RC 90% alcohol extracts (RC-treated) for
15 min at 37°C. Subsequently, platelets were washed three times with PBS-glucose buffer [PBS (pH 7.4); 5% (w/v) glucose =1:9, isosmotic solution] and centrifuged at 3246 × g for 10 min. The pellets were dissolved in lysis buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 40 mM DTT and 0.02% (w/v) protease-inhibitor for 30 min on ice, then the protein solution was purified by 2-D Clean-up kit. Finally, the pellet was re-suspended in 150 μL lysis buffer [7 M urea, 2 M thiourea, 2% CHAPS, 40 mM DTT, 0.25% (v/v) pharmalyte] for 2-DE analysis.

**Two-dimensional electrophoresis**

Two-dimensional electrophoresis analysis was performed on a GE 2-DE system. Briefly, 150 μL protein samples were applied for isoelectric focusing (IEF) using Drystrip IPG strips (13 cm, pH 4–7). Before IEF, IPG strips were rehydrated 20 h in hydration solution [7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, 0.3% pharmalyte pH 4–7, 0.2% pharmalyte pH 3–10]. Then, the IPG strips were placed into Ettan IPI Ghpor Manifold and the proteins were separated based on their pI. After IEF, the IPG strips were equilibrated and then directly transferred onto 10% SDS-PAGE gels (gel dimensions 14 × 16 cm). Control and RC-treated platelet protein samples for three independent experiments were analyzed by 2-DE. The gels were then silver stained and the images were processed by the PDQuest (Bio-rad, Hercules, CA). The gel images from control and RC-treated and the images were processed by the PDQuest software, and the matching rates were air-dried and analyzed with an ABI 4800 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, Wilmington, DE). All databases were searched using Mascot search engine in the NCBInr protein sequence database restricted to *Oryctolagus cuniculus*. Mass tolerance for peptides is 100 ppm and mass tolerance of TOF/TOF fragments is 0.6 Da using cysteine carboxymethylation for a fixed modification. Meanwhile, methionine oxidation was used as a variable modification. The confidence in the peptide mass fingerprinting matches (p < 0.05) was determined based on the MOWSE score.

**Identifications of target proteins**

The differentially expressed proteins were manually selected and extracted from gels. MS analysis was performed based on the reported method (Sui et al., 2015). Briefly, gels were destained with 30 mM KFe(CN)6 in 50% 100 mM Na2S2O3 and 100 mM NH4HCO3, dried in ACN and digested with trypsin. Then, peptides were extracted by 0.1% TFA in 50% ACN, and the samples were air-dried and analyzed with an ABI 4800 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, Wilmington, DE). All databases were searched using Mascot search engine in the NCBInr protein sequence database restricted to *Oryctolagus cuniculus*. Mass tolerance for peptides is 100 ppm and mass tolerance of TOF/TOF fragments is 0.6 Da using cysteine carboxymethylation for a fixed modification. Meanwhile, methionine oxidation was used as a variable modification. The confidence in the peptide mass fingerprinting matches (p < 0.05) was determined based on the MOWSE score.

**Determination of intracellular Ca2+ levels of platelet**

Intracellular Ca2+ concentration transients were measured by Fura-2/AM fluorescence according to the previous report (Xia et al., 2012). In brief, rabbit PRP platelet was incubated with Fura-2/AM (final concentration of 2 μM) for 45 min at 37°C. Before re-suspended at 3 × 10^6 cells/mL with the Tyrode-Hepes, the above platelets were washed twice by washing buffer. Afterwards, platelets were incubated with various concentrations of RC (final concentrations of 0.0156–0.1563 mg/mL), and blank control PBS solution (0.01 mol/L) at 37°C for 5 min, and the Fura-2 fluorescence was monitored at an excitation wavelength of 340/380 nm and an emission wavelength of 500 nm. Finally, the fluorescence intensity after THR-induced (0.25 u/mL) platelet aggregation was measured by the same method.

**Quantification of platelet P-selectin and cGMP by ELISA**

Competitive enzyme-linked immunosorbent assay (ELISA) method was carried out to measure the levels of P-selectin and cGMP. Rabbit PRP platelets at a concentration of 3 × 10^8 cells/mL were incubated with 10 μL RC extracts (final concentrations of 0.0156–1.5625 mg/mL), 0.25 u/mL THR and blank control PBS solution (0.01 mol/mL) at 37°C for 5 min, respectively, and then the RC-treated groups were incubated with 10 μL THR (0.25 u/mL) for 5 min at 37°C. Then, 13 mM EDTA-Na2 was added to the tubes to terminate the progress on ice. After centrifuged at 8232 × g for 5 min, the supernatants were collected. Finally, soluble P-selectin and cGMP contents were analyzed using a microplate reader at a wavelength of 450 nm according to the manufactures’ instructions, respectively.

**Measurement of platelet 5-HT**

Platelet 5-HT content was measured based on the previous report with minor modifications (Jin et al., 2004). Firstly, rabbit PRP platelets were incubated with RC extracts (final concentrations of 0.3125–1.5625 mg/mL), and control PBS solution (0.01 mol/L) for 3 min at 37°C, and then immediately stimulated with agonists (THR 0.25 u/mL, AA 0.205 mmol/L and ADP 10 μmol/L). After 5 min, the reaction was stopped by adding 1% precooled EDTA-Na2 on ice. Supernatants were obtained by centrifugation at 6961 × g for 10 min, 300 μL solution of each sample was used for measuring fluorescence intensity. Meanwhile, the fluorescence intensity of 0.3 mL 0.6 mg/L 5-HT standard solution was also measured. Secondly, three concentrations (0.3125, 0.7813 and 1.5625 mg/mL) of RC-treated platelet samples (respectively activated by three agonists) were mixed with TCA and Tyrode-Hepes. After centrifuged at 6961 × g for 10 min, supernatants were incubated with OPT/HCl at 100°C for 10 min. The solution was cooled on ice for 10 min and then mixed with trichloromethane. The fluorescence intensity of solution was monitored at excitation wavelength of 340 nm and emission wavelength of 470 nm after centrifuged at 2325 × g for 10 min.

**Statistical analysis**

PDQuest (Bio-rad, Hercules, CA) software was applied to process the 2-DE images, the quantitative analysis of protein spots were performed using the Student’s t-test between protein gels for RC-treated and control group. All data were analyzed by origin 8.5.1, and presented as mean ± SD, p values of .05 or less were considered to be statistically significant.

**Results**

**2-DE analysis of control and RC-treated platelets**

Protein profiles of control and RC-treated platelets are shown in Figure 1, up to 900 protein spots were monitored after each image processed by PDQuest software, and the matching rates up to 90% among three 2-DE gel maps indicate good
reproducibility of three independent experiments. Twenty-six downregulated and 26 upregulated protein spots were found by comparing 2-DE gel maps of control and RC-treated groups. The differentially expressed proteins are indicated by the arrows in Figure 1, and the enlarged spots are shown in Figure 2. The fold differences, indicating the ratio of the protein spots’ intensity values of control to RC-treated group are shown in Table 1.

**Figure 1.** The 2-DE proteome images of control (A) and RC-treated (B) platelets. The differentially expressed protein spots were shown by the arrows.
Identification of the differentially expressed proteins

The protein spots (Figure 2) with more than twofold differences in RC-treated and control group were identified by MALDI-TOF-MS/MS. The NCBI accession number, theoretical molecular weight, pI, protein score and coverage of each spot of 51 protein spots are shown in Table 1. There were 12 cytoskeleton proteins, i.e., gamma non-muscle actin, lamin isoform X1 and prohibitin; 7 cell signalling proteins, such as Ras-related protein Rap-1b, annexin A4 and ADP-ribosylation factor 3; three molecular chaperone proteins, including heat shock cognate 71 kDa protein, protein disulphide isomerase-associated 3 precursor and chaperonin-containing T-complex; 6 directly related to the platelet function proteins, 16 enzymes and 7 other proteins.

Effect of RC on platelet calcium level

As shown in Figure 3(A), the Ca^{2+} contents of both resting and THR-induced platelets were affected by RC 90% alcohol extracts. From the Figure 3(A), RC extracts with middle (0.0313 mg/mL) and high (0.1563 mg/mL) concentrations can significantly inhibit the intracellular calcium levels against the resting platelet (p < 0.001, vs. resting platelet), and the Ca^{2+} concentration was decreased from 130.28 ± 5.96 to 89.59 ± 7.04 nM (resting platelet group: 130.28 ± 5.96 nM). Furthermore, compared with the THR-activated group (172.73 ± 5.07 nM), the RC-treated group has strong inhibition function on the release of Ca^{2+} increased by THR. For example, RC extracts at the concentration of 0.0313 mg/mL can obviously inhibit the Ca^{2+} release in THR-induced platelets and the Ca^{2+} levels was decreased from 172.73 ± 5.07 to 113.56 ± 5.46 nM (p < 0.001, vs. THR-activated group).

Effects of RC extract on the platelet P-selectin and cGMP levels

As shown in Figure 3(B), compared with the resting platelets, the content of P-selectin was obvious increased in THR-stimulated platelet (resting group: 11.12 ± 0.84 ng/3 × 10^{9}, THR-activated group: 13.48 ± 0.96 ng/3 × 10^{9}). And RC extracts with final concentration 0.0156 mg/mL treatment were decreased the content of P-selectin to 11.64 ± 0.17 ng/3 × 10^{9} (p < 0.05, vs. THR-activated group), but the inhibition function with middle (0.1563 mg/mL) and high (1.5625 mg/mL) concentrations of RC were not obvious. On the other hand, one of the major inhibitory pathways in platelets is represented by the NO/cGMP/PKG pathway (Li et al. 2010), and the activation of PKG and PKA depended on the availability of cGMP. In the present study, platelet cGMP levels were greatly increased after RC treatment (Figure 3(C)) as compared to that of THR-activated platelets,
Table 1. The results of proteins identification of differentially expressed proteins using MALDI-TOF-MS/MS.

| Spot no. | Protein name | NCBI acc. no. | MW/PI | Protein score | Sequence coverage | Fold changed | Function |
|----------|--------------|----------------|-------|---------------|------------------|--------------|----------|
| 2        | Gamma non-muscle actin | gi|1703 | 42.1/5.30 | 188 | 8% | 0.33 | Involved in cell mobility, exchanges nucleotide faster than α skeletal muscle actin and also binds profilin with greater affinity |
| 4        | Lamin isoform X1 | gi|655846237 | 74.3/6.73 | 240 | 8% | 3.21 | Involved in cell mobility, keeps the nucleus shape and strength |
| 16       | Prohibitin | gi|291405834 | 29.8/5.57 | 574 | 31% | 0.35 | Binds to monomeric actin and phophatidyl inositol phosphates, increases the rate of nucleotide exchange on actin |
| 17       | Alpha-smooth muscle actin | gi|1701 | 42.3/5.23 | 53 | 4% | 2.19 | Involved in cell mobility, activates and dedifferentiates mesangial cells |
| 21       | Tropomodulin-3 | gi|291402996 | 39.6/4.86 | 204 | 13% | 1.91 | The only Tmod isoform detected in platelets and megakaryocytes (MKs), caps actin filament (F-actin) pointed ends and binds tropomyosins (TMs), regulates actin polymerization and stability |
| 35       | Alpha-smooth muscle actin | gi|1701 | 42.3/5.23 | 75 | 4% | 0.15 | Involved in cell mobility, activates and dedifferentiates mesangial cells |
| 40       | Low quality protein: zyxin | gi|655815444 | 62.8/6.04 | 132 | 8% | 0.27 | A actin-binding protein, interacts with α-actinin and enables activated phosphoprotein |
| 43       | WD repeat-containing protein 1 | gi|655604311 | 77.0/6.02 | 146 | 12% | 4.35 | Regulates the components of multi-protein complexes involved in transcriptional activation or repression, signal transduction |
| 44       | WD repeat-containing protein 1 | gi|655604311 | 66.9/6.02 | 500 | 19% | 95.68 | Regulates the components of multi-protein complexes involved in transcriptional activation or repression, signal transduction, etc. |
| 46       | Actin-related protein 3 | gi|291391454 | 47.7/5.61 | 167 | 11% | 3.08 | Major constituent of the ARP2/3 complex, generates new barbed ends by stimulating nucleation, required for actin assembly |
| 47       | Alpha-smooth muscle actin | gi|1701 | 42.4/5.23 | 34 | 4% | 3.06 | Involved in cell mobility, activates and dedifferentiates mesangial cells |
| 49       | Cullin-2 isoform X1 | gi|291409897 | 87.5/6.45 | 30 | 2% | 0.21 | The help of F-box or SOCS box proteins attaches to the substrate |

Cell signalling

| Spot no. | Protein name | NCBI acc. no. | MW/PI | Protein score | Sequence coverage | Fold changed | Function |
|----------|--------------|----------------|-------|---------------|------------------|--------------|----------|
| 6        | Ras-related protein Rap-1b | gi|291389525 | 21.0/6.69 | 99 | 7% | 0.35 | Rap 1 isoform and the most abundant Ras family member, regulates crosstalk between platelet integrin α2[β1] and integrin α3[β1] |
| 12       | Annexin A4 | gi|655606395 | 36.1/5.30 | 163 | 18% | 0.27 | A cytosolic calcium-binding protein with four repeat domains, interacts with the phospholipids membrane to regulate the signal transduction |
| 15       | Guanine nucleotide-binding protein G(i) subunit alpha-2 | gi|655834605 | 40.9/5.35 | 293 | 20% | 0.49 | Involved in platelet activation signal pathways, such as binds P2Y12 receptor to active the downstream transfiguring signal. |
| 26       | ADP-ribosylation factor 3 | gi|291389073 | 20.6/6.84 | 113 | 23% | 0.55 | The member of Ras GTP, binds G protein to regulate transfiguring signal |
| 28       | Synaptotagmin-7 | gi|655882206 | 71.2/9.63 | 33 | 2% | 0.13 | Calcium signalling |
| 30       | Transient receptor potential cation channel subfamily M member 3 | gi|291383356 | 197.5/7.56 | 36 | 1% | 5.95 | Calcium binding and signalling |

Molecular chaperones

| Spot no. | Protein name | NCBI acc. no. | MW/PI | Protein score | Sequence coverage | Fold changed | Function |
|----------|--------------|----------------|-------|---------------|------------------|--------------|----------|
| 9        | Heat shock cognate 71 kDa protein | gi|291383777 | 71.1/5.37 | 423 | 6% | 0.3 | May be involved in protecting the centrosome and intermediating filaments during heat shock |
| 39       | Protein disulphide isomerase-associated 3 precursor | gi|217030873 | 56.7/5.98 | 146 | 11% | 0.32 | Catalyzes disulphide exchange reaction, mediates platelet aggregation and secretion |
| 42       | Chaperonin-containing T-complex polypeptide beta subunit | gi|209981451 | 57.8/6.01 | 79 | 10% | 3.1 | Mediates the folding of proteins and folding pathway from denatured state to correctly folded product; assembles various newly synthesized polypeptides |

Directly related to the platelet function

| Spot no. | Protein name | NCBI acc. no. | MW/PI | Protein score | Sequence coverage | Fold changed | Function |
|----------|--------------|----------------|-------|---------------|------------------|--------------|----------|
| 11       | Fibrinogen beta chain | gi|291401109 | 56.7/8.42 | 113 | 6% | 2.09 | Polymerizes into fibrin; acts as cofactor in aggregation |
| 19       | Zinc-finger protein 64 homologue, isoforms | gi|655881403 | 78.8/8.88 | 43 | 2% | 4.24 | Transcription/transcription regulation, involved in various cellular process |
| 23       | Peroxiredoxin-6 | gi|291397244 | 25.0/5.73 | 435 | 27% | 0.22 | Oxidase activity (direct oxidation of H2O2) |
| 34       | Fibrinogen beta chain | gi|291401109 | 56.7/8.42 | 44 | 4% | 0.24 | Polymers into fibrin; acts as cofactor in aggregation |
| 37       | Serum albumin precursor | gi|29653363 | 70.8/5.85 | 295 | 18% | 3.78 | Transports and depots protein for numerous endogenous and exogenous compounds in the circulation system, maintains the osmotic pressure of plasma and scavenges oxygen free radicals (platelet function; associated with α granules) |

(continued)
and the content of cGMP increased from 38.93 ± 0.57 to 50.26 ± 4.05 ng/3 × 10^8 (p < 0.05, vs. THR-activated group). Hence, the NO/cGMP/PKG pathway might be one of the inhibitory aggregation pathways in platelets of RC.

### Table 1. Continued

| Spot no. | Protein name | NCBI acc. no. | MW/PI | Protein score | Sequence coverage | Fold changed | Function |
|----------|--------------|---------------|-------|---------------|-------------------|--------------|----------|
| 45       | Fibrinogen gamma chain isoform X1 | gi|291401113 | 51.0/5.68 | 185 | 17% | 0.26 | Polymerizes into fibrin; acts as cofactor in aggregation |
| 3        | Succinyl-CoA:3-ketoacid coenzyme A trans-ferase 1 | gi|291395294 | 56.5/8.62 | 213 | 8% | 3.65 | Activates acetoacetate by transferring the CoA group to produce acetoacetyl-CoA and succinate |
| 5        | Isocitrate dehydrogenase [NADP] cytoplasmic | gi|291392139 | 47.1/6.61 | 137 | 13% | 0.3 | An essential cofactor for many enzymatic reactions including glutathione metabolism and cholesterol biosynthesis |
| 8        | V-type proton ATPase subunit B, brain isomorf | gi|291401067 | 56.7/5.57 | 376 | 13% | 6.93 | ATPase, couples ATP hydrolysis to proton transport, acidizes a variety of intracellular compartments in eukaryotic cells |
| 14       | Pyruvate carboxylase | gi|65589056 | 35.2/9.60 | 149 | 6% | 0.19 | Anaplerosis influences insulin secretion in beta cells; catalyzes the ATP-dependent carboxylation of pyruvate to form oxaloacetate |
| 18       | Medium-chain specific acyl-CoA dehydrogenase | gi|291398674 | 46.5/8.65 | 178 | 6% | 0.19 | Catalyzes the rate-determining step of fatty acid ß-oxidation in mitochondria |
| 20       | V-type proton ATPase catalytic subunit A | gi|655854861 | 68.8/5.36 | 202 | 6% | 3.12 | ATPase, responsible for acidifying a variety of intracellular compartments in eukaryotic cells, may participate in maintenance of cytoplasmic Ca^{2+} homeostasis. |
| 22       | Methylcrotonoyl-CoA carboxylase subunit alpha | gi|655853635 | 80.8/6.90 | 90 | 4% | 0.13 | Catalyzes the fourth step in the leucine catabolic pathway |
| 25       | Acyl-coenzyme A synthetase ACSM3 | gi|291390708 | 66.0/8.76 | 43 | 2% | 0.32 | Esterifies free fatty acids to CoA to form acyl-CoAs |
| 29       | Guanlylate cyclase soluble subunit beta-1 | gi|655856074 | 71.2/5.23 | 98 | 4% | 0.35 | Converts GTP into cGMP |
| 31       | Protein monoglycylase TILL8 | gi|655894895 | 108.4/9.35 | 33 | 2% | 3.21 | Modifies both tubulin and non-tubulin proteins to generate side chains of glycine on the gamma-carboxyl groups of specific glutamate residues of target proteins |
| 32       | arf-GAP with GTPase, ANK repeat and PH domain-containing protein 3 | gi|655844868 | 93.8/7.86 | 39 | 1% | 3.43 | GTPase-activating protein for ARF1, involved in ARF GTPase activator activity, regulates the adapter protein 3 (AP-3)-dependent trafficking of proteins in the endosomal-lysosomal system |
| 33       | Beta beta enolase | gi|14141143 | 47.3/7.63 | 64 | 4% | 0.32 | Catalyzes the production of phosphoenolpyruvic acid |
| 38       | Arachidonate 12-lipoxygenase, 12S-type | gi|291405193 | 76.3/8.52 | 55 | 5% | 0.29 | Shows broad substrate specificity and blocks platelets aggregation induced by agonists |
| 50       | V-type proton ATPase subunit B, brain isomorf | gi|291401067 | 56.7/5.57 | 108 | 10% | 2.53 | ATPase, acidulated cellular organelles |
| 51       | Succinyl-CoA:3-ketoacid coenzyme A trans-ferase 1 | gi|291395294 | 56.5/8.62 | 142 | 3% | 46.03 | Rate-limiting enzyme, converts GAP into GTP and succinic acid |
| 52       | Mitochondrial ATP synthase, H = transporting F1 complex beta subunit, partial | gi|89574025 | 45.6/5.21 | 356 | 10% | 103.83 | Catalyzes ATP synthesis, |
| 1        | Mitochondrial inner membrane protein isoform X1 | gi|655606040 | 84.0/5.92 | 170 | 5% | 0.16 | Synthesizes ATP and provides energy, eliminates oxygen free radicals |
| 10       | Ubiquinone biosynthesis protein COQ9 | gi|655788788 | 44.0/9.07 | 95 | 6% | 2.35 | Involved in biosynthesis of COQ10 |
| 13       | Elongation factor 1 delta | gi|1134985 | 31.1/5.06 | 81 | 22% | 2.35 | EF-Tu: catalyzes the production of GDP |
| 24       | Initiation factor 4AI | gi|227238 | 46.3/5.32 | 324 | 23% | 0.2 | Participate in initiation process of eukaryotic translation |
| 27       | Elongation factor Tu | gi|291390870 | 49.7/6.94 | 196 | 17% | 0.3 | Controls protein synthesis and translation |
| 36       | Transcription factor HIVEP3 | gi|655850235 | 257.8/8.5 | 47 | 20% | 3.96 | Regulates gene transcription |
| 41       | NSFL1 cofactor p47 isoform X2 | gi|291388803 | 40.6/5.03 | 231 | 14% | 0.19 | Determines the reaction type |

**Effect of RC extract on the platelet 5-HT level**

As an indolamine platelet agonist, platelet or extracellular 5-HT can act on the platelet serotonin receptor, 5-HT2A, and stimulates Gq-mediated PLC[β] activation that results in the increased...
production of inositol triphosphate (IP_3) and diacylglycerol (DAG) (Offermanns 2006). As shown in Figure 3(D), the intracellular 5-HT contents in platelet activated by AA, ADP and THR can be downregulated by RC treatment, respectively. RC-treated groups had a strong inhibition effect on the THR-induced intracellular 5-HT release, and the inhibition rates of three concentrations (0.3125, 0.7813 and 1.5625 mg/mL) were 83.40, 96.80 and 96.05\%, respectively. Furthermore, RC can also suppressed AA-induced platelets 5-HT release at the three concentrations with the inhibiting rates of 63.51, 60.31 and 71.54\%, respectively. Relatively, RC-treatment had a mild inhibition effect on ADP-induced intracellular 5-HT release (16.41, 22.26 and 21.05\%, respectively).

**Discussion**

According to our previous reported, RC extracts could inhibit ADP, AA and THR-induced rabbit platelets aggregation (Chen et al. 2016). Understanding the mechanism of RC on antiplatelet aggregation would be important for RC to be developed as a natural antiplatelet drug. In the present study, platelet proteomic analysis based on 2-DE combined with MLDAI-TOF-MS/MS analysis was used to separate and identify target-related proteins in RC-treated platelets (Table 1). Based on their biological functions, 51 identified proteins were classified into following five categories: (1) cytoskeleton structure proteins; (2) cell signalling proteins; (3) platelet function directly related proteins; (4) molecular chaperones proteins; (5) enzymes and other proteins.

Cytoskeletal and actin-associated proteins (such as profiling, lamin) were cytoskeleton proteins, which shared cruel roles in maintenance of platelet shape. The remodelling of the resting cytoskeleton and the assembly of new cytoskeleton are required when platelet shape change, this physiological process is associated with a sturdy internal cytoskeleton composed of the polymers of actin and tubulin and their associated proteins (Boyles et al. 1985; Patel-Hett et al. 2008; Michelson 2013). After exposure of rabbit washed platelets to RC, lamin isoform X1, tropomodulin-3 and actin-related protein 3 were upregulated, which may directly result in signal transduction pathways to stimulate or regulate actin polymerization and its stability (Rohatgi et al. 1999; Schmidt & Hall 1999; Sui et al. 2015).

Some cell signalling proteins have been previously reported to be associated with calcium signalling pathways, such as Annexin A4, synaptotagmin-7 and transient receptor potential cation channel subfamily M member 3. G-protein-couple receptors (GPCRs), a family of seven-transmembrane domain receptors, play a pivotal role in the transmembrane signalling process as they take part in processing and sorting of incoming signals, and are centrally involved in the second phase of platelet-dependent thrombus formation (Offermanns 2003, 2006). Meanwhile,
G-protein consist of an α-subunit which binds and hydrolyzes guanosine triphosphate (GTP) as well as an β- and a γ-subunit, and three major G-protein-mediated signalling pathways involved in platelet activation via GPCRs that are initiated by the activation of the G protein Gq, Gi13 and Gi. Importantly, platelet stimulated by ADP is mediated by two G protein-coupled receptors, P2Y12 and P2Y1 (P2Y1 couples to Gq, P2Y12 couples to G-type G proteins). Interestingly, P2Y12 receptor binding Gi protein is a major mechanism responsible for the activation of phosphoinositide 3-kinase (PI3K) (Hollopeter et al. 2001; Gachet 2006). Therefore, the downregulation of guanine nucleotide-binding protein Gi(1) subunit α-2 after RC-treated platelets indicated that P2Y12 receptor might be one of the direct target proteins of RC in platelet to mediate Gαi signalling pathways for the inhibition of platelet aggregation. Ras-related protein Rap-1b (an abundant small GTPase), which is the predominant Rap 1 isoform and the most abundant Ras family member in platelets, has been shown to activate downstream GPCRs and upstream integrin αIIbβ3. Predictantly, ADP stimulates Rap 1b activation mainly via a calcium-independent pathway to activate downstream Gαi-coupled P2Y12 receptor, and also activates phosphoinositide 3-kinase and its lipid product phosphatidylinositol 3,4,5-trisphosphate (Lova et al. 2002; Crittenden et al. 2004; Chrzanowska-Wodnicka et al. 2005). Hence, the expression of Ras-related protein Rap-1b down regulated after treated with RC is corresponded to CalDAG-GEFi (calcium-DAG-GEF/RasGRP protein family) and PI3K signalling pathways. Especially, Annexin A4 was cytosolic calcium-binding protein with four repeat domains, each containing one calcium-binding site, and interacted with lipid membranes in a calcium-dependent manner (Rescher & Gerke 2004; Arii et al. 2015). On the other hand, different isoforms of adenylyl cyclase (AC, AC1-AC9) have wide range of physiological regulations, the enzymatic activity of ACs are responsible for the synthesis of cAMP from ATP stimulated through GPCRs (Defer et al. 2000). It had been reported that annexin A4 was a novel regulator of adenylyl cyclase 5 (AC5), occupying critical functions associated with stimulation of the β-adrenoceptor in multiple tissues (Heinick et al. 2015). So the downregulation of annexin A4 after RC treatment might be directly interacting with membrane-bound AC5 to regulate GPCRs signal pathways. In addition, ADP-ribosylation factor 3 (ARFs 3) was also downregulated after RC treatment. ARFs 3 is a member of ARF family with small GTPase, which are associated with vesicle formation from different intracellular site as well as in the regulation of phospholipase D activity and cytoskeleton modifications (Shome et al. 1998; Belov et al. 2005).

Three molecular chaperones (heat shock cognate 71 kDa protein, protein disulphide isomerase-associated 3 precur sor and chaperonin-containing T-complex polypeptide beta subunit) were also identified and listed in Table 1. Molecular chaperones are a group of structurally diverse proteins including heat shock proteins, t-complex polypeptide 1 (TCP-1 complex), which play a crucial role in facilitating the correct folding of protein by preventing protein aggregation or facilitating the forward folding and assembly of proteins into higher order structure (Hartl & Hayer-Hartl 2002; Young et al. 2004). A previous report showed that Heat shock protein 70 protected the centrosome and perhaps intermediated filaments during heat shock and played cooperative roles in the formation and function of the eukaryotic cell cytoskeleton (Liang & MacRae 1997), it is supposed that the downregulation of heat shock cognate 71 kDa protein may have similar functions with Heat shock protein 70. Protein disulphide isomerase-associated 3, a membrane of protein disulphide isomerase (PDI), was also downregulated by RC treatment. Evidence indicates that the oxidation state of labile disulphide bonds play critical roles in regulating the process of thrombus formation, and important for fibrin generation and platelet accumulation (Cho et al. 2008; Jasuja et al. 2010).

Some of the identified proteins are directly related to platelet functions. Fibrinogen consists of three pairs of polypeptide chains (Aα, Bβ and γ) linked by disulphide bonds, which can bind αIIbβ3 receptor resulted in platelet aggregation after platelets being activated (Mustard et al. 1978; Marguerie et al. 1980). Furthermore, fibrinogen binds through its γ chains (i.e., fibrinogen γ chain isoform X1, protein spot 47) to growth factors and coagulation factors to perform its key roles in fibrin clot formation, platelet aggregation and wound healing (Farrell 2004; Lovely et al. 2007). Peroxiredoxin-6, which can be downregulated in platelet treated with RC, is a family member of peroxiredoxins for antioxidant proteins related to either protection against oxidation or participation in signalling by the reduction of H2O2 (Da Silva-Azevedo et al. 2009; Ambruso 2013).

As listed in Table 1, nearly 30% identified proteins were enzymes that catalyzed several reactions in antiplatelet signalling cascades, for example, succinyl-CoA catalyzed GAP to GTP and succinic anhydride (12-lipoxigenase 12S-type (protein spot 38), an isoform of lipoxigenase, which showed broad substrate specificity and could directly oxygenate polyunsaturated fatty acids esterified to cholesterol in low-density lipoprotein (LDL) particle (Kühn et al. 1994; Brash 1999). Arachidonic 12- lipoxigenase also could catalysis AA to 12(S)-hydroxyeicosatetraenoic acid (12-HETE), which can block the platelets aggregation induced by collage and arachidonate, or negative feedback to prevent excess aggregation by interfering with the liberation of arachidonic acid from membrane phospholipids (Siegel et al. 1979; Sekiya et al. 1990). Hence, the down-regulated expression of arachidonate 12-lipoxigenase (12S-type) in RC treated rabbit platelets may mediate arachidonic acid signalling pathway to inhibit platelet aggregation. Guanylate cyclase soluble (sGC) subunit β-1 (protein spot 29), a GTP-binding protein, which was downregulated in platelet after treated with RC, could catalyze the formation of cGMP from GTP (Tomita et al. 1997; Li et al. 2010). Interestingly, sGC played important roles in the activation of NO/cGMP/PKG/MAPK signalling pathway for GPIIb-IX-mediated platelet activation (Li et al. 2010).

When platelets stimulated by physiological agonists, the release of granule secretions including P-selectin, 5-HT, ADP and Ca2+ will amplify platelet activation and to recruit circulating platelet response to agonists. Among which calcium was sequentially activated integrin αIIbβ3, this process is called ‘inside-out signalling’ (Li et al. 2010), in addition, numerous signalling pathways and molecules were stimulated by calcium levels, including signalling actin-myosin interaction, PKC, calmodulin, NO synthase, etc. (Hassock et al. 2002; Bergmeier & Stefanini 2009). During the inside-out signalling, endothelial nitric oxide synthase (eNOS) was simulated and continually activated soluble guanylyl cyclase, etc. (Hassock et al. 2002; Bergmeier & Stefanini 2009). During the inside-out signalling, endothelial nitric oxide synthase (eNOS) was simulated and continually activated soluble guanylyl cyclase (sGC), then catalyzed guanylic acid to cGMP. And the activated cGMP as an important secondary messenger was sequentially induced the downstream receptor PKG and PLA2 (phospholipase) activation to amplify platelet activation signalling pathway (Li et al. 2003, 2010). In the present study, RC can decrease the levels of P-selectin, 5-HT and Ca2+, and increase the level of cGMP, especially RC has strong inhibition function for platelet Ca2+ release.

In summary, RC-treated rabbit platelets could alter 51 target-regulated proteins’ expression, those proteins play important
roles in platelet activation, oxidation stress and cytoskeleton structure. Importantly, four proteins closely related to platelet activation, i.e., fibrinogen beta chain and serum albumin precursor, were upregulated in RC-treated platelets. Furthermore, guanine nucleotide-binding protein G(i) subunit α-2, which plays crucial role in platelet-activated signalling cascades, can be downregulated by RC extract. On the other hand, RC treatment showed obvious effect on the contents of the calcium, P-selectin, cGMP and 5-HT in AA, THR and AA-activated platelet. Based on the results of the present study and the previous reported about the signalling pathways of platelet activation, the possible signalling pathways of RC antiplatelet aggregation were summarized in Figure 4. One of the signal cascades of RC after binding with P2Y12 receptor might mediate the Gαi proteins to activate downstream AC signalling pathway, and the other was Gαi-mediated PI3K signalling pathway to activate downstream proteins. Therefore, P2Y12 receptor might be one of the direct target proteins of RC in platelet and then to mediate Gαi signalling pathways for the inhibition of platelet functions.

**Conclusion**

In the present study, platelets treated by RC was analyzed using 2-DE based comparative proteomics, and 52 proteins altered in the expression level after RC extract treatment were identified by MALDI-TOF-MS/MS. Some of the identified proteins play crucial roles in the platelet signalling cascades as shown in Figure 4. Based on the present study, the effect of RC on platelet function might be related to its binding to P2Y12 receptor and mediated Gαi signalling pathways. Furthermore, RC can decrease the levels of 5-HT, Ca2+ and P-selectin, and increase cGMP levels in ADP, THR and AA induced platelets. Up to date, this is the first study to identify the possible target-related proteins and suggest the possible antiplatelet signalling cascades of RC by platelet proteomics. The results of present study provide evidences for further study on the antiplatelet aggregation signal pathways of active components in RC.

**Disclosure statement**

The authors declared that they have no conflicts of interest.

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