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Original Article

Synthesis of huaicarbon A/B and their activating effects on platelet glycoprotein VI receptor to mediate collagen-induced platelet aggregation

Hongli Yu[1,2,3*], Yeqing Chen[1*], Hao Wu[1,2,3*], Kuilong Wang[1], Liping Liu[1], Xingde Zhang[1]

[1]School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing 210023, P. R. China; [2]Jiangsu Key Laboratory of Chinese Medicine Processing, Nanjing University of Chinese Medicine, Nanjing 210023, P. R. China; [3]Engineering Center of State Ministry of Education for Standardization of Chinese Medicine Processing, Nanjing 210023, P. R. China. [*]Equal contributors.

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Abstract: Quercetin and rhamnose were efficiently converted into huaicarbon A/B by heating at 250°C for 10-15 min or at 200°C for 25-30 min. With the optimum molar ratio of quercetin/rhamnose (1:3), huaicarbon A and B yields reached 25% and 16% respectively after heating at 250°C, with 55% quercetin conversion. Huaicarbon A/B both promoted washed platelet aggregation dose-dependently, which was antagonized by an inhibitor of glycoprotein VI (GPVI) receptor. Similarly, they both promoted collagen-induced platelet aggregation in platelet-rich plasma in dose-dependent manners. According to the S type dose-response model, EC_{50} values of huaicarbon A and huaicarbon B were calculated as 33.48 μM and 48.73 μM respectively. They induced intracellular Ca^{2+} accumulation that was specifically blocked by GPVI antagonist. Huaicarbon A/B enhanced intracellular Ca^{2+} accumulation and facilitated collagen-induced platelet aggregation, which were blocked by GPVI antagonist. They were conducive to collagen-induced platelet aggregation by activating platelet GPVI receptor.

Keywords: Heating reaction, huaicarbon A/B, quercetin, rhamnose

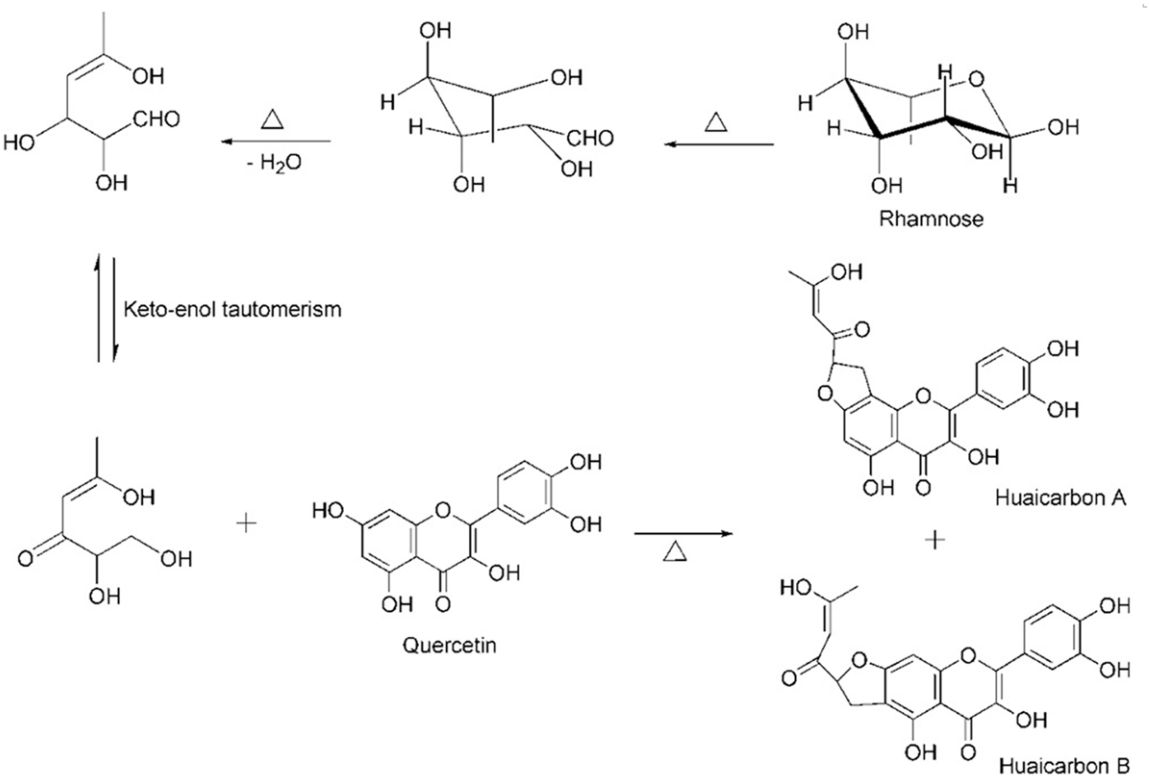
Introduction

We have previously processed (heated) total flavonoids from *Flos Sophorae* (FFS), *Platycladi Cacumen* (FPC) and *Pollen Typhae* (FPT) to generate two new compounds huaicarbon A/B, with significant hemostatic activities [1]. However, the flavonoids that are converted during heating or the precursors for these new compounds remain unknown. Thus, it is necessary to find efficient precursors for huaicarbon A/B.

Conversion of the two compounds was tentatively proposed (**Scheme 1**). The flavonoids in FFS, FPC and FPT, which are O-tri-, O-di- and O-mono-glycosylated 3-O-substituted flavonols respectively, are generated by quercetin and isorhamnetin bound with sugar moieties from rhamnose and glucose. Upon heating, O-glycosidic bonds in the flavonoids are cleaved into aglycone quercetin, isorhamnetin as well as rhamnose and glucose moieties. According to chemical structures, huaicarbon A and huaicarbon B are the products of quercetin/isorhamnetin and rhamnose/glucose reactions respectively.

In our previous study, rutin was heated to generate huaicarbon A and huaicarbon B. Therefore, quercetin and monosaccharide glucose or rhamnose, which are generated by the decomposition of rutin, may be precursors for the two compounds again. Herein, we investigated the heating reaction of quercetin/isorhamnetin mixed with rhamnose/glucose. The experimental results well support our assumption.

Huaicarbon A/B can promote platelet aggregation *in vitro* [1]. It is well known that platelets play a vital role in initial hemostasis [2]. Platelet aggregation is one of the important factors participating in thrombosis and hemostasis. At least five pathways are involved in platelet activation [3], which are mediated by ADP, throm-



Scheme 1. Postulated reaction for the conversion of huaicarbon A/B.

boxane A2, thrombin, 5-hydroxytryptamine and collagen respectively. Huaicarbon A/B promote platelet aggregation mainly by mediating collagen but not ADP or thrombin [1].

Fibrillar collagen, as the main component of vascular subendothelial matrix, is the most potent vascular wall component that initiates platelet adhesion and aggregation [4]. It is also a strong inducer for platelet activation. There are two main collagen-binding receptors on the platelet membrane, i.e. glycoprotein VI (GPVI) and integrin α2β1. GPVI receptor predominantly mediates platelet activation [5, 6]. After vascular damage, collagen in the subcutaneous extracellular matrix is exposed to the blood to directly bind GPVI of collagen on the platelet surface. As a result, GPVI receptor is activated to induce transduction of downstream signals and platelet activation, leading to platelet adhesion and aggregation finally [5, 7, 8]. As a specific inhibitor for GPVI receptor, GPVI antagonist can suppress GPVI-mediated platelet activation [9], so it has been applied in clinical practice to treat thrombosis [10].

Thereby motivated, the effects of huaicarbon A/B on platelet aggregation and intracellular Ca^{2+} level before and after addition of GPVI antagonist were evaluated, aiming to clarify their roles in promoting platelet aggregation.

Materials and methods

Materials and reagents

Glucose (99%) was purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Rhamnose (99%) was purchased from Nanjing Oddofoni Biology Technology Co., Ltd. (China). Quercetin (95%) and isorhamnetin (99%) were purchased from Sigma-Aldrich (USA). Analytical reference standards for huaicarbon A (95%) and huaicarbon B (93%) were provided by Jiangsu Key Laboratory of Chinese Medicine Processing, Nanjing University of Chinese Medicine, China. LC-MS-grade methanol and MS-grade formic acid (98%) were purchased from Merck (Darmstadt, Germany). Deionized water was purified by a Milli-Q system (Millipore, Bedford, MA, USA).

Huaicarbon A/B were synthesized and purified in our group. Sephadex LH-20 column was bought from GE (Sweden). Horm collagen was obtained from Nycomed (Norway). GPVI-FC

fusion protein (an antagonist of collagen-mediated platelet activation) was provided by Professor Farndale from Cambridge University (UK). Fluo-4 NW dye was purchased from Molecular Probes® (USA).

HPLC-MS/MS

Sample preparation: In a typical run, 0.180 g glucose (0.001 mol) or 0.164 g rhamnose (0.001 mol) was well mixed with 0.302 g quercetin (0.001 mol) or 0.316 g isorhamnetin (0.001 mol) in a 100 ml crucible and heated at 200°C or 250°C using a muffle furnace. The heating temperature and time were selected based on the processing conditions for medicinal materials.

Analysis

Mass chromatographic separation was performed using an HPLC-MS/MS system (Triple TOFTM 5600, AB SCIEX, CA) equipped with an electrospray ionization source to analyze the heating samples. Chromatographic separations were performed by a SHIMADZU 20DXR HPLC system connected with a Hanbon Dubhe C18 column using a mobile phase comprising methanol and 0.1% aqueous formic acid solution (60:40) at a flow rate of 1.0 ml/min with the split ratio of 1:1. The column oven temperature was 30°C. The mass spectrometer was operated in the negative ion mode under the following conditions: nebulizer gas (gas 1) of 55 psi; heater gas (gas 2) of 55 psi; curtain gas of 35; ion spray voltage of 7 eV; turbo spray temperature of 550°C; declustering potential (DP) of 60 V for MS; DP of 100 V for MS/MS; collision energy (CE) of 10 for MS; CE of 35 and CE spread of 15 for MS/MS. The data were acquired and analyzed using PeakView® software.

Separation and purification of huaicarbon A/B

Reaction products were separated by a Sephadex LH-20 column (1200 mm × 10 mm i.d.) and eluted by CH_2Cl_2-CH_3OH (1:1) as huaicarbon A/B, with the purities of over 92%.

Platelet aggregation assay

Preparation of platelets: Blood was drawn from healthy volunteers who did not take aspirin by using a syringe containing 4% citric acid (1:9). The anticoagulated whole blood was centrifuged at 800 rpm for 10 min, and the upper layer of platelet-rich plasma (PRP) was used to determine Ca^{2+} and cyclic nucleotide levels. After 12.5 ng/mL PGI2 was added, PRP was further centrifuged at 3000 rpm for 10 min, giving platelet granules that were thereafter redissolved in modified Tyrode's-HEPES buffer (134 mM NaCl, 2.9 mM KCl, 0.34 mM $Na_2HPO_4{\cdot}12H_2O$, 12 mM $NaHCO_3$, 20 mM HEPES, 1 mM $MgCl_2$, pH7.3), centrifuged at 3000 rpm again for 10 min and washed. On the other hand, platelet granules were redissolved in modified Tyrode's-HEPES buffer at the density of 4×10^8 platelets/mL for aggregation assay.

Promotive effects of huaicarbon A/B on platelet aggregation

Collagen as the inducer and GPVI-Fc fusion protein as the inhibitor were added in samples with different concentrations (dissolved in 0.01% DMSO). All the aggregation experiments were performed on a Chrono-Log Model 700 whole blood/optical lumiaggregometer (USA). All measurements were conducted according to kit's instructions and the methods described in a previous study.

Detection of intracellular Ca^{2+} levels in platelets

Intracellular Ca^{2+} levels in platelets were detected by a fluorescent microplate reader. Staining solution was prepared according to kit's instructions. Afterwards, 40 μL of PRP (4×10^8 platelets/mL) and 3690 μL of staining solution were mixed, added in 96-well plates (100 μL each well) and incubated at 37°C for 30 min. After 5 μL of samples with different concentrations (dissolved in the presence of 0.01% DMSO; final concentrations: EC_{50} values; final concentration of GPVI-Fc fusion protein: 20 μg/mL) were added, intracellular Ca^{2+} fluorescence intensity was detected immediately at 37°C. The calcium release rate was calculated based on the average fluorescence intensity.

Statistical analysis

All data were analyzed by SPSS16.0 and GraphPad Prism 5, and expressed as mean ± SD. Each experimental group was compared with the control group by one-way analysis of variance and then Dunnett's or Tukey's test. $P<0.05$ was considered statistically significant.

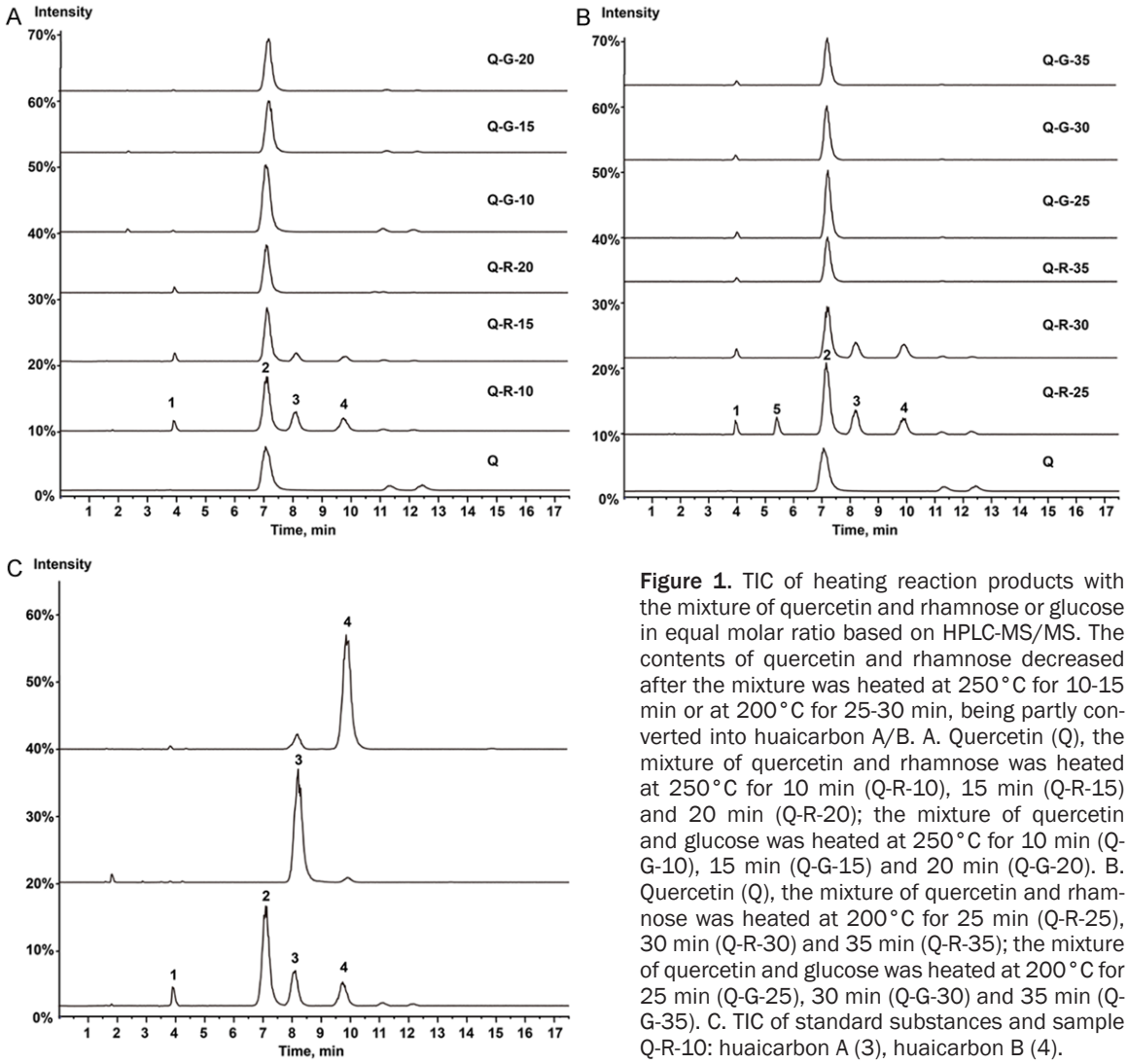


Figure 1. TIC of heating reaction products with the mixture of quercetin and rhamnose or glucose in equal molar ratio based on HPLC-MS/MS. The contents of quercetin and rhamnose decreased after the mixture was heated at 250°C for 10-15 min or at 200°C for 25-30 min, being partly converted into huaicarbon A/B. A. Quercetin (Q), the mixture of quercetin and rhamnose was heated at 250°C for 10 min (Q-R-10), 15 min (Q-R-15) and 20 min (Q-R-20); the mixture of quercetin and glucose was heated at 250°C for 10 min (Q-G-10), 15 min (Q-G-15) and 20 min (Q-G-20). B. Quercetin (Q), the mixture of quercetin and rhamnose was heated at 200°C for 25 min (Q-R-25), 30 min (Q-R-30) and 35 min (Q-R-35); the mixture of quercetin and glucose was heated at 200°C for 25 min (Q-G-25), 30 min (Q-G-30) and 35 min (Q-G-35). C. TIC of standard substances and sample Q-R-10: huaicarbon A (3), huaicarbon B (4).

Results and discussion

Heating reaction of quercetin and glucose or rhamnose at 250°C or 200°C

The contents of quercetin and rhamnose decreased after their mixture was heated at 250°C for 10-15 min or at 200°C for 25-30 min, being partly converted into peaks 1, 3 and 4 (**Figure 1A** and **1B**). Peaks 3 and 4 were identified as huaicarbon A and huaicarbon B respectively by comparing the retention times with those of the reference substances (**Figure 1C**). After heating at 250°C for 10 min, the conversion of quercetin was close to 56%, and the yields of peaks 3 and 4 were about 10% and 6% respectively. After heating at 200°C for 25 min, the conversion of quercetin approached 38%, and the yields of peaks 3 and 4 were about 11% and 8% respectively.

However, when the mixture was further heated for 20 min at 250°C or for 35 min at 200°C, huaicarbon A or huaicarbon B disappeared, being in accordance with the heating transformation rules of total flavonoids in FFS [11]. Thus, the reaction time evidently affected the conversion of the two compounds. When glucose was added, no new components formed after heating at 250°C for 10-20 min or at 200°C for 25-35 min. In short, the mixture of quercetin and rhamnose but not glucose was converted to huaicarbon A and huaicarbon B after heating at 250°C or 200°C.

Effect of initial molar ratio of quercetin to glucose/rhamnose

As described above, the conversion of quercetin approached 18% and the yields of huaicar-

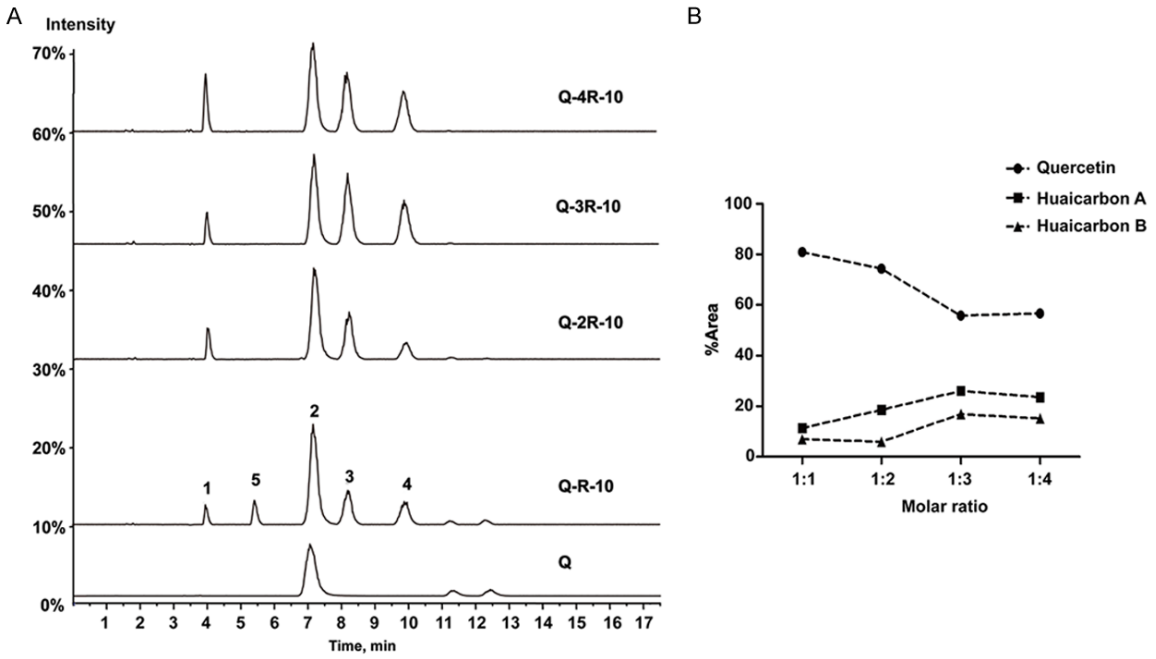


Figure 2. Quercetin conversions and huaicarbon A/B yields in the presence of quercetin and rhamnose with various molar ratios. The mixture was heated at 250°C for 10 min. When the molar ratio of quercetin to rhamnose was 1:3, the yields of huaicarbon A/B reached maxima. A. TIC of heating reaction products with different molar ratios of quercetin to rhamnose based on HPLC-MS/MS. Quercetin (Q), molar ratio of quercetin to rhamnose: 1:1 (Q-R-10), molar ratio: 1:2 (Q-2R-10), molar ratio: 1:3 (Q-3R-10), molar ratio: 1:4 (Q-4R-10). B. Conversion of quercetin and rhamnose with various molar ratios into huaicarbon A/B. Quercetin conversions and huaicarbon A/B yields which are represented by %area are based on HPLC-MS/MS.

bon A and huaicarbon B were only 10% and 5% respectively. Probably, rhamnose and glucose themselves had already been converted, so they did not respond well to quercetin. For comparison, the reaction was carried out with different molar ratios of quercetin to rhamnose or glucose under identical conditions at two temperatures.

Adding 2-, 3- and 4-fold rhamnose elevated the formation of huaicarbon A by about 15%, 25% and 23% respectively and that of huaicarbon B by about 6%, 16% and 15% respectively (**Figure 2**). When the molar ratio of quercetin to rhamnose was 1:3, the yields of huaicarbon A and huaicarbon B reached maxima. Huaicarbon A or huaicarbon B did not form even if more glucose was mixed with quercetin and heated at 250°C for 10 min (**Figure 3A**).

Heating reaction of isorhamnetin and glucose or rhamnose

The chemical compositions of mixtures of isorhamnetin and rhamnose or glucose with different molar ratios changed mildly after heating at 250°C for 10 min (**Figure 3B**). Isorhamnetin mixed neither with rhamnose nor glucose could be converted into huaicarbon A or huaicarbon B when heated at 250°C and 200°C (data not shown).

Promotive effects of huaicarbon A/B on platelet aggregation

After human washed platelets were co-incubated with huaicarbon A/B for 5 min, the aggregation rate was detected by adding 0.03 μg/mL collagen. As shown in **Figure 4A**, huaicarbon A/B both promote washed platelet aggregation dose-dependently. Collagen (1 μg/mL) alone induced the aggregation of 74% platelets (**Figure 4B**), which, however, was blocked by GPVI-Fc fusion protein. Similarly, this protein also blocked the promotive roles of huaicarbon A/B in platelet aggregation, being associated with GPVI receptor.

Besides, whether these two compounds exerted promotive effects on the aggregation of

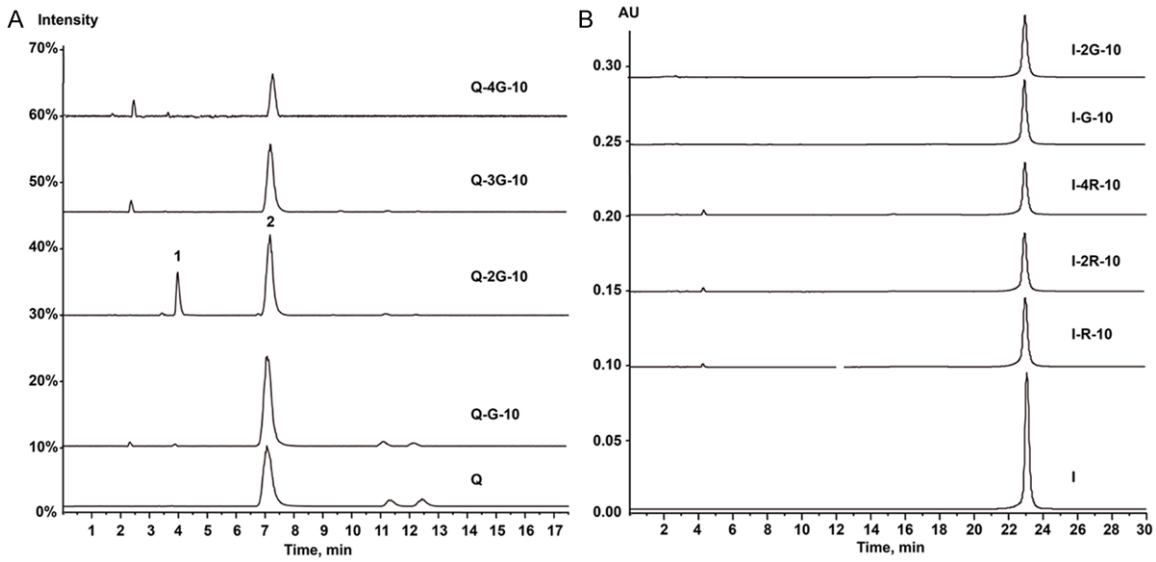


Figure 3. Huaicarbon A/B were not produced either by heating the mixture of quercetin and glucose or the mixture of isorhamnetin and rhamnose or glucose. A. TIC of heating reaction products with different molar ratios of quercetin to glucose based on HPLC-MS/MS. Quercetin (Q), the mixture of quercetin and glucose was heated at 250°C for 10 min (molar ratio: 1:1, Q-G-10), (molar ratio: 1:2, Q-2G-10), (molar ratio: 1:3, Q-3G-10), (molar ratio: 1:4, Q-4G-10). B. HPLC chromatogram of heating reaction products with different molar ratios of isorhamnetin to rhamnose and glucose. Isorhamnetin (I), the mixture of isorhamnetin and rhamnose was heated at 250°C for 10 min (molar ratio: 1:1, I-R-10), (molar ratio: 1:2, I-2R-10), (molar ratio: 1:4, I-4R-10); the mixture of isorhamnetin and glucose was heated at 250°C for 10 min (molar ratio: 1:1, I-G-10), (molar ratio: 1:2, I-2G-10).

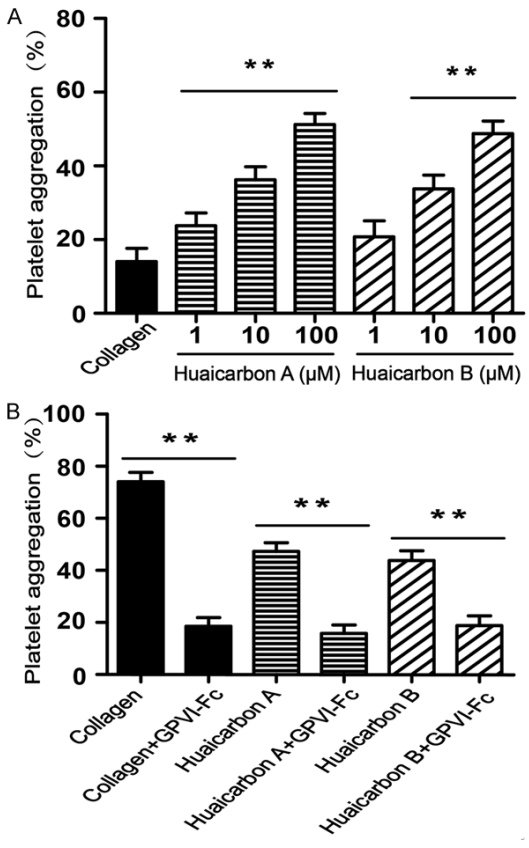


Figure 4. Huaicarbon A/B induced washed platelet aggregation, and GPVI-Fc fusion protein blocked the effect. A. Huaicarbon A/B (1 μM, 10 μM, 100 μM) promoted collagen (0.03 μg/mL)-induced platelet aggregation. B. GPVI-Fc fusion protein (20 μg/mL) inhibited the platelet aggregation induced by collagen (1 μg/mL) and huaicarbon A/B (100 μM) respectively. Data were expressed as mean ± SD of six measurements. **represents statistical significance of $P<0.01$.

platelets in PRP was explored. **Figure 5** exhibits that huaicarbon A/B both facilitate collagen-induced platelet aggregation in dose-dependent manners, indicating that their activities were not affected by plasma protein. EC_{50} values of huaicarbon A and huaicarbon B were calculated as 33.48 μM and 48.73 μM respectively.

Regulatory effects of huaicarbon A/B on intracellular Ca^{2+} recruitment

GPVI is the main receptor mediating platelet aggregation. When it is activated, a series of molecules are involved in signaling transduction [12]. For instance, an instant increase in intracellular free Ca^{2+} level initiates the related

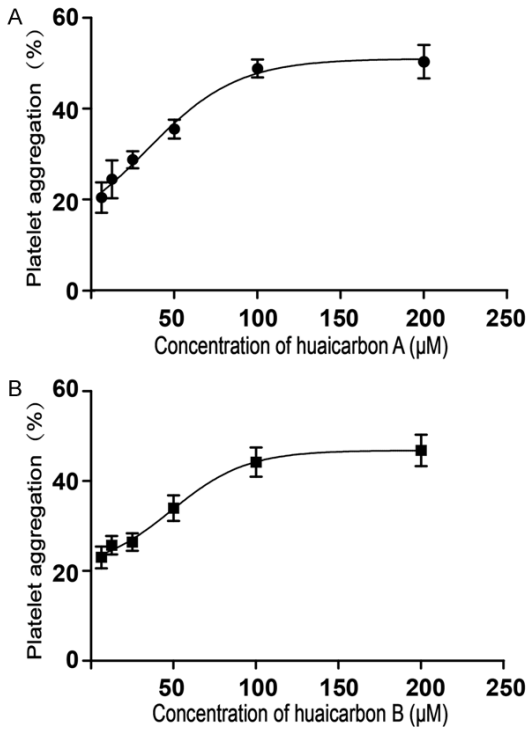


Figure 5. Dose-response curves for huaicarbon A/B on human PRP platelet aggregation rate *in vitro*. Data were expressed as mean ± SD of six measurements. EC_{50} values of huaicarbon A and huaicarbon B were calculated as 33.48 μM and 48.73 μM respectively.

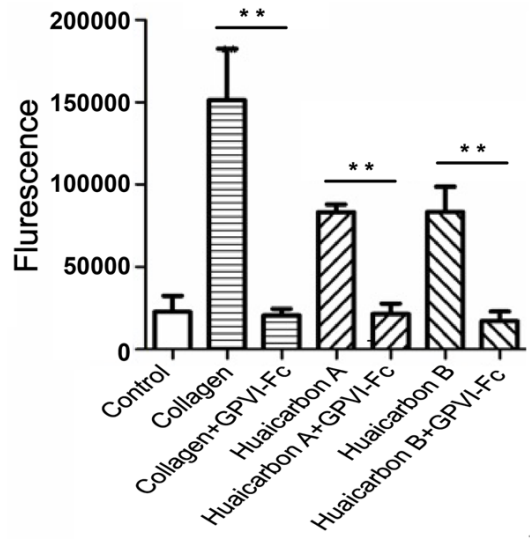


Figure 6. GPVI-Fc fusion protein inhibited huaicarbon A/B-induced intracellular calcium accumulation within platelets. Collagen (1 μg/mL), 33.5 μM huaicarbon A and 48.7 μM huaicarbon B all significantly enhanced intracellular calcium accumulation in platelets. Adding GPVI-Fc fusion protein significantly reduced such levels. Data were expressed as mean ± SD of six measurements. **represents statistical significance of $P<0.01$.

signaling system [13, 14], further inducing cascade reactions including phospholipase 2 activation, platelet morphological changes, dense granule release and eventually platelet aggregation. To analyze the effects of these two compounds on intracellular Ca^{2+} recruitment, the Ca^{2+} level in PRP was detected. As presented in **Figure 6**, 1 μg/mL collagen, 33.5 μM huaicarbon A and 48.7 μM huaicarbon B all evidently enhance Ca^{2+} accumulation in platelets. The increases of intracellular Ca^{2+} level was blocked by adding inhibitor. In addition, huaicarbon A/B induced platelet aggregation by facilitating the elevation of Ca^{2+} level, probably due to the mediating role of GPVI receptor.

Quercetin and rhamnose were the precursors for huaicarbon A/B. The mixture of quercetin and rhamnose was efficiently converted into huaicarbon A and huaicarbon B by heating at 250°C for 10-15 min or at 200°C for 25-30 min. The yields of huaicarbon A and huaicarbon B, which increased with rising initial molar ratio of quercetin to rhamnose, reached maxima at the ratio of 1:3. This heating reaction can thus be used to prepare huaicarbon A/B.

Huaicarbon A/B augmented intracellular Ca^{2+} accumulation and promoted collagen-induced platelet aggregation, which were blocked by GPVI receptor. Therefore, the two compounds were conducive to collagen-induced platelet aggregation by activating GPVI-mediated signaling pathways.

Huaicarbon A/B, which are derived by heating traditional Chinese medicine FFS [15], the dried flower and bud of *Sophora japonica* L., are capable of hemostasis [1]. Thus, hemorrhage diseases including hematochezia, hematemesis, epistaxis, dysfunctional uterine bleeding and hemorrhoidal bleeding can be treated by FFS after heating [16, 17] owing to the hemostatic activities of heating products. Unraveling the synthetic pathways and conditions for huaicarbon A/B as well as their promotive effects on collagen-induced platelet aggregation provides valuable evidence for studying their hemostatic functions.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Hao Wu, School of Pharmacy, Nanjing University of Chinese Medicine, Xianlin Road 138#, Nanjing 210023, P. R. China. Tel: +86-25-86798281; E-mail: whao5795@126.com

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