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Full Length Research Paper

Comparative effects of Hirudo, Frankincense and Motherwort on the inhibition of blood platelet aggregation and P-selectin secretion

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Blood harmonizing *Leonurus japonicus Houtt.* (Motherwort), blood activating *Boswellia carterii Birdw.* (Frankincense), and blood breaking and stasis expelling *Whitmania pigra Whitman* (Hirudo), which all belong to traditional medicines of activating blood and resolving stasis (ABARS), have been used in the treatment of tumors and tumor metastasis for years. However, their eventual efficacies have never been clearly investigated. This paper described and compared the effects of the water extracts of Hirudo, Frankincense and Motherwort on human platelet functions including platelet aggregation and protein secretion. It has been demonstrated that Hirudo for "break blood and expel stasis" extremely suppressed platelet activation and adhesion, but Motherwort for "Harmonize the Blood" did not exhibit the same effects on CD41 and CD62p levels, implying that the platelet inhibiting potency of the ABARS drug for "break blood and expel stasis" was indeed stronger than that for "Harmonize the Blood". The results promoted us to choose a different drug on the basis of the P-selectin level of patients, which will assist clinicians to select drugs rationally.

Key words: Hirudo, Motherwort, Frankincense, platelet aggregation, CD41, CD62p.

INTRODUCTION

Blood harmonizing *Leonurus japonicus Houtt*. (Motherwort) (Zou et al., 1989), blood activating *Boswellia carterii Birdw.* (Frankincense) (Mazzio and Soliman, 2009; Yadav et al., 2011), and blood breaking and stasis expelling *Whitmania pigra Whitman* (Hirudo) (Huang et al., 2003) are herbal, animal and mineral drugs, respectively, which have been used in the treatment of tumors and tumor metastasis for years (Deckmyn et al., 1995; Eldor et al., 1996; Mazzio and Soliman, 2009). They all belong to the scope of traditional medicine in activating blood and resolving stasis (ABARS). In the theory of traditional Chinese medicine (TCM), they all exhibit same effects with different roles (Table 1).

However, accurate quantification and description of the differences have not been thoroughly assessed and thus, confused the clinicians. Therefore, this study aimed to find out reasons for the same role of different types of drugs and the underlying mechanisms of the drugs. On

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Abbreviations: ADP, Adenosine 5'-diphosphate; **PAF,** platelet activating factor; **PRP,** platelet-rich plasma; **PPP,** platelet-poor plasma; **TCM,** traditional Chinese medicine; **ABARS,** activating blood and resolving stasis;

[#]These authors contributed equally to this work.

Table 1. ABARS list under research.

Pharmaceutical name	Abbreviation	Power for ABARS
L. japonicus Houtt.	Motherwort	Harmonize the blood, a therapeutic method to relieve or cure blood disorders
B. carterii Birdw.	Frankincense	Activate blood, a general term for promoting blood flow in the treatment of blood stasis
W. pigra Whitman	Hirudo	Break blood and expel stasis, a therapeutic method to treat severe cases of blood stasis with intact health by using drastic blood-activating medicinal

the other hand, blood coagulation has been reported to be closely related to tumor metastasis (Gay and Felding-Habermann, 2011). Platelets play essential role in many diseases such as vascular remodeling, inflammation, atherosclerosis, acute coronary syndrome, wound healing and other pathological conditions (Jain et al., 2010; Laubli,Borsig, 2010; Thomas et al., 2009; Xue et al., 2010). Besides, platelet aggregation in vascular vessels is one of the crucial steps during haematogenous metastasis. Hypercoagulability of the blood is an important incentive leading to tumor progression (Kanz et al., 2011), which have also been treated utilizing the ABARS drugs.

In a sense, ABARS drug commonly functions in the regulation of platelet state in blood circulation (Qian and Wang, 2009). Generally, platelets may be activated in response to many factors including vessel wall injury and different agonists such as adenosine 5'-diphosphate (ADP), platelet activating factor (PAF) and thrombin that lead to platelet aggregation (Jain et al., 2010).

However, the eventual efficacies of these three drugs on platelet function(s) have never been clearly investigated. This paper described and compared the effects of the water extracts of Hirudo, Frankincense and Motherwort on human platelet functions including platelet aggregation and protein secretion.

MATERIALS AND METHODS

Materials

ADP, PAF and thrombin were obtained from Sigma-Aldrich Corp. (St Louis, MO, USA); Fluorescent-labeled monoclonal antibodies including fluorescein isothiocyanate (FITC) conjugated CD41, phycoerythrin (PE)-conjugated $CD62_P$ and IgG1 (as negative control) were purchased from Amersham Pharmacia Biotech (UK).

Plant materials

Motherwort, Hirudo and Frankincense were purchased from Nanjing Tongrentang Pharmacy. The materials were dried far from direct sunlight, and the resulting dried drugs were powdered and kept in a sealed container in a cold room.

Extraction

Herbs (10 g) were boiled in 100 ml of distilled water for 30 min for isolation. The boiled suspension was then clarified by centrifugation at 5000 g for 15 min. Concentration of the aqueous extract was adjusted by phosphate buffered saline (PBS) to a final concentration at 10 mg/ml and stored at -20°C. The pH of the sample was adjusted to 7.4 prior to experimental studies.

Isolation of blood platelets

Human blood sample was purchased from Jiangsu Province Blood Center. The sample was collected into ACD (78 mMcitric acid, 117 mM sodium citrate and 111 mM dextrose) solution in a 5:1 (v/v) ratio, and the platelets were isolated by differential centrifugation of the blood (20 min at 200 × g). Platelet-rich plasma (PRP) was separated and centrifuged for 20 min at 1000 × g to sedimentate the platelets. The resulting pellet was gently resuspended in Ca^{2+}/Mg^{2+} free modified Tyrode's buffer (140 mM NaCl, 10 mM glucose and 15 mM Tris/HCl, pH 7.4). Then the platelets were subsequently washed three times with the same buffer. Blood platelets were suspended in $Ca^{2*}/^{Mg2*}$ free Tyrode'sbuffer at a final concentration of 10^9 platelets/ml. Platelet-poor plasma (PPP) was prepared by centrifuging the sample for 10 min at 1500 × g after PRP collection.

Platelet aggregation assay

Platelet aggregation was monitored by optical method using Chronolog Lumi aggregometer (Chrono-log, Havertown, PA). PRP (450 µl) was incubated with 50 µl of pH 7.4 adjusted extracts at different concentrations (50 to 200 µg/ml at 37°C) for 5 min prior to the addition of an aggregating agent. Concentrations of the agonists used for aggregation were 2 mg/ml for PAF, 10 mM for ADP and 0.5 U/L for thrombin, respectively.

Controls replacing the extract with 50 µl of PBS were performed in parallel. Inhibition of platelet aggregation was expressed as the decrease in the area under the curve of the sample compared to that of the control. Effect of the herbal extracts on the thrombin-induced platelet aggregation was investigated in the washed platelet suspensions. Their products prepared from PRP were taken as the blank.

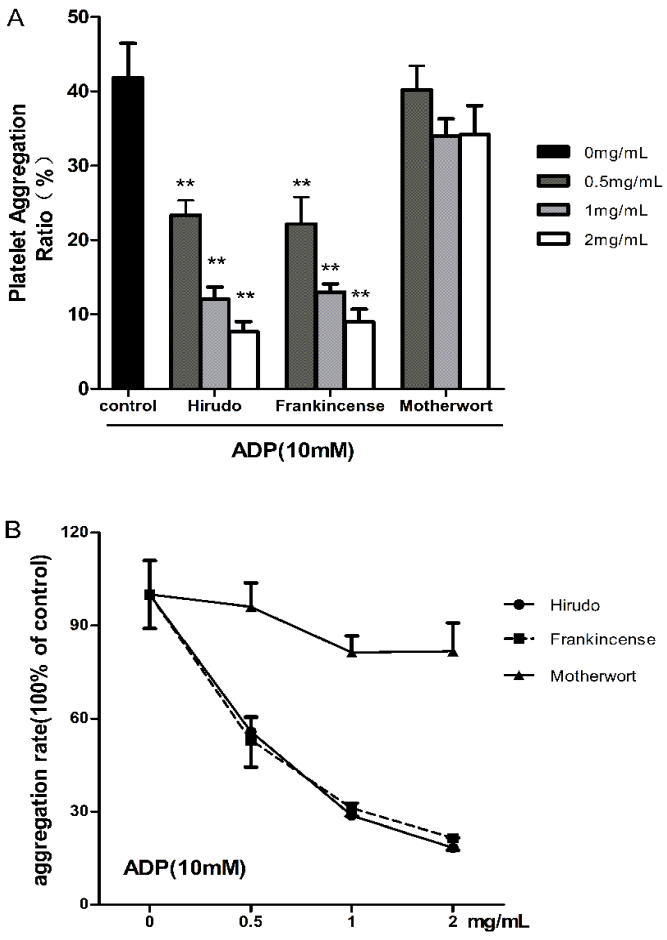

Figure 1. Inhibitory effect of different aqueous extracts of medicinal plants at different concentrations (0.5 to 2 mg/ml) on the aggregation of ADP-activated platelets. Effect of the extracts on ADP induced platelet aggregation was studied by pretreating PRP without or with the extracts at the indicated concentrations for 5 min. After incubation, platelet aggregation was induced by 10 mM ADP and measured with aggregometer. (A). ADP led to a decrease in the optical density significantly, indicating the occurrence of platelet aggregation. (B) Relative aggregation rate was calculated by 100% of control. Each value represents the means's. (n=6).

Protein secretion assay

Flow cytometry analysis of the CD41 and CD62p expressions of the activated platelets was performed on FACsCalibur FC500 (Becton Dickinson, USA). To identify the molecules on the cell surface, 5 µl of PRP was incubated with 5 µl of thrombin (0.5 U/ml) for 10 min at room temperature (RT), which was followed by the addition and incubation of 5 µl of sample herb extracts or PBS control for 5 min at RT.

Samples loaded with IgG1-PE were utilized as the negative control, and then were reacted with CD41-FITC or CD62p-PE in dark for 15 min at RT. 1 ml of PBS was added and mixed in tube for analysis. The labeled platelets were detected by a FACScan (Beckman-Counter, USA). The extent of antibody binding was expressed as the ratio of fluorescence intensity and total platelet population and was used to measure the expression of surface receptors quantitatively.

Statistical analysis

All the data were presented as means ± SD of at least three independent measurements. Comparisons of means were performed by T-test with Graphpad prism 5.0 and values were considered to be significantly different when $P< 0.05$.

RESULTS

Effect of the extracts on PAF or ADP induced platelet aggregation

The effect of the extracts on PAF or ADP induced platelet aggregation was studied by pretreating PRP without or with them at the indicated concentrations for 5 min (Figures 1 and 2). After incubation, platelet aggregation was induced by ADP or PAF and measured with aggregometer. Addition of PAF or ADP to the suspension of human platelets led to the significant decrease of optical density, indicating the occurrence of platelet aggregation. As shown in Figures 1A and 2A, dose-dependent anti-aggregation is associated with each of the extracts, the crude extract of Hirudo exhibits the highest anti-aggregation activity compared to other two extracts at the concentrations beyond 0.5 mg plant powder per milliliter of the test environment. ADP induced platelet aggregation in the PRP samples was not inhibited by Motherwort up to the concentration of 2 mg/ml (Figure1A).

In the platelet activation by PAF, Hirudo and Motherwort showed significant inhibition of platelet aggregation at the concentrations of 0.5 to 2 mg/ml (Figure 2B). Meanwhile, various concentrations of Frankincense did not significantly inhibit platelet aggregation at the maximal concentration of 2 mg/ml in the PAF activated platelet aggregation (Figure 2A).

Effect of the extracts on thrombin induced platelet aggregation

Partial inhibition by the extracts was observed at the concentrations of 0.5, 1 and 2 mg/ml. Figure 3 shows that dose-dependent anti-aggregation is associated with each of the extracts. The results clearly showed that the addition of thrombin to the suspension of human platelets resulted in a remarkable decrease in optical density, indicating the aggregation of platelets. Besides, the aggregation effects of the three extracts on the activated platelets were also studied by incubating the platelets with individual extracts for 5 min. The results showed that Hirudo, Frankincense and Motherwort exhibited descending inhibition effects on platelet aggregation, which is consistent with their effects as traditional medicines.

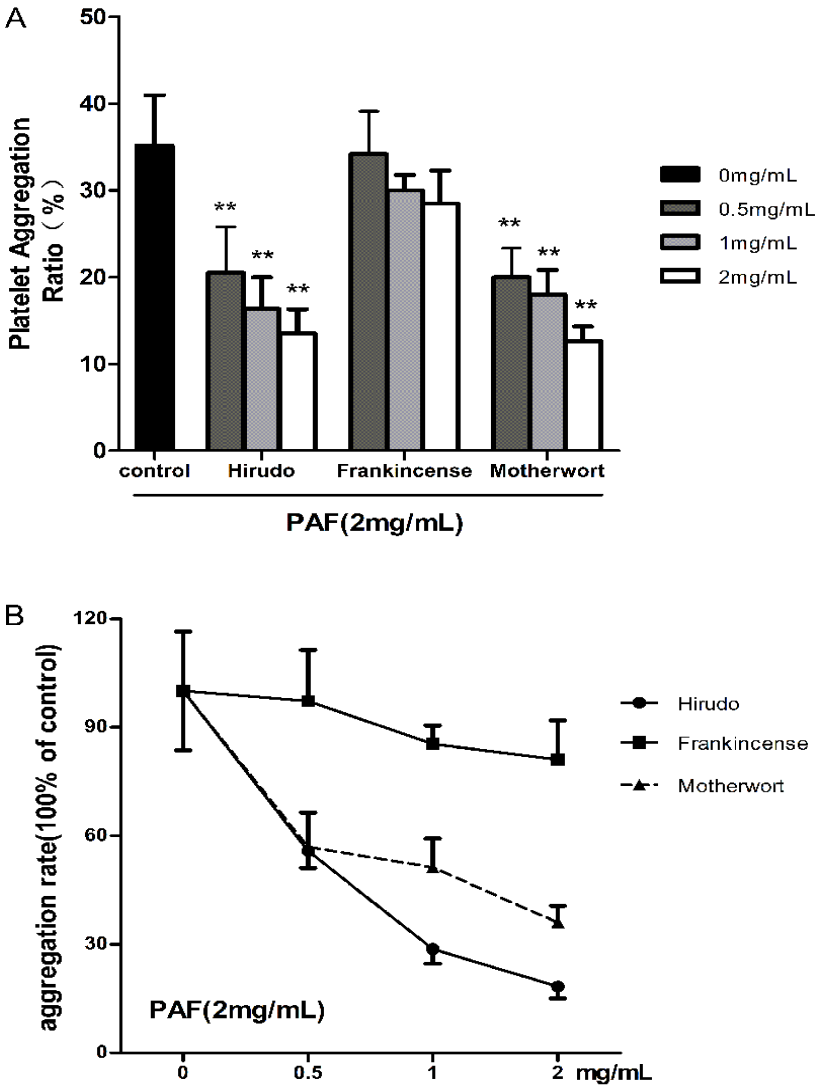

Figure 2. Inhibitory effect of different aqueous extracts of medicinal plants at different concentrations (0.5 to 2 mg/ml) on the aggregation of PAF-activated platelets. Effect of the extracts on PAF induced platelet aggregation was studied by pretreating PRP without or with the extracts at the indicated concentrations for 5 min. After incubation, platelet aggregation was induced by 2 mg/ml PAF and measured with aggregometer. (A). PAF resulted in a decrease in the optical density significantly, indicating the occurrence of platelet aggregation. (B) Relative aggregation rate was calculated by 100% of control. Each value represents the means's (n=6).

Effect of the extracts on protein secretion

To explore the mechanism differences of thrombin-induced platelet aggregation among the three aqueous extracts, the effects of each aqueous extracts on protein secretion were investigated. Human platelets were incubated with various extracts at the same concentration (1 mg/ml), which were all followed by thrombin activation (0.5 U/ml). Figure 4 shows the inhibitory profile of each plant extracts, in which the protein secretions of CD41 decreased by almost 60, 50 and 30% in the presence of 1 mg/ml of the crude extract of Hirudo, Frankincense and Motherwort, respectively. Similarly, the protein secretions of CD62p decreased by almost 30, 20 and 10%, respectively. The results herein clearly suggested that the crude extract of Hirudo at the concentration of 1 mg/ml exhibited a higher potency in the inhibition of protein secretion compared to other two plant extracts.

DISCUSSION

Blood platelets play an important role both in the process of haemostasis and thrombosis and that of tumor

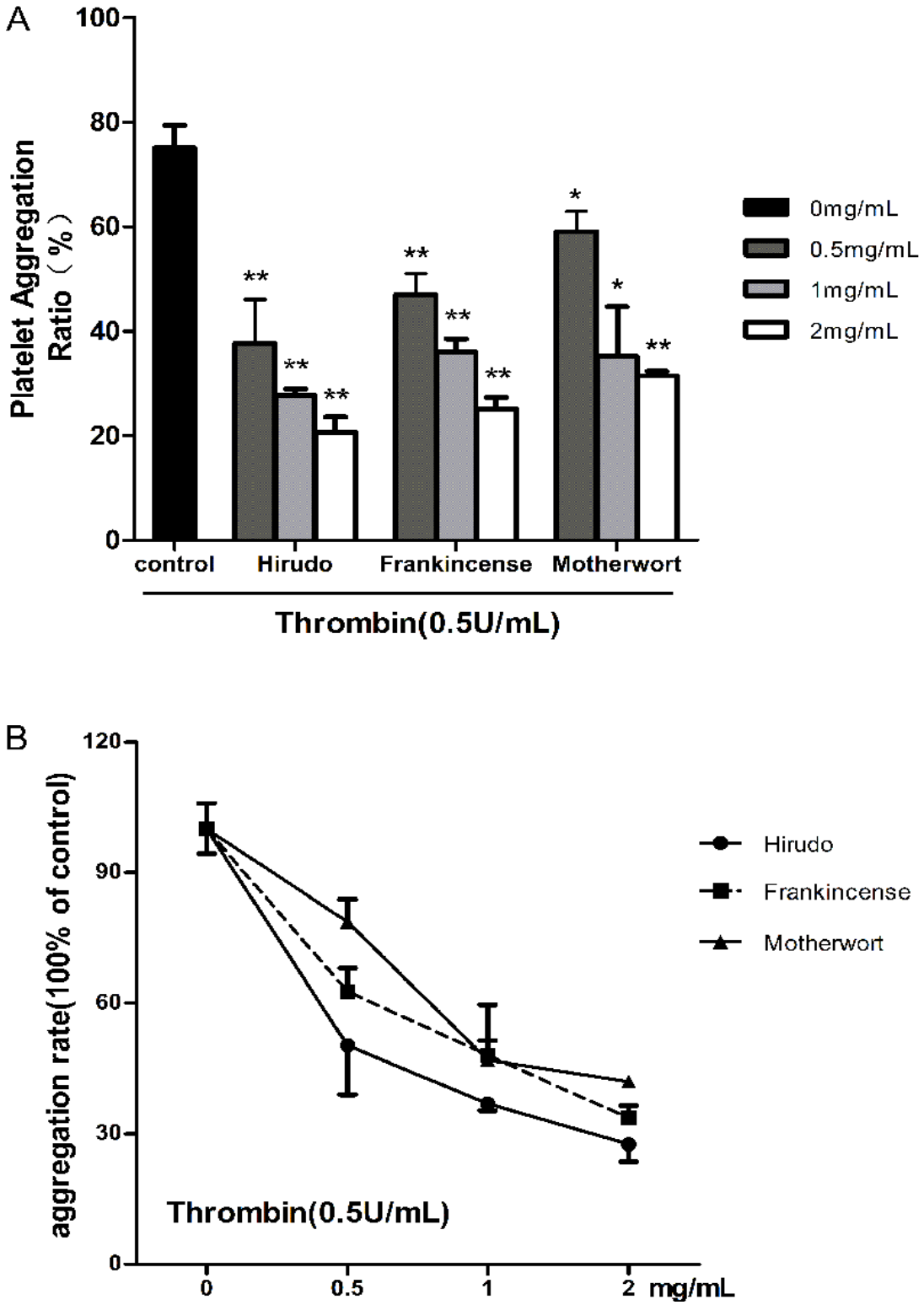

Figure 3. Inhibitory effect of different aqueous extracts of medicinal plants at different concentrations (0.5–2 mg/ml) on the aggregation of thrombin-activated platelets. Effect of the extracts on thrombin induced platelet aggregation was studied by pretreating PRP without or with extracts at the indicated concentrations for 5 min. After incubation, platelet aggregation was induced by 0.5 U/ml thrombin and measured with aggregometer. (A) Thrombin led to a decrease in the optical density significantly, indicating the occurrence of platelet aggregation. (B) Relative aggregation rate was calculated by 100% of control. Each value represents the means's (n=6).

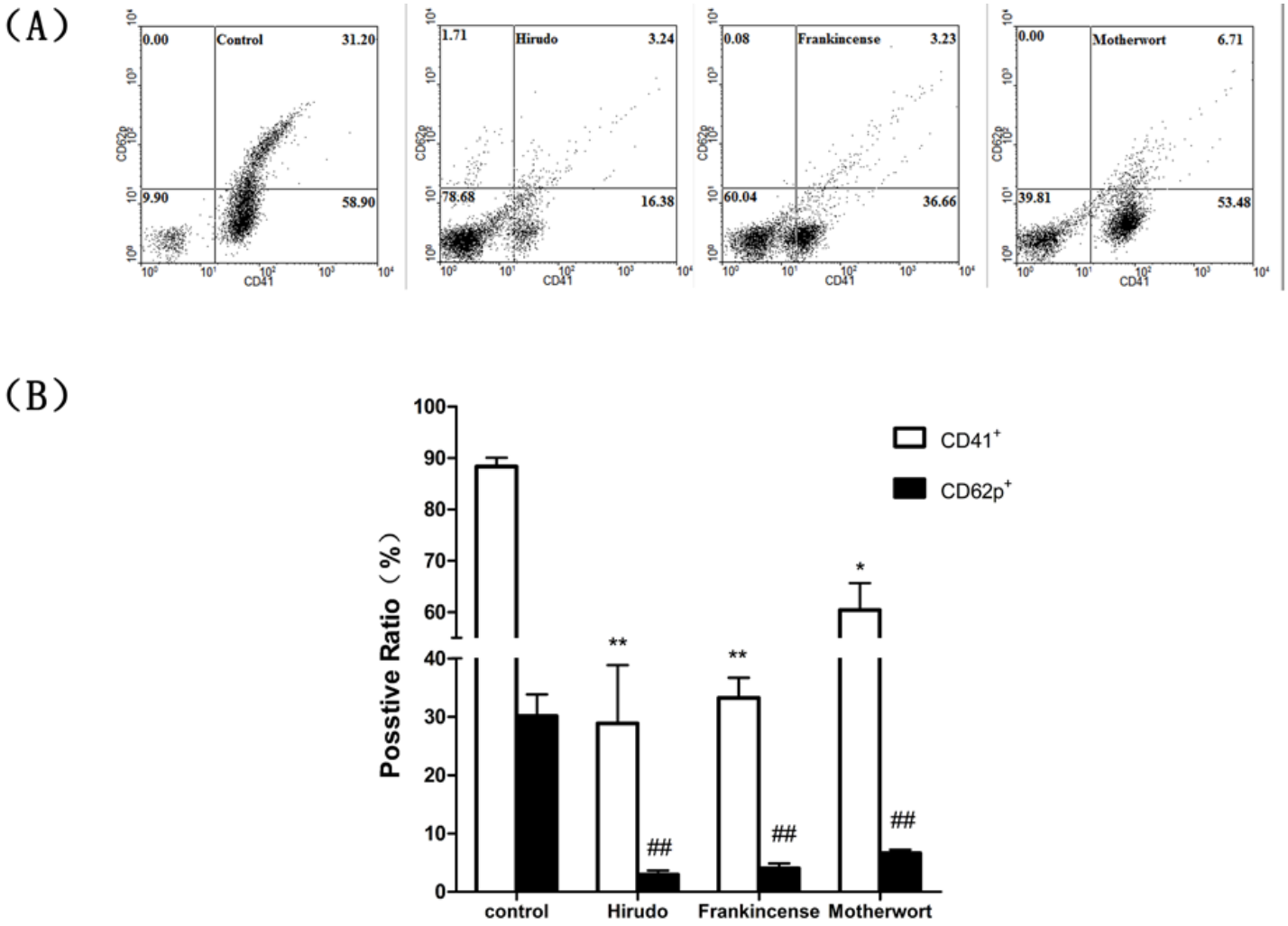

Figure 4. Effect of different aqueous extracts of medicinal plants at different concentrations (0.5 to 2 mg/ml) on the release of proteins from the thrombin-activated platelets. Human platelets were incubated with various extracts at the same concentration (1 mg/ml) and followed by thrombin activation (0.5 U/ml). The labeled platelets were detected by a FACScan (Beckman-Counter, USA). The extent of antibody binding was expressed as the ratio of fluorescence intensity and total platelet population, and was used as a quantitative measure for the expression of surface receptors. Each value represents the means (n=6).

metastasis (Gay and Felding-Habermann, 2011; Jain et al., 2010; Laubli and Borsig, 2010). Excessive platelet activation will result in thrombosis, whereas insufficient platelet activation will lead to bleeding. Thus, evaluating platelet function under various conditions is crucial for estimating the risk of thrombosis or bleeding.

In Chinese medicine, ABARS drugs have been widely used in the treatment of anti-metastatic tumors. Motherwort, Hirudo and Frankincense have been massively applied in the treatment of breast cancer (Eldor et al., 1996; Lu and Li, 2009; Mazzio and Soliman, 2009; Tao et al., 2009; Yadav et al., 2011). It is traditionally considered that Motherwort, Frankincense and Hirudo are able to harmonize blood, activate blood, and break blood and expel stasis, respectively. Therefore, ADP, PAF and thrombin that can induce a primary aggregation of platelets were adopted to evaluate the anti-platelet activities of ABARS drugs, which have been demonstrated to be comparable with diverse platelet activating agents to different extent.

In the present study, we have tested and compared the anti-platelet effects of the aqueous crude extracts of the three medicinal plants on human platelet aggregation and protein secretion *in vivo*. The results showed that treating the platelets with different concentrations of the plant extracts significantly modulated the aggregation behaviors of ADP, PAF or thrombin induced platelets (Figure 1 to 3). Besides, our results also indicated that Hirudo was more active in inhibiting platelet aggregation stimulated by ADP, PAF or thrombin than Frankincense and Motherwort.

In addition, the crude aqueous extracts of the plants inhibited protein secretion induced by thrombin. Flow cytometry assay exhibited significantly higher expressions of the two platelet activation biomarkers CD41 and CD62p. Meanwhile, it has been verified that Hirudo showed most apparent inhibitory effect on both of the mentioned indicators as well as its “break blood and expel stasis” characteristic (Figure 4). Though, Motherwort could inhibit PAF and thrombin-induced platelet aggregation, it failed to perform the same as Hirudo on CD41 and CD62p expressions, which incorporated its intrinsic “harmonize the blood” property. Frankincense also inhibited CD41 and CD62p, but its performance could not be comparable with that of Hirudo, which could be interpreted as an “activate blood” property. The obtained results clearly revealed the correlation among the ABARS drugs.

Moreover, Hirudin isolated from Hirudo is a potent non-specific inhibitor of platelet aggregation induced by thrombin, collagen, ADP, epinephrine, platelet-activating factor and arachidonic acid (Eldor et al., 1996). Hirudin is a peptide component that can be easily destroyed by heat. Considering that the water extract of Hirudo was verified to be able to inhibit platelet aggregation, other components of Hirudo were also of inhibitory effect on platelet aggregation. The photochemical data showed that Motherwort and Frankincense contained alkaloidal, aromatic acid and flavone compounds, representing broad-spectrum therapeutic potentials (Kale et al., 2008). Meanwhile, it has also been reported that these chemicals were able to significantly inhibit the adhesion, aggregation and secretion of platelets (Hwang et al., 2008; Jantan et al., 2006; Phuwapraisirisan et al., 2007; Tang et al., 2010). Thus, the anti-platelet performance of the plants tested in this study was originated from these chemicals. It was believed that coumarone could inhibit the platelet functions through a variety of mechanisms.

Conclusion

The present work have demonstrated that all the water extracts of the three ABARS drugs could significantly inhibit ADP, PAF and thrombin induced platelet aggregations, but their influences on the expressions of the activated platelet membrane surface makers CD41 and CD62p varied. For the ABARS drugs investigated herein, we found that “break blood and expel stasis” drug Hirudo was able to extremely suppressed platelet activation and adhesion, but “harmonize the blood” drug Motherwort did not affect CD41 and CD62p levels, implying that the potency to inhibit platelet of TCM for “break blood and expel stasis” is indeed stronger than that for “harmonize the blood”. Besides, three drugs could inhibit platelet aggregation. However, their effects on P-selectin secretion of platelets differed. P-selectin expression is relative to tumor metastasis (Gay and Felding-Habermann, 2011; Green and Karpatkin, 2010; Kohler et al., 2010; Laubli and Borsig, 2010; Xue et al., 2010), which promotes us to choose a different drug on the basis of the p-selection levels of patients and will assist clinicians to rationally select drugs in the future. Taking into consideration that most previous studies focused on the alcohol extracts, the satisfactory results obtained in our study employing the water extracts are still in need of thorough mechanism investigations.

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