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Original Research

Screening for hemostatic activities of popular Chinese medicinal herbs *in vitro*

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ABSTRACT

Aims: This study aimed to identify new hemostyptics by assessing the coagulation enhancing activity of 114 Chinese herbal extracts *in vitro*. **Methods:** Herbs were boiled in water for 30 min, filtered and then lyophilized filtrates (10 mg/mL) were dissolved in water. Coagulation was assayed as prothrombin time (PT). Plasma diluted in saline was incubated with each extract for 5 min and then PT reagent was added, followed by $CaCl_2$ solution and the time taken to form clots was measured. Extracts that decreased coagulation time were regarded as containing active compounds. The abilities of extracts to activate Factor XII were assessed and the activated form of factor XII (XIIa) was resolved by SDS-PAGE and visualized by silver staining. **Results:** Coagulation time was obviously shortened by extracts of Alpinia Rhizome, Areca, Artemisia Leaf, Cassia Bark, Danshen Root, Ephedra Herb, Epimedium Herb, Forsythia Fruit, Great Burdock Achene, Moutan Bark, Perilla Herb, Red Paeony Root, Schizonepeta Spike, Senticosus Rhizome, Sweet Annie, Uncaria Thorn and Zanthoxylum Peel. Factor XII was obviously activated by extracts of Artemisia Leaf and Great Burdock Achene, and slightly by Perilla herb. **Conclusion:** Some popular Chinese medicinal herbs have potential as hemostatic agents and could thus be develope as new strategies for the treatment and prevention of bleeding.

KEY WORDS: Hemostatic, herbal treatment, screening

INTRODUCTION

Many Chinese medicinal herbs contain substances that promote hemostasis and when administered orally or externally, can help to support the process through which bleeding stops. The hemostatic components and mechanisms of action in some of these herbs have been investigated using the mouse bleeding model [1-5]. Hemostyptics are specific hemostatic agents that retard or stop bleeding by causing blood vessels to contract or by accelerating blood clotting when externally applied [3,6,7]. The hemostatic activities of herbal hemostyptics are often due to mechanisms such as tannin astringency [8]. Some hemostatic herbs can shorten bleeding and blood coagulation times [9], thus preventing bleeding from fragile capillaries, and they can also inhibit infection and inflammation that leads to vessel leakage and damage. We previously showed that Cat-tail pollen (*Pollen Typhae*), a traditional Chinese medicinal herb, has hemostyptic effects *in vitro* and in the mouse bleeding model *in vivo* [10]. That study also showed that the hemostyptic properties of externally applied Cat-tail pollen were attributable to the activation of intrinsic coagulation [10]. Acidic polysaccharide in pollen extract directly activates factor XII in the intrinsic coagulation cascade [10]. The present study searched for novel hemostyptics by assessing ability of 114 Chinese herbal extracts to enhance coagulation activity *in vitro*.

MATERIALS AND METHODS

Materials

Extracts from 114 Chinese medical herbs were supplied by the Cooperative Research Project at the Joint Usage/Research Center (Joint Usage/Research Center for Science-Based Natural Medicine), Institute of Natural Medicine, University of Toyama [Table 1]. Human factor XII was purchased from Hematologic Technologies Inc. (Essex Junction, Vermont, USA). Pooled normal human plasma was obtained from Axis-Shield (Oslo, Norway). The coagulation assay reagent, Thromborel S was purchased from Dade Behring (Marburg, Germany) and Sysmex (Kobe, Japan), respectively.

Preparation of Extract

Extracts were prepared by boiling 45 g of each herb in 900 mL of water for 30 min and then passed through a cotton plug.

Table 1: Chinese medicinal herbs

Acanthopanax Bark (*Acanthopanacis Cortex*)	Achyranthes Root (*Achyranthis Radix*)
Aconite Root (*Aconiti Radix*)	Acorus Gramineus Rhizome (*Acori Graminei Rhizoma*)
Alpinia Rhizome (*Alpiniae Officinari Rhizoma*)	Angelica Dahurica Root (*Angelicae Dahuricae Radix*)
Apricot Kernel (*Armeniacae Semen*)	Araria Root (*Araliae Cordatae Rhizoma*)
Areca (*Arecae Semen*)	Artemisia Capillaris Flower (*Artemisiae Capillaris Herba*)
Artemisia Leaf (*Artemisiae Folium*)	Asiasarum Root (*Asiasari Radix*)
Asparagus Tubers (*Asparagi Tuber*)	Astragalus Root (*Astragali Radix*)
Atractylodes Lancea Rhizome (*Atractylodis Lanceae Rhizoma*)	Atractylodes Rhizome (*Atractylodis Rhizoma*)
Biond Magnolia Flower (*Magnoliae Flos*)	Bitter Cardamon (*Alpiniae Fructus*)
Bupleurum Root (*Bupleuri Radix*)	Burningbush (*Dictamni Radicis Cortex*)
Cassia Bark (*Cinnamomi Cortex*)	Chinese Bugbane Rhizome (*Cimicifugae Rhizoma*)
Chinese Wolfberry Root-bark (*Lycii Cortex*)	Chrysanthemum flower (*Chrysanthemi Flos*)
Cnidium Rhizome (*Cnidii Rhizoma*)	Codonopsis Root (*Codonopsitis Radix*)
Coix Seed (*Coicis Semen*)	Common Anemarrhena Rhizome (*Anemarrhenae Rhizoma*)
Cornus fruit (*Corni Fructus*)	Corydalis Tuber (*Corydalis Tuber*)
Cyperus Rhizome (*Cyperi Rhizoma*)	Danshen Root (*Salviae Miltiorhizae Radix*)
Desertliving Cistanche (*Cistanchis Herba*)	Dried Tangerine Peel (*Citri Reticulatae Pericarpium*)
Ephedra Herb (*Ephedrae Herba*)	Epimedium Herb (*Epimedii Herba*)
Eucommia Bark (*Eucommiae Cortex*)	Evodia Fruit (*Euodiae Fructus*)
Figwortflower Picrorrhiza Rhizome (*Picrorrhizae Rhizoma*)	Forsythia Fruit (*Forsythiae Fructus*)
Fritillary Bulb (*Fritillariae Bulbus*)	Ganoderma (*Ganoderma*)
Gardenia Fruit (*Gardeniae Fructus*)	Gentiana Macrophylla Root (*Gentianae macrophyllae radix*)
Ginger (*Zingiberis Rhizoma*)	Ginseng Root (*Ginseng Radix*)
Glycyrrhiza (*Glycyrrhizae Radix*)	Great Burdock (*Achene Arctii Fructus*)
Hemp Fruit (*Cannabidis Fructus*)	Himalayan Teasel Root (*Dipsaci Radix*)
Immature Orange (*Aurantii Fructus Immaturus*)	Imperata Rhizome (*Imperatae Rhizoma*)
Japanese Angelica Root (*Angelicae Radix*)	Japanese Gentian (*Gentianae Scabrae Radix*)
Jujube (*Zizyphi Fructus*)	Leonurus Herb (*Leonuri Herba*)
Lindera Root (*Linderae Radix*)	Loquat Leaf (*Eriobotryae Folium*)
Magnolia Bark (*Magnoliae Cortex*)	Malaytea Scurfpea Fruit (*Psoraleae Semen*)
Mentha Herb (*Menthae Herba*)	Moutan Bark (*Moutan Cortex*)
Mulberry Bark (*Mori Cortex*)	Mulberry Leaf (*Mori Folium*)
Myrrha (*Myrrha*)	Notoginseng Root (*Notoginseng Radix*)
Notopterygium (*Notopterygii Rhizoma*)	Ophiopogon Tuber (*Ophiopogonis Tuber*)
Oriental Waterplantain (*Rhizome Alismatis Rhizoma*)	Panax Rhizome (*Panacis* Japonici *Rhizoma*)
Peach Kernel (*Persicae Semen*)	Peony Root (*Paeoniae Radix*)
Perilla Herb (*Perilla Herba*)	Phellodendron Bark (*Phellodendri Cortex*)
Pinellia Tuber (*Pinelliae Tuber*)	Plantago Seed (*Plantaginis Semen*)
Platycodon Root (*Platycodi Radix*)	Polygala Root (*Polygalae Radix*)
Polygonum Root (*Polygoni Multiflori Radix*)	Polyporus Sclerotium (*Polyporus*)
Poria Sclerotium (*Poria Sclerotium*)	Prepared Rehmannia Root (*Processi Rehmanniae Radix*)
Processed Ginger (*Zingiberis Processum Rhizoma*)	Pueraria Root (*Puerariae Radix*)
Red Ginseng (*Ginseng Radix Rubra*)	Red Paeony Root (*Paeoniae Radix Rubra*)
Rehmannia Root (*Rehmanniae Radix*)	Safflower (*Carthami Flos*)
Saffron (*Crocus*)	Saposhnikovia Root (*Saposhnikoviae Radix*)
Sasa Leaf (*Sasa Folium*)	Saussurea Root (*Saussureae Radix*)
Schisandra Fruit (*Schisandrae Fructus*)	Schizonepeta Spike (*Schizonepetae Spica*)
Scutellaria Root (*Scutellariae Radix*)	Senticosus Rhizome (*Senticosus Rhizome*)
Sesame (*Sesami Semen*)	Shrub Chaste Tree Fruit (*Viticis Fructus*)
Sinomenium Stem (*Sinomeni Caulis Et Rhizoma*)	Smilax Rhizome (*Smilacis Rhizoma*)
Sophora Root (*Sophorae Radix*)	Sparganium Rhizome (*Sparganii Rhizoma*)
Stemona Root (*Stemonae Radix*)	Sweet Annie (*Artemisiae* Annuae *Herba*)
Sweet Flag Root (*Acori Rhizoma*)	Swertia Herb (*Swertiae Herba*)
Tall Gastrodia Tuber (*Gastrodia elata*)	Tribulus Fruit (*Tribuli Fructus*)
Trichosanthes Root (*Trichosanthis Radix*)	Turmeric (*Curcumae Rhizoma*)
Uncaria Thorn (*Uncariae Uncis Cam Ramlus*)	Zanthoxylum Peel (*Zanthoxyli Fructus*)
Zedoary (*Zedoriae Rhizoma*)	Zizyphus Seed (*Zizyphi Semen*)

Filtrates were lyophilized and dissolved in water to a final concentration of 10 mg/mL.

Coagulation Assays

Coagulation was assayed as prothrombin time (PT). Plasma diluted five-fold with isotonic sodium chloride solution (100 μL) was incubated with extracts (50 μL) for 5 min. The PT reagent (50 μL), was added and then coagulation was started by adding 25 mmol/L $CaCl_2$. The time required to form clots was measured using a KC4A coagulometer (Amelung, Lemgo, Germany). The plasma coagulation time of the control was about 350 s. Extracts that shortened coagulation time were regarded as containing

active compounds. Extracts that shorted coagulation time to <230 s were selected. However, some herb extracts that were very turbid or contained precipitates were not further investigated.

Factor XII Activation Assay

Factor XII activation was assayed [6]. A volume of 70 μL of purified human factor XII (0.14 mg/mL) and 70 μL of extracts (10 mg/mL) were incubated for 5 h and then the mixtures were resolved by SDS-PAGE and stained with silver.

RESULTS

We determined the potentiating effect of herbal extracts on the coagulation time of the extrinsic pathway to assess hemostatic activity *in vitro*. Trace amounts of PT reagent were used to start the reaction. A shortened coagulation time was taken to indicate hemostatic activity. Extracts of Alpinia Rhizome, Areca, Artemisia Leaf, Cassia Bark, Danshen Root, Ephedra Herb, Epimedium Herb, Forsythia Fruit, Great Burdock Achene, Moutan Bark, Perilla Herb, Red Paeony Root, Schizonepeta Spike, Senticosus Rhizome, Sweet Annie,

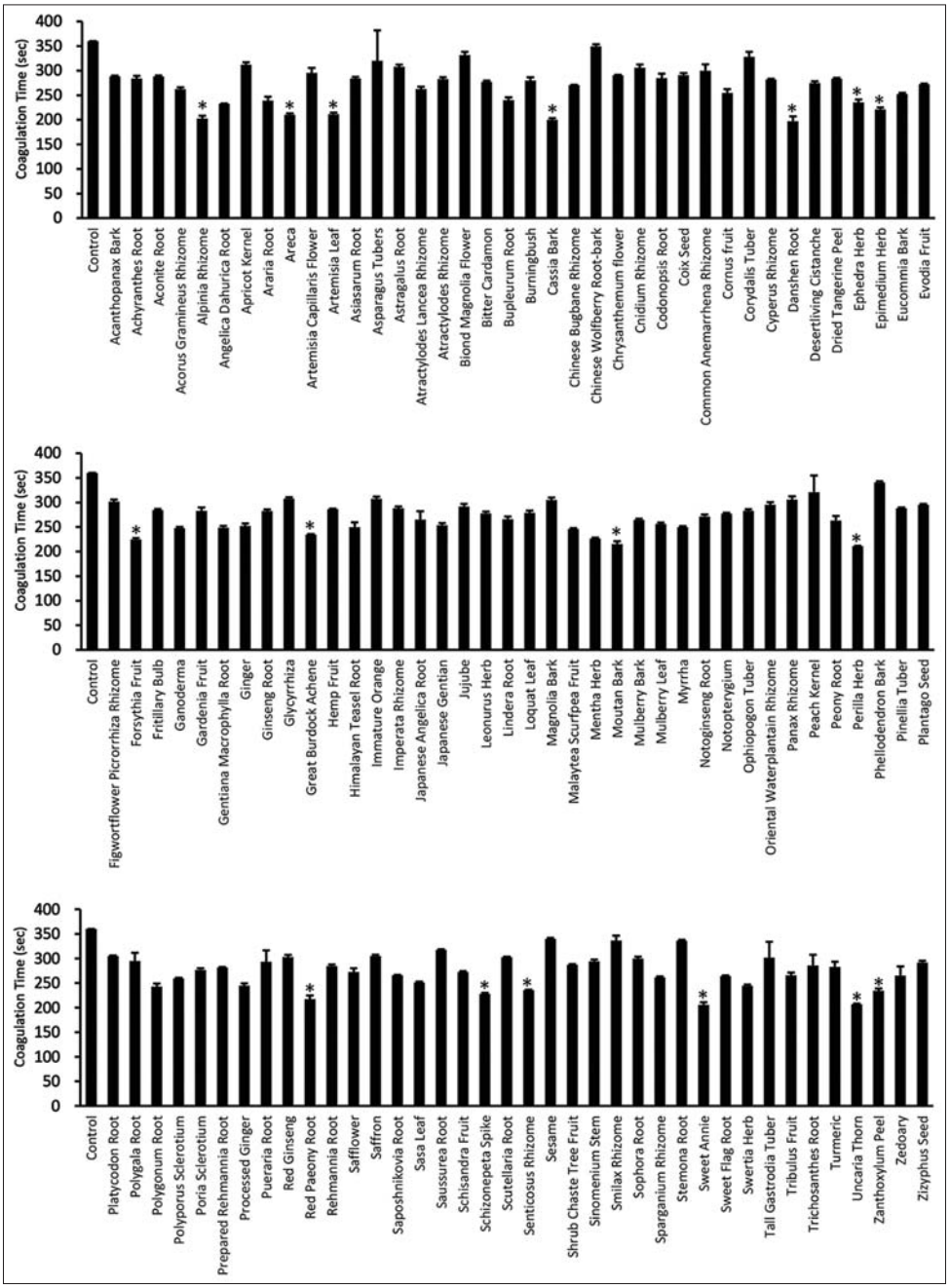

Figure 1: Effects of extracts of Chinese medicinal herbs on prothrombin time, Herbal extracts were incubated with plasma for 5 min before $CaCl_2$ solution was added and amount of time required for clot formation was measured. Data are expressed as means ± standard deviation (n = 3). *Coagulation time was under 230 s.

Uncaria Thorn and Zanthoxylum Peel shortened coagulation time [Figure 1]. We then assessed the ability of these extracts to activate factor XII. Purified human factor XII was incubated with the extracts for 5 h at 37°C and then the presence of Factor XIIa was assessed. Proteins in the mixture were resolved by SDS-PAGE and stained with silver. The light and heavy chains of factor XII were detected after adding extracts of Cat-tail pollen, Artemisia Leaf and Great Burdock Achene. A slight amount of light chain was generated by extracts of Perilla Herb [Figure 2].

DISCUSSION

The hemostatic properties of Cat-tail Pollen have been attributed to the activation of intrinsic coagulation [10,11]. Acidic polysaccharide in extracts of Cat-tail pollen directly activate factor XII in the coagulation cascade [10,11]. However, whether other hemostatic herbs such as Artemisia Leaf can activate intrinsic coagulation has remained unknown and other medicinal herbs might have as yet undiscovered hemostatic activity. Therefore, we screened active substances in 117 extracts of Chinese herbs by measuring the ability to enhance the extrinsic coagulation reaction. Extracts of Alpinia Rhizome, Areca, Artemisia Leaf, Cassia Bark, Danshen Root, Ephedra Herb, Epimedium Herb, Forsythia Fruit, Great Burdock Achene, Moutan Bark, Perilla Herb, Red Paeony Root, Schizonepeta Spike, Senticosus Rhizome, Sweet Annie, Uncaria Thorn and Zanthoxylum Peel shortened coagulation times. The ability of these extracts to activate factor XII was assessed by incubation for 1 h at 37°C, followed by resolving the reaction products by SDS-PAGE. Figure 2 shows that extracts of Artemisia Leaf and Great Burdock Achene cleaved factor XII to the light and heavy chains of factor XIIa, namely the active form of factor XII. Artemisia Leaf is a known antipyretic, insecticide, diuretic, and hemostatic agent [12]. Kneaded leaves of Artemisia Leaf have been used to treat excoriations or cuts on the skin surface. The hemostatic activity is thought to be a result of tannin astringency because mugwort contain high levels of tannin [8]. The present study found that Artemisia Leaf directly activates Factor XII. Hayakawa *et al.* reported that extracts of Artemisia Leaf leaves contain sulfated polysaccharide [13], and this probably induced the activation. However, the contribution of factor XII activation to the hemostatic activity of Artemisia Leaf is not clear. Great Burdock Achene has traditionally been used in China as an anti-inflammatory, detoxifying, or diuretic agent, and to dispel pathogenic wind-heat, promote eruption and remove toxic substances such as heavy metals [13]. The major bioactive principles in Great Burdock Achene are phenolic compounds such as lignans that have various biological properties *in vitro* and *in vivo* such as anti-cancer, antioxidant, antibacterial, antiviral anti-inflammatory and immunosuppressive activities [14-16]. However, whether Great Burdock Achene has hemostatic activity and effects on blood coagulation had not been reported. The present results showed that extracts of Great Burdock Achene and of Cat-tail pollen have essentially identical ability to activate factor XII. Negatively-charged compounds such as acidic polysaccharides in burdock fruit might contribute to the activation of factor XII. Sweet Annie and Artemisia Leaf both belong to the genus *Artemisia*. Sweet Annie (*Artemisia annua*) is a renowned antimalarial [17] and its effect on hemostasis has already been established in traditional Chinese medicine [18]. Wang *et al.* screened the hemostatic active fraction of sweet Annie using assays of plasma recalcification times and found an active fraction [19]. However, the mechanism involved in the reduction of coagulation time was not clear. Sweet Annie did not activate factor XII in the present study. Schizonepeta spike (*Schizonepeta tenuifolia*) exerts hemostatic action by promoting coagulation and inhibiting fibrinolysis [20]. Here, Schizonepeta spike shortened PT, but the activity was not dependent on factor XII activation. Extracts of Red Paeony Root also shortened blood coagulation time in the present study, which contradicted the findings of Wang and Ma, who showed that an extract of Red Paeony Root prolonged coagulation time [21]. We did not detect factor XII activation by other herbs that shorten coagulation time. These extracts might activate coagulation factors downstream of the intrinsic coagulation cascade such as factor XI and factor IX or inhibit anticoagulant proteins such as antithrombin. Our screening study showed that some popular Chinese herbs have

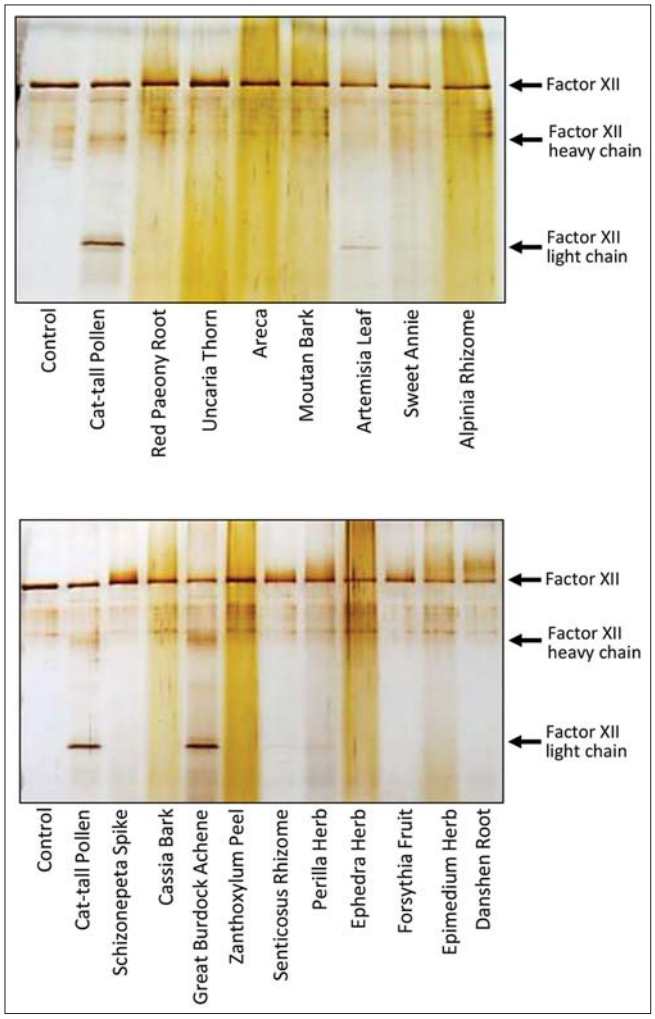

Figure 2: Analysis of factor XII activation, Chinese herbal extracts were incubated with factor XII for 5 h and then generation of activated factor XIIa was analyzed by SDS-PAGE followed by silver staining.

potential as hemostatic agents. However, to the best of our knowledge, these herbs have not been used as a treatment to preventing bleeding. The mechanisms through which these herbs shortened blood coagulation time remain also obscure. Nonetheless, these traditional herbs could be applied as a novel clinical approach to the control or prevention of bleeding.

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