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Research Paper

Aqueous extract from *Brownea grandiceps* flowers with effect on coagulation and fibrinolytic system

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ABSTRACT

Ethnopharmacological relevance: Brownea grandiceps flowers are used in Venezuelan folk medicine as anti-hemorrhagic in women with heavy menstrual blood loss (menorrhagia). However, prior to this study, there were no scientific investigations to support this fact, because the aqueous extract from *Brownea grandiceps* flowers had not been previously evaluated neither phytochemically nor biologically. The objective of this work was to evaluate *in vitro* the effects of aqueous extract from *Brownea grandiceps* flowers on the coagulation system and fibrinolysis.

Materials and methods: An infusion of *Brownea grandiceps* flowers (160 g) was performed; then, it was homogenized, centrifuged and lyophilized to obtain the aqueous extract, and this was called BGE. Subsequently, the extract was characterized on the one hand, phytochemically and on the other hand, biologically, employing prothrombin time (PT), partial thromboplastin time (PTT) and thrombin time (TT) to determine the effects on extrinsic, intrinsic and common coagulation pathways, respectively. In addition to that, the fibrinogenolytic and fibronectinase activity was evaluated by SDS-PAGE using Tris-Tricine system and analyzed by densitometric study utilizing ImageJ program. Also, by using specific chromogenic substrates for Factor Xa (FXa), thrombin, tissue plasminogen activator (t-PA), urokinase plasminogen activator (u-PA) and plasmin, it was assessed whether BGE exhibited some enzyme-like activity, and inhibitory activity of the afore mentioned enzymes. Fibrinolytic and antifibrinolytic activities were determined by a fibrin plate method. Data were analyzed by an nonparametric method.

Results: BGE presented tannins, saponins, glycosides, alkaloids, flavonoids, coumarins, and did not contain triterpenoids and steroids. Also, BGE at low concentrations (250–1250 μg/mL) reduced the PT, while higher concentrations (15000–25000 μg/mL) prolonged this time. However, BGE concentrations between 1250 and 25000 μg/mL prolonged the PTT. Prolongation of PT and PTT was observed at high concentrations and was due to FXa inhibitor found in BGE and this effect could be strengthened by degradation of fibrinogen and fibronectin, which were also produced by BGE. Moreover, BGE did not clot fibrinogen or human plasma, and neither did it cleave the chromogenic substrates specific to FXa nor thrombin. These results suggest the pro-coagulant components could be acting on some factor of the extrinsic pathway, since only PT was shortened. Furthermore, BGE did not hydrolyze the chromogenic substrate specific to plasmin, t-PA and u-PA nor did it produce fibrin degradation. However, all BGE concentrations tested inhibited the plasmin activity in a dose-dependent manner.

Conclusions: The outcomes of this study reveal the presence of fibrinogenolytic, fibronectinase and anti-FXa components in BGE, plus anti-plasmin compounds that could be acting as antifibrinolytic, thus delaying the fibrin degradation in pathophysiological processes, as it has been observed in women presenting with menorrhagia due to a high plasmin concentration. Where this anti-plasmin compound, along with pro-coagulant components also present in BGE, could be made responsible for reducing heavy menstrual bleeding in women, since a deficiency in one or more blood coagulation factors such as factor VII, V or X, is a potential cause of menorrhagia.

Abbreviations: BGE, *Brownea grandiceps* flowers aqueous extract; CI, confidence interval; FXa, Factor Xa; Fg, fibrinogen; FgC, fibrinogen control; FN, fibronectin; FNC, fibronectin control; g, grams; *g*, gravity; h, hour; L, liters; m, meters; min, minutes; MW, molecular weight; PPP, platelet poor plasma; PT, Prothrombin time; PTT, Partial thromboplastin time; s, seconds; SDS-PAGE, Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis; t-PA, tissue plasminogen activator; u-PA, urokinase plasminogen activator.

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1. Introduction

The hemostatic system requires a balance between fibrin formation (coagulation) and fibrin dissolution (fibrinolysis) to prevent blood loss by sealing sites of injury and ensure perfusion through tissues (Riddel et al., 2007). Thrombin is the enzyme that transforms fibrinogen into fibrin, while plasmin is the main enzyme capable of degrading both fibrinogen and fibrin under physiological conditions (Monroe et al., 2002). Any disruption of this equilibrium may bring about thrombosis or hemorrhage, that is, on the one hand, excessive local or systemic activation of coagulation may wind up in the development of thrombi or consumption coagulopathy. On the other hand, excessive local or systemic fibrinolytic activity may very well evolve into heavy bleeding or clotting episodes (Rasche, 2001). These coagulation and fibrinolysis disorders cause serious medical problems, which have led to isolate anti- and pro-coagulant compounds as fibrinolytic and antifibrinolytic components, resorting to various sources such as snakes, caterpillars, scorpions and plants to name but a few, and are currently being investigated aiming to control these problems (Reis et al., 2001; Swenson and Markland, 2005; Shivaprasad et al., 2009; Brazón et al., 2008; 2009; 2013; 2014).

In most developing countries, plant remedies are the most prevalent treatments, where recipes have been handed down from generation to generation and every culture has used decoctions or extracts of leaves, flowers, barks or roots to treat various medical problems (Agra et al., 2007). Several isolated plant components have a marked effect on the hemostasis. It is worth mentioning flavonoids with vasodilator effect (Victório et al., 2009), coumarins with antiaggregant platelet effects (Lee et al., 2003), phenolic compounds with inhibitory activity on platelets adhesion and secretion (Olas et al., 2005), proteases exhibiting coagulation factors-like activity or else showing fibrinolytic components-like activity (Shivaprasad et al., 2009) and inhibitors of thrombin activity (Francischetti et al., 1997).

Brownea genus belongs to the Fabaceae family, comprising evergreen small trees with capitate racemes axillary or terminal of red, yellow or white flowers which are found from the south of Mexico to Peru, including Antillean islands, Jamaica, Trinidad and Tobago (Stevermark and Huber, 1978). Species from this genus are used in the traditional medicine for treatment of hemorrhages (Otero et al., 2000; Márquez et al., 2005; Díaz and Ortega, 2006). In Colombia and Brazil, some plant species from this genus such as *Brownea ariza* Bentham and *Brownea rosademonte* are mostly employed in folk medicine as cicatrizant and to neutralize hemorrhages caused by snake venom (Otero et al., 2000). However, in Venezuela, flowers of *Brownea grandiceps* and other species of this genus (called mountain rose or rose-of-Venezuela) are used as decoctions in traditional medicine to reduce the bleeding in women with heavy menstrual blood loss (Díaz and Ortega, 2006). Nevertheless, the biological active components are yet to be identified.

Due to the scarce actual scientific information and our interest in the haemostatic activity compounds with great medical potential present in *Brownea grandiceps*, the very aim of this study was to evaluate *in vitro* the effects of aqueous extract from *Brownea grandiceps* flowers on the coagulation system and fibrinolysis in order to determine whether *Brownea grandiceps* flowers contain components that could aid in the reduction of heavy menstrual blood loss, because menorrhagia as a common clinical problem among reproductive age females, happens to have a severe impact on the impairment of their quality of life, not just by causing an iron deficiency anemia, but also altering their emotional, social and working life to a great degree (Shankar et al., 2008).

2. Materials and methods

2.1. Collection of vegetal material and preparation of aqueous extracts

Brownea grandiceps flowers were collected from 3-m-tall wild trees, fertilizers free, from the areas surrounding the Posada Mocundo, Carabobo State, Venezuela (10°14′14″N–68°15′44″W). The identity of this plant, collected in March 2014, was confirmed by Reina Gonto, and a voucher specimen of it (number 5699) was deposited in the herbarium of the Instituto Venezolano de Investigaciones Científicas (IVIC), Caracas, Venezuela.

An infusion using 116 g of *Brownea grandiceps* fresh flowers was prepared in 2.5 L of double distilled water at 55 °C for 2 h. Next, it was homogenized in a blender during 5 min. The homogenate was centrifuged at 27500*g* for 30 min at 4 °C. The supernatant was lyophilized, weighed (17 g were obtained) and stored at 4 °C until use. This was called *Brownea grandiceps* flowers aqueous extract (BGE).

2.2. Phytochemical analysis

The qualitative phytochemical analyses were done following the methods described by Barnett and Thorne (1921), Harborne (1973) and Cole (2003) for saponins, tannins, alkaloids and triterpenoids, Carvajal Rojas et al. (2009) for anthocyanins, Dubois et al. (1956) for total carbohydrates, Feigl and Anger (1966) for coumarins, and Kar (2003) for glycosides, all of them using the appropriated reagents and standards.

2.3. Plasma sampling

Blood samples were obtained from healthy volunteers and were drawn by venepuncture and mixed in a proportion 9:1 with 3.8% sodium citrate (Sigma-Aldrich, USA) as anticoagulant. The citrate blood was centrifuged at 1312*g* for 15 min at 4 °C. The supernatant corresponds to platelet poor plasma (PPP) and stored at −80 °C until use. This research followed the guidelines of the Declaration of Helsinki and Tokyo for humans and the procedure was approved by the bioethical committee of the IVIC with the bioethical allowance number DIR-1432/1527/2014.

2.4. Partial thromboplastin time (PTT)

PTT was determined using the reagents and methodology of DIAGNOSTICA STAGO Company (France). Briefly, 0.1 mL PPP, 0.1 mL Tris–NaCl buffer (0.05 M Tris–HCl/0.15 M NaCl at pH 7.4) and 0.1 mL platelet substitute (Ck-Prest, Diagnostica Stago) were mixed in a borosilicate tube (10 × 75 mm). Tubes were then incubated at 37 °C for 3 min immediately afterwards, then 0.1 mL 0.025 M $CaCl_2$ prewarmed to 37 °C was added and the clotting time was measured with a stopwatch turned on at the precise moment of the $CaCl_2$ addition. The effect of BGE on PTT was assessed by replacing the Tris–NaCl buffer volume with BGE at different final concentrations (25, 125, 250, 375, 500, 625, 1250, 2500, 5000, 7500, 10000, 15000, 20000 and 25000 μg/mL). Controls and experiments were carried out by triplicate for each concentration studied. The results were expressed in seconds. The controls used in this test were heparin (Liquemine®, 0.5 IU/mL, was purchased from Roche, Brazil) as anticoagulant and Tris–NaCl buffer (vehicle). The reference values for PTT when PPP was incubated with or without buffer are set between 28 and 36 s. Changes in PTT were considered significant when the difference between experimental and control was shortened (meaning a pro-coagulant activity) or lengthened (meaning an anticoagulant activity) more than 6 s (Langdell et al., 1953).