Per oral rat treatment with glyconjugate fractions of *Genipa americana* leaves protects thrombus formation

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The current study evaluated the effect of the arabinogalactan-glycoconjugate fractions (FI and FII) isolated from *Genipa americana* leaves given *per oral* in rat hemostasis protocols. Rats received daily treatment with FI or FII during 7 days and were evaluated for coagulation, platelet aggregation, venous thrombosis and bleeding tendency 1 h after the last treatment. FII prolonged in 5.5-fold the rat plasma coagulation time (activated partial thromboplastin time test). FI inhibited by 46% the platelet aggregation. Both FI and FII prevented thrombus formation by 33 and 28%, respectively. However, the bleeding time was not altered by any fractions, showing an advantage in relation to acetylsalicylic acid or warfarin that increased the bleeding time in 3.6 and 2.9-fold, respectively. *Per oral* treatment with the arabinogalactan-glyconjugate fractions FI and FII of *G. americana* leaves in rats prevents thrombus formation, being devoid of hemorrhagic risk. These results bring novel therapeutic possibilities for thromboembolic diseases. *Blood Coagul Fibrinolysis* 30:000–000 Copyright © 2020 Wolters Kluwer Health, Inc. All rights reserved.

Blood Coagulation and Fibrinolysis 2020, **30**:00–00

Keywords: anticoagulant, antiplatelet, antithrombotic, plant polysaccharide

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Received 28 March 2019 **Revised** 12 November 2019
Accepted 28 November 2019

Introduction

Cardiovascular diseases are the major world cause of death, corresponding to 31% of global deaths (75% in developing countries), being manifested primarily by heart attacks and strokes [1]. The main treatment of thromboembolic diseases includes anticoagulant (heparins, warfarin), antiplatelet (acetylsalicylic acid – ASA, clopidogrel) and fibrinolytic (streptokinase, alteplase) agents. Although effective, these drugs cause relevant disadvantages, such as severe bleeding, thrombocytopenia, allergy, osteoporosis, poor bioavailability, narrow therapeutic window, unpredictable pharmacodynamic and pharmacokinetic, and thrombotic events recurrence [2,3].

Among anticoagulants, heparin is of clinical importance, due to its efficacy in thromboembolic disturbances, although the restriction for the use by parenteral administration. Heparin is a glycosaminoglycan of animal source rich in sulfate and uronic acid [2,4]. Since polysaccharides or glycoconjugates isolated from higher plants present uronic acid in its structure, associated with the ability to interfere in cellular and vascular events, mostly devoid of toxicity [5], they are considered potential therapeutic alternative for the treatment of hemostasis disorders [6,7].

Genipa americana (Rubiaceae), popularly known as 'jenipapo' or 'jenipapeiro', is a medicinal plant widely distributed in Brazil, used to treat fever, diarrhea and syphilis in the form of macerate or decoction of its leaves [8]. Experimental studies have shown that the polysaccharide-rich extract of *G. americana* leaves present trypanocidal [9], antioxidant and anticonvulsant effects [10].

In addition, the glycoconjugates containing arabinogalactan and uronic acid isolated from *G. americana* leaves present in-vitro antiplatelet and anticoagulant activities, and in-vivo prevents thrombus formation in rats treated by endovenous route, being devoid of hemorrhagic risk [7].

The aim of this study was to evaluate the effect of the arabinogalactan-glycoconjugate fractions of *G. americana* leaves in coagulation, platelet aggregation and thrombus formation after *per oral* treatment in rats.

Methods

Extraction and fractioning of *Genipa americana* arabinogalactan-glycoconjugate

Leaves of *G. americana* (voucher no. 4683 – Herbarium Prisco Bezerra – Federal University of Ceara), collected at Custódio-Quixadá, Ceará, Brazil, was washed, dried at 40 °C and macerated. Five grams of dry powder were suspended in absolute methanol for depigmentation, followed by extraction with 0.1 mol/l NaOH and precipitation in ethanol, producing a polysaccharide extract of *G. americana* (PE-Ga). PE-Ga was dissolved in distilled water (2 : 1, *w*/*v*) and fractioned by ion exchange chromatography (dietilaminoetil celulose (DEAE-cellulose)), equilibrated and eluted with distilled water. The major fractions of the acidic arabinogalactan-glycoconjugate were eluted with NaCl 0.1 mol/l (FI) and 0.25 mol/l

DOI:10.1097/MBC.0000000000000880

(FII), pooled, dialyzed, lyophilized and quantified for total carbohydrate [11], uronic acid [12], protein [13] and polyphenolic compounds [14], presenting similar content, to the previously demonstrated [7].

Animals and treatment

Wistar rats (200–250 g) were maintained under 12/12 h light/dark cycle, at 25 °C, receiving food and water *ad libitum*. Protocols were performed in compliance with the Guide for the Care and Use of Laboratory Animals of the US Department of Health and Human Services (8th edition, 2011) and approved by the Animal Care and Use Committee of the Universidade Estadual do Ceará (no. 5748564/2015).

Rats received *per oral* daily treatment with FI or FII (1 mg/kg) during 7 days [15,16], warfarin sodium (0.01 mg/kg), ASA (100 mg/kg) or vehicle (distilled water). One hour later, animals received intramuscular anesthesia (5% ketamine – 90 mg/kg and xylazine – 10 mg/kg) and were submitted to the ex-vivo tests of coagulation and platelet aggregation and the in-vivo protocols of venous thrombosis and bleeding tendency.

Ex-vivo tests: coagulation and platelet aggregation

For the coagulation assay (activated partial thromboplastin time – aPTT), blood was collected from the animal orbital plexus 1 h after treatment at days 0 (zero) (D0), 3 (D3) and 7 (D7). Plasma was obtained by blood centrifugation (1008 × *g*, 15 min, 25 °C). The clotting time (s) was measured up to 300 s in coagulometer (CLOTimer DRAKE, São Paulo-SP, Brazil) and expressed as the ratio between treated or nontreated rats.

For the platelet aggregation, animal blood was collected at day 7 (D7). The platelet rich plasma was obtained by centrifugation (118 × *g*, 10 min, 25 °C) and warmed (37 °C) in aggregometer cuvettes (Qualiterm PA.04 Cesário Lange - SP, Brazil). Platelet aggregation was registered during 5 min in presence of the agonist ADP (3 μmol/l) and expressed as the maximal light transmittance (100% T) in relation to ADP [17].

In-vivo models: venous thrombosis and bleeding tendency

Venous thrombosis was induced by a method combining stasis and hypercoagulability. One hour after treatment, animals received intramuscular anesthesia before dissection and isolation of the abdominal inferior vena cava for the placement of loose sutures between right and left renal veins. Thromboplastin (5.0 mg/kg) was injected in single bolus into the isolated segment (0.7 cm) before being clamped off. After 20 min of stasis, thrombus was removed, dried (1 h, 60 °C) and weighted [18].

Bleeding was induced 1 h after treatment by transection of the tail extremity 3 mm from the tip. Tails were blotted with filter paper every 30 s to quantify bleeding cessation time (s) [19].

Statistical analysis

Results were expressed as mean ± SEM ($n = 5$) and analyzed by one-way analysis of variance and Bonferroni post test (Prism 5.0; GraphPad Software Inc., San Diego, California, USA). Differences were considered for P less than 0.05.

Results and discussion

The *per oral* treatment of rats with aqueous solution of FI and FII, prevented thrombus formation, associated with FI antiplatelet and FII anticoagulant effects. These findings are in line with the literature showing that glycoconjugates and polysaccharides of higher plants possess anticoagulant and antithrombotic effects [20].

The plasma coagulation time (aPTT) of animals treated with FII, after the 7-day treatment (D7 in Table 1), was prolonged in 5.5-fold (233.3 ± 43.1 s) compared with control (41.8 ± 1.8 s) (Table 1), similarly to the in-vitro effect demonstrated in human plasma [7]. However, the coagulation time of animals treated with FI, ASA or warfarin showed no activity (Table 1), in line with the literature [2,7,21].

In respect to the platelet aggregation, fraction FI, but not fraction FII, inhibited this activity by 46% (47.1 ± 2.7% vs. control: 88.0 ± 8.0%) in the last day of treatment (D7), as demonstrated in-vitro [7]. The reference drug ASA inhibited platelet aggregation by 94% (4.4 ± 2.1%) (Fig. 1). These effects are relevant, since most of the traditional anticoagulants are associated with adverse effects, such as bleeding and bruises, especially in association with antiplatelet agents [22].

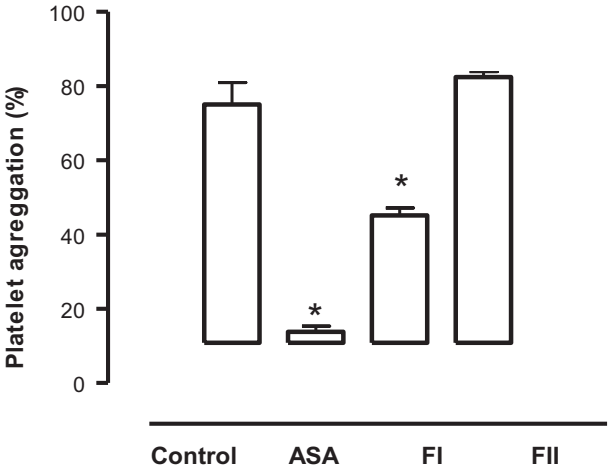
Thrombus formation was prevented either by FI (33% – 3.0 ± 0.1 mg) and FII (28% – 3.2 ± 0.3 mg) compared with control (4.5 ± 0.1 mg). The animal treatment with ASA or warfarin prevented thrombus formation by 31 (3.1 ± 0.3 mg) and 44% (2.5 ± 0 mg), respectively (Fig. 2). In respect to the bleeding time, it was not altered by any fraction (FI: 843.6 ± 51.5 s; FII: 1272 ± 182.9 s; control: 754.5 ± 94.1 s) (Fig. 2), showing an advantage in relation to ASA (2744.0 ± 264.5 s) or warfarin (2219.0 ± 537.6 s), that increased the bleeding time in 3.6 and 2.9-fold,

Table 1 The glyconjugate FII of *Genipa americana* leaves prolongs the coagulation time (activated partial thromboplastin time)

Treatment	mg/kg	D0 (s)	D3 (s)	D7 (s)	[a]T1/T0
Control	–	41.4 ± 1.4	32.2 ± 2.0	41.8 ± 1.8	–
FI	1.0	29.1 ± 1.8	32.8 ± 2.0	23.9 ± 0.9	–
FII	1.0	42.5 ± 4.3	42.5 ± 2.4	233.3 ± 43.1*	5.5
Warfarin	0.01	24.0 ± 1.1	22.0 ± 0.5	23.0 ± 0.7	–
ASA	100	39.2 ± 3.2	39.3 ± 6.1	33.0 ± 4.0	–

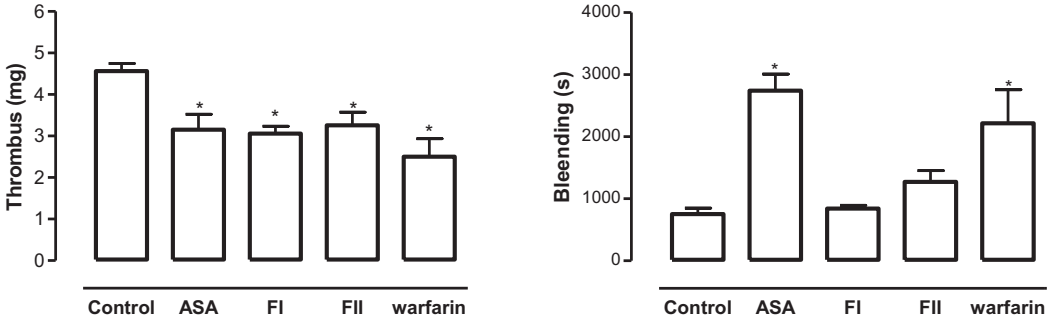
ASA, acetylsalicylic acid. [a]T1/T0: coagulation time ratio of treated (T1) or nontreated (T0) rats. Mean ± SEM ($n = 5$, duplicate). analysis of variance and Bonferroni post test. * $P < 0.05$ vs. control.

Fig. 1



The glyconjugate FI of *Genipa americana* leaves inhibits plasma platelet aggregation induced by ADP in rats. Mean ± SEM ($n = 5$, duplicate). analysis of variance and Bonferroni post test. $*P < 0.05$ vs. control.

Fig. 2



Glyconjugate fractions of *Genipa americana* leaves reduce thrombus formation and bleeding time. Mean ± SEM ($n = 5$). ANOVA and Bonferroni posttest. $*P < 0.05$ vs. control.

respectively. Unlike heparin, warfarin is an oral anticoagulant widely used in the clinical practice to reduce the risk of thromboembolic events [2]. However, despite of these benefits warfarin interacts with food and other drugs, in addition to the risk of thrombotic events recurrence and the need of coagulation monitoring and dose adjustment [2]. In this context, the arabinogalactan-glycoconjugate fractions arise as an alternative plant source capable to protect thrombotic events without hemorrhagic risk.

Our data all together, are in line with other studies using plant polysaccharides or glycoconjugates *per oral*: the plant polysaccharides of *Orbignya phalerata* mesocarp (100 mg/kg) [23] and of *Rubus* spp. (blackberry) seeds (120 mg/kg) [16] prolonged the coagulation time in the aPTT test and decreased thrombus formation, although at higher doses; the red *Ginseng radix* (500 mg/kg) exhibited antiplatelet and antithrombotic effects without alteration in the coagulation (aPTT) [24]. Despite of these, several unpublished reports indicate that certain medicinal plants present beneficial effects both for thrombosis treatment or prevention [25,26].

Moreover, the polysaccharides rich extract of *G. americana* leaves given *per oral* during 14 days to rats at 300 mg/kg did not show signs of toxicity (hematological evaluation and plasma biochemical parameters) or mortality (*unpublished data*), according to the literature referred to polysaccharides and glycoconjugates of higher plants [5]. These results bring novel therapeutic possibility for thromboembolic diseases of long-term effect.

Conclusion

Per oral treatment by 7 days with the arabinogalactan-glyconjugate fractions FI and FII of *G. americana* leaves in rats prevents thrombus formation, associated with FI

antiplatelet or FII anticoagulant effects, being devoid of hemorrhagic risk.

Acknowledgements

None.

Conflicts of interest

There are no conflicts of interest.

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