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Antiplatelet activity of *Croton celditifolius*

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Croton celtidifolius *Baill is a tree found in the Atlantic Forest South of Brazil, mainly in Santa Catarina. The bark and leaf infusions of this medicinal plant have been popularly used for the treatment of inflammatory diseases. The anti-aggregant activity of* C. celtidifolius *crude extract (CE) and the column chromatography (CC) isolated compounds flavonoids, catechin and gallocatechin were evaluated in human blood platelets. The platelet-rich plasma (PRP) was incubated with different concentrations of flavonóides (50 - 200 µg/mL) to be tested before platelet aggregation was induced by the agonists adenosine 5´diphosphate (ADP) and collagen. At 200 µg/mL the CE, catechin and gallocatechin markedly inhibited platelet aggregation with the aggregant agents. Using ATP production as an index of platelet secretory capacity, we observed a decreased production of ATP in platelets treated with flavonoids when stimulated by collagen. On the other hand, the flavonoids did not promote inhibitory effect on prothrombin time (PT), thromboplastin time (APTT) and thrombin time (TT). In conclusion, these observations suggest that* C. celtidifolius *is likely to exert an inhibitory action on platelets* in vitro *by suppressing secretion and platelet aggregation.*

Uniterms
- *Croton celtidifolius*
- Platelet aggregation
- Catechins
- Gallocatechins
- Coagulation

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INTRODUCTION

Activation of platelets plays a key role in haemostasis and circulation. Platelet aggregation is known to be a result of complex signal transduction cascade reactions brought about by stimulants. After activation, platelets provide a catalytic membrane surface for thrombin generation, which accelerates the formation of fibrin, necessary to stabilize thrombin. Key to these events is the presence, on the platelet surface, of receptors that can respond rapidly to soluble agonists, including collagen, thrombin and adenosine 5'diphosphate (Bluestein, 2004; Massberg *et al.*, 2005). Platelet dysfunction contributes in the development and progression of many cardiovascular diseases like arterial hypertension, atherosclerosis and thrombosis. Indeed, it has been reported that patients with hypertension or coronary heart disease tend to have increased platelet reactivity (Hernández Hernández *et al.*, 1997; Maeda, Bydlowski and

Lopes, 2005). Other studies in hypertensive patients showed that platelets were more sensitive to thrombin (Andrioli *et al.*, 1996; Görlach *et al.*, 2005) and exhibited an elevation in their intracellular free calcium (Erne *et al.*, 1984; Bruschi *et al.*, 1985). This later may potentiate the platelet activity and increase the risk of thrombosis. Therefore, many investigations were carried out in order to prevent this abnormal hyperactivity of platelets reported in cardiovascular disorders by using different therapies, including use of medicinal plants. In fact, it has been shown that some plants, such as garlic (Rahman and Billington, 2000) and tomato (Dutta-Roy, Crosbie and Gordon, 2001), may be beneficial in protecting against cardiovascular diseases as a result of the platelet aggregation inhibition. There are many nutritive and non-nutritive compounds present in the diet that may affect platelet function in various ways. Therefore, the compounds that inhibit platelet function are of great interest. In fruits and vegetables, there are many bioactive compounds, such as polyphenolic, vitamins and carotenoids (Olas *et al.*, 2005; Rechner and Kroner, 2005).

Flavonoids represent an interesting group of polyphenols natural compounds found ubiquitously in the plant-derivated constituents on the human diet and many studies indicates that certain flavonoids have been shown to modify eicosanoids synthesis (antiprostanoid and antiinflammatory responses), protect low-density lipoprotein from oxidation, preventing atherosclerotic plaque formation, and prevent platelet aggregation (Cody, 1988; Coni *et al.*, 2000; Olas *et al.*, 2005).

Croton celtidifolius Baill, commonly known as "Sangue-de-adave" is a tree found in the South Atlantic Forest of Brazil, mainly in Santa Catarina State. The bark and leaf infusions of this medicinal plant have been popularly used for the treatment of ulcer, rheumatism and other inflammatory diseases. Several compounds with pharmacological activity have been previously isolated from other *Croton spp*. It has been reported that latex of *C. lechleri* contains the alkaloid taspine, which may be the active principle responsible for the anti-inflammatory (Vaisberg *et al.*, 1989) and anti-tumourogenic activity (Chen, Cai and Phillipson, 1994) of this plant. However, studies about chemical constituents, as well as pharmacological effects of *C. celtidifolius*, are still limited. Recently, Nardi *et al.* (2003) showed that CE of *C. celtidifolius* and two isolated compounds, catechin and gallocatechin, have significant anti-inflammatory and antioxidant activity. Nevertheless, there is no information about the eventual influence of this medicinal plant on haemostasis. The haemostatic system depends on the interaction between wall of sanguineous vase and platelets circulating factors. The platelets reactions as aggregation, peroxidation and secretion are considered as a primary replay. The proteins that participate in the coagulation process are enclosed in the reply and will play in the haemostatic regulation. Thus, the purpose of the present study was to investigate the effects of *C. celtidifolius* CE and the isolated compounds, catechin and gallocatechin, in platelet activity and in coagulation.

MATERIALS AND METHODS

Plant material

C. celtidifolius Baill was collected in March, 2000, from Orleans city, SC, Brazil. The voucher specimen was identified with the collection documenting number 31272 at the Bottany Department, UFSC, by Prof. Dr. Daniel de Barcelos Falkenberg and deposited in the author's laboratory.

Extraction and isolation

The extraction and isolation of the catechin and gallocatechin were performed as described previously (Nardi *et al.*, 2003). Air-dried bark (154 g) was chopped into small pieces and extracted three times with 250mL of 80% aqueous EtOH at room temperature. The combined extracts were filtered and the solvent was evaporated in a vacuum to give 42,9 g of the crude hydroalcoolic extract (EB). The crude hydroalcoolic extract was successively partitioned with ether, ethyl acetate and n-butanol. The ethyl acetate fraction was chromatographed over a column of silica gel water (20%) inactivated and eluted with hexane/acetate gradient. The first fractions containing catechin and gallocatechin were further purified by flash chromatography using hexane 40%/EtOAc 59%/AcOH 1% as isocratic eluent.

Reagents

Adenosine diphosphate (ADP), collagen and luciferin/luciferase were purchased from Chronolog Corpration, USA. Thromboplastin (PT test), elagic phospholipide (APTT test) and thrombin reagents were purchased from Organon Technika, Durham, NC, USA.

Subjects

The study was approved by the Ethics Committee of the University, UFSC, Florianópolis, SC, Brazil, and included 15 healthy volunteers who gave written informed consent to participate, process 150-03/2002.

Isolation of platelets

Platelet rich plasma (PRP) was prepared by centrifugation of citrated blood at 22 °C for 6 min. at 400g. Platelets were adjusted to 3.0×10^8cell/mL with sterile saline.

Measurement of platelet aggregation (PA)

PA was determined by the turbidimetric method (Born, Cross, 1963) using a Chronolog Aggregometer. Aliquots of 400 µL of a platelet suspension were transferred into a small cuvette and stirred at a constant speed of 180 g at 37 °C. The platelets were pre-incubated with different fractions of CE, catechin, gallocatechin or vehicle (1mM-NaOH) for 5 min at 37 °C, before the addition of aggregant agents. The extent of aggregation (%) was recorded continually for 5 min after addition of the agonists.

Measurement of platelet secretion induced by collagen

Platelet secretion (%) was evaluated using the system luciferin/luciferase according Pascual and Romay (1992). The platelets were pre-incubated with different concentrations of compounds or with the vehicle (1 mM-NaOH) for 5 min at 37 °C, before the addition of 4 µg/mL of collagen.

Measurement of blood coagulation

Human blood was obtained from 15 individual healthy donors without history of bleeding or thrombosis. Nine parts of blood collected by venipuncture were drawn into one part of 3,8% trisodium citrate. Blood was centrifuged for 10 min at 900g, and the plasma stored at - 20 °C until were tested. Activated prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) were evaluated according to Triplett *et al.* (1978). The plasma was pre-incubated with different concentrations of inhibitors or vehicle (1mM-NaOH) for 2 min at 37 °C and coagulation time was measured using a Net Lab Digital Coagulation System.

Statistical Analysis

Data were expressed as mean ± SD. The Tukey-Krammer test was employed to determine differences between the groups. Differences were considered significant when the probability was $p < 0,05$. The statistical program Instat-2 was utilized.

RESULTS AND DISCUSSION

Many different herbal components in folk medicine are antiplatelet agents that inhibit platelet activation and have been used in the management of cardiovascular disorders. The objective of the present study was to determine whether *C. celtidifolius* would affect platelet function and blood coagulation. A control aggregation test induced by collagen or ADP was systematically carried out in the beginning of each experiment in order to verify the good physiological status of platelets. Human blood platelets were incubated in the presence of CE, catechin and gallocatechin for 5 min at 37 °C. Using 200 µg/mL of CE, or of its isolated compounds, was sufficient to exhibit a significant inhibition by two types of agonists. The effect of CE, catechin and gallocatechin on platelet aggregation induced by collagen is shown in Table I. Treatment with 200 µg/mL of CE, catechin and gallocatechin were sufficient to induce a significant inhibition of 75%, 60% and 65% respectively. Subsequently, we examined the effect of these flavonoids in the presence of ADP. The results obtained with these compounds were sufficient to exhibit a potent inhibitory effect on platelet aggregation induced by this agonist (Table II). The results obtained from the platelet aggregation study demonstrated that *C. Celtifoulius* promoted changes in platelet metabolism with inhibitory effect of the primary hemostasis. We observed, in PRP, that the shape change and the primary platelet aggregation phase induced by the two agonists was not affected in the presence of these flavonoids, on the other hand, the second platelet aggregation phase was significantly inhibited.

TABLE I - Effects of *Croton celtidifolius* Baill, crude extract (CE), catechin and gallocatechin on platelet aggregation induced by 4µg/mL of collagen.

Treatment		Collagen (%)
Control (1mM-NaOH)		83.0 ± 1.8
CE	50 µg/mL	78.2 ± 3.9
	100 µg/mL	60.0 ± 3.0*
	200 µg/mL	20.4 ± 1.8**
Catechin	50 µg/mL	68.2 ± 3.8
	100 µg/mL	50.0 ± 5.0*
	200 µg/mL	32.4 ± 4.0**
Gallocatechin	50 µg/mL	68.0 ± 7.0
	100 µg/mL	39.7 ± 7.0**
	200 µg/mL	29.0 ± 6.0**

Statistical: Tukey Kramer test n=15, $p < 0.05$*; $p < 0.001$**
Platelets were pre-incubated with each compound for 5min. at 37 ^{0}C before stimulation. Each value represents the mean ± SD of 15 independent experiments.

In the current study, we have found that the inhibitory effect were, in all cases, dose dependent at concentrations between 50 - 200 µg/mL. Platelet aggregation is known to be a result of complex signal transduction cascade reactions brought about by stimulants. Blood platelets contain three types of secretory organelles: lysosomes, α-granules and dense granules. In these secretory organelles, there are different biologically relevant molecules that are released in response to

TABLE II - Effects of *Croton celtidifolius* Baill, crude extract (CE), catechin and gallocatechin on platelet aggregation induced by 6 µM of ADP.

Treatment		ADP(%)
Control (1mM-NaOH)		71.0 ± 3.4
CE	50 µg/mL	68.3 ± 4.8
	100 µg/mL	53,8 ± 2.7*
	200 µg/mL	17.6 ± 2.3**
Catechin	50 µg/mL	70.0 ± 3.0
	100 µg/mL	60.4 ± 2.0*
	200 µg/mL	40.0 ± 1.4**
Gallocatechin	50 µg/mL	61.0 ± 3.1*
	100 µg/mL	24.0 ± 3.3**
	200 µg/mL	15.5 ± 1.4**

Statistical: Tukey Kramer test n=15, $p < 0.05$*; $p < 0.001$** Platelets were pre-incubated with each compound for 5 min at 37 ^{0}C before stimulation. Each value represents the mean ± SD of 15 independent experiments.

platelet agonists, like thrombin or collagen. The platelet secretory process plays a key role in thrombosis. Moreover, platelets play a very important role in the pathogenesis of cardiovascular diseases and at the site of injury or in atherosclerosis vessel wall stability of atherosclerotic plaques (Varughese, Lip, 2005; Vorchheimer, Becker, 2006). Using ATP production as an index of platelet secretion capacity, we observed a decreased platelet ATP production. A significant effect was observed after cells were incubated with the flavonoids at a concentration of 200 µg/mL (Table III). The second platelet aggregation phase, which is dependent on platelet secretion, let us to conclude that the reaction release inhibition could be, in this study, the mechanism involved in the antiaggregatory effects of these compounds. Our results are in agreement with many studies that have shown that flavonoids significantly inhibited platelet adhesion, aggregation and secretion (Polette *et al.*, 1996; Mardla, Kobzar, Samel, 2004; Guglielmone *et al.*, 2005; Vitseva *et al.*, 2005). Polette *et al.* (1996) reported that catechins inhibited N-3 fatty acid-induced aggregation in washed platelets. Furthermore, the most frequently studied flavonoid, quercetin, has been shown to have biological properties consistent with a sparing effect on the cardiovascular system. Quercetin and other flavonoids modify eicosanoid biosynthesis, prevent platelet aggregation and promote relaxation of cardiovascular smooth muscle (Cody, 1988; Mardla, Kobzar, Samel, 2004). The results obtained in the present study demonstrate that both, catechin and gallocatechin, were effective in preventing platelet aggregation in the presence of two agonists.

TABLE III - Effects of *Croton celtidifolius* Baill, crude extract (CE), catechin and gallocatechin on platelet secretion stimulated by 4 µg/mL collagen.

Treatment		Secretion (%)
Control (1mM-NaOH)		100 ± 0.0
CE	50 µg/mL	73.3 ± 4.0*
	200 µg/mL	43.3 ± 4.1**
Catechin	50 µg/mL	83.0 ± 2.6*
	200 µg/mL	42.0 ± 2.0**
Gallocatechin	50 µg/mL	72.0 ± 1.5*
	200 µg/mL	36.3 ± 6.6**

Statistical: Tukey Kramer test n=5, $p < 0.05$*; $p < 0.001$** Platelets were pre-incubated with each compound for 5 min at 37 ^{0}C before stimulation. Each value represents the mean ± SD of 5 independent experiments.

Finally, to evaluate the effects of flavonoids on the enzymes of blood coagulation, screening tests were carried out in relation to activated PT, activated partial APTT and TT. High concentration of flavonoids (200 µg/mL) did not increase the clotting time of the three parameters evaluated under the experimental conditions (Table IV). It is worth noting that the pre-incubation of plasma at a maximum

TABLE IV - Effects of *Croton celtidifolius* Baill, crude extract (CE), catechin and gallocatechin (200µg/mL) on prothrombin time (PT), partial thromboplastin time (APTT) and thrombin time (TT). The plasma was incubated with each compound for 5 min. at 37 ^{0}C. Each value represents the mean ± SD of 6 independent experiments.

Test/ Treatment	Time (s)
PT/	
Control	12.4 ± 0.5
CE	11.8 ± 0.5
Catechin	11.4 ± 0.6
Gallocatechin	12.1 ± 0.3
APTT/	
Control	40.0 ± 1.8
CE	40.3 ± 2.0
Catechin	41.2 ± 1.0
Gallocatechin	41.6 ± 1.4
TT/	
Control	15.5 ± 0.3
CE	14.4 ± 0.2
Catechin	14.0 ± 0.2
Gallocatechin	14.8 ± 0.3

Statistical: Tukey Kramer test n= 6 $p > 0.05$

dosage (200 µg/mL) did not promote inhibitory action on fibrinogen as observed on TT. In addition, neither had an inhibitory effect upon the activity of the other plasma proteins as measured by PT and APTT.

CONCLUSION

The obtained data demonstrate that the activity of *C. celtidifolius* crude hidroalcoolic extract and its isolated compounds, catechin and gallocatechin, are limited to primary haemostasis in human blood. However, future studies are necessary to elucidate the biochemical platel*et al.*terations which are associated with the effects of these compounds to promote inhibition of platelet secretion and aggregation

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RESUMO

Atividade antiplaquetária do *Croton celtidifolius*

Croton celtidifolius *Baill é uma árvore encontrada na Mata Atlântica, no sul do Brasil, principalmente no estado de Santa Catarina. A infusão da casca e folhas dessa planta medicinal é utilizada na medicina popular para o tratamento de doenças inflamatórias. A atividade antiagregante do extrato bruto de* C. celtidifolius *(CE) e de seus flavonóides isolados por coluna cromatográfica (CC), catequina e galocatequina, foi avaliada em plaquetas humanas. O plasma rico em plaquetas (PRP) foi incubado com diferentes concentrações dos flavonóides testados (50 - 200 µg/mL) e posteriormente a agregação foi induzida pelos agonistas adenosina 5'difosfato (ADP) e colágeno. Na concentração de 200 µg/mL o CE, a catequina e a galocatequina inibiram a agregação plaquetária induzida pelos agonistas. A produção de ATP foi utilizada como um índice de capacidade de secreção plaquetária e observamos uma diminuição na produção de ATP nas plaquetas tratadas com os flavonóides e estimuladas com o colágeno. Por outro lado, os flavonóides não promoveram um efeito inibitório no tempo de protrombina (PT), tempo de tromboplastina parcial ativada (APTT) e tempo de trombina (TT). Essas observações sugerem que o* C. celtidifolius *exerce, in vitro, uma ação inibitória nas plaquetas através da inibição da secreção e agregação plaquetária.*

UNITERMOS: Croton celtidifolius. Agregação plaquetária. Catequinas. Galocatequinas. Coagulação.

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