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Original Article

Buddleja globosa (matico) prevents collagen-induced platelet activation by decreasing phospholipase C-gamma 2 and protein kinase C phosphorylation signaling

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ABSTRACT

Platelets play a key role in thrombosis and cardiovascular diseases. Medicinal plants could be one of the most important factors that influence risks for platelet activation. *Buddleja globosa* (known as "matico") is a medicinal plant with many biological activities. The high content of polyphenols suggest that matico could have antiplatelet activity. The present study was aimed at evaluating mechanisms of antiplatelet action of an extract of matico. We demonstrated that matico extract at low concentrations and in a concentration dependent manner (0.05−1 mg/mL) was a potent inhibitor of platelet aggregation in response to collagen, convulsion and ADP (IC_{50} values was 61 μg/mL, 72 μg/mL and 290 μg/mL, respectively). In this sense matico extract exerted the greatest antiaggregant activity induced by collagen. Similarly, matico showed a decrease in % of positive platelet for P-selectina (vehicle, 0.01, 0.05, 0.1, 0.5 and 1 mg/mL were 32 ± 2%, 29 ± 2 ($p < 0.05$), 19 ± 1 ($p < 0.01$), 15 ± 2 ($p < 0.01$), 10 ± 1% ($p < 0.01$) and 7 ± 2% ($p < 0.01$), respectively) and PAC-1 binding (vehicle, 0.01, 0.05, 0.1, 0.5 and 1 mg/mL were 59 ± 1, 58 ± 3 (n.s), 55 ± 2 ($p < 0.05$), 50 ± 2 ($p < 0.01$), 38 ± 1 ($p < 0.01$), 36 ± 2 ($p < 0.01$). The cellular mechanism for the antiplatelet activity of matico might be mediated by the inhibition of phospholipase C-gamma 2 and protein kinase C phosphorylation. This beneficial property of matico may be of importance in thrombosis, in which platelet activation and aggregation are important determinants of thrombus initiation and development, and may contribute to the beneficial effects of matico intake in the prevention of cardiovascular diseases.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death throughout the world and in recent years has increased in frequency.[1] Platelets play an important role in thrombosis and CVD; where a state of hyper-aggregation is associated with the presence of cardiovascular risk factors.[2,3]

Patients with CVD using antiplatelet drugs present adverse effects,[4] for this reason the search for new strategies to modulate platelet activity are needed. Different extracts have been shown to be relevant in the prevention of CVD, probably due to their phytoconstituents.[5,6]

Buddleja globosa, known as "matico", is a medicinal plant that mainly grows in the central zone of Chile, but also in Bolivia, Peru and Argentina. Matico is a plant often used in Mapuche culture applied for the treatment of wounds, intestinal and liver problems.[7] Matico has bioactive compounds such as flavonoids (luteolin, luteolin 7-glucoside, linarina and caffeic acid derivatives), phenylethanoids, sterols, phenolic fatty acid ester and terpenes.[8–12]

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Matico extract may be used topically or also as an infusion.[11,13] It has been reported that matico has effects on healing and wound closure modulating inflammation and oxidative stress.[12] In this regard, a study in which mice consumed an extract of matico for 12 days showed that intake did not alter the blood count and biochemical parameters of the animals under study.[14] In mice, the topical application of matico favors wound healing, correlating with decreased histological expression of ciclooxigenase (COX)-2 enzyme.[14] In addition, antinociceptive effects of matico have been attributed to β-sitosterol, α and β-amyrins, luteolin 7-glucoside and verbascoside.[11,13]

The use of matico extract for the treatment of gastric ulcers and other gastric disorders has been suggested.[15] Furthermore, the protective effects in hepatocyte cultures exposed to cytotoxic compounds have been described. These effects are primarily mediated by flavonoids from matico.[16]

Studies evaluating the biological effects of matico are guided by empirical knowledge; however, no anti-thrombotic effects have been documented. Matico has a high content of compounds with anti-platelet effects, such as luteonin and verbascoside. This fact suggests that it could have effects on hemostasis. In this sense, other species of the genus Buddleja, such as *Buddleja crispa*[17] and *Buddleja thyrsoides*[18] have shown antiplatelet action. The present study aimed to determine the antiplatelet effects of *B. globosa* and evaluate possible mechanisms of action.

2. Materials and methods

2.1. Reagents

Adenosine 5′- diphosphate (ADP), collagen and prostaglandin E1 (PGE-1) were obtained from Sigma-Aldrich (St. Louis, Missouri/MO, U.S.A). Convulxin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Sodium chloride (p.a.) was obtained from Arquimed (Santiago, Chile). Antiphospho (S660)-PKC-β2 and anti-phospho (Tyr753)-PLC-γ2 antibodies were obtained from Santa Cruz (Biotechnology, CA, USA). Anti γ-tubulin monoclonal antibody (4D11) was obtained from Thermo Scientific (Thermo Scientific, Pierce, Rockford, IL, USA). Antibodies (anti-CD62P-PE, anti-CD61-FITC, anti-GPIIb/IIIa-FITC PAC-1 and anti-CD61-PE) were obtained from BD Pharmingen (BD Biosciences, San Diego, CA, USA).

2.2. Matico recollection and authentication

B. globbosa leaves were collected from Linares city, Maule region, Chile, in January (summer) in 2016 and identified by an agricultural engineer. A voucher specimen (PRL-V03) was stored in our laboratory.

2.3. Preparation of extract from matico

The air-dried leaves of matico (50 g) were ground and successively extracted at room temperature with ethanol and after concentration under reduced pressure 1.4 g of extract was obtained (yield 2.8%). Then the extract was lyophilized and stored at −70 °C until use. Matico extract was dissolved in DMSO. The final concentration of DMSO used required as diluent was 0.2% (v/v), which does not affect platelet function.

2.4. Human platelet

The samples were taken from young healthy volunteers in 3.2% sodium. Platelet-rich plasma (PRP) was obtained by centrifugation at 240 g (DCS-16 Centrifugal Presvac RV) for 10 min and adjusted to 200×10^9 platelets/L with platelet-poor plasma (PPP). Samples for washing platelet were collected with ACD-A containing 50 ng/mL of PGE-1 (sample/anticoagulant ratio 9:1). Platelet was pellet with 50 ng/mL PGE-1 by centrifugation at 750 g for 5 min, resuspended in solution ($CaCl_2$, $MgCl_2$ 1, 0.1% dextrose, pH 7.4) and adjusted to 200×10^9 platelets/L. Hematologic counter (Bayer Advia 60 Hematology System, Tarrytown, NY, USA) was used for platelet counts. Protocol was approved by the ethics committee of the Universidad de Talca.

2.5. Flow cytometry study

Expression of P-selectin and glycoprotein (GP)IIb/IIIa on platelet surface were analyzed by double-label flow cytometry by method previously described by Fuentes et al.[19] 480 μL of PRP (200×10^9 platelets/L) were incubated with concentrations of matico ranged from 0.01 to 1 mg/mL for 3 min. Then, each sample was treated for 6 min at 37 °C with ADP 8 μmol/L. An aliquot of 50 μL was taken and mixed with saturated concentrations of anti-CD62P-PE and anti-CD61-FITC for P-selectin expression, or incubated with anti-GPIIb/IIIa antibody PAC-1 and anti-CD61-PE for GPIIb/IIIa activation. The sample was incubated for 25 min in the dark. Platelet populations were gated on cell size using CD61 positivity and forward scatter (FSC) vs. side scatter (SSC), and analyzed over 5000 events in Accuri C6 flow cytometer (BD, Biosciences, USA). The results represent the mean and standard deviation of three independent determinations.

2.6. Platelet aggregation study

Effects of matico on platelet aggregation were evaluated using a lumi-aggregometer (Chrono-Log, Havertown, PA, USA). Matico (final concentration 0.01−1 mg/mL) was added to 480 μL of PRP (200×10^9 platelets/L) and incubated 3 min at 37 °C in constant agitation before addition of agonist collagen (1.5 μg/mL), convulxin (20 ng/mL) or ADP (8 μmol/L). The effect of matico on platelet aggregation was followed for 6 min and compared with saline control. Maximal amplitude (%) was measured by AGGRO/LINK software (Chrono-Log, Havertown, PA, USA).

The results represent the mean and standard deviation of three independent determinations.

2.7. Western blotting study

The samples were treated as in aggregation assay and these were activates with collagen or ADP (1.5 μg/mL or 8 μmol/L, respectively). When the reaction was stopped the samples were centrifuged and lised with lysis bufferr (50 mM Tris-HCl, 50 mM NaCl, 1 mM MgCl2, 1 mM EDTA, 0.1% Triton® X-100 at pH 7.4). 20 μg of protein from each samples were subjected to SDS/PAGE, immunoblotted and probed with antiphospho (Tyr753)- PLC-γ2, anti-phospho (S660)-PKC-β2 or an anti-γ-tubulin antibody overnight at 4 °C. Membranes were incubated with a goat anti-rabbit secondary antibody linked to horseradish peroxidase and phosphoproteins were detected using an enhanced chemiluminescence method. Analysis of individual protein was quantified by densitometry with ImageJ software. The results represent the mean and standard deviation of three independent determinations.

2.8. Statistical analysis

Data were expressed as mean ± standard deviation (SD) and analyzed using Prism 6.0 software (GraphPad Inc., San Diego CA, USA). All measurements were performed from six separate platelet donors. Results were expressed as percentage of vehicle (as 100%). Fifty-percent inhibitory concentration (IC_{50}) of matico extract against agonist-induced platelet aggregation was calculated from the dose-response curves. Differences between groups were

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