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

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A B S T R A C T

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 “mático”) 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 (IC50 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.

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1. Introduction

Cardiovascular disease (CVD) is the leading cause of death throughout the world and in recent years has increased in frequency. Platelets play an important role in thrombosis and CVD; where a state of hyper-aggregation is associated with the presence of cardiovascular risk factors. Patients with CVD using antiplatelet drugs present adverse effects, 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.

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. Matico has bioactive compounds such as flavonoids (uteolin, luteolin 7-glucoside, linarina and caffeic acid derivatives), phenylethanoids, sterols, phenolic fatty acid ester and terpenes.6–12

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Matício extract may be used topically or also as an infusion.\textsuperscript{11,13} It has been reported that matício has effects on healing and wound closure modulating inflammation and oxidative stress.\textsuperscript{12} In this regard, a study in which mice consumed an extract of matício for 12 days showed that intake did not alter the blood count and biochemical parameters of the animals under study.\textsuperscript{1,3} In mice, the topical application of matício favors healing wound, correlating with decreased histological expression of ciclooxigenase (COX)-2 enzyme.\textsuperscript{14} In addition, antinociceptive effects of matício have been attributed to β-sitosterol, α and β-amyrins, luteolin 7-glucoside and verbascoside.\textsuperscript{11,13}

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

Studies evaluating the biological effects of matício 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 luteolin 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\textsuperscript{17} and Buddleja thyrsoides\textsuperscript{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-γ 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-C6D2P-PE, anti-C6D1-FITC, anti-GPIIb/IIIa-FITC PAC-1 and anti-C6D1-PE) were obtained from BD Pharmingen (BD Biosciences, San Diego, CA, USA).

2.2. Matício recollection and authentication

B. globosa 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 matício

The air-dried leaves of matício (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. Matício 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 $200 \times 10^9$ 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 pelleted 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 $200 \times 10^9$ 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.\textsuperscript{19} 480 µL of PRP ($200 \times 10^9$ platelets/L) were incubated with concentrations of matício ranging 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-GPIIb/IIIa antibody PAC-1 and anti-C6D1-PE for GPIIb/IIIa activation. The sample was incubated for 25 min in the dark. Platelet populations were gated on cell size using C6D1 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 matício on platelet aggregation were evaluated using a lumi-aggregometer (Chrono-Log, Haverton, PA, USA). Matício (final concentration 0.01–1 mg/mL) was added to 480 µL of PRP ($200 \times 10^9$ 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 matício on platelet aggregation was followed for 6 min and compared with saline control. Maximal amplitude (%) was measured by AGGRO/LINK software (Chrono-Log, Haverton, 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 buffer (50 mM Tris-HCL, 50 mM NaCl, 1 mM MgCl$_2$, 1 mM EDTA, 0.1% Triton X-100 at pH 7.4). 20 µg of protein from each samples was subjected to SDS/PAGE, immunoblotted and probed with anti-phospho (Tyr753)- PLC-γ2, anti-phospho (S660)-PKC-β2 and 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 GraphPad 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 matício extract against agonist-induced platelet aggregation was calculated from the dose-response curves. Differences between groups were
analyzed by nonparametric Kruskal–Wallis statistical test and the Mann–Whitney as post-test for cases where there were significant differences. P values < 0.05 were considered significant.

3. Results

3.1. Matico inhibits platelet P-selectin expression in human platelets

Platelet P-selectin expression is a thrombo-inflammatory protein involved in platelet activation and thrombus formation. In this study, we used matico extract at 0.01, 0.05, 0.1, 0.5 and 1 mg/mL to evaluate its inhibitory activity on platelet P-selectin expression ADP-induced using flow cytometry. As shown in Fig. 1A, ADP 8 μmol/L induced platelet P-selectin expression was inhibited by different concentrations of matico extract. Thus P-selectin expression in presence of matico extract at 0.01, 0.05, 0.1, 0.5 and 1 mg/mL was inhibited from 32 ± 2% in the control group to 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.

3.2. Matico inhibits GPIIb/IIIa activation in human platelets

The GPIIb/IIIa is an integrin complex found on platelet membrane. GPIIb/IIIa activation represents the first phase of the platelet-aggregation process. In this assay we used PAC-1 that reacts with activation-induced conformational epitope of the GPIIb/IIIa complex. Matico extract (0.01, 0.05, 0.1, 0.5 and 1 mg/mL) concentration-dependently attenuated ADP induced platelet GPIIb/IIIa activation at 3 min after ADP stimulation (Fig. 1B). The above results indicate that inhibition of GPIIb/IIIa activation may contribute to matico extract action in activated platelets.

3.3. Matico inhibits human platelet aggregation

We determined whether matico extract affected platelet aggregation induced by collagen 1.5 μg/mL, convulxin 20 ng/mL or ADP 8 μmol/L. Matico extract (0.01–1 mg/mL) exhibited potent activity of inhibiting platelet aggregation stimulated by collagen 1.5 μg/mL, convulxin 20 ng/mL and ADP 8 μmol/L (Fig. 2). The IC50 values of matico extract for platelet aggregation induced by collagen, convulxin and ADP were approximately 61 μg/mL, 72 μg/mL and 290 μg/mL, respectively.

3.4. Matico inhibits PLC-γ2 and PKC-β2 phosphorylation in human platelets

Phosphorylation of PLC-γ2 and PKC-β2 is one of the key mechanisms regulating the activity of activated platelet in human platelets. In order to elucidate the mechanism underlying the effects of matico extract on platelets, we further examined the phosphorylation of downstream signaling protein of collagen including PLC-γ2 and PKC-β2. Our immunoblot study revealed that phosphorylation of PLC gamma 2 and PKC beta 2 was almost completely blocked by pre-incubation with different concentrations of matico extract (Fig. 3).

4. Discussion

In this study we demonstrated for first time that matico extract (B. globosa), at a low concentration and in a concentration dependent manner, was a potent inhibitor of platelet aggregation in response to collagen, convulsion and ADP. In this sense matico extract exerted the greatest antiaggregant activity induced by collagen. Similarly, matico showed a decrease of P-selectin expression from α-granules and GPIIb/IIIa expression. The cellular mechanism for the antiplatelet activity of matico might be mediated by the inhibition of PLC-γ2 and PKC-β2 phosphorylation.

Matico extract is rich in polyphenols and flavonoids compound (Table 1) with high anti-oxidant and anti-inflammatory activity. This is able to modulate platelet via a common mechanism. Reactive oxygen species (ROS) are produced in platelet mostly by NADPH oxidase enzyme. ROS are important in platelet activity. Polyphenols can counter them acting as scavengers and also modulating NADPH oxidase activity. Certain flavonoids are able to bind and cause antagonism in thromboxane receptors, among them luteolin, the principal flavonoid present in B. globosa, can inhibit TXA2-R mediated activation, impair release of Ca2+ and ERK 1/2 phosphorylation. TXA2-R is important in collagen-induced activation. Luteolin, among other flavonoids, reduces collagen activation acting on TXA2-R. Furthermore, luteolin is able to cause inhibition of PI3K, a pivotal enzyme in collagen-induced activation.

Recent studies have shown that verbascoside possesses anti-platelet effects, inhibiting the aggregation induced by ADP and AA in vitro, as well as in patients with high cardiovascular risk. Verbascoside acts on the arachidonic acid pathway, particularly in the synthesis of TXA2, probably interfering with the enzyme thromboxane synthase. Its role as a PKC inhibitor has also been described. Verbascoside inhibits the activity of this enzyme in a concentration-dependent manner by competing with ATP.

Alpha and beta amyrin: in vitro assays have shown that amyrins inhibited platelet aggregation induced by different agonists, in...
particular β-amyrin inhibited collagen induced aggregation with IC50 lower than Aspirin.\textsuperscript{30} α-Amyrin, has also shown to be a competitive inhibitor of PKC.\textsuperscript{31} In another model, Amyrins disrupted both MAPK and AA metabolisms related to PKC upstream inhibition.\textsuperscript{32} Mechanism of platelet inhibition of compounds present in matico is summarized in Fig. 4.

In platelet aggregation study, matico inhibited collagen and convulxin-mediated platelet aggregation in a concentration-
dependent manner, and the IC50 value for collagen and convulxin was more potent than that for ADP. In addition, matico plays an important role in the inhibition of GPIIb/IIIa in activated platelets. Accordingly the granule secretion, which was determined as the level of platelet P-selectin expression, was inhibited by matico in the same pattern as inhibition of platelet aggregation. Because the collagen-mediated platelet activation is believed to be caused by activation of PLC-$\gamma_2$ and PKC-$\beta_2$, we further examined the effect of matico extract on PLC-$\gamma_2$ and PKC-$\beta_2$ activation.

Convulxin, a potent platelet aggregating protein from snake venom of the Crotalus durissus terrificus is known to bind to the platelet collagen receptor, GPVI. The downstream signaling pathway of collagen and convulxin induces platelet activation of GPVI through a tyrosine kinase-based signaling pathway that involves the kinase Syk and PLC-$\gamma_2$, which results in PKC-$\beta_2$ activation and (Ca$^{2+}$)$\_i$ increase. In fact, the activation of this signaling pathway results in shape change and granule release (expression of P-selectin), and platelet adhesion and aggregation (activation of GPIIb/IIIa). In this sense, collagen-induced time-dependent increase of PLC-$\gamma_2$ and PKC-$\beta_2$ phosphorylation was completely abrogated after treatment of matico extract. These results suggest that inhibition of PLC-$\gamma_2$ phosphorylation may be important in the inhibition of matico on collagen-mediated platelet aggregation.

In summary, our study showed that matico was able to inhibit the phosphorylation of PLC-$\gamma_2$ and PKC-$\beta_2$, both are downstream signaling pathways of collagen induced platelet activation. This suggests that $B.\ globosa$ may be useful in CVD prevention, since platelet Hyperreactivity is an important determinant of thrombosis related diseases.

This beneficial property of matico must be tested by in vivo assays. Studies that have evaluated the in vivo anti-inflammatory and anti-nociceptive effects of $B.\ globosa$ use a range from 200 to 600 mg/kg. Future studies on the antiplatelet effects of matico would consider this range of concentrations. In clinical trials, different plant extracts have shown to induce protection against CVD development. In view of our results, $B.\ globosa$ consumption could induce cardioprotective effects in addition to their known properties. Unfortunately, to date, the biological effects are not

| Compound                  | mg/g | Solvent extract | Type compound | Plant part | Place            | Reference |
|---------------------------|------|-----------------|---------------|------------|------------------|-----------|
| Luteolin                  | 3.3  | Water           | Flavonoids    | Fresh leaves | London garden    | 9         |
| 6-hydroxyluteolin         | 2.5  | Water           | Flavonoids    | Fresh leaves | London garden    | 9         |
| Linarin                   | 58   | Water           | Flavonoids    | Fresh leaves | London garden    | 9         |
| Echinacoside              | 75   | Water           | Phenylethanoids | Dried leaves | Temuco, IX Region, Chile | 11 |
| 2-arylmethylnaphthalene   | 4.5  | Hexane          | Triterpenoids | Dried leaves | Temuco, IX Region, Chile | 11 |
| 2-arylmethylnaphthalene   | 6.7  | Hexane          | Triterpenoids | Dried leaves | Temuco, IX Region, Chile | 11 |
| 2-arylmethylnaphthalene   | 6.5  | CH2Cl2          | Steroid       | Dried leaves | Temuco, IX Region, Chile | 11 |
| 2-arylmethylnaphthalene   | 1    | Methanol        | Steroid       | Dried leaves | Temuco, IX Region, Chile | 11 |
| Luteolin 7-O-glucoside    | 11   | Methanol        | Flavonoids    | Dried leaves | Temuco, IX Region, Chile | 11 |
| Apigenin 7-O-glucoside    | 0.25 | Methanol        | Flavonoids    | Dried leaves | Temuco, IX Region, Chile | 11 |
| Buddledin A               | 47   | CHCl3           | Sequiterpenoids | Roots        | London garden    | 12 |
| Buddledin B               | 1.3  | CHCl3           | Sequiterpenoids | Roots        | London garden    | 12 |
| Buddledin C               | 1.5  | CHCl3           | Sequiterpenoids | Roots        | London garden    | 12 |
| Zerumbone                 | 1    | CHCl3           | Sequiterpenoids | Roots        | London garden    | 12 |
| Dihydrobuddledin A        | 3.5  | CHCl3           | Terpenoid     | Roots        | London garden    | 12 |
| Buddledone A              | 0.4  | CHCl3           | Diterpenoid   | Roots        | London garden    | 12 |
| Buddledone B              | 0.4  | CHCl3           | Diterpenoid   | Roots        | London garden    | 12 |
| Verbascoside              | 92   | Water           | Phenylethanoids | Fresh leaves | London garden    | 9         |
| 1.2                       | Ethanol       | Phenylethanoids | Dried leaves  | Cunco, IX Region, Chile | 8         |
| 17.5                      | Methanol      | Phenylethanoids | Dried leaves  | Temuco, IX Region, Chile | 11 |
supported by any rigorous clinical studies. Only one study has focused on evaluating the healing effects of topical use of this plant in patients with wounds, however results are not yet available. Our results suggest a potential cardioprotective effect not previously described, supporting the evaluation of these effects on people. It is also necessary to contrast empirical knowledge and evaluate toxicological effects.

Conflict of interest

The authors report no conflict of interest.

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