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Anti-platelet effects of bioactive compounds isolated from the bark of *Rhus verniciflua* Stokes

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Abstract

It has previously been shown that EtOAc extracts of *Rhus verniciflua* Stokes (RVS) inhibit the platelet aggregation response. In this report, bioassay-guided fractionation using ADP-, arachidonic acid-, and collagen-induced human platelet aggregation by a whole blood aggregometer yielded the bioactive compounds isomaltol and pentagalloyl glucose from different highly effective fractions. In addition, column chromatography of fractions from RVS yielded another five compounds: butin, fisetin, sulfuretin, butein and 3,4′,7,8-tetrahydroxyflavone. We investigated the effects of bioactive compounds from RVS fractions on several markers of platelet activation using receptor expression on platelet membranes, including glycoprotein IIb/IIIa (CD41), GPIIb/IIIa-like expression (PAC-1) and P-selectin (CD62), and intracelluar calcium mobilization responses by flow cytometry in healthy subjects. Dose-dependent inhibition of platelet aggregation and significantly decreased platelet activation were observed for the isomaltol- and pentagalloyl glucose-treated platelets, respectively. These results show that isomaltol and pentagalloyl glucose from the bark of *Rhus verniciflua* Stokes have potent anti-platelet activity and emphasize the need to further examine the mechanism of these active compounds for platelet modulation.

Keywords: *Rhus verniciflua* Stokes (Anacardiaceae); Isomaltol; Pentagalloyl glucose; GPIIb/IIIa-like expression; P-selectin; Calcium mobilization

1. Introduction

Platelet aggregates, stabilized by fibrin, rapidly form hemostatic plugs when blood vessels are severed or upon the occurrence of arterial thrombi at sites of vessel injury, such as ruptured atherosclerotic plaques, or at regions where blood flow is disturbed, such as at stenoses. These thrombi cause thromboembolic complications of atherosclerosis: heart attacks, strokes, and peripheral vascular disease (Packham, 1994).

The modulation of platelet activity using specific pharmacological agents has proven to be a successful strategy for the prevention of thrombosis (Hubbard et al., 2003). Inhibitors of aggregation can provide protection against these symptoms that affect millions of people worldwide. Aspirin (acetylsalicylic acid, ASP) is one such inhibitor. The chances of a second heart attack can be reduced by as much as 40% by taking aspirin daily (Patrono, 1994).

In terms of traditional Korean medicine, thrombosis is a type of blood indigestion caused by circulation problems in blood veins. For the treatment of thrombosis, herbs for promoting blood flow and removing blood stasis are used in Korea. We previously reported a study on the screening of medicinal herbs

Abbreviations: AA, arachidonic acid; ADP, adenosine-5′-diphosphate; FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity; PRP, platelet-rich plasma; PE, phycoerythrin

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used for promoting blood flow and removing blood stasis for anti-platelet activity (Jeon et al., 2003). From the results, we found that *Rhus verniciflua* Stokes (RVS) extract was the most effective candidate for further study.

Rhus verniciflua Stokes is an indigenous plant to Korea, China, and Japan, and is used as a food supplement and traditional herbal medicine for promoting blood circulation and removing blood stasis (Kim, 1996; Lee et al., 2004). RVS has been found to have various biological activities: it exhibits antioxidant activity (Lee et al., 2001; Lim et al., 2001), anti-obesity activity (Jeon et al., 2003), anti-inflammatory effects (Kim et al., 2004) and anti-mutagenic activity (Park et al., 2004). It also inhibits proliferation and apoptosis in human cancer cell lines (Lee et al., 2002, 2004; Son et al., 2005; Ko et al., 2005).

However, there is no information about the eventual influence of this plant on platelet activity. Thus, the aim of the current investigation was to evaluate RVS in terms of the inhibition of whole-blood platelet aggregation and platelet membrane receptor expression and intracellular calcium mobilization. We performed whole-blood flow cytometry assays on samples from healthy volunteers to determine the effects of in vitro incubation with bioactive compounds on multiple markers of platelet activation. We report here for the first time the most active compounds, isomaltol and pentagalloyl glucose, that exhibited anti-platelet properties.

2. Materials and methods

2.1. Plant material

The bark of *Rhus verniciflua* Stokes (Anacardiaceae) was purchased from a commercial supplier in Seoul, Korea, in 2001 and was identified by Prof. W.K. Whang (Department of Pharmacy, Chung-Ang University, Seoul, Korea). A voucher specimen (No. 2001-02) was deposited at the herbarium of the Quality Control of Herbal Medicine Department, KIOM (Korea).

2.2. Reagents and instruments

Aspirin was purchased from Sigma Chemical Co. (USA). Adenosine-5′-diphosphate (ADP), arachidonic acid (AA), collagen, and epinephrine were obtained from Chrono-Log Co. (USA). Fluo-3AM and fluorescent-labeled monoclonal antibodies, including CyChrom-labeled CD41a, fluorescein isothiocyanate (FITC)-labeled PAC-1, and phycoerythrin (PE)-labeled CD62P, were purchased from Becton Dickinson. Recycling preparative HPLC was carried out on an LC-908 system (Sunil JAI, Japan). ^{1}H NMR, ^{13}C NMR, ^{1}H COSY, HMQC and HMBC spectra were measured with a Bruker AM-600 instrument (Bruker, Germany). Mass spectra were taken with a Hewlett Packard model 5989 B GC/MS system and VG70-VSEQ (UK). Column chromatography was conducted using Kiesel gel 60 (70–230 mesh, Merck, Germany), ODS (Lichroprep RP-18, Merck, Germany), and Sephadex LH-20 (Pharmacia, USA) columns. Other chemicals were of analytical grade.

2.3. Extraction and isolation

Dried and chopped bark of *Rhus verniciflua* Stokes was extracted with 70% MeOH and then concentrated under vacuum using a rotary evaporator. The residue from the methanol extract was then extracted with methylene chloride, followed by ethyl acetate and *n*-butanol. The dried ethyl acetate extract was chromatographed on an ODS column. Elution was performed using a solvent mixture of 50% MeOH. This process yielded five fractions (fractions 1–5), which were tested for biological activity. Inert fractions (fractions 3–5) were identified as flavonoids using NMR, high-resolution GC/MS and literature comparisons. Fraction 3 was chromatographed on an ODS column with 60% MeOH to isolate compound **1**, identified as butin. Fraction 4 was recrystallized in 40% MeOH to yield compound **2**, identified as fisetin. Fraction 5 was chromatographed by recycling preparative HPLC to yield compound **3** (sulfuretin) and compound **4** (butein). The biologically active fraction 2 yielded compounds **5**, **6** and **7**, which were identified as 3,4′,7,8-tetrahydroxyflavone, isomaltol, and pentagalloyl glucose, respectively.

Compound **6** (isomaltol): Brown (10 mg), crystals from water or ether, mp 98–103 °C ($C_6H_6O_3$); FAB(–) mass (*m*/*z*): 125 $[M-H]^-$; ^{1}H NMR (600 MHz, CD_3OD): δ 7.88 (1H, d, J = 1.8 Hz, H-5), 6.84 (1H, d, J = 8.7 Hz, H-4), 2.52 (3H, s, H-1′Methyl); ^{13}C NMR (CD_3OD, 150 MHz): δ 199.7 (C-3), 164.3 (C-5), 132.3 (C-2), 130.2 (C-1′), 116.4 (C-4), 26.4 (C-1′Methyl).

Compound **7** (pentagalloyl glucose): White powder (190 mg), $[\alpha]_D$ +16.9 (*c* 0.76, MeOD); ESI-MS *m*/*z* 941 $[M]^+$; ^{1}H NMR (300 MHz, MeOD): 4.40–4.68 (3H, m, H-5 and H-6), 5.60 (1H, dd, J = 8.1, 9.0 Hz, H-2), 5.63 (1H, t, J = 9.0 Hz, H-4), 6.00 (1H, t, J = 9.0 Hz, H-3), 6.27 (1H, d, J = 8.1 Hz, H-1), 6.91, 7.00, 7.01, 7.07, 7.13 (each 2H, s, galloyl H); ^{13}C NMR (75 MHz, MeOD): 62.7 (C-6), 69.3 (C-4), 71.7 (C-2), 73.3 (C-3), 74.0 (C-5), 93.3 (C-1).

2.4. Volunteers

A total of 15 healthy volunteers (age range 21–36 years, 4 males and 11 females) entered the study. All subjects gave written informed consent before participation. The study was approved by the Ethics Committee of the Korea Institute of Oriental Medicine, Daejeon, Korea. For this study, blood was collected in Vacutainer tubes (Becton Dickinson, USA) containing 3.8% sodium citrate with a draw volume of 2.7 ml using standard phlebotomy techniques. All subjects had no history of bleeding disorders or cardiovascular disease, and refrained from any pharmacological therapy for at least 2 weeks before enrolment. None of the subjects smoked, or exhibited hypertension, diabetes or abnormal hematocrit.

2.5. Whole-blood platelet aggregation

Aggregation studies were performed with a 500VS Chrono-Log aggregometer (Chrono-Log, USA) using the impedance method (Armida et al., 1995), which allows the quantification of aggregation in whole blood. Whole blood was incubated at 37 °C in 450-μl aliquots until the time of use; during aggregation