Arch Pharm Res Vol 28, No 9, 1037-1041, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

Anticoagulant 1,2,3,4,6-Pentagalloyl-β-D-Glucopyranose Isolated from Geranium (*Pelargonium inquinans* Ait)

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(Received May 26, 2005)

Geranium (*Pelargonium inquinans* Ait) leaves were extracted with 80% MeOH, and partitioned into n-hexane, ethyl acetate, BuOH and H_2O to isolate the anticoagulant principles. The EtOAc fraction was found to be the most active, and was further purified using silica and octadecylsilane column chromatography employing a bioassay-guided fractionation method. The active compound was isolated and identified as 1,2,3,4,6-pentagalloyl-β-D-glucopyranose (PGG) (compound I). The isolated anticoagulant significantly prolonged the activated partial thrombin time (APTT) and thrombin time (TT) using normal human plasma. One microgram of 1,2,3,4,6-pentagalloyl-β-D-glucopyranose showed 0.063 heparin units in the APTT and 2.73 heparin units in the TT for anti-thrombosis. This is the first report of the isolation of PGG from geranium plants.

Key words: Geranium, *Pelargonium inquinans* Ait, Anticoagulant activity, 1,2,3,4,6-Pentagalloyl-β-D-glucopyranose, Activated partial thrombin time (APTT), Thrombin time (TT)

INTRODUCTION

Coagulation consists of a series of zymogens that can be converted into active enzymes *via* limited proteolysis leading to the generation of thrombin, which in turn converts fibrinogen into fibrin (Rand *et al.*, 1996). Thrombin is also responsible for the feedback activation of other coagulation factors and is believed to be the pivotal enzyme in the coagulation pathways (Mann, 1999). Consequently, the control of thrombin generation regulates the plasma coagulant activity. Antithrombin III (ATIII) and heparin cofactor II (HCII) are two of the most important physiological anticoagulants and members of the serpin superfamily. Both molecules share a similar mechanism of action to inactivate thrombin. Certain glycosaminoglycans accelerate the rate of thrombin inhibition by both molecules (Janciauskiene, 2001). ATIII is activated exclusively by heparin (Desai *et al.*, 1998), while HCII is activated by many polyanionic macromolecules (Farias *et al.*, 2000; Mauray *et al.*, 1995; Hayakawa *et al.*, 2000), mainly by heparin and dermatan sulphate (Bauman and Church, 1999). Glycosaminoglycans, such as heparin and dermatan sulphate, are used clinically for both prophylaxis and treatment of thrombosis.

Although several synthetic anticoagulants have been used to prevent and treat various diseases, they have various side effects as well as toxicity. The disadvantages of these compounds, to a different extent, include the risk of hemorrhage, resistance of the clot-bound thrombin, and short intravenous half-life compared with the plasma protein inhibitors (ATIII and HCII) (Plummer *et al.*, 1999). Natural anticoagulants have received a great deal of attention, and significant effort has been made to discover safe and effective therapeutic agents for treating coagulant-related diseases.

There is compelling evidence indicating that the increased consumption of dietary anticoagulants or natural products with anticoagulant properties can improve the quality of life by delaying the onset and reducing the risk of many degenerative diseases (Matsubara *et al.*, 2001; Guglielmone *et al.*, 2002). The increased consumption of natural products, such as fruits and vegetables containing high levels of phytochemicals, are recommended to prevent

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chronic diseases related to coagulable stress in the human body (Dismore *et al.*, 2003; Barcellona *et al.*, 2002). However, the anticoagulant activity of these natural products has not been clearly demonstrated.

As part of an ongoing investigation into the coagulant scavenging effect and the anticoagulant capacity of natural products, the geranium (*Pelargonium inquinans* Ait) plant was found to have excellent anticoagulant potential. Geranium was reported to exhibit diuretic, hemostatic, tonic, stomachic, antidiabetic and antidiarrheic activity (Akdemir *et al.*, 2001). Geranium contains many active components, including antocyanin, flovonol and polyphenols (Asen and Griesbach, 1983; Guo *et al.*, 1987). This study focused on isolating the active components in geranium using a bioassay-guided fractionation method and verifying the anticoagulant activity.

MATERIALS AND METHODS

Materials

Geranium (*P. inquinans* Ait, variety Lingo 2000) leaves were collected from a farm near Gwangju city, Korea. Porcine intestinal mucosa heparin, thrombin, fibrinogen from bovine plasma, and the activated partial thromboplastin time (APTT) reagent were purchased from Sigma. All the coagulation assays (APTT, PT and TT) were performed using a coagulometer (Coatron M1, TECO GmbH, Germany). The silica gel (Kieselgel 60, 230~400 mesh ASTM, Merck Art. 9385) and TLC plates (Kieselgel 60 F_{254}) were purchased from Merck (Darmastadt, Germany). The NMR spectra were measured on a ^{Unit}INOVA 500 spectrometer (Varian, Walnut Creek, CA, U.S.A.). The CD_3OD for NMR was purchased from Aldrich (Aldrich Chemical Co., U.S.A.). The Fab-MS was recorded on a micromass PLATFORM II (Micromass, Mc, UK).

Extract preparation and fractions from geranium

The fresh leaves (2.6 kg) were cut into approximately 1 cm, submerged in 80% methanol (12 L) for 5 days and extracted twice. The organic solvent was removed under vacuum to give 165 g of the MeOH extract. The MeOH extract was dissolved in water and further partitioned in succession with n-hexane, EtOAc and n-BuOH. Seventy grams of the crude preparation was obtained from the EtOAc fraction.

Bioactivity-guided fractionation and isolation

Activity-guided isolation of the EtOAc fraction (40 g) with silica gel chromatography (5 × 50 cm) eluted with *n*-hexane-ethylacetate (5:1 to 1:1, v/v) and chloromethane-methanol (15:1 to 1:1) gave 13 fractions. Fraction 7 (3.5 g) showed the most potent activity. A second silica gel chromatography (2.5 × 60 cm) step was carried out with chloromethane-MeOH-H_2O (gradient elution) to give 11 fractions. Fraction 9 (0.5 g) was further purified over octadecylsilane (ODS) using H_2O-MeOH (4:1 to 0:1) and HPLC (Senshu-Pak C_{18} column, 8 × 250 mm) to yield compound **I** (123 mg). Compound **I** was identified as 1,2,3,4,6-pentagalloyl-â-D-glucopyranose (Fig. 1) based on the spectral results (Kim and Song, 2000).

1,2,3,4,6-Pentagalloyl-β-D-glucopyranose (I)

Amorphous powder, $C_{41}H_{32}O_{26}$, FAB-MS *m/z*: 963 $(M+Na)^+$; ^{1}H-NMR (500 MHz, CD_3OD) δ: 6.23 (1H, d, *J*=8 Hz, H-1), 5.62 (1H, t, *J*=9.5 Hz, H-2), 5.90 (1H, t, *J*=9.5 Hz, H-3), 5.58 (1H, t, *J*=9.5 Hz, H-4), 4.51 (1H, d, *J*=10.5 Hz, H-5), 4.40 (2H, m, H-6); ^{13}C-NMR (125 MHz, CD_3OD) δ: 93.96 (C-1), 72.32 (C-2), 74.24 (C-3), 69.94 (C-4), 74.56 (C-5), 63.26 (C-6), 119.59~121.43 (each C-1'), 110.16~110.73 (each C-2', 6'), 146.45~146.74 (each C-3',5'), 140.09~141.29 (each 4'), 166.42~168.10 (each COO).

Anticoagulant activity assay

Activated partial thromboplastin time (APTT) clotting assays were carried out as described elsewhere (Mourao *et al.*, 1996). Normal human plasma (100 μL) was incubated with 25 μL of sample solution in a test tube. One hundred μL of the APTT assay reagent was then added to the mixture and incubated for 2 min at 37°C. Warmed 20 mM $CaCl_2$ (100 μL) was then added and the clotting time was recorded. The coagulant time was compared with a blank solution without the sample at 37°C. The activity is expressed in international units/mg using a parallel standard curve based on the international heparin standard (160 units/mg). For the prothrombin time (PT) assay, citrated normal human plasma (100 μL) was mixed with 25 μL of a sample and incubated for 2 min at 37°C. The PT assay reagent (100 μL), which had been preincubated for 5 min at 37°C, was then added and the clotting time was recorded. For the thrombin time (TT)

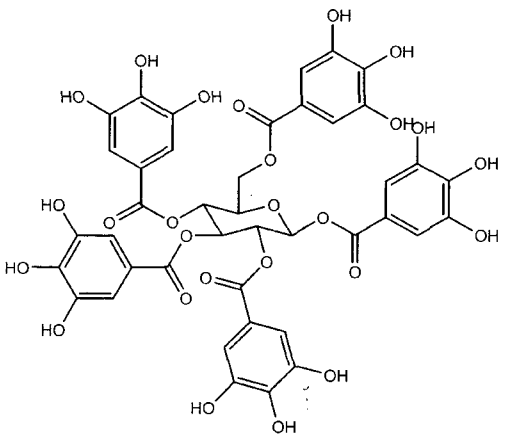

Fig. 1. Structure of 1,2,3,4,6-pentagalloyl-β-D-glucopyranose

assay, 250 μL of a fibrinogen solution (0.8% in 0.2 M borate buffer) was mixed with a 25 μL sample and incubated for 3 min at 37°C. Twenty five μL of thrombin (10 unit/mL), which had been preincubated for 5 min at 37°C, was added and the clotting time was recorded.

RESULTS

Protective effect from coagulation induced by APTT and TT reagents

As shown in Fig. 2, the methanol extract of geranium (*P. inquinans* Ait) leaves exhibited anticoagulant activity on the APTT and TT reagents in a dose-dependent manner. The APTT of the methanol extract of geranium was > 300 s at 0.5 mg/mL and the TT was > 75 s at 0.5 mg/mL. However, PT was barely affected. Fig. 3 shows the anti-coagulant activity of the n-hexane, EtOAc, BuOH and H_2O fractions from the methanol extract. The EtOAc fraction showed the strongest inhibitory effect on fibroin genera-

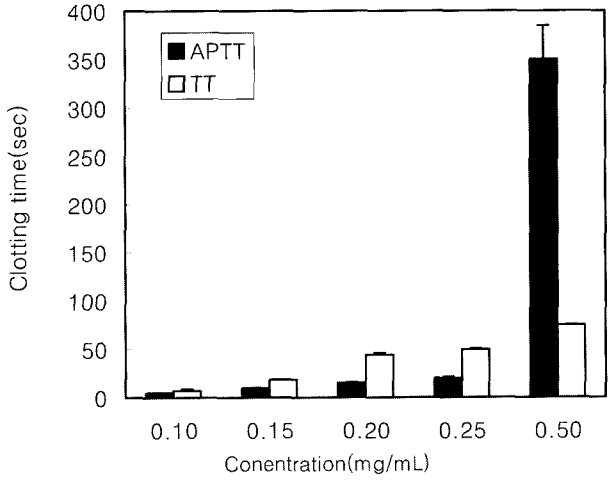

Fig. 2. Anticoagulant activity of the 80% MeOH extract from *P. inquinans* at various concentrations

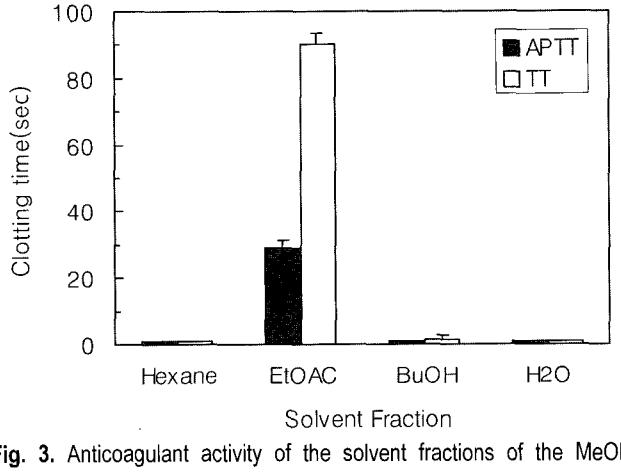

Fig. 3. Anticoagulant activity of the solvent fractions of the MeOH extract from geranium leaves at 0.125 mg/mL

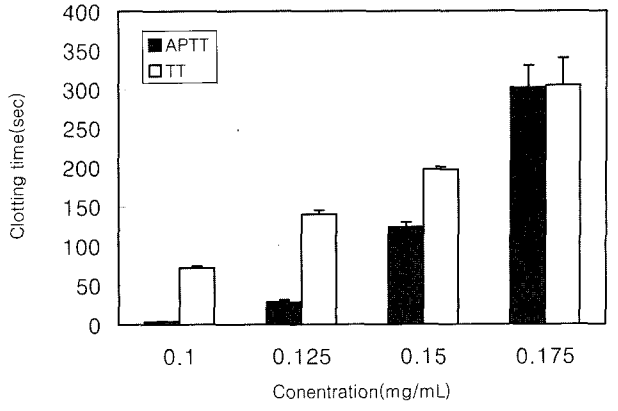

Fig. 4. Anticoagulant activity of the ethyl acetate fraction of MeOH extract from *P. inquinans* at various concentrations

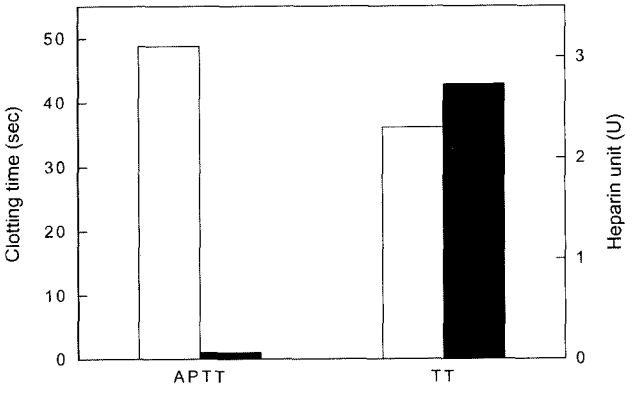

Fig. 5. Prolonged APTT and TT times (□) and their heparin units (■) of 1 μg 1,2,3,4,6-pentagalloyl-β-D-glucopyranose isolated from *P. inquinans*

tion. The anticoagulant activity was dose-dependent, and the APTT and TT were > 300 s at 0.175 mg/mL (Fig. 4).

Identification of 1,2,3,4,6-pentagalloyl-β-D-glucopyranose from geranium

A pentagalloyl glucopyranose (compound **I**) was isolated from the EtOAc fraction of geranium using the bioassay-linked fractionation method. Its structure was characterized using various spectroscopic techniques, including mass and nuclear magnetic resonance spectroscopy. Fig. 1 shows the chemical structure.

Anticoagulant effects of 1,2,3,4,6-pentagalloyl-β-D-glucopyranose

This is shown that the anti-fibrin generation effect of the active component. PGG showed 0.063 heparin units in the APTT and 2.73 heparin units in the TT for anti-thrombosis at 1 μg.

DISCUSSION

Currently, thrombosis is regulated using either heparin

or its derivatives or oral anticoagulants of the dicumarol type, which all indirectly inhibit thrombin. Hirudin and hirulog-1, which are hirudin-derived peptides, have reached the advanced stages in clinical development (Fenton, 1992; Markwardt, 1994; Johnson, 1994; Greinacher *et al.*, 1999). However, they are expensive parental anticoagulants with a relatively short half live and are currently only suitable for acute care. The ideal clinical anticoagulant would both reliably and predictably inhibit thrombin (in particular, the clot-bound thrombin) without substantially increasing the risk of hemorrhage.

Biologically active compounds can be found in a variety of plants and may play a role in reducing the risk of degenerative diseases associated with coagulation stress (Chevolot *et al.*, 1999; Araujo *et al.*, 2001). Geranium (*P. inquinans* Ait) is becoming an increasingly popular source of biologically active components including antocyanin, flovonol and polyphenols (Asen and Griesbach, 1983; Guo *et al.*, 1987).

1,2,3,4,6-Pentagalloyl-β-D-glucopyranose (PGG) was isolated as the major anticoagulating principle using the bioassay-guided fractionation of the MeOH extract of the *Pelargonium inquinans* leaves. The PGG isolated from *Geum japonicum* exhibited potent anticoagulant activity by significantly prolonging the clotting of rabbit plasma, and was found to be a mixed noncompetitive inhibitor of thrombin (Dong *et al.*, 1998). PGG was also reported to be potent inhibitor of the acetyl-CoA:1-alkyl-sn-glycero-3-phosphocholine acetyltransferase, which is a key enzyme in platelet-activating factor biosynthesis, and platelet-activating factor-induced platelet aggregation (Sugatani *et al.*, 2004).

Under these experimental conditions, the purified 1,2,3,4,6-pentagalloyl-β-D-glucopyranose showed significant *in vitro* anticoagulant activity. The APTT and TT using human plasma were significantly extended. The prolongation of the APTT suggests the inhibition of the intrinsic and/or common pathway, whereas the prolongation of TT indicates the inhibition of thrombin activity or fibrin polymerization. This is the first report of the isolation of PGG from *P. inquinans*.

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