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ORIGINAL ARTICLE

Antithrombotic Activity and Saponin Composition of the Roots of *Panax bipinnatifidus* Seem. Growing in Vietnam

Vu Thi Thom, Nguyen Huu Tung, Dang Van Diep[1], Dang Thi Thuy, Nguyen Thi Hue, Dinh Doan Long, Bui Thanh Tung, Pham Thanh Huyen[2], Duong Thi Ly Huong

School of Medicine and Pharmacy, Vietnam National University, Hanoi, [1]Vietnam Military Medical University, [2]Vietnam National Institute of Medicinal Materials, Hanoi, Vietnam

ABSTRACT

Background: *Panax bipinnatifidus* (PB) Seem. is a highly valuable and indigenous medicinal plant wildly distributed in the northwest region of Vietnam. The PB root has been used as a tonic in Vietnamese traditional medicine, but its chemical profile and pharmacological activity have not been extensively studied. **Objective:** In this study, we investigated the major saponin components and antithrombosis activity of the root of PB. **Materials and Methods:** Individual saponins were isolated by column chromatography. The structural elucidation of the isolated saponins was based on analyses of nuclear magnetic resonance and mass spectrometry spectra. Chromatographic fingerprint analysis of the isolated compounds was run on an Agilent 1260 high-performance liquid chromatography (HPLC) system. To evaluate antithrombotic activity, *in vitro* anticoagulant, platelet aggregation, and clot lysis effects were successively tested by the respective protocols. **Results:** Two principal saponins were isolated from the butanolic fraction of the root of PB, and their structures were identified as stipuleanoside R2 (1) and araloside A methyl ester (2). The HPLC analysis suggested that the two isolated saponins are major saponins of the title plant. The biological testing showed that both the crude and butanolic extracts of PB exhibited significantly antiplatelet aggregation activity. The efficacy linearly increased according to the tested doses (0.5–5 mg/mL). On anticoagulant activity, only butanolic extract exhibited positive action via prolonged activated partial thromboplastin time and prothrombin time at the high dose. **Conclusion:** These results suggested that PB Seem. might bring benefits for preventing cardiovascular events through inhibition of platelet aggregation.

Key words: Anticoagulant, *Araliaceae*, araloside A methyl ester, *Panax bipinnatifidus*. Seem, platelet aggregation, stipuleanoside R2

SUMMARY

- Two major saponins, stipuleanoside R2 (1) and araloside A methyl ester (2), were the first time isolated from the butanolic fraction of the root of *Panax bipinnatifidus* (PB) Seem. The biological evaluation showed that the butanolic extraction of PB exerted significantly inhibition on platelet aggregation at all tested doses of 0.5, 1, 2, and 5 mg/mL and anticoagulation property with the higher doses of 2 and 5 mg/mL. These results suggested that PB Seem. might give benefits for preventing embolism and thrombosis.

Abbreviations Used: ADP: Adenosine diphosphate; APTT: Activated partial thromboplastin time; DMSO: Dimethyl sulfoxide; ESI-MS: Electrospray ionization-mass spectrometry; HPLC: High-performance liquid chromatography; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; MAPK: Mitogen-activated protein kinase; MPA: Maximum platelet aggregation; NIMM: National Institute of Medicinal Materials; NMR: Nuclear magnetic resonance; PPP: Platelet-poor plasma;; PRP: Platelet-rich plasma; PT: Prothrombin time; TLC: Thin-layer chromatography; VNU: Vietnam National University.

Correspondence:
Dr. Duong Thi Ly Huong,
School of Medicine and Pharmacy,
Vietnam National University, Hanoi.
E-mail: lyhuong.smp@vnu.edu.vn

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INTRODUCTION

Panax bipinnatifidus (PB) Seem. (*Araliaceae*), known as "*Truc-tiet-nhan-sam*," "*Tam-that-la-xe*," "*Sam-hai-lan-xe*," "*Vu-diep-tam-that*," or "*Hoang-lien-that*" in Vietnamese, is a hygrophilous and shade-enduring plant, preferring cool- and wet-climate conditions with an average temperature of about 12°C–15°C. In nature, this plant is becoming relatively rare and mostly found in the high mountainous region (1800–2400 m) of Hoang Lien Son in the Northwest of Vietnam. The root of PB has been used in Vietnamese traditional medicine as a valuable tonic to increase mental and physical performance, increase antiaging, improve thinking and memory, immunomodulatory properties,[1] decrease lipoproteinemia, prevent atherosclerosis,[2,3] and lower blood sugar in diabetes.[2-4]

To date, phytochemical and pharmacological studies on this plant have not been undertaken extensively. Like other *Panax* spp., the major components of the title plant are triterpene saponins with several individual compounds structurally reported.[5] In our study on potential medicinal plants in the Norwest region of Vietnam, we recently reported the bioactive triterpenes[6] and tanshinone diterpenes[7] from Danshen (*Salvia miltiorrhiza*). As part of our ongoing study, the current work on phytochemical profile and bioactivity of the roots of PB led to the isolation of two major saponins, stipuleanoside R2 (1) and araloside

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A methyl ester (2) for the first time. Herein, this paper deals with the isolation and structural elucidation of the two obtained saponins, in addition to the new results of antithrombotic activity of PB total and butanolic extracts.

MATERIALS AND METHODS

General procedures

Optical rotations were measured with a DIP-360 digital polarimeter (JASCO, Easton, MD, USA). Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL ECX 400 FT-NMR spectrometer (JEOL, Tokyo, Japan) at 20°C using Joel's standard pulse program, with tetramethylsilane as the internal standard, and the chemical shift values were expressed in δ (ppm). Electrospray ionization-mass spectrometry (ESI-MS) experiments employed in Agilent 1260 liquid chromatography-tandem mass spectrometry mass spectrometer (Agilent Technologies, USA). Column chromatography was performed on silica gel 60 (230–400 mesh, Nacalai Tesque Inc., Kyoto, Japan) and YMC ODS-A gel (50 μm, YMC Co. Ltd., Kyoto, Japan). Thin-layer chromatography (TLC) was performed on Kieselgel 60 F_{254} and TLC silica gel 60 RP-18 F_{254S} (Merck, Darmstadt, Germany) plates. Spots were visualized by spraying with 1% $Ce(SO_4)_2$ to 10% aqueous H_2SO_4 solution, followed by heating.

Plant materials

The roots of PB were collected in Sapa, Laocai, in March 2016 and were identified taxonomically by the botanist, Dr. Pham Thanh Huyen, the Department of Medicinal Material Resources, the National Institute of Medicinal Materials (NIMM). The specimens (DL-150716) were deposited at the herbarium of NIMM and Vietnam National University (VNU) School of Pharmacy and Medicine, Hanoi, Vietnam.

Extraction and isolation

The air-dried roots of PB (500 g) were extracted in 70% EtOH, using Soxhlet extraction apparatus (3 × 3 h × 1500 mL), and the combined extracts were concentrated *in vacuo* to dryness. The obtained EtOH residue (95.9 g) was suspended in H_2O (500 mL), then partitioned successively with hexane and EtOAc and saturated *n*-BuOH (each 3 × 500 mL) to obtain hexane (5.82 g), EtOAc (2.70 g), and BuOH (21.70 g) residues. Next, the BuOH portion (90 g) was subjected to a silica gel column (Φ85 mm × 80 mm) with stepwise gradient of CH_2Cl_2-MeOH (5:1→1:1, v/v, 600 mL/fraction) to obtain four fractions (B1–B4).

Fr. B2 (4.3 g) was then loaded onto a silica gel column (Φ 45 mm × 350 mm) with the eluent of $CHCl_3$-MeOH-H_2O (3:1:0.1, v/v/v, 1500 mL) to yield four subfractions (fr. B2.1–B2.4). Fr. B2.2 (920 mg) was further purified on a reversed-phase (RP) C_{18} column (Φ30 mm × 350 mm) with MeOH-H_2O (1:1, v/v, 1400 mL) to obtain compound 1 (white powder, 100 mg). Finally, compound 2 (white powder, 180 mg) was obtained from fr. B4 (5.1 g) using RP C_{18} column chromatography (Φ50 mm × 350 mm) with the eluent of MeOH-H_2O (1:1, v/v, 1600 ml).

Stipuleanoside R2 (1)

White powder; $[\alpha]^{25}_D$ = +7,5 (c 0.2, MeOH); ESI-MS: m/z 1089 [M + H] +; ^{1}H-NMR (400 MHz, CD_3OD): δ 0.87, 0.90, 0.98, 1.00, 1.00, 1.11, 1.22 (7× CH_3, all s, CH_3-25, 26, 24, 23, 30, 29, 27), 3.20 (1H, m, H-3), 4.42 (1H, d, J = 7.6 Hz, H-1′), 4.92 (1H, d, J = 8.0 Hz, H-1″), 5.25 (1H, brs, H-1‴), 5.32 (1H, brs, H-12), 5.45 (1H, d, J = 8.0 Hz, H-1⁗); ^{13}C NMR (100 MHz, CD_3OD): δ 38.7 (C-1), 27.0 (C-2), 89.5 (C-3), 39.3 (C-4), 55.7 (C-5), 18.1 (C-6), 32.1 (C-7), 39.3 (C-8), 48.2 (C-9), 36.5 (C-10), 24.8 (C-11), 123.1 (C-12), 143.4 (C-13), 41.5 (C-14), 27.3 (C-15), 22.6 (C-16), 46.8 (C-17), 41.2 (C-18), 46.1 (C-19), 30.1 (C-20), 33.8 (C-21), 32.1 (C-22), 27.3 (C-23), 14.9 (C-24), 15.6 (C-25), 16.3 (C-26), 25.0 (C-27), 176.7 (C-28), 32 (C-29), 23.1 (C-30). GlcA: 106.2 (C-1), 76.6 (C-2), 81.0 (C-3), 78.1 (C-4), 75.1 (C-5), 174.9 (C-6). Glc-I: 103.7 (C-1), 75.0 (C-2), 77.9 (C-3), 69.8 (C-4), 77.2 (C-5), 61.9 (C-6). Ara: 107.7 (C-1), 81.1 (C-2), 74.2 (C-3), 85.8 (C-4), 61.2 (C-5). Glc-II: 95.7 (C-1), 74.0 (C-2), 77.2 (C-3), 69.7 (C-4), 77.9 (C-5), 61.2 (C-6).

Araloside A methyl ester (2)

White powder; $[\alpha]^{25}_D$ = +16 (c 0.3, MeOH); ESI-MS: m/z 941 [M + H] +; ^{1}H-NMR (400 MHz, CD_3OD): δ 0.75, 0.81, 0.88, 0.91, 1.00, 1.11, 1.22 (7× CH_3, all s, CH_3-25, 26, 24, 23, 30, 29, 27), 3.22 (1H, m, H-3), 3.64 (3H, s, $COOCH_3$), 4.35 (1H, d, J = 7.6 Hz, H-1′), 5.03 (1H, brs, H-1″), 5.22 (1H, brs, H-12), 5.38 (1H, d, J = 8.0 Hz, H-1‴); ^{13}C-NMR (100 MHz, CD_3OD): δ 38.5 (C-1), 27.0 (C-2), 89.6 (C-3), 39.1 (C-4), 55.3 (C-5), 18.1 (C-6), 32.3 (C-7), 39.1 (C-8), 48.2 (C-9), 36.2 (C-10), 24.6 (C-11), 122.2 (C-12), 143.2 (C-13), 41.3 (C-14), 27.3 (C-15), 22.4 (C-16), 46.8 (C-17), 41.0 (C-18), 46.1 (C-19), 31.5 (C-20), 33.3 (C-21), 31.9 (C-22), 26.8 (C-23), 14.4 (C-24), 15.3 (C-25), 16.0 (C-26), 25.4 (C-27), 176.4 (C-28), 31.8 (C-29), 22.9 (C-30). GlcA: 105.3 (C-1), 74.5 (C-2), 76.4 (C-3), 77.0 (C-4), 76.4 (C-5), 170.9 (C-6). 53.0 ($COOCH_3$); Glc: 94.1 (C-1), 73.5 (C-2), 76.8 (C-3), 69.5 (C-4), 77.0 (C-5), 60.8 (C-6). Ara: 107.4 (C-1), 81.2 (C-2), 74.2 (C-3), 85.4 (C-4), 61.5 (C-5).

High-performance liquid chromatographic fingerprint of the two major saponins in the root of *Panax bipinnatifidus*

The high-performance liquid chromatography (HPLC) analyses were performed on a HPLC Model Agilent 1260 Infinity (Agilent Technologies, USA) equipped with a photodiode array detector (PDA) detector and automatic sampler and on an Agilent Eclipse Plus C18 HPLC column (4.6 mm i.d × 100 mm L, 3.5 μm). Separation was conducted using a mobile phase of acetonitrile (A) and 0.2% acetic acid aqueous solution (B) following a gradient program (30 min): 0–5 min (20% A), 5–15 min (20%→60% A), 15–20 min (60% A), 20–25 min (60%→80% A), 25–30 min (80% A); UV detection: 203 and 208 nm; flow rate: 0.8 mL/min; and injection volume: 20 μL. The pure isolated saponins (1 and 2) were dissolved in MeOH at a stock concentration of 1 mg/mL and kept at 4°C until use. The EtOH and saponin extracts (BuOH) from the PB roots were prepared at 30 mg/mL and filtered by 0.45 μm syringe filter before injection.

Bioassays

Reagents

Main reagents used in this study including activated partial thromboplastin time (APTT), prothrombin time (PT) reagent kit (Sysmex), dimethyl sulfoxide (DMSO), heparin, aspirin, and streptokinase (Sigma).

The total and butanolic extracts stored in 4°C were diluted in DMSO to reach stock solution concentration of 500 mmol/l. These stock solutions were stored in −20°C. Final concentration used for bioassays was obtained by dilution stock solution in NaCl 0.9% and freshly prepared before using.

Blood collection and plasma preparation

Total blood was withdrawn from the median cubital vein of healthy volunteers, strictly according to the standard protocol approved by Ethical Committee, VNU, Hanoi, School of Medicine and Pharmacy (code: IRB-VN01016). The whole blood was mixed with 3.8% sodium citrate (1:10, v/v) to evaluate anticoagulant and antiplatelet activities. For assessing clot lysis, the whole blood was used without anticoagulation by sodium citrate.

In vitro plasma coagulation assays

The action of PB and fractions on coagulation process was evaluated by PT and APTT tests. The test was carried out using commercial reagent kits (Sysmex CA 530) and followed to the manufacturer's recommended protocols. Briefly, 10 mL the whole blood was taken out and mixed with 3.8% sodium citrate (1:10, v/v) and then centrifuged with 3000 ×*g* for 15 min. The serum was moved out and divided into 10 Eppendorf tubes (450 µl per each). We added 50 µl of total or butanolic extracts of PB with the range of dose 0.5, 1, 2, and 5 mg/mL, respectively. DMSO 0.1% and heparin 200 IU/mL were served as a negative and positive control. We placed microcentrifuge tubes that contained serum and control or tested agents into the rank of machine, set up the program to determine PT and APTT, and repeated the test in five healthy volunteers.

In vitro platelet aggregation assay

Platelet aggregation was evaluated by light transmission aggregometry (Chrono-Log Co., USA). Blood was withdrawn from the median cubital vein of healthy volunteers and collected directly into 3.8% sodium citrate microcentrifuge tubes. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as follows: we centrifuged blood samples at 500 ×*g* for 10 min to get PRP and then centrifuged the rest blood at 3000 g for 10 min to get PPP. Both PRP and PPP were used within 4 h after getting. We contributed 450 µL PRP to each of 10 microcentrifuge tubes with magnet and then added 50 µL total or butanolic extracts with different dose of 0.5, 1, 2, and 5 mg/mL, respectively. DMSO 0.1% and aspirin 1 mg/mL were served as a negative and positive control. We incubated tubes in Chrono-Log at 37°C for 5 min. We added 5 µL adenosine diphosphate (ADP) to activate platelet. We determined continuously the light transmission at 697 nm and presumed PPP equates to 100% light transmission until the data were constant.

The result was calculated as maximum platelet aggregation (MPA) after normalized with DMSO 0.1%.

The MPA was determined as follows:

$$\text{MPA}\,(\%) = \frac{\text{Platelet aggregation percentage of aspirin or test agents}}{\text{Platelet aggregation percentage of DMSO 0.1\%}} \times 100\%$$

In vitro clot lysis assay

Based on the method reported by Prasad *et al.*,[8] 8 mL total blood was taken out from the healthy volunteers and quickly divided into 10 different preweighed sterile microcentrifuge tubes (500 µL of total blood per each tube) and incubated at 37°C for 45 min to form the natural clot. Plasma was gently removed out by a micropipette. We weighted the tube that contains clot before incubating with 100 µL of total or butanolic extracts of PD with the range of dose 0.5, 1, 2, 5 mg/mL, respectively. DMSO 0.1% and streptokinase 7.500 UI were added into two other tubes and served as a negative and positive thrombolytic control. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. We removed again the whole liquid after incubating with thrombolytic agents. Percentage of clot lysis was calculated as follows:

$$\text{Clot lysis}\,(\%) = \left(1 - \frac{\text{the weight of tube after the clot lysis} - \text{the weight of tube}}{\text{the weight of tube before the clot lysis} - \text{the weight of tube}}\right) \times 100\%$$

Data analysis

The experimental results were expressed as the mean ± standard error. A one-way analysis of variance was used for multiple comparisons followed by LSD test. A $P \leq 0.05$ was considered statistically significant.

RESULTS

Saponin constituents of *Panax bipinnatifidus* Seem

Isolation and structural elucidation of saponins

The roots of PD were extracted, partitioned, and then followed by combined column chromatography to yield two saponins, whose structures were identified on the basis of extensive spectroscopic analyses and comparison with reported data.

Compound **1**, a white powder, has the specific rotation ($[\alpha]^{25}_{D}$) of + 7.5 (c 0.2, MeOH) and reacted with 10% H_2SO_4 under heating to appear pink then turned into purple typical of triterpene saponin. The ^{1}H and ^{13}C NMR spectra of **1** showed the features of anoleanane-type saponin in agreement with the reported saponin components from PD.[6] In detail, There were the presence of seven singlet signals of tertiary methyl groups at δ 0.87, 0.90, 0.98, 1.00, 1.00, 1.11, and 1.22 (7× CH_3, all s, CH_3-25, 26, 24, 23, 30, 29, 27) and a double bond at C-12/C-13 at δ 5,32 (1H, brs, H-12) in the ^{1}H NMR spectrum of **1**. In addition, the clustered signals at δ 3.0–4.0 in the ^{1}H NMR spectrum suggested the occurrence of oxymetin and oxymethylen groups of four sugar components of a glucuronic acid (GluA), two glucose (Glc), and a arabifuranose (Ara[f]), which were confirmed by the presence of the four anomeric proton signals at δ 4.42 (1H, d, J = 7.6 Hz, H-1′), 4.92 (1H, d, J = 8.0 Hz, H-1″), 5.25 (1H, brs, H-1‴), and 5.45 (1H, d, J = 8.0 Hz, H-1⁗). The ^{13}C NMR spectrum revealed the appearance of 53 carbon signals including 23 signals of four sugar units (GluA, 2×Glc, and Ara[f])[7] and other 30 carbons belonging to the aglycon moiety of oleanoic acid with an oxymethyl signal at δ 89.3 (C-3), a typical double bond C-12/C-13 at δ 123.1 and 143.4, and a carbonyl carboxylic group at δ 176.7 (C-28).[6,9] Furthermore, the ESI-MS spectrum of **1** showed amolecular ion peak [M + H] + at m/z 1089 in consistent with the molecular formula $C_{53}H_{84}O_{23}$ (M = 1088). According to the above evidence and in comparison with the reported NMR spectral data,[10] the compound **1** was identified as stipuleanoside R2 [Figure 1], which has not been reported from PB Seem. to date. However, stipuleanoside R2 methyl ester was declared from the title plant in a previous research.[6]

Compound **2**, also a white powder, was isolated from the BuOH fraction of PD. Similarity to **1**, the ^{1}H and ^{13}C NMR spectra of **2** revealed its aglycon/sapogeninmoiety of oleanolic acid as evidenced by the double bond signals at C-12/C-13 (δ 122.2 [C-12], 143.2 [C-13], and 5.22 [1H, br s, H-12]), an oxymethine group at δ 89.6 (C-3), and a carbonyl carboxylic signal at δ 176.4 (C-28), at which linked with a sugar chain on the basis of its chemical shift moving to upfiled.[9] The sugar components of **2** were different from those of **1**. The NMR spectra of **2** exhibited the occurrence of three sugar units including a GluA methyl ester, a Glc and an Ara (f) as confirmed by the presence of three anomeric protons at δ 4.35 (1H, d, J = 7.6 Hz, H-1′), δ 5.03 (1H, br s, H-1″) and δ 5.38 (1H, d, J = 8.0 Hz, H-1‴).[10] Moreover, a

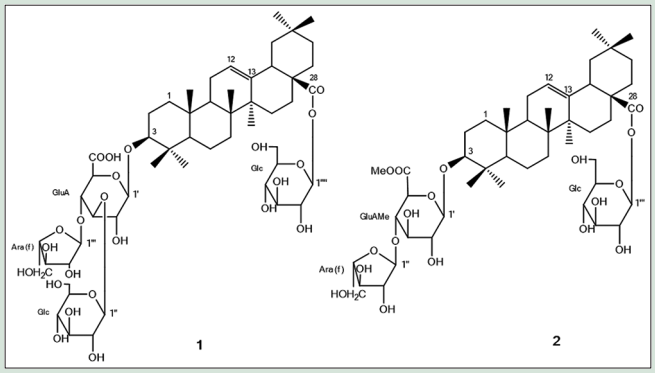

Figure 1: Chemical structures of the two isolated saponin from *Panax bipinnatifidus*

quasimolecular ion peak at m/z 941 (M + H) + in the ESI-MS spectrum of **2** was observed and supported the molecular formula $C_{48}H_{76}O_{18}$ (M = 940). On the basis of these findings, compound **2** was characterized as araloside A methyl ester [Figure 1].[10,11] To our best knowledge, this is the first isolation of araloside A methyl ester from PD.

High-performance liquid chromatographic fingerprint profile of the two isolated saponin

The two saponins (**1** and **2**) were isolated in high yields and are expected among marked compounds for quality control of PD so that we performed a fingerprinting of these two saponins in the PD roots. The results of HPLC analysis [Figure 2] showed that **1** and **2** occurred naturally in PD as evidenced by their respective retention times and co-injections. The relative peak areas of the two saponins in the chromatogram revealed that **1** and **2** were the principal saponins in the roots of PD growing in Vietnam and both the two saponins were able to be considered as marker compounds and chemical fingerprints of this title plant.

From the accumulated literature on *Panax* spp. including PD, saponins are the main bioactive constituents contributing to medicinal uses in the traditional medicine as well as the modern medicine.[12] Standardization of medicinal herbs and herbal products is based on qualitative/quantitative analyses of the major active ingredients playing as the marker compounds such as tanshinone IIA in Danshen (*S. miltiorrhiza*),[13] majonoside R2 in Vietnamese ginseng (*Panax vietnamensis*).[14] Subsequently, the present results of our study on the saponins components of the title plant contributed partly to the phytochemical database, quality control, and chemotaxonomy of PD and *Panax* sp.

Biological activity of *Panax bipinnatifidus*

Anticoagulant activity

The PT and the APTT are used commonly to evaluate coagulation disorders. While PT is performed to measure the extrinsic pathway, the APTT is used to investigate the intrinsic pathway.

Effect of PD on blood coagulation is presented in Table 1. Heparin, a positive anticoagulant agent, affects to the intrinsic pathway, but no longer to the extrinsic pathway, thus significantly prolonged the APTT in comparison to the negative control. The total extract had no effect to blood coagulation; only butanolic extract exhibited anticoagulant activity. At the highest dose, butanolic extract inhibited blood coagulation in both intrinsic and extrinsic pathways.

Antiplatelet aggregation

Both total and the butanolic extracts of PD exhibited significantly platelet aggregation inhibition in all tested doses (0.5, 1, 2, and 5 mg/mL). This effect linearly increased according to the dose. Butanolic extract tended to inhibit platelet aggregation stronger than the total extract. At the high dose of 5 mg/mL, this effect was better than that of aspirin 1 mg/mL.

Table 1: Effect of extractions of *Panax bipinnatifidus* on prothrombin time and activated partial thromboplastin time

Agent	Dose	*n*	PT (s)	APTT (s)
DMSO 0.1%		5	11.86±0.20	34.94±0.41
Heparin	200 IU/mL	5	11.82±0.28	56.94±1.36*
PB total	0.5 mg/mL	5	11.82±0.11	34.62±0.37
PB total	1 mg/mL	5	11.76±0.14	35.12±0.33
PB total	2 mg/mL	5	11.84±0.14	35.52±0.33
PB total	5 mg/mL	5	12.04±0.19	36.80±0.27
PB butanol	0.5 mg/mL	5	11.42±0.22	36.72±0.76
PB butanol	1 mg/mL	5	11.52±0.28	38.78±1.11
PB butanol	2 mg/mL	5	12.24±0.26	43.80±1.32*
PB butanol	5 mg/mL	5	14.34±0.51*	60.02±2.83*
P (ANOVA)			<0.01	<0.001

*P<0.05 in comparison to negative control DMSO 0.1%. PB: *Panax bipinnatifidus* seem; APTT: Activated partial thromboplastin time; PT: Prothrombin time; ANOVA: Analysis of variance

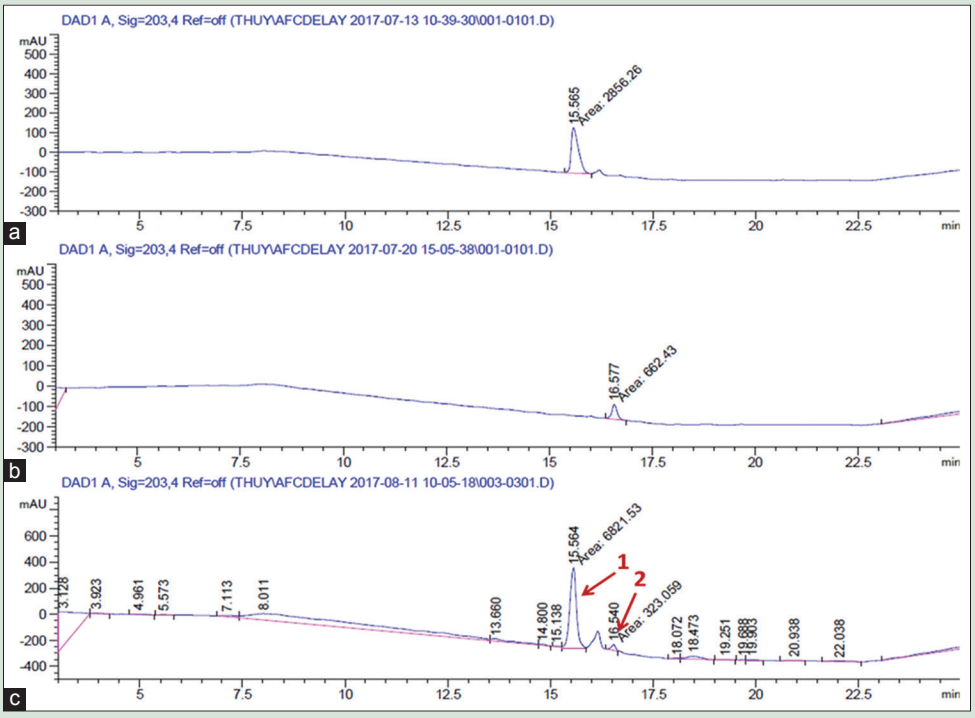

Figure 2: High-performance liquid chromatography chromatograms of the two isolated saponins, 1 (a) and 2 (b), and the saponin extract (c) from the roots of *Panax bipinnatifidius*

Clot lysis activity

The results in Table 2 expressed that no total neither butanolic extract of PD showed the thrombolysis effect via enhancing the percentage of clot lysis (%).

Table 2: Percentage of clot lysis (%) of *Panax bipinnatifidus*

Agent	Dose	*n*	Mean±SD
DMSO 0.1%		5	3.30±0.36
Streptokinase	7.500 IU/ml	5	34.37±1.87***
PB total	0.5 mg/mL	5	6.47±2.18
PB total	1 mg/mL	5	8.92±2.39
PB total	2 mg/mL	5	10.21±2.48
PB total	5 mg/mL	5	12.28±3.03
PB butanol	0.5 mg/mL	5	6.63±1.47
PB butanol	1 mg/mL	5	8.89±2.01
PB butanol	2 mg/mL	5	9.58±3.91
PB butanol	5 mg/mL	5	10.62±3.20
P (ANOVA)			>0.05

***$P<0.001$ in comparison to negative control DMSO 0.1%. PB: *Panax bipinnatifidus* seem; ANOVA: Analysis of variance; SD: Standard deviation

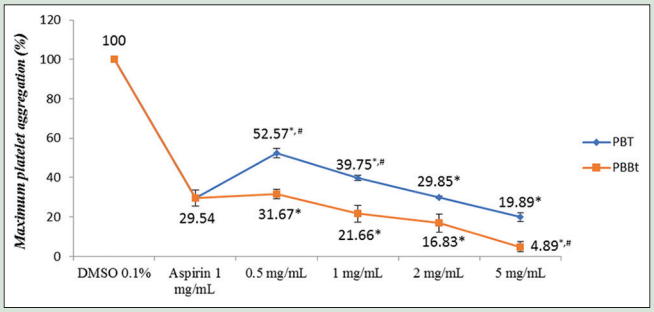

Figure 3: The maximum platelet aggregation of *Panax bipinnatifidus* PBT: Total extract of *Panax bipinnatifidus*; PBBt: Butanol extract of *Panax bipinnatifidus*; *: $P < 0.05$ in comparison to negative control DMSO 0.1%; #: $P < 0.05$ in comparison to positive control aspirin 1 mg/mL)

DISCUSSION

Panax genus is a large genus belonging to the *Araliaceae* family. Most of them have been extensively studied on both phytochemistry and biological activity, and it becomes evident and be noteworthy that *Panax* sp. exerts important pharmacological effects, such as immunomodulatory effect, antidiabetes, and antioxidant activities of *Panax ginseng* and *Panax notoginseng*.[12,15] Recently, antithrombotic and antiplatelet aggregation effects of *Panax* sp. tended to be markedly researched. Jin *et al.* studied the actions of Korean red ginseng extract (KRGE) on rat artery thrombosis *in vivo* and platelet aggregation *in vitro* and *ex vivo*.[16] The results showed that KRGE significantly inhibited ADP- and collagen-induced platelet aggregation *ex vivo* but not prolonged PT or APTT, indicating that the antithrombotic effect of the KRGE may be due to its antiplatelet aggregation rather than anticoagulation effect.[16] Compared the antiplatelet and anticoagulant effects of raw and steamed *P. notoginseng* with *P. ginseng* and *Panax quinquefolius*, Lau *et al.* realized both raw and steamed of *P. notoginseng* significantly inhibited platelet aggregation and plasma coagulation, but the steamed extract had more potent effects than the raw extract. Compared among three the *Panax* species, *P. notoginseng* showed the highest antiplatelet effect.[17] Coagulation and platelet aggregation are the corresponding factors of thrombosis forming. Agents that against or prevent thrombosis have an important role in decreasing heart attack or stroke. Many herbs exert the cardioprotective properties, such as *Ginseng* sp., *Allium sp.*, *Terminalia arjuna, Clematis species, Glycyrrhiza glabra, Ilex cornuta, Crataegus oxyacantha,* and *Astragalus membranaceus*. They are all saponin-enriched medicinal plants.[18] Some other natural polyphenols also have demonstrated beneficial effects in animal models of several cardiovascular disorders such as hypertension, atherosclerosis, endothelial dysfunction, dyslipidemia, and diabetes-related cardiovascular complications.[19,20]

Our study is the first evidence indicated the effect of PD on platelet aggregation, blood coagulation, and clot lysis. Saponins were demonstrated as the main components occurred in the butanolic extract were the main active compounds for these actions in blood. Figure 3 shows clearly effects of both total extract and the butanolic extract on ADP-induced platelet aggregation with various doses. Interestingly, butanolic extract expressed higher inhibition activity on ADP-induced platelet aggregation in comparison with total extract. These findings are in line with Lau *et al.* when studying the platelet aggregation effects of *P. notoginseng*, *P. ginseng*, and *P. quinquefolius* extracted by methanol with dose of 3, 3, and 1.5 mg/mL, respectively.[17] Different dose efficacy could be resulted from different saponin content in these extracts. Moreover, high dose of butanolic extract of PD seemed to prolong the PT and APTT that was not observed in total extract [Table 1]. To test whether extracts of PD have effect on blood clotting, we performed the blood clotting lysis assay. Although the effect did not reach significantly difference, it tended to increase of blood clotting lysis by dose-dependent manner [Table 2]. These results were in line with many other studies. Jin *et al.* performed experiments on rat arterial thrombosis model feeding with red ginseng extract found that ginsenosides isolated from red ginseng had thrombosis effects and mostly depend on antiplatelet aggregation rather than anticoagulation pathways.[15] This result was in consistent with other studies both on ADP-induced rat platelet aggregation assay[21] and on human platelet aggregation.[22,23] In our current study, the total saponins from the butanolic extraction seem to have potential beneficial effect for antithrombosis via antiplatelet aggregation. The platelet aggregation process is highly complex with the platelet activation process following by the release process.

In practice, there are several agonists that could induce platelet aggregation such as ADP, collagen, thrombin, thromboxane, or ristocetin. Among various agonists, ADP is most commonly used to examine the platelet aggregation assay *in vitro*. From our results, PD expressed clearly inhibition on ADP-induced human platelet aggregation. It could be explained by the modulation of intracellular signaling cascade by calcium homeostasis and P-selectin expression via mitogen-activated protein kinase and PI3K/Akt phosphorylation-dependent manner.[24] Whether saponin extracted from PD suppressed the ADP-induced platelet aggregation through this mechanism or not, further study needs to be elucidated. And also, the effects of isolated saponin components should be further tested to confirm that they are real active components.

CONCLUSION

From the roots of PD growing in Vietnam and by extraction, partition, and chromatographic separation, the two major saponins, stipuleanoside R2 (1) and araloside A methyl ester (2), were isolated for the first time and structurally identified by spectroscopic evidence. In addition, the HPLC fingerprint of the two saponins was carried out and contributed to quality control and chemotaxonomy of PD. In addition, the butanolic extraction of PD showed clearly beneficial effects on platelet aggregation at the tested doses and anticoagulation with high doses that implied the potential medicinal material for antithrombosis treatment.

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Conflicts of interest

There are no conflicts of interest.

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