BIOACTIVE SAPONINS FROM Panax bipinnatifidus Seem. GROWING IN VIET NAM

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Abstract. Panax bipinnatifidus Seem. belonging to the family Araliaceae has been used as a folk medicine in Viet Nam. Our ongoing study on bioactive compounds from Panax bipinnatifidus Seem. collected in Sapa, Lao Cai resulted in the isolation of two saponins together with the well-known saponin Stipuleanoside R2. Their structures were identified as 3-O-β-D-glucopyranosyl-(1→3)-β-D-glucuronopyranosyleanolic acid (1) and Stipuleanoside R1 (2) based on NMR spectroscopic data as well as comparison with the literature data. For the first time, 3-O-β-D-glucopyranosyl-(1→3)-β-D-glucuronopyranosyleanolic acid (1) was found in genus Panax. On biological screening, the crude extract and the principal saponin, stipuleanoside R2 showed weak inhibitory effect on markedly nitric oxide production in lipopolysaccharide-treated RAW 264.7 cells.

Keywords: Panax bipinnatifidus Seem., 3-O-β-d-glucopyranosyl-(1→3)-β-d-glucuronopyranosyleanolic acid, Stipuleanoside R1.

Classification numbers: 1.1.1, 1.2.1.

1. INTRODUCTION

Panax bipinnatifidus Seem., which is naturally distributed and growing in some North Western provinces of Viet Nam such as Lao Cai and Ha Giang, has been used as a medicinal
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plant and a functional food for many years [1, 2]. Up-to-date investigation on phytochemical profile of Panax spp. indicated the occurrence of saponins as main constituents. There are more than 300 saponins that have been found [3]. There are some studies on the chemical constituents and bioactivities in different parts of the Panax bipinnatifidus Seem. In our research, we have focused on the root of Panax bipinnatifidus Seem [4 - 8] and recently reported the two main saponins, stipuleanoside R2 and araloside A methyl ester. As part of our study course on the title plant, the present paper deals with identification of the two more saponins of 3-O-β-D-glucopyranosyl(1→3)-β-D-glucuronopyranosyloleanolic acid (1) and Stipuleanoside R1 (2). In addition, the preliminary evaluation on the anti-inflammamatory activity of the extract and the principle saponins of the title plants are also discussed in this paper.

2. MATERIALS AND METHODS

2.1. General procedures

Optical rotations were recorded on a DIP-360 digital polarimeter (JASCO, Easton, USA). NMR spectra were recorded on a JEOL ECX 400 FT-NMR spectrometer (JEOL, Tokyo, Japan) and a Bruker Avance 500 FT-NMR Spectrometer (BrukerSpin, Germany) with the internal standard of tetramethylsilane. ESI-MS spectra were recorded on an Agilent 1260 TripleQuad 6420 LC-MS/MS (Agilent Technologies, USA). Column chromatography utilized silica gel 60 (230–400 mesh, Nacalai Tesque Inc., Kyoto, Japan) and YMC ODS-A gel (50 μm, YMC Co. Ltd., Kyoto, Japan). TLC was performed on Kieselgel 60 F254 and TLC Silica gel 60 RP-18 F254S (Merck, Damstadt, Germany) plates. Spots were visualized by spraying with 1 % Ce(SO4)2-10 % aqueous H2SO4 solution, followed by heating.

2.2. Plant materials

The roots of Panax bipinnatifidus were collected in Sapa, Lao Cai in May 2019 and were taxonomically identified by Dr. Do Ngoc Dai, Department of Forestry, Nghe An University of Economics, Nghe An province, Viet Nam. A voucher specimen was kept at the Department of Agriculture Chemistry, Center for High Technology Development, Vietnam Academy of Science and Technology.

2.3. Extraction and isolation

The air-dried and powdered P. bipinnatifidus roots (900 g) were extracted with 80 % ethanol (EtOH) 3 times. The combined extracts were concentrated to give 350 g of crude extract.

The obtained EtOH residue (350 g) was suspended in water (H2O, 1000 mL), then partitioned successively with n-hexane, ethyl acetate (each 3 × 1000 mL), followed by removal of solvents, to obtain 82.50 g and 35.72 g of n-hexane and ethyl acetate residues, respectively. The water residue was filtered through a Diaion HP-20 column (Φ67 mm × 890 mm) with a gradient of H2O–ethanol (100:0→4:96, v/v, 2000 ml) to obtain 5 fractions (Fr.1–Fr.5) based on TLC monitoring.

Fr. 3 (670 mg) was further purified on a reversed-phase C18 column (Φ20 mm × 300 mm) with MeOH-H2O (1:1, v/v) to obtain stipuleanoside R2 (white powder, 40 mg) as reported in our previous paper [6]. Fr.4 (2.4 g) was chromatographed on a silica gel column with a gradient of CH2Cl2–MeOH – H2O– (90/10/0→0/100/1, v/v/v) to furnish 5 fractions (Fr.4.1–4.8). Fr.4.6 was
repeatedly chromatographed on a silica gel column with CH₂Cl₂–MeOH–CH₃COOH (90/10/1→0/100/1, v/v/v), to yield compound 1 (10 mg) and compound 2 (8.7 mg).

3-O-β-D-glucopyranosyl-(1→3)-β-D-glucuronopyanosyloleanolic acid (1): white powder; [α]²⁵_D = +12 (c 0.3, MeOH); ESI-MS: m/z 795.3 [M+H]⁺; ¹H-NMR (500 MHz, C₅D₅N): δ 0.86, 0.86, 0.91, 0.96, 0.96, 1.07, 1.07, 1.17 (7 × CH₃, all s, CH₃-25, 26, 24, 23, 30, 29, 27), 3.23 (1H, m, H-3), 4.41 (1H, d, J = 8.0 Hz, H-1ʹ), 4.69 (1H, d, J = 8.0 Hz, H-1ʺ), 5.24 (1H, br t, H-12, J = 3.0 Hz); ¹³C-NMR (125 MHz, C₅D₅N): see Table 1.

Stipuleanoside R1 (2): white powder; [α]²⁵_D = +12 (c 0.3, MeOH); ESI-MS: m/z 927.4 [M+H]⁺; ¹H-NMR (500 MHz, C₅D₅N): δ 0.85, 0.86, 0.91, 0.95, 0.96, 1.06, 1.16 (7 × CH₃, all s, CH₃-25, 26, 24, 23, 30, 29, 27), 3.23 (1H, m, H-3), 4.41 (1H, d, J = 8.0 Hz, H-1ʹ), 4.69 (1H, d, J = 8.0 Hz, H-1ʺ), 5.16 (1H, br s, H-1ʺʹ), 5.24 (1H, br t, H-12, J = 3.0 Hz); ¹³C-NMR (125 MHz, C₅D₅N): see Table 1.

Stipuleanoside R2: white powder; [α]²⁵_D = +7.5 (c 0.2, MeOH); ESI-MS: m/z 1089 [M+H]⁺; ¹H-NMR (400 MHz, CD₃OD): δ 0.87, 0.90, 0.98, 1.00, 1.00, 1.11, 1.22 (7 × CH₃, all s, CH₃-25, 26, 24, 23, 30, 29, 27), 3.20 (1H, m, H-3), 4.42 (1H, d, J = 7.6 Hz, H-1ʹ), 4.92 (1H, d, J = 8.0 Hz, H-1ʺ), 5.25 (1H, brs, H-1ʺʺ), 5.32 (1H, brs, H-12), 5.45 (1H, d, J = 8.0 Hz, H-1ʺʺ) [6].

2.4. Anti-inflammatory evaluation

2.4.1. Cell culture

The RAW 264.7 cells was obtained from the RIKEN BioResource Center Cell Bank and cultured at 37 °C in a 5 % CO₂ atmosphere in Dulbecco’s modified Eagle’s medium (DMEM) containing 10 % FBS (Fetal bovine serum). To stimulate the cells, the medium was replaced with fresh DMEM and then lipopolysaccharide (LPS) was added in the presence or absence of compounds to the indicated concentrations. Celastrol was used as positive control and assessed the test validity of the anti-inflammatory activity of the isolated compounds.

2.4.2. Cell viability assay

The cytotoxic effect of samples was determined by MTT assay. RAW 264.7 cells were plated in 96-well plates (2 × 10⁴ cells/well) and treated with or without the compounds before exposure to 50 ng/ml LPS. After 12 h of incubation, MTT solution was added to each well, and the cells were incubated for another 4 h. The resulting MTT–formazan product was lysed by adding 100 μl of 0.04 N HCl–isopropanol. The amount of formazan was determined by measuring the absorbance at 595 nm with a microplate reader. The results are expressed as the optical density ratio of the treatment to that of LPS [9, 10].

2.4.3. Measurement of NO/Griess assay

RAW 264.7 cells were plated at 4 × 10⁵ cells/well in 24-well plates and treated with or without the compounds before exposure to 50 ng/ml LPS. After 12 h of incubation, the level of nitrite (an indicator of NO synthesis) was measured using the Griess reaction. Briefly, the cell culture medium was mixed with an equal volume of Griess reagent (1 % sulfanilamide in 0.1 % naphthylethlenediamine dihydrochloride and 5 % phosphoric acid in water). The absorbance was measured with a microplate reader at 540 nm [9, 10].

2.4.4. Statistical analyses

The experimental results are presented as mean ± SE. Each experiment was repeated at least three times.
3. RESULTS AND DISCUSSION

3.1. Isolation and structural elucidation of the two saponins

The roots of *P. bipinnatifidus* were extracted, partitioned and then followed by combined column chromatography to yield two saponins in addition to stipuleanoside R2, whose structures were identified as shown in Figure 1 based on extensive spectroscopic analyses and comparison with reported data.

![Figure 1. The chemical structure of compounds 1 and 2.](image-url)

Compound 1 was obtained as a white powder. The $^1$H- and $^{13}$C-NMR spectra of 1 revealed the features of a typical oleanane-type saponin with signals of seven tertiary methyl groups, a double bond and oxygenated carbons C-3 and C-28 [5, 11, 12]. The $^1$H-NMR (500 MHz, CD$_3$OD) spectrum of the aglycone moiety of 1 showed 7 tertiary methyl groups at $\delta_H$ 0.86, 0.86, 0.91, 0.96, 0.96, 1.07, 1.17 that correlated in the heteronuclear single quantum coherence (HSQC) experiments with the carbon signals at $\delta_C$ 33.7, 29.0, 26.4, 24.0, 18.0, 17.0, and 15.9, respectively (Table 1). In addition, the signals at $\delta_H$ 5.24 (1H, t, $J = 3$ Hz, H-12) and $\delta_C$ 123.1 (C-12) and 145.9 (C-13) further supports the $\Delta^{12}$ oleanane skeleton [10, 11]. The $^1$H-NMR and $^{13}$C-NMR (500 MHz, MeOD) spectra of 1 showed the presence of two anomeric protons at $\delta_H$ 4.41 (1H, d, $J = 8.0$ Hz, H-1’), 4.69 (1H, d, $J = 8.0$ Hz, H-1”) and carbons at $\delta_C$ 106.3 and 104.9 respectively, accounting for a $\beta$-D-glucuronic acid GlcA(I) and a $\beta$-D-glucopyranosyl(II) units [12]. The carbon signal C-28 not observed in the $^{13}$C-NMR could be explained by low sample concentration and this phenomenon has been reported in the literature. Overall assignments of all the protons in each sugar unit were achieved by double-quantum filtered correlation spectroscopy (DQF-COSY) and heteronuclear multiple bond correlation (HMBC) spectra, starting from their respective anomeric proton signals. In addition, the HMBC spectrum assured a sugar chain at the C-3. Furthermore, the sugar sequence at C-3 was evidenced by the HMBC cross-peaks of H-1’GlcA(I) ($\delta_H$ 4.41)/C-3 ($\delta_C$ 90.8), H-3 ($\delta_H$ 3.23)/C-1’GlcA(I) ($\delta_C$ 106.3), H-3’GlcA(I) ($\delta_H$ 3.63)/C-1”Glc(II) ($\delta_C$ 104.9) and H-1”Glc(II) ($\delta_H$ 4.69)/C-3’GlcA(I) ($\delta_C$ 86.4) (Figure 2).

HMBC spectra also showed that an anomeric proton at $\delta_H$ 4.69 had long-range coupling with the $^{13}$C peak at $\delta_C$ 86.4, indicating that glucuronic acid connected to one glucose at C-3’ [13]. Using CD$_3$OD as a solvent, some carbon signals were not observed in $^{13}$C-NMR spectra. Using C$_5$D$_3$N as a solvent, the $^{13}$C-NMR spectra exhibited one carboxyl group at $\delta_C$ 179.5 (C-
and one carboxyl group at 171.7 (C-6’’). Consequently, compound 1 was deduced as 3-O-β-D-glucopyranosyl-(1→3)-β-D-glucuronopyranosyleanolic acid, an oleanane-type saponin isolated for the first time from the genus *Panax* [14].

Table 1. $^{13}$C-NMR (C$_5$D$_5$N, 125 MHz) data for compounds 1 and 2.

| Carbon | 1$^{ab}$ (δC, DEPT) | 2$^{ab}$ (δC, DEPT) | 1$^{ab}$ (δC, DEPT) | 2$^{ab}$ (δC, DEPT) |
|--------|----------------------|----------------------|----------------------|----------------------|
| Aglycone | Sugar Moiety | 3-GluAMe (I) | 28-Glc (II) | 28-Glc (II) |
| 1 | 39.8 CH$_2$ | 39.8 CH$_2$ | 106.3 CH | 106.4 CH |
| 2 | 26.8 CH$_3$ | 26.9 CH$_2$ | 72.2 CH | 76.5 CH |
| 3 | 90.8 CH | 90.9 CH | 86.4 CH | 81.9 CH |
| 4 | 40.2 C | 40.2 C | 76.7 CH | 79.3 CH |
| 5 | 57.1 CH | 57.1 CH | 75.4 CH | 78.0 CH |
| 6 | 19.4 CH$_2$ | 19.4 CH$_2$ | ND* | 176.5 C |
| 7 | 34.1 CH$_2$ | 34.0 CH$_2$ | 28-Glc (II) | 28-Glc (II) |
| 8 | 40.6 C | 40.6 C | 104.9 CH | 104.4 CH |
| 9 | 49.2 CH | 49.2 CH | 75.3 CH | 75.6 CH |
| 10 | 37.9 C | 37.9 C | 77.8 CH | 78.4 CH |
| 11 | 24.6 CH$_2$ | 24.5 CH$_2$ | 71.3 CH | 71.1 CH |
| 12 | 123.1 CH | 123.1 CH | 78.1 CH | 78.2 CH |
| 13 | 145.9 C | 145.9 C | 62.3 CH$_2$ | 63.3 CH$_2$ |
| 14 | 43.0 C | 43.0 C | Ara(f) (III) | |
| 15 | 28.5 CH$_2$ | 28.5 CH$_2$ | 108.3 CH | |
| 16 | 24.0 CH$_2$ | 24.1 CH$_2$ | 82.2 CH | |
| 17 | 48.5 C | 48.5 C | 75.4 CH | |
| 18 | 43.0 CH | 43.1 CH | 87.2 CH | |
| 19 | 47.7 CH$_2$ | 47.7 CH$_2$ | 62.3 CH$_2$ | |
| 20 | 31.7 C | 31.7 C | | |
| 21 | 35.2 CH$_2$ | 35.2 CH$_2$ | | |
| 22 | 33.7 CH$_2$ | 33.7 CH$_2$ | | |
| 23 | 29.0 CH$_3$ | 29.1 CH$_3$ | | |
| 24 | 17.0 CH$_3$ | 17.0 CH$_3$ | | |
| 25 | 15.9 CH$_3$ | 15.9 CH$_3$ | | |
| 26 | 18.0 CH$_3$ | 18.0 CH$_3$ | | |
| 27 | 26.4 CH$_3$ | 26.4 CH$_3$ | | |
| 28 | ND* | ND* | | |
| 29 | 33.7 CH$_3$ | 33.7 CH$_3$ | | |
| 30 | 24.0 CH$_3$ | 24.2 CH$_3$ | | |

$^a$ Measured in Pyridine-$d_5$ and at 125 MHz. $^b$ Assignments were established by DEPT, HMQC, DQF-COSY, and HMBC spectra. ND: Not determined.
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Figure 2. The chemical structure of compound 1 with HMBC correlations (arrows).

Compound 2 was also obtained as a white powder. The NMR data suggested that compound 2 belongs to oleanane-type triterpene saponin that is commonly reported in *P. bipinnatifidus* Seem. The signals of thirty carbons in the aglycone of 2 were similar to those of 1 which indicated compounds 1 and 2 containing the same aglycone with the features of oleanane-type saponin with typical signals of methyl groups (especially tertiary methyl), a double bond, and oxygenated carbons of C-3 and C-28 [5, 11, 12]. The $^1$H-NMR spectrum 2 showed similar signals to compound 1 with seven singlet methyl groups at $\delta_H$ 0.85, 0.86, 0.91, 0.95, 0.96, 1.06, 1.16 (s, CH$_3$-29, 23, 27, 30, 26, 24, 25). Another feature was the proton signals at $\delta_H$ 5.25 (1H, t, $J = 3.0$ Hz, H-12) and the carbon signals at $\delta_C$ 121.9 (C-12) and 145.4 (C-13). Furthermore, of 47 carbon signals, apart from 30 carbon atoms belonging to the aglycone, seventeen remaining carbon atoms were account for a sugar chain of three sugar units at $\delta_C$ 90.9 (C-3). Furthermore, the presence of a carbonyl carbon ($\delta_C$ 176.5) suggested a glucuronic acid. Anomeric carbon signal ($\delta_C$ 108.3) was characterized for an arabinofuranosyl group and another monosaccharide was proposed to be a glucopyranosyl unit by the array of six carbon signals ($\delta_C$ 104.4, 75.6, 78.4, 71.1, 78.1, 63.3). Hence, compound 2 was identified as Stipuleanaside R1 on the basis of NMR spectroscopic data as well as comparison with those reported in the literature [11].

3.2. Anti-inflammatory screening of the extract and compounds from the roots of *P. bipinnatifidus*

Together with our previous studies, it becomes evident that the oleanane-type saponins are major components in the roots of *P. bipinnatifidus*. The HPLC analyses supported that saponin content in *P. bipinnatifidus* is relatively rich in the roots and, especially, stipuleanoside R2 is more than 2 % (wt/wt) [6].

To evaluate the anti-inflammatory potential of the title plant, the extract and stipuleanosid R2 were screened for whether an inhibitory effect on nitric oxide (NO) production in LPS (lipopolysaccharide)-treated RAW 264.7 cells using the Griess reagent [9, 10]. The samples were first screened for the cytotoxicity against RAW 264.7 cells using MTT assay. Cell viability was not affected by concentration below 100 μM (for compound) and 100 μg/ml (for extract) (data not shown). Therefore, it supported to treat the cells with the samples from *P. bipinnatifidus* at concentrations below 100 μM/μg for testing their anti-inflammatory effects. It is noteworthy
that both the crude extract and stipuleanoside R2 relatively reduced NO production (Figure 3). Although their inhibition rates were not strong, the crude extract with high yield (20%, wt/wt) and the high concentration of stipuleanoside R2 contributed in part to biological and pharmacological evidence for pharmaceutical basis and medicinal use of P. bipinnatifidus.

![Figure 3](image)

**Figure 3.** Effect of the crude extract and stipuleanoside R2 on LPS-induced production of NO in RAW 264.7 cells.

The principal occurrence of bioactive oleanane-type saponins in P. bipinnatifidus contributes to phytochemical database of ginseng associated with both chemotaxonomic and pharmacological meanings.

### 4. CONCLUSIONS

*P. bipinnatifidus* is an important medicinal plant of the North West region and the title plant has been ongoing researched to develop applications in modern medicine and pharmacy. The study of bioactive ingredients is very important because the use of medicinal herbs in modern medicine is based on the scientific basis of the active ingredient. The present study resulted in the new scientific information regarding both phytochemical compounds and the biological data. It is noteworthy that 3-O-β-D-glucopyranosyl-(1→3)-β-D-glucuronopyranosyloleanolic acid (I) was isolated for the first time in genus *Panax*.

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**Declaration of competing interest.** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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