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Isolation, identification, and quantification of triterpene saponins in the fresh fruits of *Panax notoginseng*

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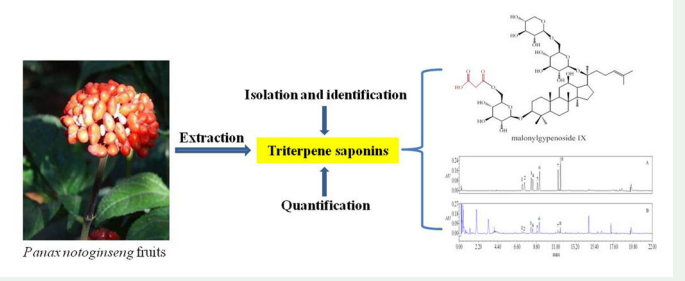
ABSTRACT

This study is to develop a method for isolation, identification, and quantitative determination of dammarane-type triterpene saponins in the *Panax notoginseng* fruits (PNF). The saponins were isolated by a serious of chromatographic methods, and their structures were elucidated on the basis of spectroscopic evidence and comparison with those of literature reports. Quantitative assay was performed on an ultra-performance liquid chromatography-UV (UPLC-UV) method. As a result, 22 saponins were isolated from the extract of PNF, among them, compound **1** was a new saponin, named as malonylgypenoside IX, compounds **3**–**10**, and **14–18** were isolated from the PNF for the first time. As to quantitative analysis, the calibration curves showed good linearity ($r > 0.998$) within the concentration range, and the method validation provided good reproducibility and sensitivity for the quantification of eight major saponins with precision and accuracy of less than 3.0%.



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Panax notoginseng; fruits; saponins; NMR; quantification

1. Introduction

Panax notoginseng (Burk.) F. H. Chen is a species of the genus *Panax*, family Araliaceae, and has been widely used as a traditional Chinese medicine for the treatment of cardiovascular diseases (Guo et al. 2010). *Panax notoginseng* saponins (PNS) are the major bioactive constituents of *P. notoginseng* (Wang et al. 2006; Jin et al.

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2017; Huang et al. 2019a). The known PNS are divided into 3 major groups due to the structure differentiation of sapogenins, protopanaxadiol (PPD), protopanaxatriol (PPT), and C-17 side-chain varied type (Yang et al. 2014). The majority of previous isolation and quantification studies have focused on the roots and leaves parts of *P. notoginseng* (Sakah et al. 2013; Liu et al. 2017; Gu et al. 2018; Xu et al. 2019), however, there are very few research on the *P. notoginseng* fruits (PNF) (Shi et al. 2010). In the interest of further exploitation and utilization of this precious medicinal plant, we recently reported six malonyl-substituted ginsenosides, which were isolated from the H_2O fraction of PNF extraction (Qu et al. 2021). A continuation of phytochemical investigation on PNF led to the isolation of another new malonyl-substituted ginsenoside, named as malonylgypenoside IX (**1**), along with 21 known saponins. Meanwhile, an ultra-performance liquid chromatography-UV (UPLC-UV) method was successfully applied to the quantification of eight major saponins in PNF.

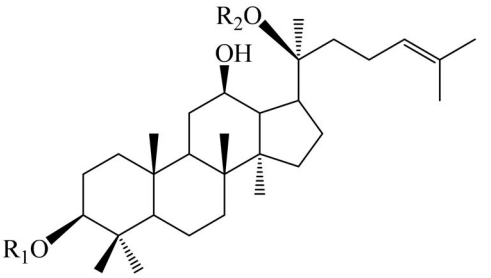
2. Results and discussion

2.1. Extraction, isolation, and identification of saponins

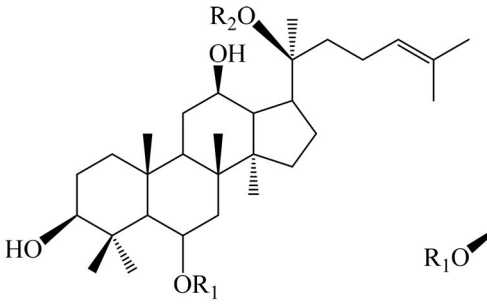
In MS^1 spectra, the deprotonated molecular ion $[M–H]^-$ in negative ion mode provided accurate molecular weight, and in MS^2 spectra, ginsenosides can be rapidly identified through the sapogenin ions at *m/z* 459 (PPD-type) and 475 (PPT-type), together with the loss of 162 Da (glucose), 146 Da (rhamnose), and 132 Da (xylose or arabinose) sugar units. On this basis, the saponins were purified from PNF with LC-MS guided, a scheme of the total procedure was as follows: First of all, the PNS were extracted by 85% aqueous EtOH, and then enriched by liquid-liquid extraction with *n*-butanol (*n*-BuOH), after that, the saponins were further isolated by a serious of chromatographic methods, eventually, their structures were established by MS and NMR spectroscopies, and comparison with those of literature date.

Following the approach described above, 22 saponins were separated from the *n*-butanol fraction of PNF extraction (Figure 1). Among them, malonylgypenoside IX (**1**), a new acidic saponin, which was belonged to a malonyl-substituted ginsenoside. It is noteworthy that malonyl-substituted ginsenosides commonly acylation on the appended sugar moieties and widely distributed in fresh or dried *Panax* genus (Wan et al. 2015; Shi et al. 2018), and exhibit potential antidiabetic activity (Liu et al. 2009; Liu et al. 2013; Qiu et al. 2017). However, malonylginsenosides are thermal instability with hydrolysis or decarboxylation to their respective neutral ginsenosides or acetylated ginsenosides, therefore, they are prone to structure transformation during extraction and separation process, and the isolated malonylginsenosides were probably much lower than actually occurs (Shi et al. 2018). Up to now, no more than 30 malonylginsenosides were isolated from *Panax* species, thus, the isolation of this new malonylginsenoside is quite significant for studying the diversity of their structures and laying the foundation for further research on other malonylginsenosides.

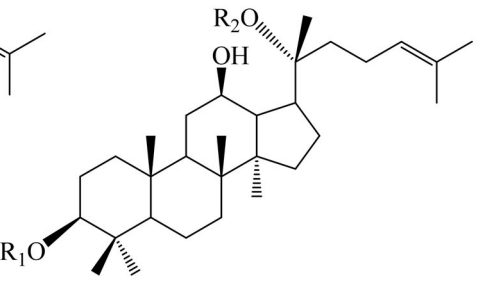
For the rest of 21 known saponins, including 20(*R*)-ginsenoside Rg_3 (**2**) (Li and Gong 2019), ginsenoside Mc (**3**) (Han et al. 2007), notoginsenoside Fe (**4**) (Yang et al. 1983), gypenoside IX (**5**) (Takemoto et al. 1983), gypenoside XIII (**6**) (Takemoto et al. 1983), notoginsenoside ST-4 (**7**) (Pei et al. 2011), notoginsenoside Ft_1 (**8**) (Chen et al. 2006), notoginsenoside K (**9**) (Ma et al. 1999), ginsenoside Rb_2 (**10**) (Zhou et al. 2016),



	R_1	R_2
1	(6-Malonyl)Glc	Xyl(1⟶6)Glc
3	H	Ara(*f*)(1⟶6)Glc
4	Glc	Ara(*f*)(1⟶6)Glc
5	Glc	Xyl(1⟶6)Glc
6	H	Xyl(1⟶6)Glc
7	Xyl(1⟶2)Glc(1⟶2)Glc	H
9	Glc(1⟶6)Glc	Glc
10	Glc(1⟶2)Glc	Ara(*p*)(1⟶6)Glc
11	Glc(1⟶2)Glc	Glc
12	Glc(1⟶2)Glc	Xyl(1⟶6)Glc
13	Xyl(1⟶2)Glc(1⟶2)Glc	Xyl(1⟶6)Glc
14	Xyl(1⟶2)Glc(1⟶2)Glc	Ara(*f*)(1⟶6)Glc
15	Glc(1⟶2)Glc	Ara(*f*)(1⟶6)Glc
16	Xyl(1⟶2)Glc(1⟶2)Glc	Glc(1⟶6)Glc
17	Xyl(1⟶2)Glc(1⟶2)Glc	Xyl(1⟶6)Glc(1⟶6)Glc
18	Xyl(1⟶2)Glc(1⟶2)Glc	Xyl(1⟶5)Ara (*f*)(1⟶6)Glc
19	Glc(1⟶2)Glc	Glc(1⟶6)Glc



	R_1	R_2
20	Xyl(1⟶2)Glc	Glc
21	Glc	Glc
22	Rha(1⟶2)Glc	Glc



	R_1	R_2
2	Glc(1⟶2)Glc	H
8	Xyl(1⟶2)Glc(1⟶2)Glc	H

Figure 1. The structures of compounds **1**–**22**. Glc, β-D-glucopyranosyl; Xyl, β-D-xylopyranosyl. Ara (*p*), α-L-arabinopyranosyl; Ara (*f*), α-L-arabinofuranosyl.

ginsenoside Rd (**11**) (Zhou et al. 2016), ginsenoside Rb_3 (**12**) (Sanada and Shoji 1978), notoginsenoside Fc (**13**) (Yang et al. 1983), notoginsenoside FP_2 (**14**) (Wang et al. 2008), ginsenoside Rc (**15**) (Zhou et al. 2016), notoginsenoside Fa (**16**) (Huang et al. 2019b), notoginsenoside D (**17**) (Yoshikawa et al. 1997), notoginsenoside S (**18**) (Yoshikawa et al. 2003), ginsenoside Rb_1 (**19**) (Zhou et al. 2016), notoginsenoside R_1 (**20**) (Zhou et al. 1981), ginsenoside Rg_1 (**21**) (Nagai et al. 1971), ginsenoside Re (**22**) (Sanada et al. 1974). As far as we know, compounds **3–10**, and **14–18** were obtained from PNF for the first time.

Compound **1** was obtained as a white amorphous powder, the molecular formula of $C_{50}H_{82}O_{20}$ was determined by the HRESIMS at *m/z* 1001.53448 $[M–H]^-$ (calcd for $C_{50}H_{81}O_{20}$, 1001.53267) with 10 degrees of unsaturation. The characteristic fragmentation pattern was identified as 1001 $[M–H]^-$, $^{44Da}\bar{}_{44Da}$957 $[M–H]^-$, $^{42Da}\bar{}_{42Da}$915 $[M–H]^-$, $^{132Da}\bar{}_{132Da}$783 $[M–H]^-$, $^{162Da}\bar{}_{162Da}$621 $[M–H]^-$, and $^{162Da}\bar{}_{162Da}$459 $[M–H]^-$, indicating the molecule was belonged to a PPD sapogenin. Analysis of the ^{13}C NMR and DEPT spectra showed the presence of 50 carbon resonances, including 3 carbon signals for a malonyl group, 17 carbons for 3 sugar moieties and 30 carbons for aglycone. The ^1H NMR date of **1** included 8 methyls [δ_H 0.81 (3H, s), 0.96 (6H, s), 0.98 (3H, s), 1.31 (3H, s), 1.62 (3H, s), 1.66 (6H, s)], one olefinic proton [δ_H 5.33 (1H, t-like)], and 3 anomeric protons [δ_H 4.92 (1H, d, $J = 7.5$ Hz), δ_H 5.16 (1H, d, $J = 7.5$ Hz), δ_H 5.01 (1H, d, $J = 7.3$ Hz)]. The NMR data (Table S1) of **1** were similar to those of gypenoside IX (Takemoto et al. 1983) except for an additional malonyl substituent, including 2 carbonyl groups at δ_C 168.1 and δ_C 169.6 and 1 methylene [δ_C 42.8/δ_H 3.82 (2H, d, $J = 6.3$ Hz)], further verified by the HMBC correlations from δ_H 3.82 to δ_C 168.1. Malonylation were observed at 6′-OH of the 3-Glc by the long-range HMBC correlation from H-6′ [δ_H 5.14 (1H, m)] to the carbonyl carbon (δ_C 168.1) (Figure S1), representing the sigals of C-5′ shifts to upfield with 3.3 ppm and C-6′ shifts to downfield with 2.2 ppm compared with those of gypenoside IX. The glycosyl moieties of **1** were confirmed to be two β-D-glucopyranosyl and one β-D-xylopyranosyl due to the coupling constant [(H-1′, $J = 7.5$ Hz), (H-1″, $J = 7.5$ Hz), (H-1‴, $J = 7.3$ Hz)] and UPLC analysis of the acid hydrolysate. Furthermore, the linkages of sugar units were proved by the long-range HMBC correlations from H-1′ to C-3 (δ_C 89.2), H-1″ to C-20 (δ_C 83.4), and H-1‴ to C-6″ (δ_C 70.1). The relative configuration of 3-OH and C-20 were deduced to be β and *S* according to the ROESY correlations from H-3 [δ_H 3.38 (dd, $J = 11.6$, 4.1 Hz)] to H-5 (δ_H 0.77, m) and H-17 (δ_H 2.59, m) to H-21 (δ_H 1.66, s), respectively. Based on the evidence above, the structure of compound **1** was established as 3-*O*-[(6-*O*-malonyl)-β-D-glucopyranosyl]-(3β,12β,20*S*)-trihydroxydammarane-24-ene-20-*O*-β-D-xylopyranosyl(1→6)-β-D-glucopyranoside, and named as malonylgypenoside IX.

2.2. Quantification of saponins

2.2.1. Optimization of UPLC separation

A good UPLC conditions was required for obtaining chromatograms with high resolution of adjacent peaks in a short time. The most common organic solvent of the

mobile phase for the separation of saponins was methanol or acetonitrile, however, no satisfied separation was found when the organic solvent was methanol, so the acetonitrile was chosen as organic solvent, and the resolution was improved. Phosphoric acid, phosphorate buffer, and acetic acid were also added to the aqueous phase for optimizing the separation, the results indicated that phosphoric acid could show higher resolution and better peak shapes. The column temperature was maintained at 35 °C for its good baseline resolution and suitable analysis duration. Under the above optimization conditions, a good separation of the saponins in mixed standards and sample was shown in Figure S11.

2.2.2. Quantitation of eight major saponins in PNF

The developed UPLC-UV method was applied to quantitative determination of the eight major saponins in PNF, and giving the dry weight contents of 2.04 ± 0.07 mg/g for notoginsenosides FP_2, 1.57 ± 0.11 mg/g for ginsenoside Rb_1, 2.99 ± 0.14 mg/g for notoginsenoside Fc, 2.02 ± 0.13 mg/g for ginsenoside Rc, 1.76 ± 0.12 mg/g for ginsenoside Rb_2, 3.01 ± 0.14 mg/g for ginsenoside Rb_3, 0.88 ± 0.15 mg/g for notoginsenoside Fe, and 0.94 ± 0.12 mg/g for gypenoside IX, respectively. The result showed that the major saponins were PPD type in PNF, which were similar to those of *P. notoginseng* leaves (Liu et al. 2018).

2.2.3. Validation of UPLC-UV method

As shown in Table S2. The calibration curves showed good linearity ($r > 0.998$) in a relatively wide concentration range. The limit of detection (LOD) and limit of quantification (LOQ) were in the range of 2.38–3.4 μg/mL and 5.13–8.5 μg/mL, respectively. These results revealed that the method exhibited good sensitivity. The relative standard deviation (RSD) values of intra-day and inter-day precision were less than 3.0% for all the analytes, which indicated that the precision of the analytical method was excellent. The repeatability of the method was good with a RSD of $< 3.0\%$. The recoveries of the method were in the range of 93.9–106.8%, with RSD value less than 3.0% as shown in Table S3, which suggested the good accuracy of this method.

3. Experimental

3.1. General experimental procedures

One-dimensional (1 D) and two-dimensional (2 D) nuclear magnetic resonance (NMR) spectra were determined on Bruker Avance Neo 600 MHz NMR spectrometer (Bruker Co., Fällanden, Switzerland) with tetramethylsilane as an internal standard. IR spectra were run on Bruker Vertex 70 FT-IR spectrophotometer (Bruker Co., Ettlingen, Germany). The electrospray ionization mass spectrometry (ESI-MS) and high resolution electrospray ionization mass spectrometry (HRESIMS) spectra were obtained from an Agilent 1200 HPLC with a 6300 Ion-trap liquid chromatography-mass spectrophotometry and a Vanquish UHPLC instrument connected to the Orbitrap Fusion mass spectrometer. Medium pressure liquid chromatography (MPLC) system was composed of a C-660 fraction collector (Büchi), two C-601 pumps (Büchi), and a RP-C_{18} column (Merck, 40-63μm, 49 × 460 mm). Reversed-phase semi-preparative HPLC was performed

on a Prostar/Dynamax system consisting of a Prostar 325 UV-Vis detector and two Varian PS-218 pump, UV detection was carried out at 203 nm with a flow rate of 3 mL/min. Column chromatographies (CC) were carried out on silica gel (200-300 mesh, Qingdao Marine Chemical Group Co., Qingdao, China), RP-C_{18} (Merck Co., 40-63 μm, Damstadt, Germany). Thin-layer chromatography (TLC) was carried out on silica gel 60 RP-18 F_{254s} and silica gel 60 F_{254}. Spots were detected under UV light or by spraying with 10% sulfuric acid in ethanol (EtOH) followed by heating at 105 °C.

3.2. Plant material

The fresh fruits of *Panax notoginseng* (Burk.) F. H. Chen were collected from Wenshan prefecture, Yunnan province, China in December 2018, and authenticated by Prof. Peihe Zheng. A voucher specimen (No. FN20181225) was deposited at Institute of Special Wild Economic Animals and Plants, Chinese Academy of Agricultural Sciences.

3.3. Extraction and isolation

The fresh fruits of *P. notoginseng* (18 kg) were rubbed with appropriate amount H_2O and removed the seeds, after filtration, the remaining residue was extracted twice in 85% EtOH-H_2O at 25 °C. Combined the aqueous and EtOH solutions and evaporated under vacuum at < 40 °C, the residue (605 g) was dissolved in H_2O and partitioned successively with petroleum ether (PE, 60–90 °C) and *n*-BuOH. The *n*-BuOH fraction (165 g) was separated via silica gel CC with a gradient of CH_2Cl_2-MeOH-H_2O (20:1:0.1-1:1:0.1, v/v) to afford 17 fractions (Frs.1–17), Fraction 5 (26.5 g) was separated by MPLC system (MeOH-H_2O, 40:60 − 90:10, 6 mL/min) to yield 6 fractions (Frs.5.1 − 5.6). Fraction 5.5 (6.1 g) was purified via RP-C_{18} CC again with a constant gradient of MeOH-H_2O (60:40, v/v) to yield compounds **2** (14 mg), **3** (22 mg), and **8** (25 mg). Fraction 5.4 (4.3 g) was isolated by semi-preparative HPLC on a XBridge BEH C_{18} OBD column (10 × 250 mm, 5 μm; Waters) with a constant gradient of CH_3CN-H_2O (45:55, v/v) to afford compounds **4** (51 mg, t_R = 8.9 min), **5** (42 mg, t_R = 10.8 min), **11** (30 mg, t_R = 6.3 min), and **12** (46 mg, t_R = 5.2 min). Fraction 5.2 (2.9 g) was isolated by semi-preparative HPLC on a Gemini C_{18} Column (10 × 250 mm, 5 μm; Phenomenex) with a constant gradient of CH_3CN-H_2O (45:55, v/v) to afford compounds **6** (56 mg, t_R = 8.9 min) and **7** (32 mg, t_R = 10.8 min). Fraction 11 (23.4 g) was separated by MPLC system (MeOH-H_2O, 30:60 − 90:10, 6 mL/min) to yield 8 fractions (Frs.11.1 − 11.8). Fraction 11.4 (12.2 g) was isolated by semi-preparative HPLC on a Gemini C_{18} Column (10 × 250 mm, 5 μm; Phenomenex) with a constant gradient of CH_3CN-H_2O (35:65, v/v) to afford compounds **9** (15 mg, t_R = 23.0 min) and **14** (73 mg, t_R = 16.4 min). Fraction 11.6 (2.6 g) was isolated by semi-preparative HPLC on a ZORBAX SB-C_{18} column (9.4 × 250 mm, 5 μm; Agilent) with a constant gradient of CH_3CN-0.05% TFA (43:57, v/v) to afford compounds **1** (21 mg, t_R = 12.2 min). Fraction 12 (6.7 g) was chromatographed over RP-C_{18}, eluted with MeOH-H_2O (28:72–80:20, v/v) to yield compounds **10** (24 mg), **15** (39 mg), and **19** (88 mg). Fraction 14 (17.4 g) was separated by MPLC system (MeOH-H_2O, 25:75–70:30, 6 mL/min) to yield 7 fractions (Frs.14.1–14.7). Fraction 14.3 (9.6 g) was purified by semi-preparative HPLC on a Gemini C_{18} Column (10 × 250 mm, 5 μm;

Phenomenex) with a constant gradient of CH_3CN-H_2O (30:70, v/v) to afford compounds **13** (38 mg, t_R = 33.2 min) and **16** (8 mg, t_R = 25.0 min). Fraction 14.5 (1.6 g) was then purified by semi-preparative HPLC on a ZORBAX SB-C_{18} column (9.4 × 250 mm, 5 μm; Agilent) with a constant gradient of CH_3CN-H_2O (30:70, v/v) to afford compounds **17** (18 mg, t_R = 8.4 min) and **18** (12 mg, t_R = 11.5 min). Fraction 16 (30.8 g) was separated by was repeated separation on RP-C_{18}, eluted with MeOH-H_2O (20:80–60:40, v/v) to yield compounds **20** (33 mg), **21** (18 mg), and **22** (12 mg).

3.3.1. Malonylgypenoside IX (1)

White amorphous powder; $[\alpha]_D^{25}$ +6.3 (c 0.1 MeOH); IR (KBr) ν_{max} 3401, 2946, 1752, 1676, 1385, 1168, 1077, 1041 cm^{-1}; HRESIMS *m/z* 1001.53448 $[M\text{-}H]^-$ (calcd for $C_{50}H_{81}O_{20}$, 1001.53267). ^1H NMR (600 MHz, pyridine-d_5, δ in ppm, *J* in Hz), δ_H: [1.74 (1H, m), 1.01 (1H, m), H-1], [2.33 (1H, m), 1.86 (1H, m), H-2], 3.38 (1H, dd, *J* = 11.6, 4.1 Hz, H-3), 0.77 (1H, m, H-5), [1.49 (1H, m), 1.36 (1H, m), H-6], [1.49 (1H, m), 1.21 (1H, m), H-7], 1.45 (1H, m, H-9), [2.04 (1H, m), 1.57 (1H, m), H-11], 4.21 (1H, m, H-12), 2.00 (1H, m, H-13), [1.57 (1H, m), 1.01 (1H, m), H-15], [1.86 (1H, m), 1.36 (1H, m), H-16], 2.59 (1H, m, H-17), 0.96 (3H, s, H-18), 0.81 (3H, s, H-19), 1.66 (3H, s, H-21), [2.42 (1H, m), 1.83 (1H, m), H-22], [2.63 (1H, m), 2.38 (1H, m), H-23], 5.33 (1H, t-like, H-24), 1.62 (3H, s, H-26), 1.66 (3H, s, H-27), 1.31 (3H, s, H-28), 0.96 (3H, s, H-29), 0.98 (3H, s, H-30), 4.92 (1H, d, *J* = 7.5 Hz, 3-Glc-1′), 4.05 (1H, m, 3-Glc-2′), 4.18 (1H, m, 3-Glc-3′), 4.09 (1H, m, 3-Glc-4′), 4.11 (1H, m, 3-Glc-5′), [5.14 (1H, m), 4.92 (1H, d, *J* = 7.5 Hz), 3-Glc-6′], 5.16 (1H, d, *J* = 7.5 Hz, 20-Glc-1″), 3.94 (1H, m, 20-Glc-2″), 4.22 (1H, m, 20-Glc-3″), 4.09 (1H, m, 20-Glc-4″), 4.09 (1H, m, 20-Glc-5″), [4.76 (1H, d, *J* = 10.9 Hz), 4.33 (1H, m), 20-Glc-6″], 5.01 (1H, d, *J* = 7.3 Hz, Xyl-1‴), 4.05 (1H, m, Xyl-2‴), 4.16 (1H, m, Xyl-3‴), 4.22 (1H, m, Xyl-4‴), [4.35 (1H, m), 3.70 (1H, m), Xyl-5‴], 3.82 (2H, d, *J* = 6.3 Hz, 6′-Mal). ^{13}C NMR (150 MHz, pyridine-d_5, δ in ppm), δ_C: 39.2 (C-1), 26.7 (C-2), 88.2 (C-3), 39.7 (C-4), 56.5 (C-5), 18.5 (C-6), 35.1 (C-7), 40.1 (C-8), 50.2 (C-9), 37.0 (C-10), 30.8 (C-11), 70.1 (C-12), 49.5 (C-13), 51.7 (C-14), 31.0 (C-15), 26.8 (C-16), 51.4 (C-17), 16.0 (C-18), 16.3 (C-19), 83.4 (C-20), 22.3 (C-21), 36.2 (C-22), 23.2 (C-23), 126.1 (C-24), 131.0 (C-25), 25.8 (C-26), 18.0 (C-27), 28.2 (C-28), 16.8 (C-29), 17.4 (C-30), 107.0 (3-O-Glc-1′), 75.6 (3-O-Glc-2′), 79.4 (3-O-Glc-3′), 71.6 (3-O-Glc-4′), 74.9 (3-O-Glc-5′), 65.5 (3-O-Glc-6′), 98.2 (20-O-Glc-1″), 74.9 (20-O-Glc-2″), 78.6 (20-O-Glc-3″), 71.7 (20-O-Glc-4″), 77.0 (20-O-Glc-5″), 70.1 (20-O-Glc-6″), 105.9 (Xyl-1‴), 74.9 (Xyl-2‴), 78.0 (Xyl-3‴), 71.1 (Xyl-4‴), 67.0 (Xyl-5‴), 168.1 (-O-CO), 42.8 (-CH_2), 169.6 (-COOH).

$$[\alpha]_D^{25}$$

3.3.2. Acid hydrolysis and UPLC analysis

Acid hydrolysis was carried out as reported previously (Li et al. 2012; Qu et al. 2021), in brief, compound **1** (2 mg) were hydrolyzed in 2 M HCl (3 mL) and stirred at 90 °C for 3 h. The resultant of reaction was diluted with water and extracted by EtOAc (3 mL), the aqueous solution was neutralized with Ag_2CO_3 and subsequently analyzed by UPLC equipped with evaporative light scattering detector (ELSD), and an Acquity BEH Amide column (2.1 × 50 mm, 1.7 μm; Waters). The configurations of D-xylose (2.41 min)

and D-glucose (3.33 min) were determined by comparison of the retention time of authentic standard.

3.4. Quantification of saponins

3.4.1. Calibration curves

The mixed standards solution were prepared by dissolving the accurately weighed of eight standard reference compounds (i.e., notoginsenosides FP_2, Fc, Fe, and ginsenosides Rb_1, Rc, Rb_2, Rb_3, and gypenoside IX. All standards were isolated from this study, and the purity of all standards $>$ 95%) with methanol, and giving a appropriate concentration ranges for the establishment of calibration curves. Five concentrations of the mixed standards solution were determined under the same chromatographic conditions, and their calibration curves were obtained by plotting the peak area versus the corresponding concentration of saponin concerned. The LOD and LOQ were determined based on signal-to-noise ratios of 3 and 10, respectively.

3.4.2. Sample preparation

The fresh sample (drying rate, 27.1%) was accurately weighed (40.0 g), and extracted with 500 mL ethanol in an ultrasonic bath (KQ-500DE, Kunshan, China) for 30 min and filtered. This extraction was repeated two additional times, the combined filtrate was evaporated to dryness in vacuo. The residue was dissolved with water, and then subjected to a HP-20 macroporous resin (30.0 g, Mitsubishi Chemical Co., Tokyo, Japan) CC, eluting with H_2O (100 mL) and 90% ethanol solution (100 mL) at a flow rate of 2 BV/h, the 90% ethanol fraction was collected and evaporated to dryness, and then dissolved and diluted to the volume scale by methanol in a 25 mL volumetric flask. This solution was filtered through 0.22 μm nylon filter membrane prior to UPLC analysis.

3.4.3. Chromatographic conditions

UPLC analysis was performed on an ACQUITY UPLC system (Waters Corp., Milford, USA), equipped with a PDA detector and a binary pump solvent management system. Chromatographic separation was carried out on a Waters ACQUITY UPLC BEH C_{18} (2.1 $\times$ 50 mm, 1.7 μm; Waters) at a flow rate of 0.5 mL/min. A gradient elution system consisted of solvent A (acetonitrile) and solvent B (0.1% aqueous phosphoric acid, v/v) was as follows: 0–3 min, 10% A; 3–4 min, 10–28% A; 4–7 min, 28% A; 7–9 min, 28–32% A; 9–10 min, 32% A; 10–11 min, 32–40% A; 11–12 min, 40–50% A; 12 – 14 min, 50% A; 14 – 14.5 min, 50 – 65% A; 14.5 – 16.5 min, 65% A; 16.5 – 17 min, 65 – 95% A; 17 – 19 min, 95% A; 19 – 19.5 min, 95 – 10% A; 19.5 – 22 min, 10% A. The detection wavelength and column temperature were set at 203 nm and 35 °C.

3.4.4. Method validations

The method precision was evaluated by intra-day and inter-day tests. Intra-day tests were performed by analysis of six times (0 h, 5 h, 10 h, 15 h, 20 h, and 24 h) of the same sample within one day. Inter-day experiments were carried out on three consecutive days in the same method as intra-day experiments. The RSD was taken as a measure of precision. The method repeatability was evaluated with parallelly divided

the sample into six parts, and extracted and analyzed by the mentioned above. Recovery test was used to evaluate the accuracy of this method. Accurate weights of standard compounds were added to the sample of known amounts of these compounds, the resultant samples were extracted and analysed with the described method. The average recovery and RSD were taken as a measure of recovery.

4. Conclusion

In conclusion, a continuation of phytochemical study on PNF led to yield a new malonyl-substituted ginsenoside, named as malonylgypenoside IX (**1**). Meanwhile, other known saponins (**2–22**) were also isolated, among these known isolates, thirteen (**3–10**, and **14–18**) saponins were obtained from PNF for the first time. In addition, this is the first report on the simultaneous determination of eight major saponins [notoginsenoside FP_2 (**14**), ginsenoside Rb_1 (**19**), notoginsenoside Fc (**13**), ginsenoside Rc (**15**), ginsenoside Rb_2 (**10**), ginsenoside Rb_3 (**12**), notoginsenoside Fe (**4**), and gypenoside IX (**5**)] in PNF with UPLC-UV method, which was proved to be simple, rapid and accurate via method validation. This study would provide scientific foundation for the comprehensive utilization of PNF.

Supplementary material

Supplementary material relating to this article is available online, alongside Figures S1−S11 and Tables S1−S3.

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