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Converting ginsenosides from stems and leaves of *Panax notoginseng* by microwave processing and improving their anticoagulant and anticancer activities

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A microwave processing technology was applied to degrade saponins from the stems and leaves of *Panax notoginseng*. Six transformation products (1–6), named 20(*S*)-ginsenoside Rg₃ (1), 20(*R*)-ginsenoside Rg₃ (2), notoginsenoside SFT₃ (3), ginsenoside Rk₁ (4), ginsenoside Rg₅ (5), and 20(*S*)-ginsenoside Rh₂ (6) were isolated and identified from a microwave processed extract of the stems and leaves of *P. notoginseng* (MEL). This transformation method was also applied for producing the minor ginsenosides in flowers, seeds and pedicels of *P. notoginseng*. The extract and compounds 1–6 in MEL were evaluated *in vitro* for anticancer and anticoagulant activities. The results showed that the MEL extract and transformation products had outstanding inhibitory activities against human cervical cancer Hela and lung cancer A549 cells. The strongest inhibitory effect was observed for 20(*S*)-Rh₂ (6) with an IC₅₀ value of 8.23 μM in Hela cells. Moreover, the results showed that the MEL significantly prolonged prothrombin time in a concentration-dependent manner. The anticoagulant effect of the MEL improved with the increased contents of Rk₁, Rg₅, and SFT₃.

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1 Introduction

Panax notoginseng (Burk.) F. H. Chen has been widely used as a herbal medicine in China. It was recorded in the Compendium of Materia Medica that the stems and leaves of *P. notoginseng* (PNL) were traditionally used to treat bone fractures, eliminate swelling, and stop bleeding. The PNL is also used as a functional food in folk medicine, as PNL is rich in nutrients and characterized by high protein, high dietary fiber, and low fat.¹ Fresh leaves are suitable for eating as a vegetable and dried as a tea.²

More than 40 chemical constituents have been isolated from the PNL. The main components are protopanaxadiol type saponins, such as notoginsenosides Fa, Fc, and Fe, and the ginsenosides Rb₁, Rc, Rb₂, and Rb₃ (Fig. 1).³ The saponins have gained prominence owing to their potential pharmaceutical values of analgesic, hypolipidemic, anticancer, anti-arrhythmic,

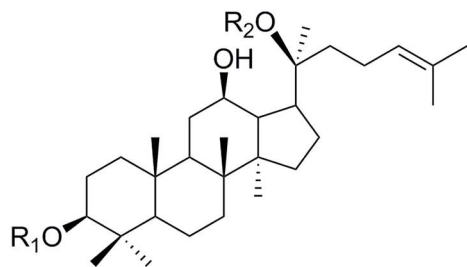
anticoagulatory, anti-inflammatory, and anti-aging activities.^{4,5} The interesting structures are less-polar saponins losing some glycosyl moieties such as 20(*S/R*)-ginsenosides Rg₃ and Rh₂ because of more effective anticancer activities.^{6,7} The Shenyi capsule containing ginsenoside Rg₃ was clinically applied for treating non-small cell lung cancer in China.⁸ The common methods used for producing less-polar saponins are acid-base degradation,⁹ enzymatic degradation,¹⁰ microbial transformation,¹¹ and steam processing.¹² At present, microwave processing method as a novel “green” solvent extraction technology is developed to increase the content of minor ginsenosides from white ginseng.^{13,14} This green extraction process is often used for extracting natural products owing to a faster extraction rate, less consumption of organic solvent, and lower costs of sample preparation,^{15,16} but more rarely in the case of transformation. The heat processing by microwave method for producing the minor ginsenosides gained high yields within about 60 minutes.¹³ The microwave time was much shorter than used in the traditional heating method (over 20 hours).¹²

We applied the microwave processing method to accelerate the transformation of minor ginsenosides from the roots of *P. notoginseng* and determined the optimal conversion conditions using response surface methodology.¹⁷ The roots of *P. notoginseng* are traditionally used as medicine, but a huge amount of PNL is often considered waste. In this study, the microwave method was applied to effectively obtain a microwave

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	R1	R2
notoginsenoside-Fa	Glc(2-I)Glc(2-I)Xyl	Glc(2-I)Glc
ginsenoside-Rb ₁	Glc(2-I)Glc	Glc(6-I)Glc
ginsenoside-Rc	Glc(2-I)Glc	Glc(6-I)Araf
notoginsenoside-Fc	Glc(2-I)Glc2-IXyl	Glc(6-I)Xyl
ginsenoside-Rb ₂	Glc(2-I)Glc	Glc(6-I)Arap
ginsenoside-Rb ₃	Glc(2-I)Glc	Glc(6-I)Xyl
ginsenoside-Rd	Glc(2-I)Glc	Glc
notoginsenoside-Fe	Glc	Glc(6-I)Araf
ginsenoside-Rd ₂	Glc	Glc(6-I)Arap
gypenoside-IX	Glc	Glc(6-I)Xyl

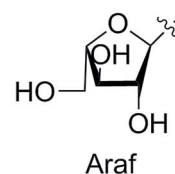
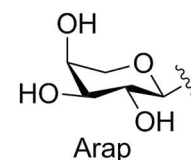
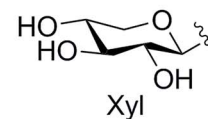
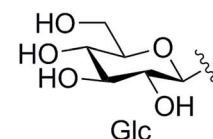


Fig. 1 Structures of saponins in the stems and leaves of *Panax notoginseng*. Glc, D-glucopyranosyl; Xyl, D-xylopyranosyl; Ara(f), L-arabinofuranosyl; Ara(p), L-arabinopyranosyl.

processing extract from stems and leaves of *P. notoginseng* (MEL). Six known compounds (1–6) were isolated from the MEL and identified by nuclear magnetic resonance spectroscopic data (Fig. 2). Moreover, the anticancer and anticoagulant effects of the MEL and the transformation products (1–6) were evaluated during microwave processing *in vitro*.

2 Materials and methods

2.1 Materials and reagents

The different parts of *P. notoginseng* (including stems and leaves, flowers, fruits, and pedicels) were purchased from the wenshan market in Yunnan province of China in October 2016. The samples were identified by Dr Xiuming Cui of Kunming University of Science and Technology. The nuclear magnetic

resonance spectra (NMR) was recorded by a Bruker Avance III 500 spectrometer (Bruker Corporation, Berlin, Germany). Formic acid (HPLC grade), acetonitrile (HPLC grade), dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), penicillin, streptomycin, L-glutamine and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and trypsin were purchased from Gibco BRL (Gland Island, NY, USA). All the other reagents and solvents were of analytical grade.

2.2 Preparation of processed PNL using the microwave processing method

The dried stems and leaves of *P. notoginseng* (PNL, 50 g) were powdered and ultrasonically extracted with 70% aqueous



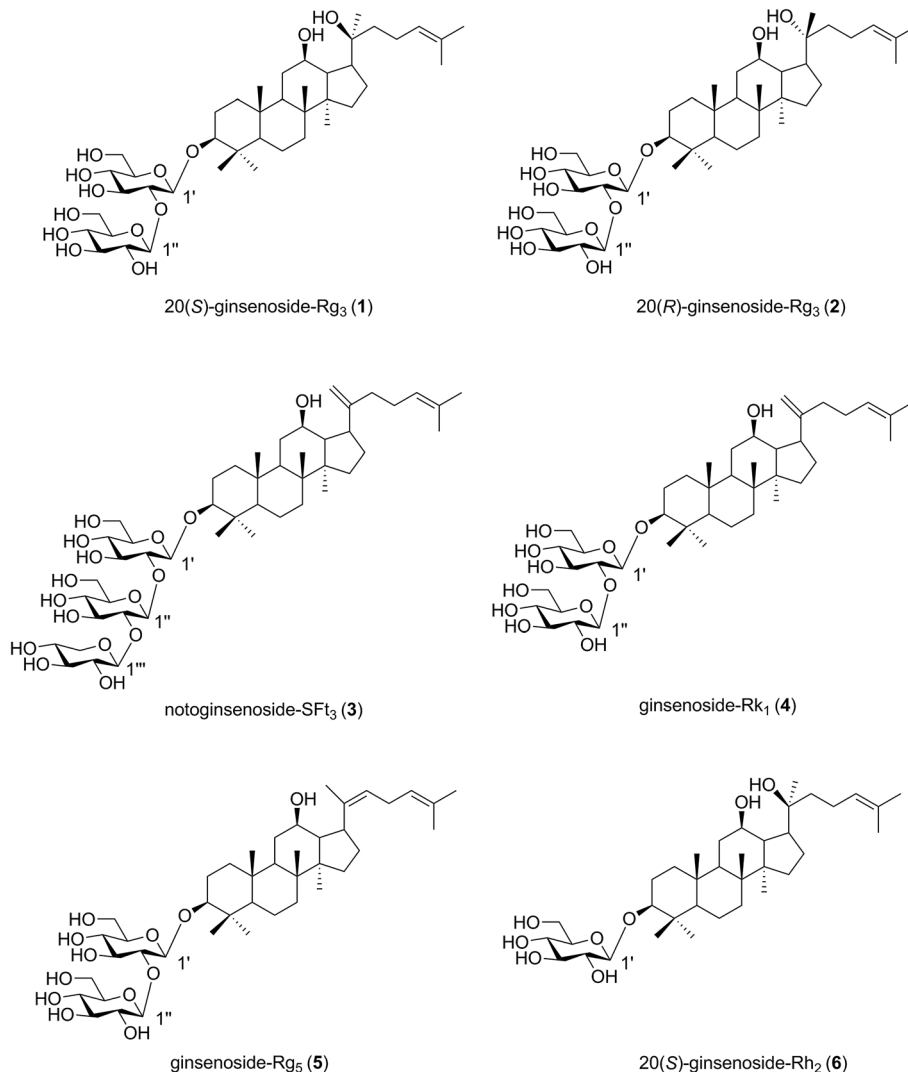


Fig. 2 Structures of transformation products 1–6 isolated from microwave processing extract of the stems and leaves of *Panax notoginseng*.

MeOH, three times for 45 min. After filtration and concentration, the extract of stems and leaves of *P. notoginseng* (EL, 11.25 g) was obtained with a yield of 22.5%. In the same way, the yields of the extracts of flowers (EF), seeds (ES), and pedicels (EP) were 36.36%, 6.48% and 25.48%, respectively.

The extract samples from different parts of *P. notoginseng* (EL, EF, ES and EP) was treated using microwave processing method as reported previously.¹⁷ 160 mg of the extract was added to the water (10 mL) in a microwave extraction system (model no: MDS-6G) manufactured by Sineo microwave chemical technology Co. Ltd. (Shanghai, China). Considering the influence of microwave temperature, microwave power and time, the temperature ranged from 60 °C to 180 °C, microwave power ranged from 300 W to 1000 W, and time ranged from 5 min to 50 min. After microwave treatment, the sample solution was centrifuged and the supernatant was added to the water for 10 mL. The obtained samples were filtered through a 0.45 µm filter membrane for HPLC analysis. The yields of transformation products were obtained from HPLC data.

$$\text{Yield (\%)} = M_g (\text{g}) / M_s (\text{g})$$

M_g is the content of transformation products and M_s is total amount of the sample.

2.3 Identification of MEL from the stems and leaves of *P. notoginseng* during microwave processing method by UHPLC-ESI-Q-TOF-MS

The EL extract (10 g) was processed at a temperature of 150 °C and microwave power of 500 W for 20 min. After removal of the solvent, microwave processing extract of stems and leaves of *P. notoginseng* (MEL, 9.04 g) was obtained with a yield of 90.4%. The composition of MEL was performed on an Agilent 1290 Infinity II LC system (UHPLC) coupled to an Agilent 6530 quadrupole time-of-flight mass spectrometry system (Q-TOF/MS) (Agilent Technologies, California, America). Samples were separated on an Agilent Poroshell 120 EC-C18 column (4.6 mm × 75 mm, 2.7 µm) at a rate of 0.6 mL min⁻¹ using an Agilent 1290 Infinity II LC system. The mobile phases consisted of 0.1%



(v/v) formic acid water (A) and 0.1% (v/v) formic acid acetonitrile (B) in the gradient program: 0–10 min, 10–25% (B); 10–50 min, 25–40% (B); 50–65 min, 40–60% (B); 65–70 min, 60–100% (B). For MS detector performed on a Q-TOF/MS, mass spectrometry conditions of electrospray ionization source (ESI) in positive ion detection mode were as follows: capillary voltage 4000 V; gas temperature, 350 °C; during gas N₂ flow rate, 10 L min^{−1}; pressure of nebulizer, 35 psi. The mass analyzer scanned over *m/z* 100–3000 Da.

2.4 Purification of transformation products (1–6)

5 g of the MEL extract was subjected to silica gel column chromatography eluted stepwise with MeOH/CHCl₃ (2 : 98, 5 : 95, 7 : 93, 10 : 90, 15 : 85, 20 : 80, 25 : 75, 30 : 70, 40 : 60, v/v) to afford 5 fractions (Fr. 1–5). Fr. 1 and Fr. 2 were purified by recrystallization to yield compound 1 (126.2 mg) and compound 2 (60 mg), respectively. Fr. 3 was subjected to pre-HPLC [YMC-Pack ODS-A (φ10 × 250 mm), 2 mL min^{−1} flow rate, MeCN (B)/H₂O (A) (0–20 min, 40–50% B; 20–65 min, 50–50% B) mobile phase] to give compound 3 (12.8 mg, *t_R* = 54.0 min). Fr. 4 was successively purified by pre-HPLC [YMC-Pack ODS-A (φ10 × 250 mm), 2 mL min^{−1} flow rate, MeCN (B)/H₂O (A) (0–40 min, 50–55% B; 40–60 min, 55–55% B) mobile phase] to yield compounds 4 (9.8 mg, *t_R* = 54.2 min) and 5 (40.5 mg, *t_R* = 58.4 min). Compounds 6 (11.6 mg, *t_R* = 58.5 min) was obtained from Fr. 5 separated by pre-HPLC [YMC-Pack ODS-A (φ10 × 250 mm), 2 mL min^{−1} flow rate, MeCN/H₂O (55 : 45) mobile phase].

2.4.1 20(S)-ginsenoside Rg₃ (1). White amorphous powder; HRESIMS *m/z* 807.4663 [M + Na]⁺. ¹H-NMR (C₅D₅N, 500 MHz) δ: 0.94 (3H, s, H-18), 0.82 (3H, s, H-19), 1.40 (1H, s, H-21), 1.60 (3H, s, H-26), 1.60 (3H, s, H-27), 1.30 (3H, s, H-28), 1.11 (3H, s, H-29), 0.99 (3H, s, H-30), 4.96 (1H, d, *J* = 7.8 Hz, Glc-I-H-1'), 5.38 (1H, d, *J* = 7.7 Hz, Glc-II-H-1''). ¹³C-NMR (C₅D₅N, 125 MHz) δ: 39.5(C-1), 27.1(C-2), 89.3(C-3), 40.1(C-4), 56.7(C-5), 18.8(C-6), 35.5(C-7), 40.4(C-8), 50.8(C-9), 37.3(C-10), 32.4(C-11), 71.4(C-12), 48.9(C-13), 52.1(C-14), 31.7(C-15), 27.2(C-16), 55.2(C-17), 16.2(C-18), 16.7(C-19), 73.3(C-20), 27.5(C-21), 36.3(C-22), 23.4(C-23), 126.7(C-24), 131.1(C-25), 26.2(C-26), 17.4(C-27), 28.5(C-28), 17.0(C-29), 18.1(C-30), 105.5 (Glc-I-C-1'), 83.9 (C-2'), 78.4 (C-3'), 72.1 (C-4'), 78.7 (C-5'), 63.2 (C-6'), 106.5 (Glc-II-C-1''), 77.5 (C-2''), 78.7 (C-3''), 72.0 (C-4''), 78.5 (C-5''), 63.1 (C-6''). The structure of compound 1 was identified as 20(S)-ginsenoside Rg₃ by comparing the NMR data and MS data with reported data.¹⁸

2.4.2 20(R)-ginsenoside Rg₃ (2). White amorphous powder; HRESIMS *m/z* 807.4652 [M + Na]⁺. ¹H-NMR (C₅D₅N, 500 MHz) δ: 1.01 (3H, s, H-18), 0.82 (3H, s, H-19), 1.39 (1H, s, H-21), 1.69 (3H, s, H-26), 1.65 (3H, s, H-27), 1.30 (3H, s, H-28), 1.11 (3H, s, H-29), 0.99 (3H, s, H-30), 4.94 (1H, d, *J* = 7.8 Hz, Glc-I-H-1'), 5.38 (1H, d, *J* = 7.7 Hz, Glc-II-H-1''). ¹³C-NMR (C₅D₅N, 125 MHz) δ: 39.1(C-1), 26.6(C-2), 88.9(C-3), 39.7(C-4), 56.4(C-5), 18.4(C-6), 35.1(C-7), 39.9(C-8), 50.3(C-9), 36.9(C-10), 31.3(C-11), 70.9(C-12), 49.2(C-13), 51.8(C-14), 32.2(C-15), 26.7(C-16), 50.6(C-17), 15.8(C-18), 16.4(C-19), 73.0(C-20), 22.8(C-21), 43.2(C-22), 22.6(C-23), 126.0(C-24), 130.8(C-25), 25.9(C-26), 17.3(C-27), 28.2(C-28), 16.6(C-29), 17.7(C-30), 105.2 (Glc-I-C-1'), 83.5 (C-2'),

78.0 (C-3'), 71.6 (C-4'), 78.3 (C-5'), 62.9 (C-6'), 106.1 (Glc-II-C-1''), 77.2 (C-2''), 78.4 (C-3''), 71.6 (C-4''), 78.2 (C-5''), 62.7 (C-6''). The structure of compound 2 was identified as 20(R)-ginsenoside Rg₃ by comparing the NMR data and MS data with reported data.¹⁸

2.4.3 Notoginsenoside SFT₃ (3). White amorphous powder; HRESIMS *m/z* 921.4989 [M + Na]⁺. ¹H-NMR (C₅D₅N, 500 MHz) δ: 0.95 (3H, s, H-18), 0.79 (3H, s, H-19), 5.14 (1H, s, H-21), 4.90 (1H, s, H-21), 1.65 (3H, s, H-26), 1.59 (3H, s, H-27), 1.28 (3H, s, H-28), 1.10 (3H, s, H-29), 1.00 (3H, s, H-30), 4.94 (1H, d, *J* = 7.8 Hz, Glc-I-H-1'), 5.52 (1H, d, *J* = 7.7 Hz, Glc-II-H-1''), 5.41 (1H, d, *J* = 6.7 Hz, Xyl-H-1'''). ¹³C-NMR (C₅D₅N, 125 MHz) δ: 39.8(C-1), 26.8(C-2), 88.8(C-3), 39.3(C-4), 56.4(C-5), 18.5(C-6), 35.4(C-7), 40.3(C-8), 48.3(C-9), 37.1(C-10), 32.7(C-11), 72.4(C-12), 52.5(C-13), 51.2(C-14), 32.8(C-15), 30.8(C-16), 50.8(C-17), 16.6(C-18), 15.9(C-19), 155.6(C-20), 108.2(C-21), 33.9(C-22), 27.1(C-23), 125.3(C-24), 131.2(C-25), 25.8(C-26), 17.8(C-27), 28.1(C-28), 16.7(C-29), 17.0(C-30), 104.8 (Glc-I-C-1'), 83.0 (C-2'), 78.7 (C-3'), 71.1 (C-4'), 78.3 (C-5'), 63.0 (C-6'), 103.2 (Glc-II-C-1''), 84.5 (C-2''), 78.0 (C-3''), 71.8 (C-4''), 77.9 (C-5''), 62.8 (C-6''), 106.5 (Xyl-C-1'''), 76.0 (C-2'''), 77.8 (C-3'''), 70.8 (C-4'''), 67.5 (C-5'''). The structure of compound 3 was identified as notoginsenoside SFT₃ by comparing the NMR data and MS data with reported data.¹⁹

2.4.4 Ginsenoside Rk₁ (4). White amorphous powder; HRESIMS *m/z* 789.4595 [M + Na]⁺. ¹H-NMR (C₅D₅N, 500 MHz) δ: 1.00 (3H, s, H-18), 0.80 (3H, s, H-19), 5.14 (1H, s, H-21), 4.90 (1H, s, H-21), 1.65 (3H, s, H-26), 1.59 (3H, s, H-27), 1.29 (3H, s, H-28), 1.10 (3H, s, H-29), 0.95 (3H, s, H-30), 4.93 (1H, d, *J* = 7.8 Hz, Glc-I-H-1'), 5.37 (1H, d, *J* = 7.7 Hz, Glc-II-H-1''). ¹³C-NMR (C₅D₅N, 125 MHz) δ: 39.6(C-1), 27.2(C-2), 89.2(C-3), 40.1(C-4), 56.7(C-5), 18.8(C-6), 35.7(C-7), 40.5(C-8), 48.6(C-9), 37.3(C-10), 33.1(C-11), 72.8(C-12), 52.9(C-13), 51.5(C-14), 32.9(C-15), 31.2(C-16), 51.2(C-17), 16.8(C-18), 16.1(C-19), 155.9(C-20), 108.5(C-21), 34.2(C-22), 27.4(C-23), 125.8(C-24), 131.5(C-25), 26.1(C-26), 18.1(C-27), 28.4(C-28), 17.0(C-29), 17.3(C-30), 105.5 (Glc-I-C-1'), 83.8 (C-2'), 78.7 (C-3'), 72.0 (C-4'), 78.3 (C-5'), 63.0 (C-6'), 106.4 (Glc-II-C-1''), 77.6 (C-2''), 78.7 (C-3''), 72.0 (C-4''), 78.5 (C-5''), 63.2 (C-6''). The structure of compound 4 was identified as ginsenoside Rk₁ by comparing the NMR data and MS data with reported data.²⁰

2.4.5 Ginsenoside Rg₅ (5). White amorphous powder; HRESIMS *m/z* 789.4569 [M + Na]⁺. ¹H-NMR (C₅D₅N, 500 MHz) δ: 1.00 (3H, s, H-18), 0.80 (3H, s, H-19), 1.83 (3H, s, H-21), 5.52 (1H, t, *J* = 6.8 Hz, H-22), 5.25 (1H, t, *J* = 6.8 Hz, H-24), 1.65 (3H, s, H-26), 1.59 (3H, s, H-27), 1.29 (3H, s, H-28), 1.10 (3H, s, H-29), 0.95 (3H, s, H-30), 4.93 (1H, d, *J* = 7.8 Hz, Glc-I-H-1'), 5.37 (1H, d, *J* = 7.7 Hz, Glc-II-H-1''). ¹³C-NMR (C₅D₅N, 125 MHz) δ: 39.6(C-1), 28.5(C-2), 89.3(C-3), 40.6(C-4), 56.7(C-5), 18.8(C-6), 35.7(C-7), 40.3(C-8), 51.1(C-9), 37.3(C-10), 33.1(C-11), 72.8(C-12), 50.9(C-13), 51.5(C-14), 32.9(C-15), 31.1(C-16), 51.2(C-17), 16.8(C-18), 17.0(C-19), 140.5(C-20), 13.5(C-21), 123.5(C-22), 27.7(C-23), 123.8(C-24), 131.5(C-25), 26.1(C-26), 18.1(C-27), 28.9(C-28), 16.1(C-29), 17.3(C-30), 105.5 (Glc-I-C-1'), 83.8 (C-2'), 78.7 (C-3'), 72.0 (C-4'), 78.3 (C-5'), 63.0 (C-6'), 106.4 (Glc-II-C-1''), 77.6 (C-2''), 78.7 (C-3''), 72.0 (C-4''), 78.5 (C-5''), 63.2 (C-6''). The structure of compound 5 was identified as ginsenoside Rg₅ by comparing the NMR data and MS data with reported data.²¹



2.4.6 20(S)-ginsenoside Rh₂ (6). White amorphous powder; HRESIMS m/z 1245.8682 [2 M + H]⁺. ¹H-NMR (C₅D₅N, 500 MHz) δ : 0.95 (3H, s, H-18), 0.78 (3H, s, H-19), 1.42 (1H, s, H-21), 1.61 (3H, s, H-26), 1.64 (3H, s, H-27), 1.31 (3H, s, H-28), 0.99 (3H, s, H-29), 0.95 (3H, s, H-30), 4.96 (1H, d, J = 7.8 Hz, Glc-I-H-1'). ¹³C-NMR (C₅D₅N, 125 MHz) δ : 39.1(C-1), 26.9(C-2), 88.8(C-3), 39.7(C-4), 56.4(C-5), 18.5(C-6), 35.2(C-7), 40.0(C-8), 50.3(C-9), 37.0(C-10), 32.1(C-11), 71.0(C-12), 48.5(C-13), 51.8(C-14), 31.3(C-15), 26.7(C-16), 54.8(C-17), 16.4(C-18), 15.8(C-19), 73.0(C-20), 27.1(C-21), 35.9(C-22), 23.0(C-23), 126.3(C-24), 130.8(C-25), 25.8(C-26), 17.7(C-27), 28.2(C-28), 16.8(C-29), 17.1(C-30), 107.0 (Glc-C-1'), 75.8 (C-2'), 78.8 (C-3'), 71.9 (C-4'), 78.4 (C-5'), 63.1 (C-6'). The structure of compound **6** was identified as 20(S)-ginsenoside Rh₂ by comparing the NMR data and MS data with reported data.²¹

2.5 HPLC analysis of transformation product (1–6)

Quantitative analysis of six transformation product (1–6) processed by microwave processing method was determined by high-performance liquid chromatography (HPLC) (Fig. 3). The HPLC data were recorded on a Shimadzu Analytical Instrument (Shimadzu, Kyoto, Japan), equipped with a solvent degasser, a binary pump, an auto sampler and a UV detector. The separation was performed on a VisionHT C18 HL column (250 mm \times 4.6 mm, 5 μ m, Grace, Deerfield, USA) at the flow rate of 1.0 mL min⁻¹. The mobile phase consisted of distilled water (solvent A) and acetonitrile (solvent B) using the following gradient program: 0–20 min, 20–20% B; 20–45 min, 20–46% B; 45–50 min, 46–55% B; 50–66 min, 55–55% B. The detection wavelength and the column temperature were at 203 nm and 35 °C, respectively. The calibration curves for 20(S)-Rg₃ (1), 20(R)-Rg₃ (2), SFT₃ (3), Rk₁ (4), Rg₅ (5) and 20(S)-Rh₂ (6) showed good linearity ($R^2 > 0.9990$) in the concentration ranges (Table 1). The samples were analyzed by HPLC in three times.

2.6 Anticancer activity *in vitro*

2.6.1 Cell lines and culture. Human cervical cancer (HeLa) and human lung cancer (A549) cell lines were obtained from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. HeLa cells and A549 cells were cultivated in RPMI Media 1640 (RPMI1640) containing 10% fetal bovine serum, 100 U mL⁻¹ penicillin, 0.1 mg mL⁻¹ streptomycin and 2 mmol L⁻¹ L-glutamine. All cultures were maintained with the aeration of 5% CO₂ at 37 °C.

2.6.2 Cell cytotoxicity assay. The cytotoxicity of EL, MEL and compounds 1–6 on two cancer cell lines was determined by MTT assay.²² The cells were seeded in five-well plates (1 \times 10⁴ cells per well) and incubated at 37 °C for 24 h. The cells were then treated with EL, MEL, cisplatin and compounds 1–6 at different concentrations (0.1–100 μ mol L⁻¹). Cisplatin was used as the positive control. After 48 h, 20 μ L MTT (5 mg mL⁻¹) was added to each well and the cells were incubated for 4 h. The supernatant was removed and then 150 μ L of DMSO was added to each well. After the plates were shaken at room temperature for 10 min, the absorbance was measured by PHERA star FSX Microplate Reader (BMG Labtech, Offenburg, Germany) at

a wavelength of 570 nm. The half maximal inhibitory concentration (IC₅₀) against cancer cells was obtained by curve fitting of a sigmoidal dosage–response curve using nonlinear regression model.

2.7 Anticoagulant activity *in vitro*

2.7.1 Animals. Kunming mice (18–22 g) were purchased by Changsha Tianqin Biotechnology Co., China (Qualified no. SCXK 2014-0011). The mice were maintained at a temperature of 25 \pm 2 °C, a humidity of 62 \pm 2%, and a 12 h dark/light cycle, with unrestricted access to food and water. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Kunming University of Science and Technology, and were approved by the Animal Ethics Committee of Kunming University of Science and Technology.

2.7.2 Plasma preparation. The plasma was obtained from mice whole blood mixed with 0.109 mol L⁻¹ sodium citrate (9 : 1 ratio of blood to citrate, v/v) by centrifugation at 3000 \times g for 15 min.

2.7.3 Blood plasma clotting assays. Prothrombin time (PT) was determined with a coagulation analyzer (XN06-IV, Aierfu, Wuhan, China). 100 μ L of a solution containing plasma (50 μ L) and EL, MEL and compounds 1–6 (50 μ L) were incubated at 37 °C for 3 min. The samples of EL and MEL were diluted with purified water to give the following concentrations: 25, 12.5, 5 mg mL⁻¹. The concentration of compounds 1–6 is 1 mg mL⁻¹. Purified water was used as negative control. The PT assay reagent (100 μ L) was added to the mixed samples, and the clotting time was recorded by the analyzer. All clotting tests were performed with three individual replicates.

2.8 Statistical analysis

Statistical analyses were performed with SPSS 19.0 software. All data were expressed as mean \pm standard deviation (SD). The values of $p < 0.05$ were considered to be statistically significant, and $p < 0.01$ and $p < 0.001$ being very significant.

3 Results and discussion

3.1 Purification and identification of the transformation products from the stems and leaves of *P. notoginseng* using the microwave processing method

The MEL was identified by ultra-high performance liquid chromatography-quantitative time of flight mass spectroscopy (QTOF-MS) in positive mode. The total ion chromatogram of the MEL is shown in Fig. 4. Table 2 summarizes the QTOF-MS data of 22 ginsenosides identified from the MEL. The compounds were determined by the molecular ions based on [M + H]⁺, [M + Na]⁺, or [2 M + H]⁺. The chemical composition of the MEL was mainly 20(S)-protopanaxadiol (PPD)-type ginsenosides; the PPD skeleton of which displayed characteristic ions at m/z 425.^{23,24} Eight transformation products in the MEL were identified as ginsenosides, such as F₂, 20(S)-Rg₃, 20(R)-Rg₃, SFT₃, Rk₁, Rg₅, 20(S)-Rh₂, and 20(R)-Rh₂. The others had already existed in the PNL before microwave transformation,



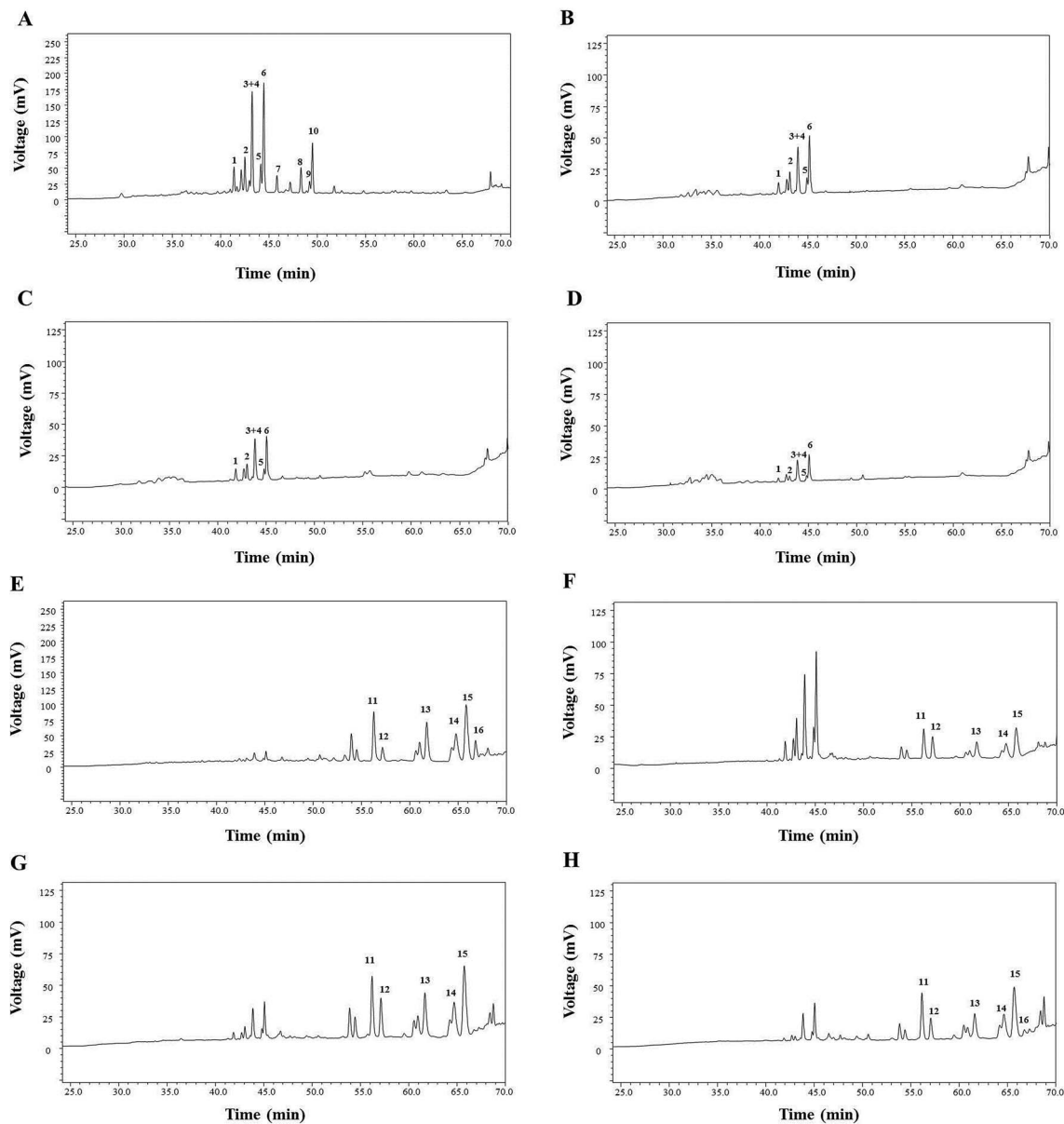


Fig. 3 Saponin transformation in different parts of *Panax notoginseng* induced by microwave processing. HPLC chromatograms of the stems and leaves (A), flowers (B), pedicels (C), and seeds (D) of *P. notoginseng*. HPLC chromatograms of the stems and leaves (E), flowers (F), pedicels (G), and seeds (H) of *P. notoginseng* after treatment with microwave processing. Peaks: (1) notoginsenoside Fa; (2) ginsenoside Rb₁; (3) ginsenoside Rc; (4) notoginsenoside Fc; (5) ginsenoside Rb₂; (6) ginsenoside Rb₃; (7) ginsenoside Rd; (8) notoginsenoside Fe; (9) ginsenoside Rd₂; (10) gypenoside IX; (11) 20(S)-ginsenoside Rg₃; (12) 20(R)-ginsenoside Rg₃; (13) notoginsenoside SFT₃; (14) ginsenoside Rk₁; (15) ginsenoside Rg₅; (16) 20(S)-ginsenoside Rh₂.

Table 1 The results of linear regression of transformation product (1–6) ($n = 6$)

Compounds	Calibration curve	R^2	Concentration range ($\mu\text{g mL}^{-1}$)
Ginsenoside 20(S)-Rg ₃ (1)	$y = 4\,000\,000x + 9678$	0.9996	1.0–1000
Ginsenoside 20(R)-Rg ₃ (2)	$y = 4\,000\,000x + 15\,317$	0.9999	4.0–1000
Notoginsenoside SFT ₃ (3)	$y = 990\,240x + 37\,027$	0.9999	3.1–3000
Ginsenoside Rk ₁ (4)	$y = 3\,000\,000x + 1465$	0.9991	4.0–1000
Ginsenoside Rg ₅ (5)	$y = 758\,099x + 45\,996$	0.9997	16.0–5000
Ginsenoside 20(S)-Rh ₂ (6)	$y = 3\,000\,000x + 10\,426$	0.9990	2.5–1000



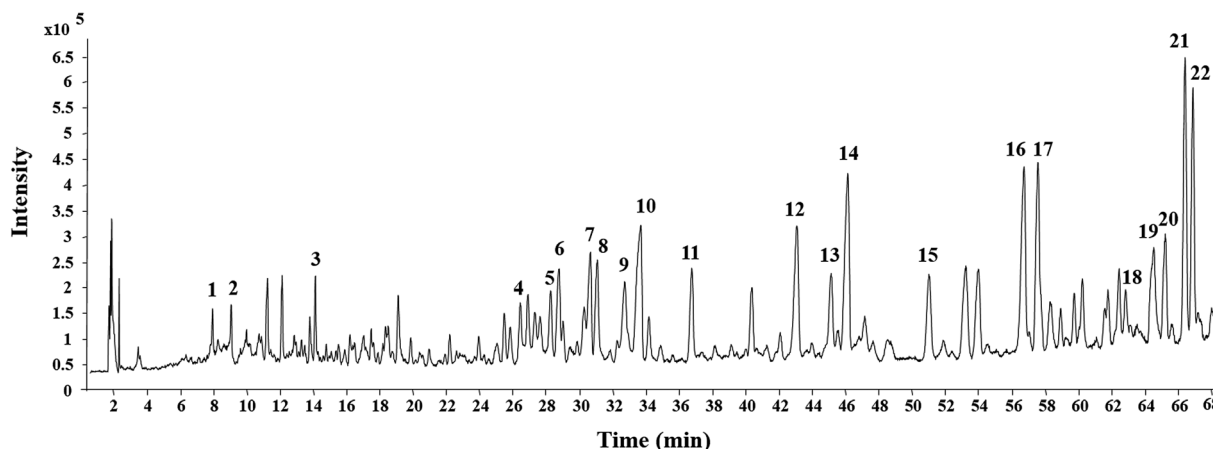


Fig. 4 Total ion current chromatogram of microwave processing extract of the stems and leaves of *Panax notoginseng* by UHPLC-ESI-Q-TOF-MS.

Table 2 Characterization of ginsenosides in microwave processing extract of stems and leaves of *P. notoginseng* using UHPLC-Q-TOF/MS

No.	Retention time (min)	Experimental m/z	Molecular formula	Diagnostic fragments (m/z)	Identification
1	7.31	627.1402 $[M + H]^+$	$C_{42}H_{26}O_6$	303.0410, 333.0460, 465.0912, 515.2338, 531.2084, 566.4141, 588.3944	Unknown
2	8.47	611.1461 $[M + H]^+$	$C_{42}H_{26}O_5$	287.0467, 449.0962, 566.4133, 588.3948	Unknown
3	13.54	823.4602 $[M + Na]^+$	$C_{42}H_{72}O_{14}$	423.3510, 441.3612, 603.4086, 621.4228, 765.4602	Ginsenoside Rg ₁
4	25.92	1263.6069 $[M + Na]^+$	$C_{59}H_{100}O_{27}$	425.3665, 587.4160, 605.4256, 749.4662, 881.5030	Notoginsenoside Fa
5	27.67	1233.5957 $[M + Na]^+$	$C_{58}H_{98}O_{26}$	425.3659, 603.4095, 749.4646, 823.4584, 881.5069, 955.5007, 1083.5334	Ginsenoside FP ₂
6	28.21	1131.5648 $[M + Na]^+$	$C_{54}H_{92}O_{23}$	425.3658, 587.4171, 749.4646, 929.5196	Ginsenoside Rb ₁
7	30.08	1101.554 $[M + Na]^+$	$C_{53}H_{90}O_{22}$	425.3664, 587.4152, 605.4269, 749.4670, 899.5157	Ginsenoside R _c
8	30.48	1233.5946 $[M + Na]^+$	$C_{58}H_{98}O_{26}$	425.3666, 587.4158, 605.4267, 749.4656, 881.5080, 916.5333	Notoginsenoside Fc
9	32.15	1101.5561 $[M + Na]^+$	$C_{53}H_{90}O_{22}$	425.3664, 559.2657, 605.4261, 749.4647, 783.4688, 881.5043	Ginsenoside Rb ₂
10	33.01	1101.5591 $[M + Na]^+$	$C_{53}H_{90}O_{22}$	425.3666, 587.4169, 605.4273, 749.4655, 881.5060, 929.5193	Ginsenoside Rb ₃
11	36.19	969.5170 $[M + Na]^+$	$C_{48}H_{82}O_{18}$	425.3663, 587.4159, 767.4730	Ginsenoside R _d
12	42.46	939.5082 $[M + Na]^+$	$C_{47}H_{80}O_{17}$	425.3672, 587.4170, 605.4255, 719.4548, 737.4652, 749.4659, 767.4736, 793.4525, 881.5041	Notoginsenoside Fe
13	44.55	939.5077 $[M + Na]^+$	$C_{47}H_{80}O_{17}$	425.3673, 587.4156, 605.4274, 719.4553, 737.4644, 749.4686, 767.4758, 785.4860, 881.5065	Ginsenoside R _{d2}
14	45.54	939.5067 $[M + Na]^+$	$C_{47}H_{80}O_{17}$	425.3670, 587.4161, 605.4256, 719.4560, 737.4651, 749.4665, 767.4736, 881.5061	Notoginsenoside Fd
15	50.42	807.4665 $[M + Na]^+$	$C_{42}H_{72}O_{13}$	425.3670, 587.4160, 605.4252, 749.4644, 765.4599	Ginsenoside F ₂
16	56.17	807.4663 $[M + Na]^+$	$C_{42}H_{72}O_{13}$	425.3663, 587.4155, 605.4249, 749.4658	20(S)-Ginsenoside Rg ₃
17	57.01	807.4652 $[M + Na]^+$	$C_{42}H_{72}O_{13}$	425.3667, 587.4162, 605.4270, 749.4646	20(R)-Ginsenoside Rg ₃
18	61.89	921.4989 $[M + Na]^+$	$C_{47}H_{78}O_{16}$	407.3562, 425.3663, 587.4162, 605.4257, 749.4642, 881.5078	Ginsenoside SFT ₃
19	63.94	789.4595 $[M + Na]^+$	$C_{42}H_{70}O_{12}$	407.3559, 425.3664, 587.4159, 605.4272, 749.4663	Ginsenoside Rk ₁
20	64.67	789.4569 $[M + Na]^+$	$C_{42}H_{70}O_{12}$	407.3563, 425.3663, 587.4163, 605.4263, 749.4657	Ginsenoside Rg ₅
21	65.85	1245.8682 $[2M + H]^+$	$C_{36}H_{62}O_8$	407.3562, 425.3663, 587.4156, 605.4254	20(S)-Ginsenoside Rh ₂
22	66.34	1245.8688 $[2M + H]^+$	$C_{36}H_{62}O_8$	407.3559, 425.3669, 587.4160, 605.4269	20(R)-Ginsenoside Rh ₂



which were identified by matching molecular formulas with reported data.^{3,4} Six transformation products were purified from the MEL, and their structures were determined by comparing their spectroscopic data with those reported in the literature (Fig. 2). The transformation products were identified as 20(*S*)-ginsenoside Rg₃ (1),¹⁸ 20(*R*)-ginsenoside Rg₃ (2),¹⁸ notoginsenoside SFT₃ (3),¹⁹ ginsenoside Rk₁ (4),²⁰ ginsenoside Rg₅ (5),²⁰ and 20(*S*)-ginsenoside Rh₂ (6).²¹

3.2 Impact of microwave processing conditions on the yields of the transformation products

Microwave processing as a “green and innovative” technique has been widely used for preservation,²⁵ transformation,^{13,14} and extraction²⁶ in research and industrial fields. The microwave processing technique has advantages of less time and energy as well as fewer solvents.¹⁶ In our study, this microwave processing method was applied to degrade the major saponins in the PNL and to produce the rare saponins with fewer sugar moieties. Microwave heating occurs within the molecule, and the glycosidic bonds in the polar molecules break easily.¹⁵ The major saponins in PNL were hydrolyzed at the glucosyl residue of C-20 to produce ginsenosides 20(*S*)-Rg₃, 20(*R*)-Rg₃, 20(*S*)-Rh₂, Rk₁, Rg₅, and notoginsenoside SFT₃ in the MEL with an increase in microwave power and temperature. The three factors of microwave power, temperature, and time were assessed for yields of the five transformation products by HPLC analysis after microwave processing (Fig. 5).

Microwave power was an important factor in the transformation process. When microwave temperature and time were set to 135 °C and 15 min, microwave power was varied from 300 to 1000 W to evaluate its impact on the yields of the transformation products (Fig. 5A). The highest yield of each transformation product was reached with microwave power of 500 W. The yields of the five transformation products in the MEL were in the order of Rg₅ > SFT₃ > 20(*R*)-Rg₃ > 20(*S*)-Rg₃ > 20(*S*)-Rh₂. When the microwave temperature range was from 60–180 °C for 20 min, the yields of the transformation products increased gradually (Fig. 5B). When the temperature was 150 °C, the yields of the transformation products were the highest in the following order: Rg₅ > SFT₃ > 20(*R*)-Rg₃ > 20(*S*)-Rg₃ > 20(*S*)-Rh₂. According to the yields of the transformation products, a suitable time for this microwave processing method was 15–30 min (Fig. 5C). The yields of the transformation products were in the following order: Rg₅ > SFT₃ > 20(*R*)-Rg₃ > 20(*S*)-Rg₃ > 20(*S*)-Rh₂. It was reported that the contents of the primary degradation products 20(*S*)-Rh₁, 20(*R*)-Rh₁, Rk₃, Rh₄, 20(*S*)-Rg₃, 20(*R*)-Rg₃, Rk₁, and Rg₅ for dried notoginseng steamed at 120 °C increase rapidly after 12 h.¹² A similar yield was achieved in 20 min when the microwave processing method was used. Transformation products are obtained by microwave processing with less energy and less waste compared with the steaming method. According to the yields of the transformation products after microwave processing, the optimum conditions of microwave power, temperature, and time were 500 W, 150 °C, and 20 min, respectively.

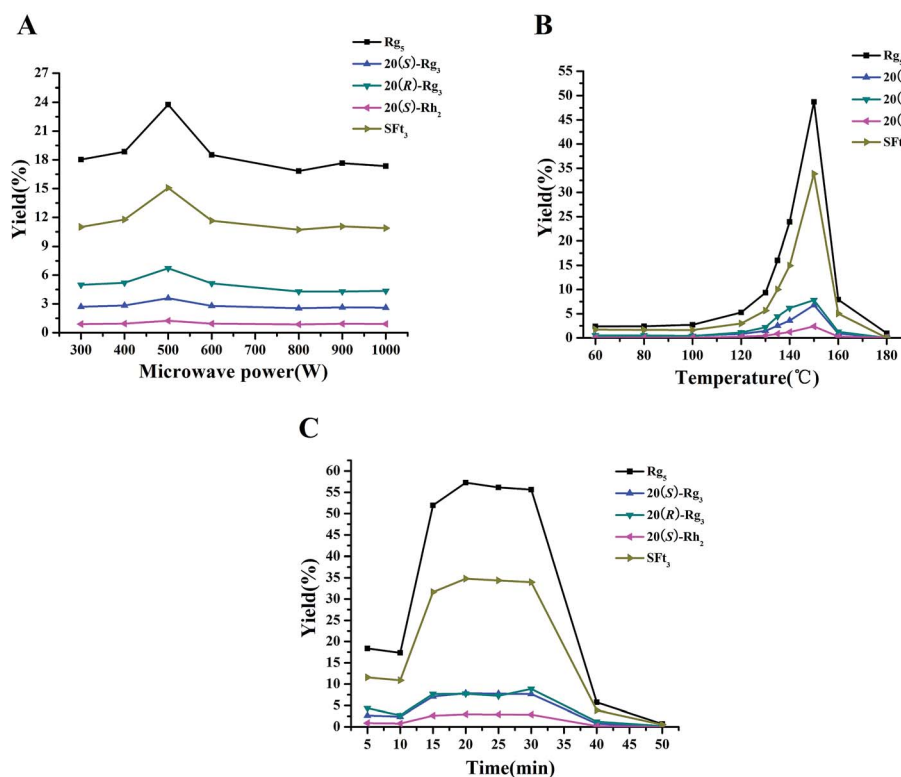


Fig. 5 Effects of microwave power (A), temperature (B), and time (C) on yields of transformation products.



3.3 Applying the microwave processing method to different parts of *P. notoginseng*

The results of saponin transformation in different parts of *P. notoginseng* (stems and leaves, flowers, seeds, and pedicels) using microwave processing are shown in Fig. 3 and 6. High performance liquid chromatography chromatograms of stems and leaves, flowers, seeds, and pedicels of *P. notoginseng* revealed similar typical ginsenosides as the PNL, consisting of notoginsenosides Fa and Fc, and ginsenosides Rc, Rb₁, Rb₂, and Rb₃ (Fig. 3A–D). After microwave processing, six newly transformed products of 20(*S*)-Rg₃, 20(*R*)-Rg₃, SFT₃, Rk₁, Rg₅, and 20(*S*)-Rh₂ were generated from the MEL, flowers (MEF), seeds (MES), and pedicels (MEP) of *P. notoginseng* (Fig. 3E–H). As shown in Fig. 6, the Rg₅ content in the microwave-treated extracts from different parts of *P. notoginseng* was the highest and the Rh₂ content was the lowest. Under the same microwave processing conditions (500 W, 500 °C, and 20 min) the yields of Rg₅ in the microwave transformed extracts from different parts of *P. notoginseng* were in the order of: MEL > MEP > MES > MEF. The 20(*S*)-Rh₂ contents in the MEL and MES were 2.58% and 0.36% respectively, while 20(*S*)-Rh₂ was not detected in the MEP or MEF. Three special saponins of notoginsenoside Fe, ginsenoside Rd₂, and gypenoside IX were detected in the PNL (Fig. 3A), which easily lost the glycosyl group at C-20 to generate 20(*S*)-Rh₂. This result indicates that 20(*S*)-Rh₂ content in the MEL was much higher than that in the MEP, MES, or MEF.

3.4 Transformation mechanism for producing the minor ginsenosides using the microwave processing method

Microwave heating occurs within molecules, which is described as “internal heating”.¹⁵ If the microwave energy matches with the molecular bond energy, the reactive activity of glycoside bond in the molecule increased and easy to break.^{15,27} In our paper, major saponins lost the glycosyl residues and produced the minor ginsenosides with increased microwave power and temperature. The proposed transformation pathway of

saponins in PNL by the microwave processing were shown in Fig. 7. The major saponins, *e.g.*, ginsenosides Rb₁, Rd, Rc, Rb₂, and Rb₃ easily lost the glycosyl residue at C-20 to produce 20(*S*)/(*R*)-Rg₃, and then formed Rk₁ and Rg₅ through dehydration at C-20. Notoginsenosides Fa and Fc were hydrolyzed and dehydrated at C-20 to obtain notoginsenoside SFT₃. When the microwave temperature was increased, a small amount of 20(*S*)/(*R*)-Rg₃ was converted to 20(*S*)/(*R*)-Rh₂ through loss of a glucosyl group at C-3. In addition, the three compounds of notoginsenoside Fe, ginsenoside Rd₂ and gypenoside IX firstly removed the arabinose or xylose residues at C-3 to produce ginsenoside F₂, and continued to lose one glucose residue at C-20 to obtain 20(*S*)-Rh₂. This indicated that the content of 20(*S*)-Rh₂ was much higher than that of 20(*R*)-Rh₂ in the microwave processing. When the glycosyl residues at C-20 were lost, 20(*S*)/(*R*)-epimers such as 20(*S*)/(*R*)-Rg₃ and 20(*S*)/(*R*)-Rh₂ were obtained by the selective attack of the OH group.²⁸

The structures of both 20(*S*)/(*R*)-Rg₃ and 20(*S*)-Rh₂ had been characterized with NMR. The ¹³C NMR analysis suggested the obvious changes of the chemical shifts in 20(*S*)/(*R*)-epimers at C-17, C-21 and C-22. The chemical shifts of 20(*S*)-Rg₃ were δ_C 55.2(C-17), 27.5(C-21) and 36.3(C-22) ppm, while those of 20(*R*)-Rg₃ were δ_C 50.6(C-17), 22.8(C-21) and 43.2(C-22) ppm. The values in 20(*S*)-Rh₂ were also observed in the peaks of δ_C 54.8(C-17), 27.1(C-21) and 35.9(C-22) ppm. The results were in agreement with the reported data.¹⁸ After dehydration at $\Delta 20(21)$ or $\Delta 20(22)$, the values were shifted to δ_C 155.75 \pm 0.15(C-20) and 108.35 \pm 0.15(C-21) ppm in SFT₃ and Rk₁, and δ_C 140.5(C-20) and 123.5(C-22) ppm in Rg₅, respectively.

3.5 Antiproliferative effect of the transformation products on the cancer cell lines

The antiproliferative activities of the PNL extract before and after microwave processing, and transformation products 1–6 were evaluated in two human cancer cell lines (HeLa and A549) by the MTT assay. The results are expressed as the IC₅₀ of the half maximal inhibitory concentration against cancer cells. As shown in Table 3, the extract of stems and leaves of *P. notoginseng* (EL) had no inhibitory effect on human cervical cancer (Hela) or human lung cancer (A549), and no IC₅₀ values were obtained. After the EL microwave treatment, microwave processing of the MEL resulted in better inhibitory activity against Hela and A549 cells, with IC₅₀ values of 42.97 and 50.47 μ g mL^{−1}, respectively. Microwave processing of ginseng enhances its anticancer activity due to the increased content of the ginsenosides Rg₃, Rg₅, and Rk₁.^{13,14} Further research showed that the transformation products of ginsenosides 20(*S*)-Rg₃, 20(*R*)-Rg₃, Rk₁, Rg₅, and 20(*S*)-Rh₂ and notoginsenoside SFT₃ in the MEL actively inhibited the Hela and A549 cells. Among compounds 1–6, ginsenoside 20(*S*)-Rh₂ (6) displayed lower IC₅₀ values (8.23 and 12.45 μ M) than cisplatin (8.43 and 16.85 μ M) in Hela and A549 cells, respectively. The ginsenoside 20(*S*)-Rh₂ effectively inhibits tumor cell growth and survival in both animal models and cell lines.^{29–31} 20(*S*)-Rh₂ directly binds to Annexin A₂, which promotes apoptosis by inhibiting NF- κ B activity and down-regulating anti-apoptosis gene expression.³¹

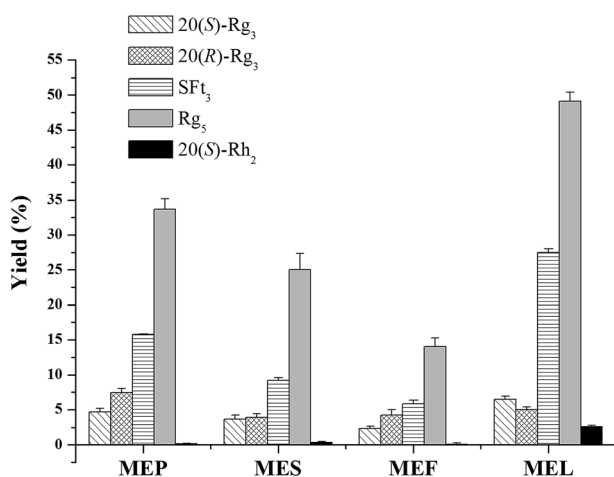


Fig. 6 The contents of five transformation products from microwave processing extracts of stems and leaves (MEL), flowers (MEF), seeds (MES), and pedicels (MEP) of *P. notoginseng*. Data are expressed as mean \pm SD ($n = 3$).



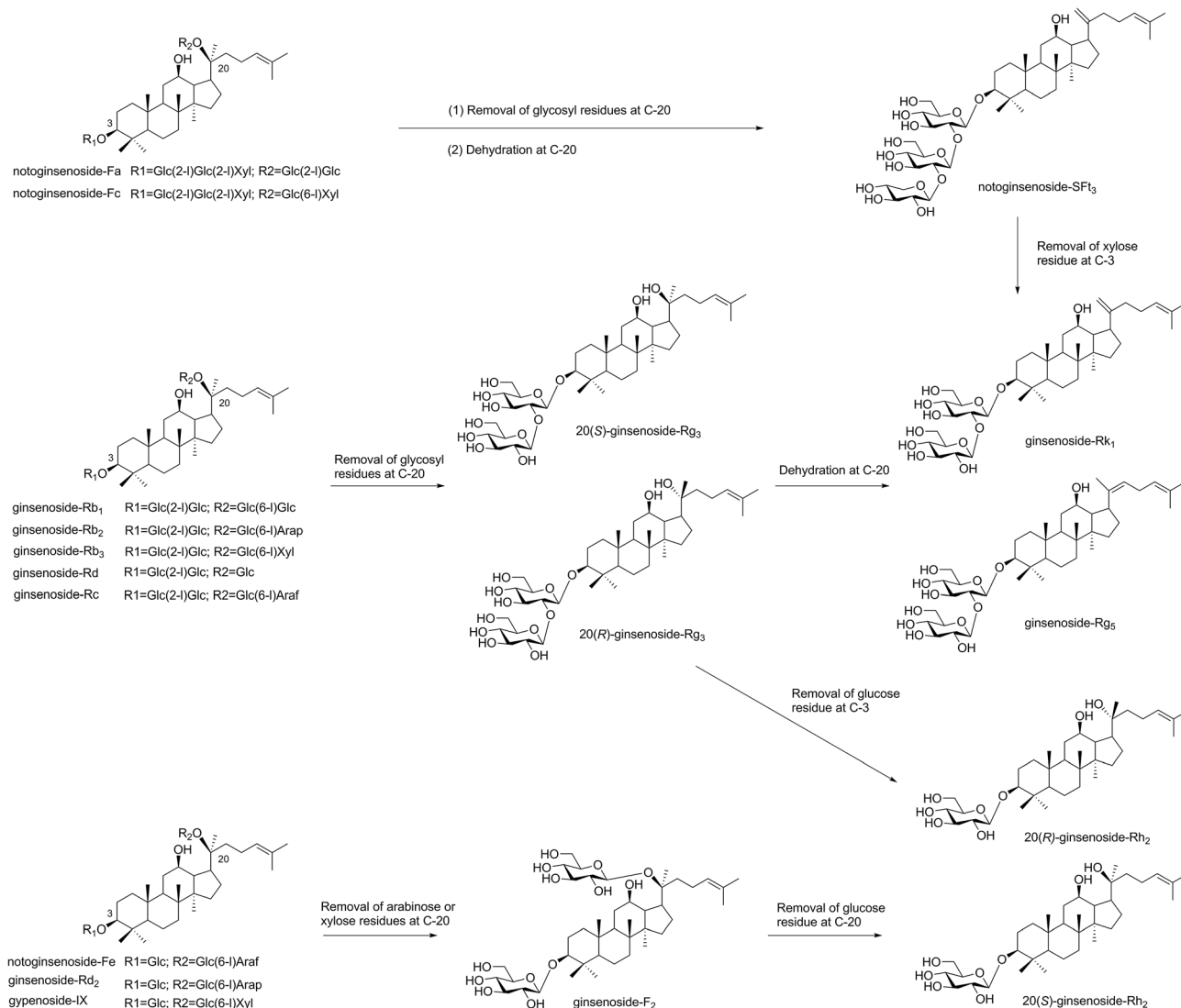


Fig. 7 Proposed transformation pathway of saponins in PNL by the microwave processing.

Table 3 Antiproliferative effect of the extracts (EL and MEL, $\mu\text{g mL}^{-1}$) and compounds 1–6 (μM) in human cancer cell lines^a

Compounds	IC ₅₀ values	
	Hela	A549
20(S)-Ginsenoside Rg ₃ (1)	23.96	20.87
20(R)-Ginsenoside Rg ₃ (2)	24.87	28.47
Notoginsenoside SF ₃ (3)	19.67	21.48
Ginsenoside Rk ₁ (4)	11.97	9.52
Ginsenoside Rg ₅ (5)	42.96	45.24
20(S)-Ginsenoside Rh ₂ (6)	8.23	12.45
Cisplatin	8.43	16.85
MEL	42.97	50.47
EL	>200	>200

^a Hela: human cervical cancer. A549: human lung cancer. IC₅₀: concentration that inhibits 50% of cell growth. EL: extract of stems and leaves of *P. notoginseng*. MEL: microwave processing extract of stems and leaves of *P. notoginseng*.

To confirm the antiproliferative effect of the transformation products, the morphologies in Hela and A549 cells treated with ginsenoside 20(S)-Rh₂ was observed under a microscope (Fig. 8). In the treatment of Hela and A549 cells, 20(S)-Rh₂ at the concentration of 1 μM had no significant effect on cell morphology. While increasing concentration to 10 or 25 μM , 20(S)-Rh₂ induced cell body shrinkage and death in Hela and A549 cells.

3.6 The anticoagulant effect of the transformation products *in vitro*

According to previous reports, different parts of *P. notoginseng* have anticoagulant activity, and the leaves possess stronger anticoagulant activity among them.³² The components of the PNL changed dramatically after the microwave processing treatment (Fig. 3), which may have resulted in the change in anticoagulant activity. Therefore, the effects of the EL, MEL and transformation products 1–6 on anticoagulation were evaluated by the PT assay (Fig. 9). The PT value reflects the activity of the



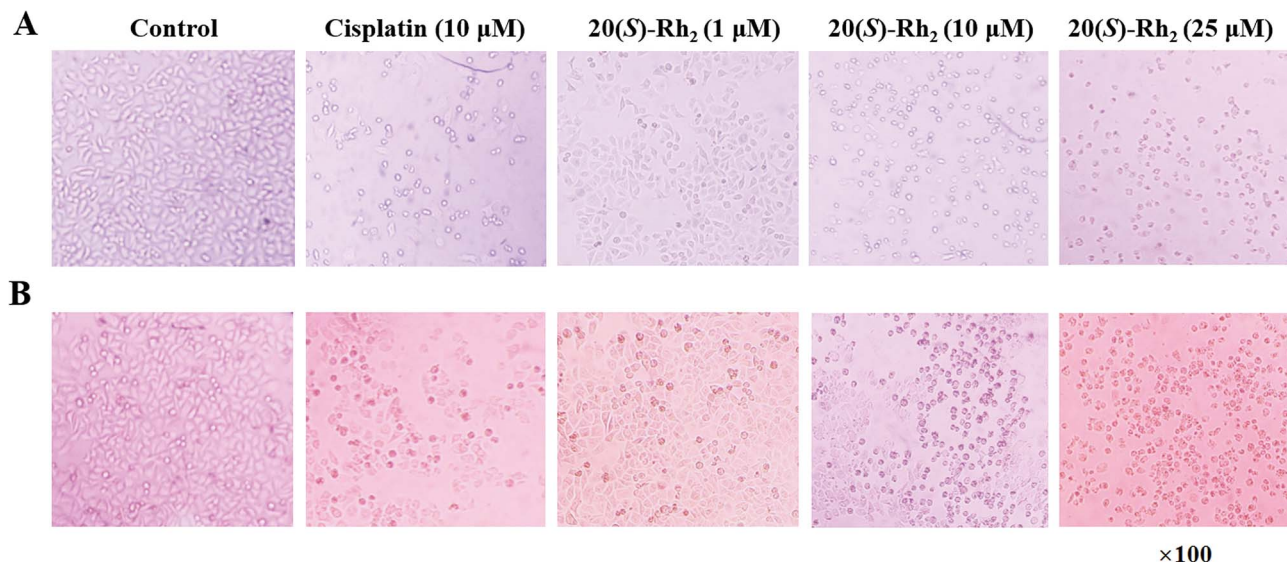


Fig. 8 20(S)-Ginsenoside Rh2 inhibits cell proliferation of human cervical cancer Hela (A) and human lung cancer A549 (B). The cells were treated with 20(S)-ginsenoside Rh2 at different concentrations.

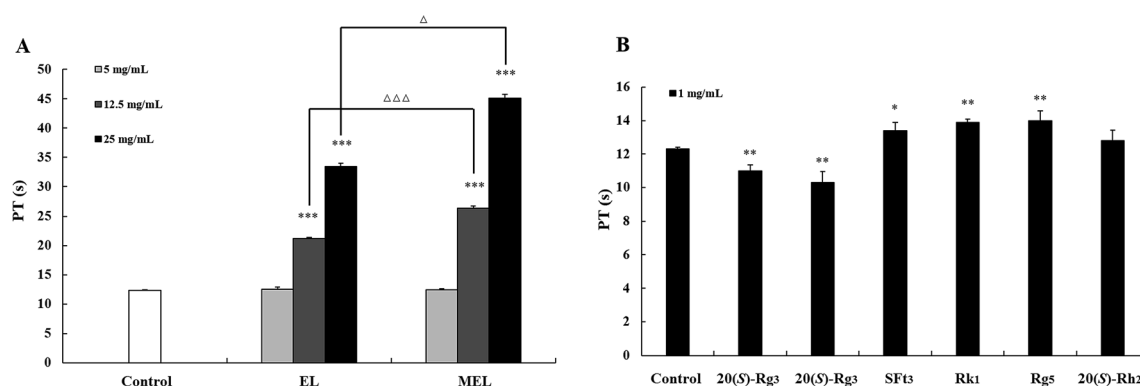


Fig. 9 Anticoagulant activities of raw and microwave processing extracts of the stems and leaves of *P. notoginseng*. (A) PT assays of the extract of stems and leaves of *P. notoginseng* (EL) and microwave processing extract of stems and leaves of *P. notoginseng* (MEL). (B) PT assays of ginsenosides 20(S)-Rg3, 20(R)-Rg3, Rk1, Rg5, 20(S)-Rh2, and notoginsenoside SFt3. Data are expressed as mean \pm SD ($n = 3$). Compared with the negative control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. EL group compared with MEL group: $\Delta p < 0.05$, $\Delta\Delta p < 0.01$, $\Delta\Delta\Delta p < 0.001$.

coagulation system *in vitro*.³³ As shown in Fig. 9A, the EL and MEL had significantly prolonged clotting times in a concentration-dependent manner ($p < 0.001$). The anticoagulant activity of the MEL was better than that of the EL after comparing the extracts of the PNL before and after the microwave treatment. Further studies showed that the degradation products of ginsenosides Rk₁ and Rg₅, as well as notoginsenoside SFt₃ prolonged coagulation time, while ginsenosides 20 (S/R)-Rg₃ shortened coagulation time (Fig. 9B). The structure–activity results revealed that the hydroxy-substituted compounds at C-20 (20 (S/R)-Rg₃) had the hemostatic activities, while Rk₁, SFt₃ and Rg₅ with double bond at $\Delta 20(21)$ or $\Delta 20(22)$ played the anticoagulant activities. The anticoagulant activity of the MEL increased due to increases in the concentrations of Rk₁, Rg₅, and SFt₃. This result was consistent with previous findings that ginsenosides Rk₁ and Rg₅ inhibit arachidonic acid-induced platelet aggregation in a dose dependent manner.³⁴

4 Conclusion

In summary, microwave processing was applied to produce rare saponins in the PNL. Six transformation products of ginsenosides 20(S)-Rg₃, 20(R)-Rg₃, Rk₁, Rg₅, 20(S)-Rh₂, and notoginsenoside SFt₃ were isolated from the MEL. Microwave power, temperature, and time affected the yields of the six transformation products. This transformation method was widely applied for producing the minor ginsenosides in stems and leaves, flowers, seeds and pedicels of *P. notoginseng*. The MEL had an obvious inhibitory effect on human cervical cancer and human lung cancer cell lines. The MEL had a stronger anticoagulant effect due to increased contents of ginsenosides Rk₁, Rg₅, and notoginsenoside SFt₃. Therefore, the PNL may be a good source of anticancer and anticoagulant therapeutics after microwave processing.



Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgements

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