https://doi.org/10.26651/allelo.j/2022-56-2-1401 0971-4693/94 Euro 20.00
Allelopathy Journal 56 (2): 237-250 (July, 2022) *International Allelopathy Foundation* 2022
Table: 4, Figs : 5

Jinping Ginseng species identification based on ITS2 bar code and HPLC technology

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(Received in revised form: March 31, 2022)

ABSTRACT

Panax vietnamensis has high medicinal and market values, is called Jinping ginseng. It is rare medicinal material in Yunnan Province, China. Since, this name is controversial, thus, we did further identification study on Jinping ginseng *P. vietnamensis* and PVF (*P. vietnamensis* var. Fuscidiscus) using morphology, the ITS2 sequence and HPLC fingerprint techniques. We found some morphological differences among the Jinping ginseng, *P. vietnamensis* and PVF. There were also variabilities in gene sequence and chemical contents. In ITS2 sequence, Jinping ginseng had 1 different position than PVF and had 5 different positions conmpaeard with *P. vietnamensis.* The ratio of (G-Rg1+G-Re): G-Rb1 in Jinping ginseng was > 4 times higher than in *P. vietnamensis*. The ITS2 bar code and HPLC fingerprint technology proved effective method to identify Jinping ginseng from *P. vietnamensis*.

Keywords: Finger printing, ginseng, ginseng species identification, HPLC, ITS2, Jinping Black Ginseng, morphology, *P. vietnamensis.*

INTRODUCTION

Jinping ginseng also known as black Sanqi/red Sanqi/Jinping Sanqi, is the rare medicinal materials native to Jinping County, Yunnan Province, China. Jinping ginseng had been known and used medicinally by ethnic minorities in Jinping area since the ancient times, for postpartum recovery of women, recovery from serious illnesses, nourishment and prolonged life of elderly. Jinping ginseng is presently grown on large scale in Jinping County and its market price in China is 10-times higher than *Panax notoginseng* (Burkill) F. H. Chen ex C. Chow & W. G. Huang. However, it has not been officially named in plants classification. Jinping ginseng is considered as a kind of *Panax. vietnamensis* Ha & Grushv and its effect is exaggerated in Chinese promotion (Fig. 1).

The author found that there were some morphological differences in disk and the basal part of the 2-styled flowers between Jinping ginseng and *P. vietnamensis*, which is closer to *P. vietnamensis* Ha & Grushv. var. fuscidiscus K. Komatsu, S. Zhu & S.Q. Cai (abbreviated as PVF) (26) (Fig. 2.). For example, their main distribution areas are different. Jinping ginseng grows mainly in Jinping County, which is the same as PVF, while *P. vietnamensis* grows mainly in Yuling Nature Reserve, central Vietnam.

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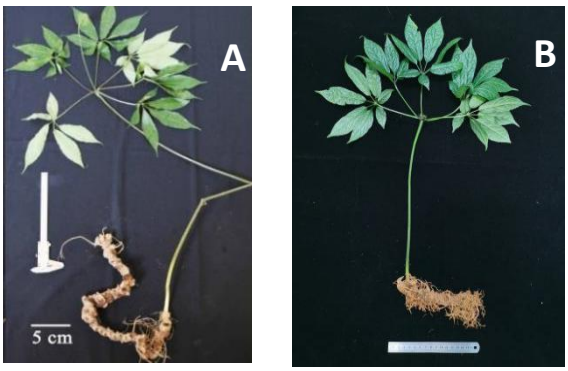

Figure 1. The plant of *P. vietnamensis* (A) and *P. notoginseng* (B). The *P. vietnamensis* piture was taken from Ngoc *et al* 2020 (13), and the picture of *P. notoginseng* was fom our research team.

Figure 2. Pictures of Jinping ginseng and PVF, picture of Jinping ginseng from our research team and the picture of PVF from Zhang *et al* 2015 (22). A: Rhizome of Jinping ginseng, B: Shoot growth of Jinping ginseng, C: Rhizome of PVF and D: Shoot growth of PVF.

Chen *et al.* (2010) found that the second internal transcribed spacer (ITS2) sequence could be a standard DNA barcode to identify the medicinal plants and their closely related species (7). Furthermore, ginseng is well known for its allelopathic effects (19). There are remarkable reports showing the physiological responses of local plants to allelopathic ginseng, while the chemical constituents needs to be further investigated (23,24,25,28). In particular, it is of great importance to study the chemical constituents in medical plants. Hence, in this study, morphological analysis, ITS2 bar code technology (2,3,4,5,10,12, 14,16,18,21) and HPLC (17,20) were used to identify and differentiate between the Jinping

ginseng and *P. vietnamensis*. Further studies needs to be done to identify the chemical constituents, its medicinal efficacy and resource development of Jinping ginseng.

MATERIALS AND METHODS

I. Experimental materials

The samples of Jinping ginseng plant were collected from Jinping Dajiangkang medicinal material company, Jinping County, Yunnan Province, China (102°55'E, 22°43'N), its major planting base. The *P. notoginseng* were collected from Qiubei County of Zhuang and Miao Autonomous Prefecture of Yunnan Province (104°40'E, 23°47'N). and identified by Zilong Zhang, Associate Researcher from Beijing University of Chinese Medicine. All the samples were collected in September 2018.

Five batches (All collected in September 2018) of Jinping ginseng and *P. notoginseng* were used in this study. All the samples were from triennial (3-years old) plants and collected in September 2018. Specimens of Jinping ginseng were deposited in museum, Traditional Chinese Medicine, Beijing University of Chinese Medicine.

Four batches (All collected in September 2018) of *P. notoginseng* and Jinping ginseng were used in this study. Four ITS2 sequences (No. J-1, J-2, J-3, J-4) of Jinping ginseng of which J-1 and J-2 came from plant sample 1; J-3 and J-4 came from plant sample 2) were obtained by DNA extraction, PCR amplification and sequencing. At the same time, two ITS2 sequences (No. S-1, S-2) of *P. notoginseng*, *P. Vietnamensis*, PVF and other ginseng plants were downloaded from GenBank database (https://www.ncbi.nlm.nih.gov/genbank).

II. DNA sequence analysis

(i). DNA extraction

The young leaves of Jinping ginseng and *P. notoginseng* stored at -70 ºC were snap-frozen in liquid nitrogen and transferred to a 2-mL eppendorf (EP) tube containing 1 mL CTAB. The leaves were pre-heated to 65 ºC for 1 h and shaken every 15 min and centrifuged at 12000 rpm for 2 min. 800 μL supernatant was transferred into the new 1.5 mL EP tube and 400 μL phenol chloroform isoamyl alcohol (25: 24: 1) was added. The mixture was shaken and set aside for few minutes until stratified. 600 μL of supernatant was transferred into the new centrifuge tube with 600 μL isopropanol and centrifuged at 12000 rpm for 5 min. Then the supernatant was removed, the pellet was washed with 1 mL 75 % ethanol and dried for 5 min. Finally, 50 μL water with RNase (0.1 %) was added to dissolve the DNA.

(ii). PCR amplification and sequencing

The ITS2 sequences were amplified with the forward primer ITS2F (5'-ATGCGATACTTGGTGTGAAT-3') and the reverse primer ITS3R (5'-GACGCTTCTCCAGACTACAAT-3') (4). The primers were synthesized by Beijing Genomics institution. PCR amplification program was as follows: 94 ˚C for 2 min, followed by 30 cycles at 94 ˚C for 30 s, 54 ˚C for 30 s, 72 ˚C for 15 s and completed with an extension at 72 ˚C for 5 min. Sequencing was performed by Tsingke Biological

Technology Company (Chongqing, China) and then the ITS2 fragments of *P. notoginseng* and Jinping ginseng with the length of 230 bp were obtained by Genbank comparison.

(iii). ITS2 sequences analysis

The four ITS2 sequences of Jinping ginseng (No. J-1, J-2, J-3, J-4) and the 2 ITS2 sequences (No. S-1, S-2) of *P. notoginseng* were obtained by DNA extraction, PCR amplification and sequencing. The ITS2 sequences of *P. vietnamensis*, PVF, *P. notoginseng* and other plants of genus *Panax* were downloaded from GenBank database of NCBI (https://www.ncbi.nlm.nih.gov/genbank). Phylogenetic and the intraspecific genetic distance analysis were performed with MEGA X software.

III. HPLC Analysis of chemical constituents

(i). Sample preparation

The ginsenoside standards used in this experiment were purchased from Shanghai Yuanye Biotechnology Company. All the Jinping ginseng and *P. notoginseng* triennial plants were washed, dried to constant weight. The root and rhizome were separated, crushed and filtered through a 60-mesh sieve. 0.5 g of sample powder was mixed with 25 ml pure methanol and incubated overnight, then boiled at 80˚C for 2 h. The mixture was filtered with 0.45 μm filter membrane and the filtrate was collected for HPLC analysis (1,9).

(ii). Chromatographic conditions

Samples were analyzed on a Thermo U3000 HPLC system with an Agilent Zorbax SBC18 (5 μm, 250 mm × 4.6 mm) column. The separation was done at solvent flow rate of 1.5 mL/min at 30˚C. Acetonitrile was used as mobile phase A and water as B. The solvent gradient was: 0-20 min, 20 % A; 20-45 min, 20 %-46 %A; 45-55 min, 46 %-55 % A; 55-60 min, 55 % A. The injection volume was 10 μL and the detection wavelength was 203 nm.

(iii). Validation of methodology

Precision was obtained by analyzing the 6-times extracted samples. Relative standard deviation (RSD) was used to express variations. As for the peak area, the RSD values of the 5 ginsenosides were 0.07-0.72 %, showing that the precision of method was acceptable.

The mixed reference solution 1 was injected at 2 μL, 4 μL, 6 μL, 8 μL, 10 μL, 15 μL and 20 μL, respectively. Thereafter, the mixed reference solution 2 was injected at 15 μL and 20 μL. A series of standard data were obtained from the standard solutions of 3-concentrations, by different injection volumes and the correction curves were prepared. The concs of G-Rg1, G-Re, G-Rb1, G-Rc and G-Rb2 in standard solution1 were as under:

Solutions	G-Rg1	G-Re	G-Rb1	G-Rc	G-Rb2
	Concentrations (g/mL)				
1	0.4000	0.4000	0.4000	0.4000	0.4000
2	0.8000	0.4000	0.8000	0.4000	0.4000
3	0.3960	0.0280	0.1800	0.0246	0.0800

Plotting the integrated peak areas of 5 different levels (or more) concentration, we got the calibration curve and results (Table 1) of each ginsenoside. All of the compounds showed good linearity within relatively wide ranges.

Table 1. Linear regression data of the five ginsenosides

Compound	Calibration curve	Linearity range (mg/mL)	Correlation coefficient (R^2)
Rg1	y = 29.84506 x + 0.57544	0.0800-1.6000	0.99909
Re	y = 28.95390 x + 0.04209	0.0800-0.4000	0.99969
Rb1	y = 25.47808 x + 0.51157	0.0800-1.6000	0.99890
Rc	y = 29.07710 x + 0.04337	0.0800-0.0600	0.99978
Rb2	y = 28.12522 x + 0.06660	0.0800-0.0600	0.99991

The freshly extracted sample solutions were analyzed at 0 h, 2 h, 4 h, 6 h, 8 h and 12 h for the stability test. The RSD values of the peak area of the 5 ginsenosides were 1.04-3.04 %. According to the results, analysis of samples within 12 h was feasible.

The accuracy of analytical method was assessed by a recovery test. The HPLC method was used to analyze the samples. One mL test solution of Jinping ginseng root was precisely absorbed, and 1 mL methanol was added to obtain the new test solution B, and 10μL was injected according to the same chromatographic conditions. Then take 1 mL solution A and add 1mL mixed control substance (approximately equal to 50-150 % of each ginsenoside amount in the extracted sample), and measure 6 times continuously under the same conditions. The recovery rates of the mean of the 5 saponins were in the range of 98.14-103.65 % with RSD < 2.76 %, showing acceptable accuracy of the method. The recovery was calculated as under:

Recovery (%) =100× (found amount– original amount) / spiked amount (20).

RESULTS AND DISCUSSION

Morphological differences in Jinping ginseng, PVF, *P. vietnamensis* and *P. notoginseng*

The author's on-site investigation found that the flower morphology of Jinping ginseng was similar to *P. vietnamensis* and PVF, with a slight difference. And there were significant differences between Jinping ginseng and *P. notoginseng* in flowers, fruits, rhizomes and leaves. All of them have more than 50 flowers (50-120) in an inflorescence and only 1 or 2 styles. There were only few differences in the disk and basal part of 2-styled flowers. Jinping ginseng had both flat and convex disks (joint surface between ovary and style) in greenish-white or fuscous colour, with 2 styles united or completely separated at the basal part (Fig. 3). *P. vietnamensis* had a convex and greenish-white disk, the 2 styles united at the basal part (8). Zhu found that PVF had a flat, fuscous or vaccinous disk and completely separated styles in the 2-styled flowers. Jinping ginseng has 2 different types of flowers: the ovary has 1 or 2 styles, we found that the growth years of Jinping ginseng increasing, the larger the proportion of flowers with 2 styles in an ovary. Almost all biennial

Figure 3. The flowers of Jinping ginseng has flat (A, B and D) or convex (C) disks in greenish-white (A) or fuscous color (B); with the styles of the 2-styled flowers united at the basal part (A, B and C) or completely separated (D). JBS which growing for three years (D) has larger proportion of flowers with 2 styles in an ovary than which growing for two years (C) (bar=1cm).

plants of Jinping ginseng have 1 style and triennial plant exists in both 1 style and 2 styles and quadrennial plants of Jinping ginseng have 2 styles. There were only a few subtle differences in disk and the basal part of 2-styles among the three, but not all the plants of Jinping ginseng had the 2-style flowers. Thus, it is difficult to identify Jinping ginseng from *P. vietnamensis* by morphological method. There were significant differences between Jinping ginseng and *P. notoginseng* in flowers, fruits, rhizomes and leaves. For example, Jinping ginseng had umbrella inflorescence, can up 150 to 200 flowers, but *P. notoginseng* is often limited to 100. The ripe fruit of Jinping ginseng was bright red and had black spots at the top, but *P. notoginseng* had no such characteristic. The rhizome of Jinping ginseng was nearly as long as the root which was different from *P. notoginseng* (Table 2).

Table 2. Morphological differences in Test Ginseng spp.

#	Morphological Character	*P. vietnamensis*	PVF	Jinping ginseng	*P. notoginseng*
1	Flowers number per inflorescence	50-120	70-100	150-200	80-100
2	Disk of 2-styled flowers	Convex and greenish-white disk	Flat, fuscous or vaccinous disk	Both flat and convex disks in greenish-white or fuscous color	Green, slightly flat, ring-shaped, scrunched disk
3	Basal of 2-styled flowers	United	Completely separated	United or completely separated	Separate into 2
4	Fruits	—	Bright red and had black spots at the top	Bright red and had black spots at the top	Bright red
5	Leaves	—	Compound leaves, generally 5, rarely 7, about 8-14 cm in length, 3-5.5 cm in width and 4-10 cm in petiole.	Compound leaves, usually 7 - 8 leaves, about 15 cm in length, 5 cm in width and 17 cm in petiole.	4-5 compound leaves, 6 - 11 cm long, 2 - 4 cm wide and 5 - 10 cm in petiole.
6	Rhizomes	—	1-2 cm diameter, nodes closely-compacted with stem scar, internode <6 mm	8 - 12 cm	2 - 4 cm
7	Root	—	3 – 12 cm	Length nearly to rhizome	6 - 9 cm

Note: The morphology of *P. vietnamensis* and PVF were from Zhu *et al* 2003 (8), Zhang *et al* 2015 (22) and Ha *et al* 1985 (26). The morphological records of PVF and *P. vietnamensis* were incomplete.

ITS2 sequence analysis

All ITS2 sequences were 230 bp in length. The average GC content of ITS2 sequences of Jinping ginseng, PVF, *P. vietnamensis* and *P. notoginseng* was 62.0 % (n = 4), 62.2 % (n = 5), 63.5 % (n = 5) and 63.4 % (n = 5), respectively. Compared with Jinping ginseng, there were differences in nucleotides at 1 different position in PVF (180), at 5 different position in *P. vietnamensis* (2, 83, 180, 208 and 216), at 10 different position in *P. notoginseng* (2, 22, 28, 35, 46, 83, 140, 180, 207 and 208) (Table 3). GC content and nucleotide sequences analysis showed that Jinping ginseng was more similar to PVF than *P. vietnamensis*.

Table 3. ITS2 sequence comparison between Jinping ginseng, PVF and *P. vietnamensis.*

Position	2	22	28	35	46	83	140	150	160	180	207	208	216
Jinping ginseng													
Jinping ginseng[a] J-1	A	T	C	A	G	T	A	A	C	A	C	A	C
Jinping ginseng J-2	.	.	.	.	.	.	.	.	.	.	.	.	.
Jinping ginseng J-3	.	.	.	.	.	.	.	C	.	.	.	.	.
Jinping ginseng J-4	.	.	.	.	.	.	.	C	.	.	.	.	.
***P. vietnamensis* var. fuscidiscus**													
PVF[b] MH345133	.	.	.	.	.	.	.	C	.	G	.	.	.
PVF MH345132	.	.	.	.	.	.	.	C	.	G	.	.	.
PVF MG604377	.	.	.	.	.	.	.	C	.	.	.	.	.
PVF MH345131	.	.	.	.	.	.	.	C	.	G	.	.	.
PVF KX768326	.	.	.	.	.	.	.	.	.	.	A	.	.
P. vietnamensis													
P. vietnamensis MK979329	G	.	.	.	.	C	.	C	.	G	.	G	T
P. vietnamensis MK979388	G	.	.	.	.	C	.	C	.	G	.	G	T
P. vietnamensis KT380922	G	.	.	.	.	C	.	C	.	G	.	G	T
P. vietnamensis MK979392	G	.	.	.	.	C	.	C	.	G	.	G	T
P. vietnamensis MK979389	G	.	.	.	.	C	.	C	.	G	.	G	T
P. notoginseng MG604373	G	C	T	G	T	C	T	C	.	G	T	G	.
P. notoginseng MG604372	G	C	T	G	T	C	T	C	T	G	T	G	.
P. notoginseng MG604374	G	C	T	G	T	C	T	C	.	G	T	G	.
P. notoginseng S-1	G	C	T	G	T	C	T	C	.	G	T	G	.
P. notoginseng S-2	G	C	T	G	T	C	T	C	.	G	T	G	.

Genetic distance : In this research, the genetic distance was calculated. The intraspecific genetic distance of Jinping ginseng was 0 - 0.0044, which of PVF, *P. vietnamensis, P. notoginseng* were 0 - 0.0133, 0, 0 - 0.0044, respectively. The genetic distances between Jinping ginseng and PVF, *P. vietnamensis*, *P. notoginseng* were 0 - 0.0088, 0.0221 - 0.0266 and 0.0451 - 0.0547, respectively. The results showed that the genetic distance between Jinping ginseng and PVF was less than that between Jinping ginseng and *P. vietnamensis*. In addition, the genetic distance between Jinping ginseng and PVF was less than the intraspecific genetic distance of PVF. Just according to ITS2 genetic distance analysis, the genetic relationship between Jinping ginseng and *P. zingiberensis* was closer than that of *P. vietnamensis*, which was not consistent with the morphological analysis. Thus, ITS2 bar code alone for species identification is not accurate, hence, use multiple methods.

The phylogenetic tree was constructed by Neighbor-Joining (NJ) method (12) and the statistical support for nodes was determined by bootstrap replication. *Eleutherococcus gracilistylus* (GenBank no. AY548182), was used as outgroup in the phylogenetic tree of genus *Panax* (Fig. 4). Genus *Panax* was divided into two sub-groups. In the first sub-group, Jinping ginseng, PVF, *P. zingiberensis* and *P. vietnamensis* formed one clade. In this clade, Jinping ginseng, PVF, *P. zingiberensis* formed one sub-clade, while only *P. vietnamensis* was in other sub-clade. Thus Jinping ginseng was more similar to PVF than *P. vietnamensis*.

It is difficult to morphologically differenciate between the Jinping ginseng and *P. vietnamensis*, large-scale cultivation may help researchers to distinguish the Jinping ginseng from *P. vietnamensis*. For example, we found some sequences of *P. vietnamensis* from genbank (https://www.ncbi.nlm.nih.gov/genbank/) database are the same as Jinping ginseng, these samples are mainly from China (MG283291, MG283294). So, ITS2 bar code can be a good method to distinguish the two, which can provide an important base for research and use of both Jinping ginseng and *P. vietnamensis*.

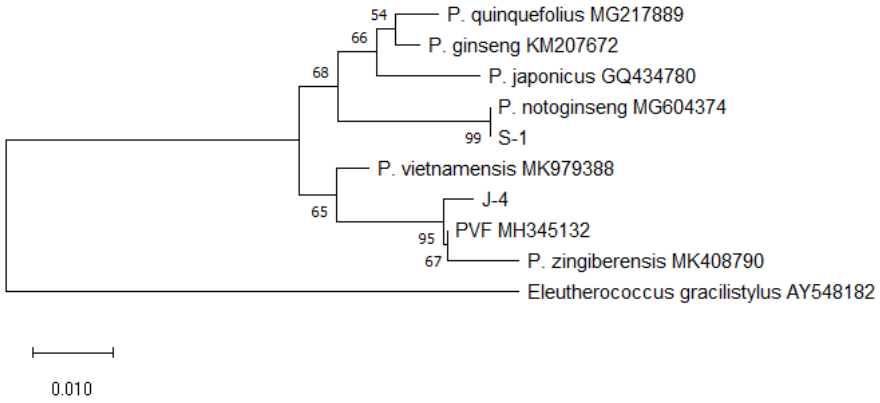

Figure 4. The neighbour-joining tree based on ITS2 sequences of 9 species of genus *Panax* with 1000 bootstrap replicates. Numbers on the branch represent the statistical support for nodes (%).

Ginsenosides in Jinping ginseng, *P. vietnamensis* and *P. notoginseng*

The contents and ratios of 5 ginsenosides in Jinping ginseng and *P. notoginseng* determined in this experiment were compared with those in *P. vietnamensis* determined by Thi Hong Van Le (9) under similar conditions. The results showed that Jinping ginseng was different from *P. vietnamensis* and *P. notoginseng*.

The total content of G-Rg1 and G-Re in Jinping ginseng was about 4 times higher than that in *P. vietnamensis*, but there was no significant differences in the contents of G-Rb1, G-Rb2 or G-Rc. The ratios of (G-Rg1+G-Re) to G-Rb1 were quite different in the two plants. As for Jinping ginseng rhizome, the ratio of (G-Rg1+G-Re) to G-Rb1 was 5.2 in root and 4.4 in rhizome. While for *P. vietnamensis*, the ratio of (G-Rg1+G-Re) to G-Rb1 was 0.8 in the mixed sample of root and rhizome (Table 4, Fig. 5).

Table 4. Contents of 5-ginsenosides in Jinping ginseng, *P. notoginseng* and *P. vietnamensis* samples

Ginseng Sample	G-Rg1	G-Re	G-Rb1	G-Rc	G-Rb2	Total
Jinping ginseng root Root Rhizome	43.1 ± 1.3	0.3 ± 0.1	8.4 ± 0.1	14.8 ± 0.5	3.5 ± 0.1	70.1
Jinping ginseng rhizome	43.4 ± 1.2	0.6 ± 0.1	10.0 ± 0.3	9.2 ± 0.3	5.5 ± 0.2	68.7
P. notoginseng root Rhizome	48.4 ± 1.7	7.5 ± 0.1	43.7 ± 1.6	2.0 ± 0.2	N. D. [c]	101.6
P. notoginseng rhizome	76.9 ± 1.2	13.2 ± 0.2	55.9 ± 2.0	2.5 ± 0.1	1.1 ± 0.1	149.6
P. vietnamensis r & r[a]	11.6 ± 0.4[b]		14.4 ± 0.5	7.0 ± 0.3	5.5 ± 0.5	38.5

Note: [a]root & rhizome. [b]calculated as G-Rg1+G-Re. [c]N.D: not detected. (n = 4 mg/g).

The contents of G-Rb1 in Jinping ginseng were lower than those in *P. notoginseng* (about 1/2), but the root and rhizome of Jinping ginseng were about three times the weight of *P. notoginseng* in triennial plants. While the contents of G-Rc and G-Rb2 in Jinping ginseng were more than three times higher than those in *P. notoginseng*.

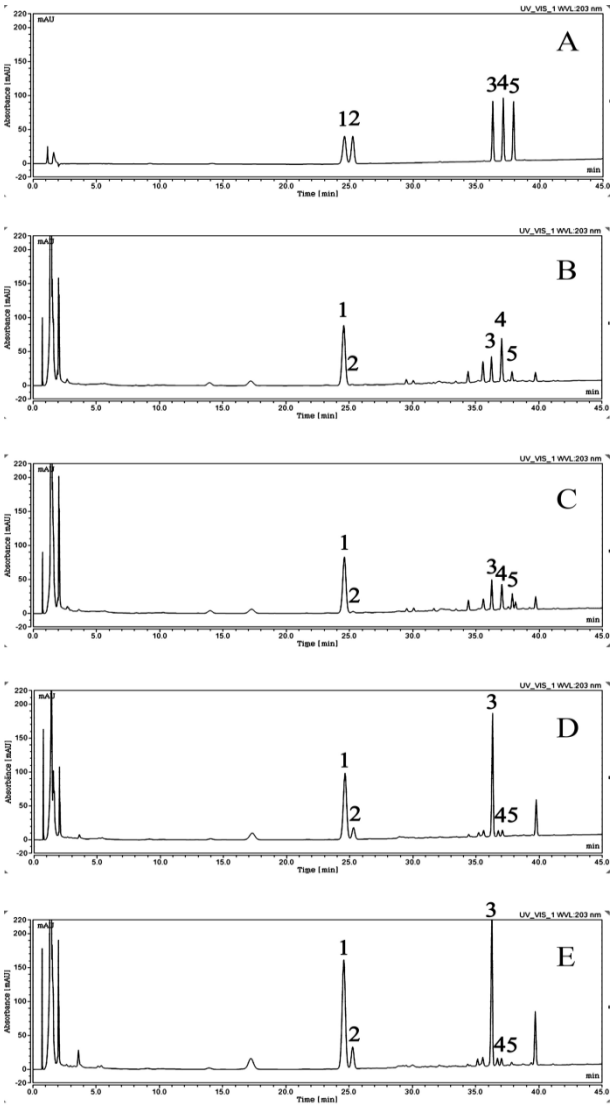

Figure 5. Analysis of 5-ginsenosides in Jinping ginseng, *P. notoginseng* by HPLC. The determination of 5 saponins in (A) reference substance; (B) root of Jinping ginseng; (C) rhizome of Jinping ginseng; (D) root of *P. notoginseng*; and (E) rhizome of *P. notoginseng* by HPLC: 1. G-Rg1; 2. G-Re; 3. G-Rb1; 4. G-Rc. 5. G-Rb2.

Le *et al*. (9) reported that the results of different experimental conditions showed that the ratio of (G-Rg1+G-Re) to G-Rb1 in *P. vietnamensis* was less than 1.5. Our experimental conditions are similar to theirs, under which the ratio was > 4. This difference can be used to distinguish the two, but whether they should be treated differently in medicine should be further studied. Zhu *et al*. (27) presented that ginsenoside Rg1 has the highest content in *Panax notoginseng*, but we found that the root and rhizome weight of Jinping ginseng was about 3-times than weight of *P. notoginseng* in triennial plants. Therefore, Jinping ginseng is an important source of G-Rg1, which has effective antiapoptotic and anti-inflammatory properties and play significant role in suppressing renal tubular cells and autophagy in cardiomyocytes (11). The data supported the hypothesis that Jinping ginseng has better nutritional value than *P. vietnamensis*.

CONCLUSIONS

These studies provided a method to identify Jinping ginseng and especially distinguishing it from *P. vietnamensis*. Firstly, the ITS2 sequences between Jinping ginseng and *P. vietnamensis* had 5-different nucleotides at position 2, 83, 180, 208 and 216. Secondly, their ginsenoside composition was different. Especially, the ratio of (G-Rg1+G-Re) to G-Rb1 in Jinping ginseng is more than 4, while the ratio is less than 1.5 in *P. vietnamensis*.

These result suggested that Jinping ginseng is different from *P. vietnamensis* and much closer to *P. vietnamensis* var. fuscidiscus. This will be helpful for the further research and popularising both Jinping ginseng and *P. vietnamensis*. The similarities and differences between Jinping ginseng and *P. vietnamensis* should be further studied in pharmacology and in medicine.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation (No. 81102751). We also thank Yunnan Jinping Great Health Medicine Co., Ltd. for providing research materials and providing convenience for our field research.

DECLARATION

We declare that all authors of this Ms. have made substantial contributions. We did not exclude any author who substantially contributed to this Ms. We have followed our ethical norms established by our respective institutions.

CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals, hence no ethical approval has been obtained from the concerned committee.

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