Original Article

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Comparison of compounds of three *Rubus* species and their antioxidant activity

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Summary ***Rubus amabilis*, *Rubus niveus Thunb.*, and *Rubus sachalinensis* are three *Rubus* species that are alternatively found in Manubzhithang, a Tibetan medicine, in different areas of China. The current study analyzed HPLC/UV chromatograms and it compared compounds of these three *Rubus* species in contrast to reference substances such as 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside, procyanidin B4, and isovitexin-7-*O*-glucoside. The three *Rubus* species produced similar peaks in chromatograms. The antioxidant activity of the three *Rubus* species was determined using an assay for DPPH free radical scavenging activity. Results indicated that the three *Rubus* species extracts had almost the same level of free radical scavenging activity. Thus, findings indicated the rationality of substituting these species for one another as an ingredient in Manubzhithang.**

Keywords: *Rubus amabilis*, *Rubus niveus Thunb.*, *Rubus sachalinensis*, antioxidant activity, Tibetan medicine

1. Introduction

Rubus species (*Rosaceae*) such as *Rubus amabilis*, *Rubus niveus Thunb.*, and *Rubus sachalinensis* are often used in herbal medicines in China. These medicines contain various chemical compounds including polyphenolics, flavanols, flavonoid glycosides, pregnane glycosides, lignin glycosides, triterpene glucosyl esters, alkanols, anthocyanins, lignans, and tannins (*1,2*). These medicines can be used in traditional medicine as a radical scavenging agent (*3-5*), an anti-inflammatory, an anticonvulsant, a muscle relaxant, an antimicrobial, an antiphlogistic, an analgesic, an antidotal, or an antitumor agent (*6-9*).

Different areas substitute *Rubus amabilis*, *Rubus niveus Thunb.*, and *Rubus sachalinensis* for one another as a key ingredient in the traditional Tibetan medicine Manubzhithang. Manubzhithang is a powder that is used to make a broth, and this medicine has been used in Tibetan medicine for 1,300 years (*10*). Manubzhithang has exceptional ability to prevent and treat inflammatory disease, "depleted heat", gastrointestinal ulcers, and vascular angina. In northwest China, Manubzhithang consists of *R. amabilis*, *Inula helenium*, *Tinospora cordifolia (Willd.) Miers*, and *Zingiber officinale Rosc.* In southwest China, Manubzhithang consists of *R. niveus Thunb.*, *I. helenium*, *T. cordifolia (Willd.) Miers*, and *Z. officinale Rosc*. In Inner Mongolia and Xinjiang, China, Manubzhithang consists of *R. sachalinensis*, *I. helenium*, *T. cordifolia (Willd.) Miers*, and *Zingiber officinale Rosc.* or *Kaempferia galanga* L.

The current study analyzed the HPLC/UV chromatograms produced by *R. amabilis*, *R. niveus Thunb.*, and *R. sachalinensis*. Compounds of the three *Rubus* species were compared to reference substances such as 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside (Compound 1), procyanidin B_4 (Compound 2), and isovitexin-7-*O*-glucoside (Compound 3). The antioxidant activity of the three *Rubus* species was also determined using an assay for DPPH free radical scavenging activity. To the extent known, the current study is the first to examine the rationality of substituting the three *Rubus* species for

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one another, based on their antioxidant activity, as a main ingredient in the traditional Tibetan medicine *Manubzhithang*.

2. Materials and Methods

2.1. *Plant materials and extract preparation*

Fresh *R. amabilis* was collected from the Northern Mountains National Forest in Huzhu, Qinghai, *R. niveus* Thunb. was collected from Nyingchi, Tibet, and *R. sachalinensis* was collected from the Dongwuqibaogeda Mountains in Inner Mongolia. Specimens of the plants were authenticated by the College of Chinese Medicine, Department of Chinese Medicinal Chemistry, Beijing University of Chinese Medicine. 2-deoxy-D-ribose, 2,2-dipheny-l-picrylhydrazyl (DPPH), and vitamin C were purchased from Sigma-Aldrich Co. (Shanghai, China). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Ethanol, phosphoric acid, and methanol were purchased from Beijing Hongxing Chemicals Co. (Beijing, China). All other reagents were of analytical grade unless otherwise noted.

The collected plants were transported to the laboratory and grouped accordingly. Plants were ground and sieved through a sieve with 0.25 mm mesh. Two g of each sample of *R. amabilis* powder was placed in a 250 mL round-bottomed flask and 50 mL of methanol was added. The contents of the flask were sonicated for 40 min. Afterwards, the contents were filtered and injected into an HPLC column. An additional 2.0 g of sample powder was also placed in a 250 mL round-bottomed flask and 50 mL of methanol was added. The contents of the flask were refluxed twice for 2 h (1 h for each reflux). Afterwards, the contents were filtered using a 0.45 mm membrane filter prior to injection into an HPLC column.

Two g of each sample of *R. amabilis* powder was placed in a 250 mL round bottomed flask and 50 mL of methanol or 95% ethanol was added. The contents of the flask were sonicated for 40 min. Afterwards, the contents were filtered and injected into an HPLC column.

2.2. *HPLC conditions*

HPLC was performed with a Waters high-pressure liquid chromatographic system equipped with a 1525 high-pressure gradient pump, a Kromasil 100-5C_{18} column (250 × 4.6mm, 5 μm), a 2998 PDA array detector, a 2707 automatic sampling injector, and a 2414 constant temperature system. The column temperature was set at 25°C. The mobile phase consisted of acetonitrile (A) and phosphoric acid:water (40:60) (B). (10-25% A; 90-75% B) (Table 1). Separation was performed by gradient elution at a total flow rate of 1.0 mL/min, a binary solvent mixture was used, and run time was adjusted to 30 min. The injection volume was 10 μL and elutes were detected at 210 nm. The standard stock solutions of isovitexin-7-*O*-glucoside, procyanidin B_4, and 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside were separated from *R. amabilis* in the laboratory.

Table 1. Gradient elution of the mobile phase

Time (min)	Acetonitrile (%)	Phosphoric acid:Water (0.4%)
0	10	90
5	15	85
30	25	75

2.3. *Comparison of compounds*

Standard stock solutions of isovitexin-7-*O*-glucoside, procyanidin B_4, and 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside were prepared in methanol. Chromatograms of the standard solutions, the standard extracts of *R. amabilis* (1.0g), and the extracts of *R. amabilis*, *R. niveus Thunb.*, and *R. sachalinensis* (1.0g each) were compared.

2.4. *Determination of antioxidant activity in vitro*

Each plant was individually washed with tap water and deionized water. Afterwards, the plants were dried at 55.8°C until a constant weight was obtained. All of the samples (5.0 g of each) were ground into powder by using a glass mortar and ground samples were stored at -4°C until use. The dried and powdered plant material of *R. amabilis*, *R. niveus Thunb.*, and *R. sachalinensis* (2.0 mg each), pure compounds of the three species, and isovitexin-7-*O*-glucoside, 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside, and procyanidin B_4 (2.0 mg each) were separately placed in a crucible, 100 μL of DMSO was added, and the mixture was stirred until all of the ingredients dissolved. Each sample was eluted with 900 μL of ethanol (100%) to yield a solution of 1.0 mg/mL. Different concentrations of samples (1.0, 0.50, 0.25, 0.125, 0.0625, 0.03125, 0.015625, and 0.0078125 mg/mL) were prepared using the 1.0-mg/mL solution. Ascorbic acid (vitamin C) was similarly prepared to serve as the positive control. Ethanol solutions of the samples (100 μL of 1.0, 0.50, 0.25, 0.125, 0.0625, 0.03125, 0.015625, and 0.0078125 mg/mL) or the positive control were mixed with a 100-μL solution of DPPH in a 96-well plate. One hundred μL of ethanol and 100 μl of DPPH ethanol served as negative control solutions. The mixtures were incubated at 37°C for 30 min and their absorbance was measured at 517 nm (DNM-9602G Micro-plate Reader, Beijing Aipu). The percentage inhibition of DPPH was calculated using the following equation:

Inhibition (%) = $[1 – (A_i – A_j) /A_c] \times 100\%$.

Here, A_c is the absorbance of the negative control (Ethanol solution of DPPH without test sample) and A_i is the absorbance of a mixture of a sample and DPPH at 517 nm. A_j is absorbance of the sample without DPPH at 517 nm. Half-maximal inhibition concentrations (IC_{50} values) were calculated from linear regression plots, where the abscissa represented the concentration of tested plant extracts and the ordinate represented the average percent of scavenging capacity from three separate tests.

3. Results

3.1. *Optimization of sample extraction*

The compounds produced more complete peaks but interference peaks were small and the peak areas were larger in chromatograms when the samples were extracted with ultrasound than with reflux. The compounds produced more complete peaks but interference peaks were small and the peak areas were larger in chromatograms when the samples were extracted using methanol as a solvent than when using 95% ethanol as a solvent. Optimal extraction was achieved with ultrasound and methanol as a solvent.

3.2. *Comparison of compounds in three Rubus species extracts*

Figure 1A is the chromatogram for standard 2,6-dimethoxym -4-hydroxyphenol-1-*O*-β-D-glucopyranoside. Figure 1B is the chromatogram for compounds from the *R. amabilis* extract. Figure 1C is chromatogram for 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside in the *R. amabilis* extract. Figure 2A is the chromatogram for standard procyanidin B_4. Figure 2C is the chromatogram for procyanidin B_4 in the *R. amabilis* extract. Figure 3A is the chromatogram for standard isovitexin-7-*O*-glucoside. Figure 3C is chromatogram for isovitexin-7-*O*-glucoside in the *R. amabilis* extract. Peaks 1, 2, and 3 were identified by adding peaks for standard 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside, procyanidin B_4, and isovitexin-7-*O*-glucoside to peaks for the *R. amabilis* extract and then comparing Figures 1B and 1C, Figures 2B and 2C, and Figures 3B and 3C. The peak area for compound 1 in Figure 1C is larger than that in Figure 1B, the peak area for compound 2 in Figure 2C is larger than that in Figure 2B, and the peak area for compound 3 in Figure 3C is larger than that in Figure 3B. In contrast, the peaks for compounds 1, 2, and 3 are that were clearly produced by 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside, procyanidin B_4, and isovitexin-7-*O*-glucoside. Figure 4B is the chromatogram for compounds from the *R. niveus Thunb.* extract while Figure 4C is the chromatogram

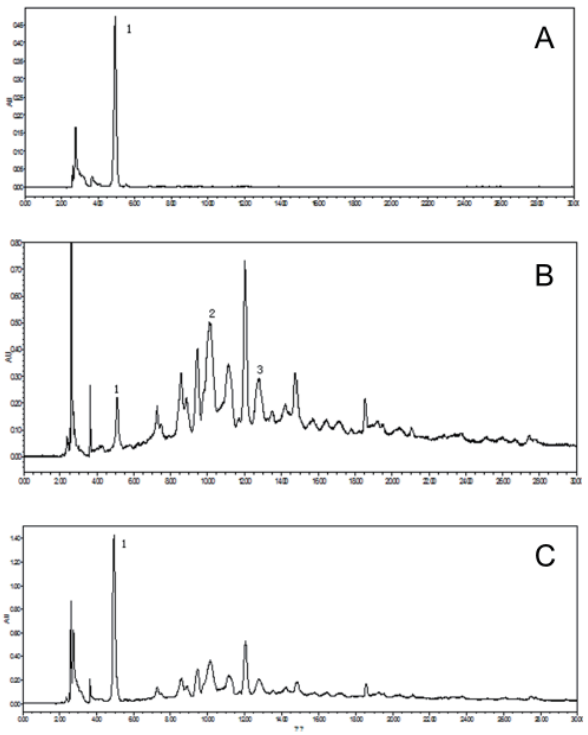

Figure 1. Chromatogram of standard 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside (A), *R. amabilis* extract (B), and *R. amabilis* extract with 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside (C).

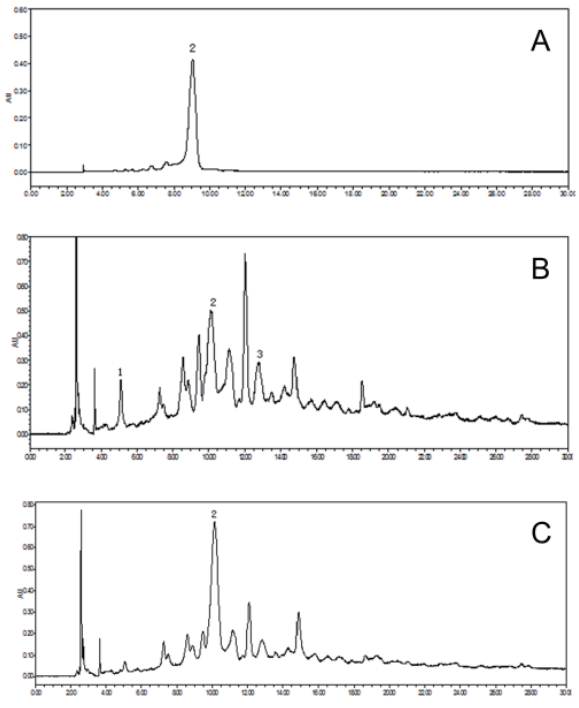

Figure 2. Chromatogram of standard procyanidin B_4 (A), *R. amabilis* extract (B), and *R. amabilis* extract with procyanidin B_4 (C).

for compounds from the *R. sachalinensis* extract. In comparison to the *R. amabilis* extract, these two *Rubus* species contained 2,6-dimethoxy-4-hydroxyphenol-1-

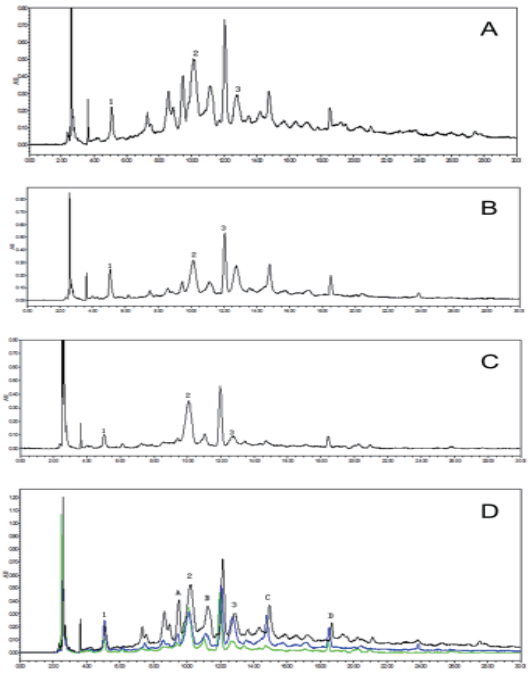

Figure 3. Chromatogram of standard isovitexin-7-*O*-glucoside (A), *R. amabilis* extract (B), and *R. amabilis* extract with isovitexin-7-*O*-glucoside (C).

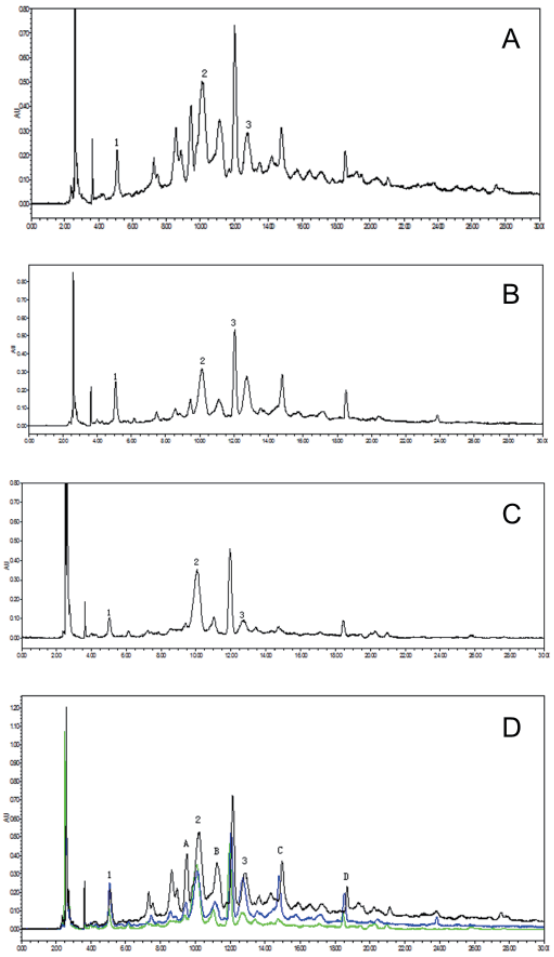

Figure 4. Chromatogram of *R. amabilis* extract (A), *R. niveus Thunb.* extract (B), and *R. sachalinensis* extract (C). Overlapping chromatograms for (A), (B), and (C) are shown in (D).

O-β-D-glucopyranoside with the same peak shape and same migration time. The three *Rubus* species contain procyanidin B_4 and isovitexin-7-*O*-glucoside with the same peak shapes and migration times. Many other similar peaks, such as peaks A, B, C, and D, are also present in Figure 4D.

3.3. *Antioxidant activity*

The antioxidant capacity of the extracts of *R. amabilis*, *R. niveus Thunb.*, and *R. sachalinensis* was evaluated by determining the radical scavenging capacity with respect to DPPH (IC_{50} value) (Figure 5; Tables 2 and 3).

Extracts of *R. amabilis*, *R. niveus Thunb.*, and *R. sachalinensis* were tested for their free radical scavenging of DPPH, and their IC_{50} values are shown in Table 2. Ascorbic acid, which was used as

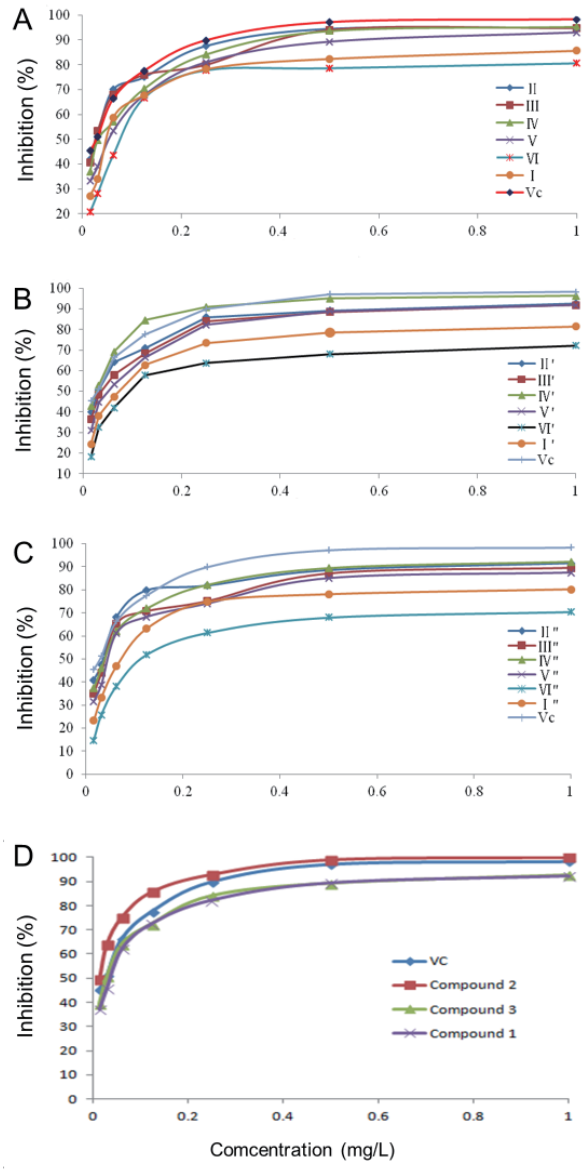

Figure 5. DPPH radical scavenging activity of *R. amabilis* I-VI and Vc (A), *R. niveus Thunb.* I′-VI′ and Vc (B), *R. sachalinensis* I″-VI″ and Vc (C), and compounds 1, 2, 3, and Vc (D).

Table 2. Radical scavenging activity of three *Rubus* species extracts

Sample	IC_{50} (mg/L)	Sample	IC_{50} (mg/L)
Vc	0.020	Vc	0.020
R. amabilis I	0.056	*R. niveus Thunb.* IV'	0.022
R. amabilis II	0.021	*R. niveus Thunb.* V'	0.046
R. amabilis III	0.025	*R. niveus Thunb.* VI'	0.108
R. amabilis IV	0.034	*R. sachalinensis* I"	0.077
R. amabilis V	0.048	*R. sachalinensis* II"	0.024
R. amabilis VI	0.083	*R. sachalinensis* III"	0.036
R. niveus Thunb. I'	0.069	*R. sachalinensis* IV"	0.033
R. niveus Thunb. II'	0.028	*R. sachalinensis* V"	0.047
R. niveus Thunb. III'	0.036	*R. sachalinensis* VI"	0.152

Note: I, I′, and I″ indicate samples extracted from 70% ethanol. II,II′, and II″ indicate samples extracted from petroleum ether. III, III′, and III″ indicate samples extracted from chloroform. IV, IV′, and IV″ indicate samples extracted from ethyl acetate. V, V, and V″ indicate samples extracted from n-butanol. VI, VI′, and VI″ indicate samples extracted from water.

Table 3. Radical scavenging activity of compounds 1-3 and Vc

Sample	IC_{50} (mg/L)
Vc	0.020
Compound 1	0.033
Compound 2	0.010
Compound 3	0.027

a positive control, had an IC_{50} value of 0.020 mg/L. All three *Rubus* species extracts had DPPH radical scavenging activity. In different solvents, *R. amabilis* extracts displayed antioxidant activity in the order of II>III>IV>V>I>VI. In different solvents, *R. niveus Thunb*. extracts displayed antioxidant activity in the order of IV'>II'>III'>V'>I'>VI'. In different solvents, *R. sachalinensis* extracts displayed antioxidant activity in the order of II">III">IV">V">I">VI". The petroleum ether, chloroform, and ethyl acetate extracts of *R. amabilis*, *R. niveus Thunb.*, and *R. sachalinensis* had significantly less scavenging of DPPH. The aqueous extracts of the three *Rubus* species had less scavenging of DPPH in comparison to non-polar solvents. The three standards had antioxidant activity in the order of compound 2 > compound 3 > compound 1.

4. Discussion

Reflux extraction has conventionally been used to extract active compounds from plant samples. However, active compounds are readily lost due to evaporation, reaction, or oxidation during the long process of extraction. There has been considerable interest in the use of ultrasound to improve extraction from plant samples. Ultrasound-assisted extraction is one of the most inexpensive, simple, and efficient techniques (*11-13*) for extraction, and it can increase the yield of extracted components, reduce extraction time, and offer a high reprocessing throughput. The current study found that ultrasound-assisted extraction was superior to reflux extraction. Compounds 1 and 3 are glycosides and compound 2 is a biflavone. All three readily dissolve in a polar solvent.

Peaks 1, 2, and 3, which were produced by 2,6-dimethoxy-4- hydroxyphenol-1-*O*-β-D-glucopyranoside (compound 1), procyanidinB_4 (compound 2), and isovitexin-7-*O*-glucoside (compound 3), can be compared to peaks produced by *R. amabilis*, as shown in Figures 1, 4, and 6, in terms of the peak shape and the migration time. Comparison of Figure 2 to Figures 1, 4, and 6 does not indicate whether peaks 1, 2, and 3 were produced by 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside, procyanidin B_4, or isovitexin-7-*O*-glucoside. However, peaks 1, 2, and 3 can be identified by adding peaks for standard compounds 1, 2, and 3 to peaks for the *R. amabilis* extract. Compounds 1, 2, and 3 are found in all three *Rubus* herbs extracts, but so are other compounds, as evident from peaks A, B, C, and D. According to the chromatograms, the three *Rubus* extracts contain 7 compounds. In conclusion, different regions substitute *R. amabilis*, *R. niveus Thunb.*, and *R. sachalinensis* for one another as a key ingredient in Manubzhithang.

Three standard compounds and three *Rubus* species extracts were compared in terms of their antioxidant activity *in vitro*. The antioxidant activity of the three *Rubus* species extracts differed with different solvents. The three *Rubus* species extracts had similar levels of antioxidant activity *in vitro*. For instance, the petroleum ether, chloroform, and ethyl acetate extracts of three *Rubus* species displayed marked antioxidant activity *in vitro* while the n-butanol and aqueous extracts displayed weak antioxidant activity *in vitro*.

The petroleum ether, chloroform, and ethyl acetate extracts of *R. amabilis*, *R. niveus Thunb.*, and *R. sachalinensis* had significantly less scavenging of DPPH in comparison to polar solvents. However, a study of *R. amabilis* separated compounds 2 and 3 from n-butanol and compound 1 from ethyl acetate (*14*). This finding suggests that the petroleum ether, chloroform, and ethyl acetate extracts contain more flavonoids such as quercetin, 8-dihydroxy-3,7-dimethoxyxanthone, 1-hydroxy -3,7,8-trimeth-oxyxanthone, and 8-dihydroxy-3,5-dimethoxyxanthone (*14*). These substances display

exceptional antioxidant properties *in vitro*. Compounds 2 and 3 had superior antioxidant properties *in vitro* when extracted with a highly polar solvent. However, the three *Rubus* species extracts could contain other compounds that do not possess radical scavenging activity when extracted with a polar solvent like n-butanol and water. The current results indicated that extracts of the three *Rubus* species had almost the same level of scavenging of DPPH despite extraction with different solvents.

The current study is the first to compare compounds of three *Rubus* species and their antioxidant activity. Findings indicated the rationality of substituting the three *Rubus* species for one another as a key ingredient in the traditional Tibetan medicine Manubzhithang.

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