



From Traditional Resource to Global Commodities:—A Comparison of *Rhodiola* Species Using NMR Spectroscopy—Metabolomics and HPTLC

Anthony Booker^{1,2}, Lixiang Zhai^{1,3}, Christina Gkouva¹, Shuyuan Li³ and Michael Heinrich^{1*}

¹ Research Cluster Biodiversity and Medicines/Centre for Pharmacognosy and Phytotherapy, UCL School of Pharmacy, University of London, London, UK, ² Division of Herbal and East Asian Medicine, Department of Life Sciences, University of Westminster, London, UK, ³ Department of Traditional Chinese Medicine, School of Traditional Chinese Medicine, Guangdong Pharmaceutical University, Guangzhou, China

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*Correspondence:

Michael Heinrich
m.heinrich@ucl.ac.uk

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The fast developing international trade of products based on traditional knowledge and their value chains has become an important aspect of the ethnopharmacological debate. The structure and diversity of value chains and their impact on the phytochemical composition of herbal medicinal products, as well as the underlying government policies and regulations, have been overlooked in the debate about quality problems in transnational trade. *Rhodiola* species, including *Rhodiola rosea* L. and *Rhodiola crenulata* (Hook. f. & Thomson) H. Ohba, are used as traditional herbal medicines. Faced with resource depletion and environment destruction, *R. rosea* and *R. crenulata* are becoming endangered, making them more economically valuable to collectors and middlemen, and also increasing the risk of adulteration and low quality. *Rhodiola* products have been subject to adulteration and we recently assessed 39 commercial products for their composition and quality. However, the range of *Rhodiola* species potentially implicated has not been assessed. Also, the ability of selected analytical techniques in differentiating these species is not known yet. Using a strategy previously developed by our group, we compare the phytochemical differences among *Rhodiola* raw materials available on the market to provide a practical method for the identification of different *Rhodiola* species from Europe and Asia and the detection of potential adulterants. Nuclear magnetic resonance spectroscopy coupled with multivariate analysis software and high performance thin layer chromatography techniques were used to analyse the samples. Rosavin and rosarin were mainly present in *R. rosea* but also in *Rosea sachalinensis* Borris. 30% of the *Rhodiola* samples purchased from the Chinese market were adulterated by other *Rhodiola* spp. The utilization of a combined platform based on ¹H-NMR and HPTLC methods resulted in an integrated analysis of different *Rhodiola* species. We identified adulteration at the earliest stage of the value chains, i.e., during collection as a key problem involving several species. This project also highlights the need to further study the links between producers and consumers in national and trans-national trade.

Keywords: *Rhodiola*, metabolomics, herb quality, adulteration, HPTLC, NMR

INTRODUCTION

While medicinal plants and spices have been traded for centuries on a global scale, the fast developing international trade of products now includes a large number of species which are used based on local and traditional knowledge and practice. The value chains of such products are starting to become an important topic in the ethnopharmacological debate. The structure and diversity of value chains, as well as their impact on the phytochemical composition of herbal medicinal products (HMPs) has been overlooked in quality issues in transnational trade. Different government policies and regulations governing trade in herbal medicinal products impact on such value chains.

Medicinal *Rhodiola* species, including *Rhodiola rosea* L. and *Rhodiola crenulata* (Hook. f. & Thomson) H. Ohba (**Figure 1**), have been used widely in Europe and Asia as traditional herbal medicines with numerous claims for their therapeutic effects. Faced with resource depletion and environment destruction, *R. rosea* and *R. crenulata* are becoming endangered, making them more economically valuable to collectors and middlemen, and also increasing the risk of adulteration and low quality. Poor quality and adulterated *R. rosea* products have been previously reported (Booker et al., 2015; Xin et al., 2015) and this paper investigates some aspects of the value chains that leads to the production of such products.

Adulteration of *R. rosea* products with *R. crenulata* has been previously reported but our fieldwork investigations suggested that other species may be implicated, and particularly *Rhodiola sachalinensis*, another species that appears to contain rosavins (the main marker compounds used for the identification of *R. rosea*).

The genus *Rhodiola* (Crassulaceae) comprises ~90 species of succulent and herbaceous perennial plants, which mainly show a circumpolar distribution across the northern hemisphere (Xia et al., 2005; Lu and Lan, 2013). *Rhodiola* species usually grow in mountainous areas such as rock ledges, precipices, tundra, brooks, and river banks (Zhu and Lou, 2010).

Ethnopharmacological Importance of Key *Rhodiola* Species

In Europe and North America, *Sedum roseum* (L.) Scop. (commonly named under its synonym *R. rosea* L.) is the most well-known and widely used among the different species. It is also known as golden root, or arctic root which reputedly demonstrates the economic importance and the geographical distribution of the plant. It has a rich history of traditional use in Russia, Europe and Asia with various uses according to the region (e.g., as shown in **Table 1**).

In Europe, the first documented medicinal use of *R. rosea* can be traced back to Dioscorides in 77 A.D. (Brown et al., 2002). In C. v. Linne's *Materia Medica*, the root of *R. rosea* was recommended for several conditions such as headaches, "hysteria," hernias and discharges (C. v. Linne, 1749 in Panossian et al., 2010). Throughout the years, it has appeared in many

pharmacopeias and medicinal books of different countries such as Sweden, France, Norway, Germany, Iceland, Estonia, and Russia (Brown et al., 2002; Alm, 2004; Panossian et al., 2010; Shikov et al., 2014).

In China, 73 different *Rhodiola* species have been reported, mainly in the northwest and southwest regions such as Tibet and the Sichuan province. The adaptogenic and tonic properties of the *Rhodiola* plants have been widely used in traditional Chinese and Tibetan medicine (Li and Zhang, 2008). They are generally referred to with the Pinyin name Hong Jing Tian 红景天 [red (or glorious) view of heaven] with slight alterations for each species (**Table 2**).

R. crenulata can be traced back to Tibetan medicine books including "The Four Medical Tantras" (*rgyud-bzhi* in Tibetan, *Si Bu Yi Dian* in Chinese), Yue Wang's Classical Medicinal Book (*Somaratsa* in Tibetan, *Yue Wang Yao Zhen* in Chinese), and Jing Zhu Materia Medica [*Shel Gong Shel Phreng* in Tibetan, *Jing Zhu Ben Cao* in Chinese (Lu and Lan, 2013)]. It is used for treatment of cough, hemoptysis, pneumonia, and abnormal vaginal discharge. In Traditional Chinese Medicine (TCM), it has effects of nourishing qi as well as promoting blood circulation and is mainly prescribed for qi deficiency and blood stasis (QDBS), stroke, hemiplegia, and fatigue. It is commonly used in China and Tibet for treating altitude sickness.

Phytochemical and Pharmacological Research

Research on the phytochemistry and pharmacology of *Rhodiola* spp. was initiated in the 1960s in the Soviet Union and Scandinavia, mainly focusing on *R. rosea* (Brown et al., 2002). After the turn of the century the interest in this plant spread globally. Intensive phytochemical research led to the detection of known and novel compounds in *R. rosea* and related species (Ma et al., 2006; Yousef et al., 2006). Between 2000 and 2015 an increased number of publications stemming from Asian research groups have focused on the detection of novel compounds from *Rhodiola* species, usually in combination with their respective pharmacological assessments (Fan et al., 2001; Nakamura et al., 2007, 2008).

There are more than a few hundred pharmacological studies on medicinal *Rhodiola* species (mainly on *R. rosea*) that show a wide range of activities reflecting their diverse traditional use. They possess adaptogenic and stress-protective (neuro-cardio and hepato protective) and antioxidant effects, as well as stimulating effects on the central nervous system, including on cognitive functions such as attention, memory and learning; anti-fatigue effects; antidepressive and anxiolytic effects; endocrine activity normalizing; and life-span increasing effects (Aslanyan et al., 2010; Sarris et al., 2011; Panossian et al., 2013). The main active compounds are reputedly phenylpropanoids (rosavin, rosarin, rosin) and phenylethanoids (salidroside and tyrosol).

Quality Issues of Medicinal *Rhodiola* spp.

Rhodiola roots and rhizomes are highly valuable products traded at an international level. Since the majority of *R. rosea* and *R.*

Abbreviations: TCM, Traditional Chinese Medicine; NMR, Nuclear magnetic resonance; HPTLC, High performance thin layer chromatography; spp, Species.

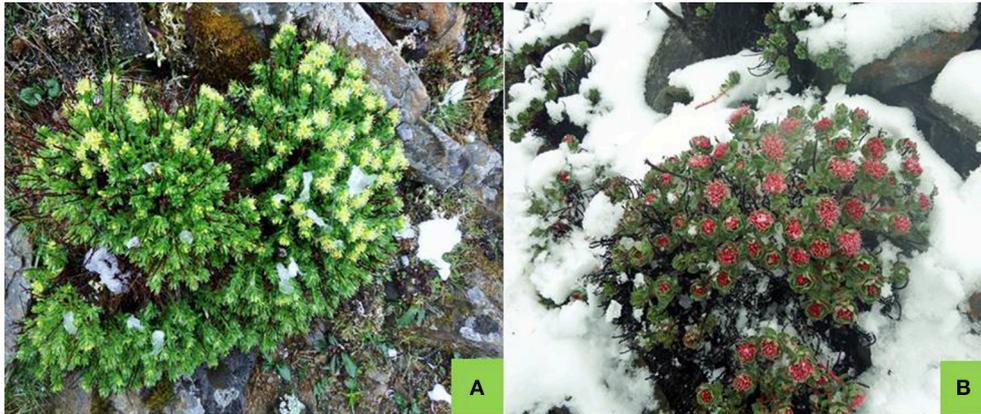


FIGURE 1 | *Rhodiola* species. (A) *R. rosea*; (B) *R. crenulata*. Photos taken by A. Booker, Sichuan-Tibet border, June, 2015.

TABLE 1 | Traditional uses of *R. rosea* in different regions.

Region	Use	References
Russia	<ul style="list-style-type: none"> Escalation of physical endurance Remedy against fatigue and high altitude sickness Aphrodisiac 	Shikov et al., 2014; Alm, 2004
Norway	<ul style="list-style-type: none"> Astringent Cure for scurvy Remedy against hair-loss and urinary tract disorders 	Alm, 2004
Iceland and Denmark	<ul style="list-style-type: none"> Alleviation of headaches 	Alm, 2004
France	<ul style="list-style-type: none"> Stimulant Astringent 	Panossian et al., 2010
Alaska	<ul style="list-style-type: none"> Cure for sores Remedy against tuberculosis 	Alm, 2004
Mongolia	<ul style="list-style-type: none"> Remedy against tuberculosis Anticancer Escalation of physical endurance Treatment for lung inflammation 	Brown et al., 2002; World Health Organization, 2013

crenulata raw material supplied still comes from wild-collection, their intensive collection leads to scarcity (Galambosi, 2006; Lu and Lan, 2013).

Herbal preparations of *Rhodiola* species (mainly *R. rosea*) are extensively utilized around the globe. There is an increasing number of commercial products available on the American, Asian and European markets, either as food supplements or herbal medicines. *R. rosea* herbal monographs have been included in many Pharmacopeias worldwide. On the other hand, *R. crenulata* is the only species used medicinally in TCM (Table 3).

Due to this rapid increase of *Rhodiola* raw material demand, other *Rhodiola* species such as *R. fastigiata*, *R. sachalinensis*,

TABLE 2 | Examples of the similar Pin Yin names of different *Rhodiola* species in China.

Scientific name	Pin Yin name
<i>R. rosea</i> L.	Qiang Wei (rose smell) Hong Jing Tian
<i>R. sachalinensis</i> Borris.	Gao Shan (high mountain) Hong Jing Tian
<i>R. quadrifida</i> (Pall.) Fisch. & C.A.Mey	Si Lie (four split) Hong Jing Tian
<i>R. crenulata</i> (Hook. f. & Thomson) H. Ohba	Da Hua (big flower) Hong Jing Tian
<i>R. yunnanensis</i> (Franch.) S.H. Fu	Yunnan (From Yunnan) Hong Jing Tian
<i>R. kirilowii</i> (Regel) Maxim.	Xia Ye (narrow leaf) Hong Jing Tian
<i>R. fastigiata</i> (Hook. f. & Thomson) S.H. Fu	Chang Bian (clustered) Hong Jing Tian

R. quadrifida, *Rhodiola sacra* (Prain ex Hamet) S. H. Fu and *Rhodiola serrata* H. Ohba have been sold on the market (Xin et al., 2015). Since there is not any consistent worldwide quality control programme, inadequate quality assessment of *Rhodiola* spp. is a common issue. This raises concerns about possible adulteration and misidentification issues. The lack of genuine drug material, confusion over the Chinese Pin Yin name of the drug when sourcing from China and accidental or deliberate adulteration during the manufacturing stage may contribute to low quality of final products.

The analytical techniques currently available focus on identifying *R. rosea* or *R. crenulata* through chromatographic methods. Other species of *Rhodiola* have generally not been considered. *R. sachalinensis* presents a particular problem as it may contain similar marker compounds to *R. rosea* (and some sources suggest that it is the same species—see <http://www.kew.org/mpns-portal>).

TABLE 3 | Generation of *Rhodiola* spp. recorded in selected pharmacopeias and publications.

Pharmacopeia/ publication	Recorded <i>Rhodiola</i> species	Medicinal use part	Herbal product
Department of Health and Ageing, Australian Government	<i>Rhodiola rosea</i>	Root (Rhizome)	Dry extract
Committee on Herbal Medicinal Products, 2012	<i>Rhodiola rosea</i>	Rhizoma et radix	Extract
United States Pharmacopeia (32th Edition)	<i>Rhodiola rosea</i>	Rhizoma et radix	Dry extract, tincture
Chinese Pharmacopoeia, 2010	<i>Rhodiola crenulata</i>	Rhizoma et radix	Extract
Russian Pharmacopoeia (12th Edition)	<i>Rhodiola rosea</i>	Rhizoma et radix	Extract

Integrated Analytical Platform Approach NMR-Based Metabolomics

NMR-based metabolic fingerprinting has been used in the analysis of numerous food and medicinal species focusing on their quality assurance as well as their pharmacology. Such comparative studies include Danggui [*Angelica sinensis* (Oliv.) Diels] and Engelwurz/European Angelica (*Angelica archangelica* L.; Li et al., 2014). Metabolomic differences between different *Tussilago farfara* L. accessions (Zhi et al., 2012) and different *Salvia miltiorrhiza* Bunge production sites (Jiang et al., 2014) were also studied by NMR fingerprinting coupled with multivariate analysis. Compared to GC-MS and LC-MS, NMR has some advantages such as non-selectiveness, high reproducibility, and good stability (Simmler et al., 2014). At the same time, structural information on metabolites can be obtained from NMR directly. Therefore, NMR can be regarded as an ideal choice for chemical comparison and identification of the phytochemical differences of medicinal plants.

HPTLC

Since the NMR-metabolomic approach is not a validated pharmacopoeial method, there is a need to be compared to a standard method like high performance thin layer chromatography (HPTLC). This method is widely used for the authentication and quality control of herbal substances (Reich et al., 2008). Compared to NMR-based metabolic fingerprinting, HPTLC could be highly effective with relatively lower price (Booker et al., 2014). HPTLC can also be helpful for the identification of specific compounds. Therefore, we chose these two complementary approaches in this study.

A third analysis strategy using DNA bar coding was used to help verify some of the samples (details are given in the Supplement S2).

MATERIALS AND METHODS

Sampling and Preparation of Plant Material

Forty-two batches of *Rhodiola* market samples (i.e., not authenticated) were collected between October 2014 and January 2015 from different suppliers including retail outlets, The internet, pharmaceutical companies in seven different locations (Beijing, Guangdong, Qinghai, Anhui, Hebei, Jilin, and Hong Kong SAR) and in China, Germany and Russia. These raw-material samples were mainly labeled as *R. rosea*, *R. crenulata*, *R. sachalinensis*, and *R. quadrifida*. 18 batches of authenticated plant material were provided by Agroscope Institute (Switzerland). The samples were rhizomes of *R. rosea* plants propagated from different wild Swiss populations (Mattmark, Carrasino, and Nomnon) or botanical gardens (Switzerland and Germany). In addition, authenticated *R. rosea* samples which were grown from seeds or provided to the institute by Dr. Bertalan Galambosi were also included. Lastly, in June 2015, samples of *R. crenulata* and *R. fastigiata* roots and rhizomes were collected from Garze, Sichuan, China (altitude 4500 m). These samples were authenticated by Professor Shuyuan Li, (Guangdong Pharmaceutical University, Guangzhou, China). Botanical reference materials (BRMs) for *R. rosea*, *R. crenulata*, and *R. sachalinensis* were obtained from the National Institute of Food and Drug Control (NIFDC, China), Dr. William Schwabe (Germany) and Agroscope (Switzerland). BRMs for *R. quadrifida* and *R. fastigiata* were provided by Professor Alexander Shikov (Saint-Petersburg Institute of Pharmacy, Russia) and Dr. Anthony Booker (UCL School of Pharmacy). *R. fastigiata* was authenticated by Professor Shuyuan Li (Guangdong Pharmaceutical University, Guangzhou, China).

All the collected samples were deposited in the herbarium of the UCL School of Pharmacy (London, UK). A detailed description of the investigated samples including their origins and representative symbols are provided in Supplement (S1).

Crude root samples were ground to powder using a household grinder (EK1665ROFOB, Salter, UK) and sieved (0.70 mm mesh). All the powder samples were kept in 1.5 ml tubes (Eppendorf AG.) at 4°C until use.

Solvents, Reagents, and Reference Compounds

Deuterium oxide (D₂O), methanol-d₄ (99.8% D, MeOD), dimethyl sulfoxide-d₆ (DMSO-d₆), and tetramethylsilane (TMS) were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). Salidroside, gallic acid, rosarin, and rosavin were purchased from Sigma-Aldrich Chemicals (St Luis, USA). Tyrosol was purchased from Acros organics (New Jersey, US). Water used in this study was purified by using ULTRAPURE water system (Millipore, Germany). All other chemicals were of analytical grade.

¹H-NMR Spectroscopy

Sample Preparation

Nine-hundred microliter of MeOD-d₄ was added for extraction. The samples were vortexed (Rodamixer, UK) for 30 s and sonicated at an ultrasound bath (Fisher, XB22, UK) for 10

min. The solutions were centrifuged for 10 min at 14,000 rpm (EBA21, Hettich, Faust Laborbedarf AG, Germany). Six-hundred microliter of supernatant was transferred to a 5 mm diameter NMR spectroscopy tube and the samples were submitted for NMR spectroscopic analysis. The one and two dimensional ^1H -NMR spectra were recorded on Bruker Avance 500 MHz spectrometer (Bruker Analytic, Germany), which was equipped with a QNP (^{31}P , ^{13}C , ^{15}N , and ^1H) 5 mm cryoprobe. The acquisition parameters were: size of the spectra 64 k data points, line broadening factor = 0.16 Hz, pulse width (PW) = 30 degrees, and the relaxation delay $d1 = 1$ s. The acquisition temperature was 298 K.

In order to assess the coherence of the results obtained, two samples from the same batch were subjected to NMR analysis on the different days of examination. To minimize the error caused by root selection during sample grinding, any samples weighing more than 500 g were analyzed twice.

Data Analysis

The resulting spectra were manually phased and auto-baseline corrected by Topspin 3.2 (Bruker, Germany) for organic fractions. Signals between δ 5.20–4.40 ppm and δ 3.35–3.22 ppm were removed prior to statistical analysis due to the presence of methanol- d_4 . The total area of peaks (δ 10.00–0.00 ppm) was integrated into small (0.04 ppm) buckets by bucketing (binning) function using AMIX or ACD-Labs in order to generate a number of integrated regions of the data set. The buckets obtained were then imported to Microsoft EXCEL (2013) where the samples were re-labeled and their species information was added.

Principal component analysis (PCA) was performed with SIMCA-P 13.0 (Umetrics, Umeå, Sweden) for metabolomic analysis of the generated dataset. Scaling mode of Pareto (Par) and Unit Variance (UV) were tested to optimize the analysis model.

HPTLC

Sample Preparation

One milliliter of ethanol was added to 50 mg of weighed samples for extraction. The solutions were then mixed on a rotary mixer (Rodamixer, UK) for 30 s, sonicated in an ultrasound bath (Fisher, XB22, UK) for 10 min and centrifuged for 10 min at 14,000 rpm. The supernatant was used for HPTLC analysis. The reference standard solutions of salidroside, rosarin, rosavin, gallic acid, and tyrosol were prepared at a concentration 1 mg/ml in methanol. Both the reference material and the test samples were stored at 4°C.

Data Analysis

Samples were applied to the plates as bands 8 mm wide by using Linomat 5 semi-automatic applicator with 100 μl syringe. The space between bands was 2.0 mm and the rate of application was 90 $\text{nl}\cdot\text{s}^{-1}$. The number of tracks per plate was 15, and 5 μl of standard and sample solutions were applied.

The temperature and relative humidity were controlled to 21–24°C and 33%, respectively. Ten milliliter of solvent was poured into the right inlet for development and 25 ml of

solvent was poured into the left inlet for saturation. Plates were previously air dried for 10 s and developed in a 20 \times 10 cm twin-trough chamber (Analtech, USA) lined with Whatman filter paper (20 \times 10 cm) and saturated with mobile phase (Ethylacetate, methanol, water, formic acid (77:13:10:2) vapor for 20 min. The development distance was 70.0 mm from the lower edge.

The developed plates were derivatised by dipping in sulfuric acid reagent, using a CAMAG chromatogram immersion device and heated at 100°C on a plate heater for 5 min. Sulfuric acid reagent was prepared with a procedure as follow: 20 ml sulfuric acid was carefully added to 180 ml ice-cold methanol and mixed. The plates were visualized using CAMAG visualizer under white light, UV 254 nm and at UV 366 nm, photographed and uploaded to HPTLC computer software (VisionCats).

RESULTS AND DISCUSSION

^1H -NMR and Multivariate Statistical Analysis

By incorporating the whole region (0–10 ppm) and Pareto (Par) scaling, a significant clustering is observed in *R. rosea* samples (Figure 2). *R. rosea* can be differentiated distinctly from the rest of the species based on their principal component variability.

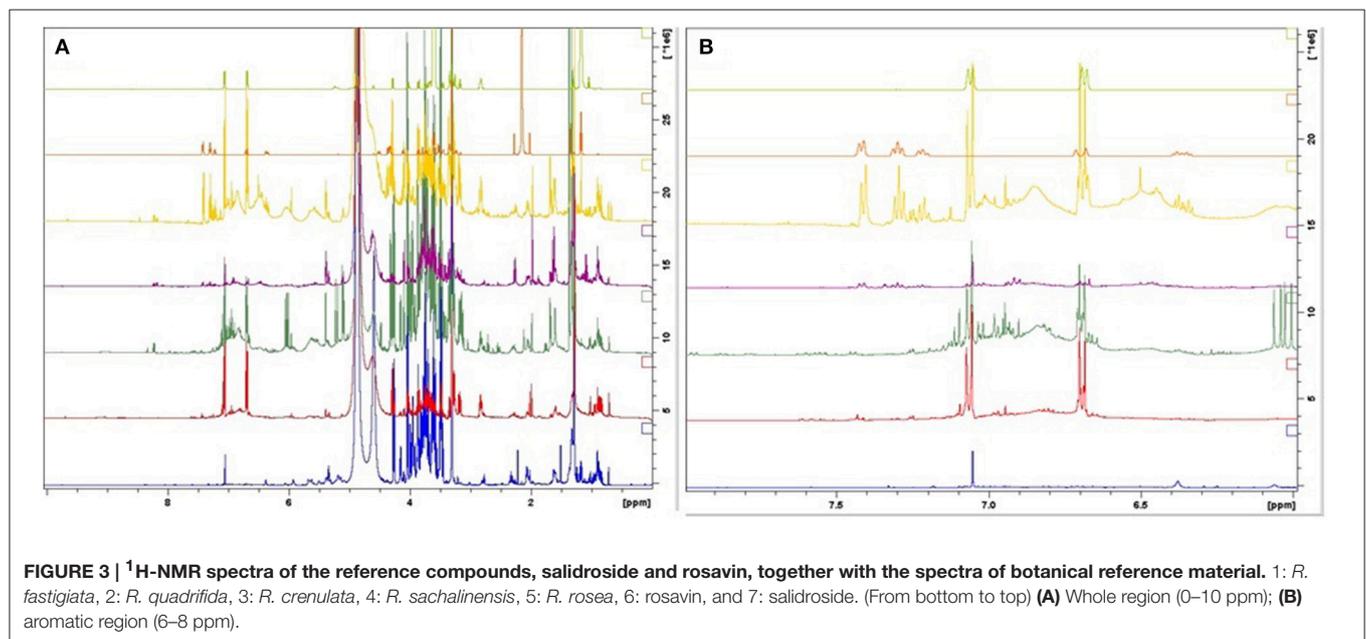
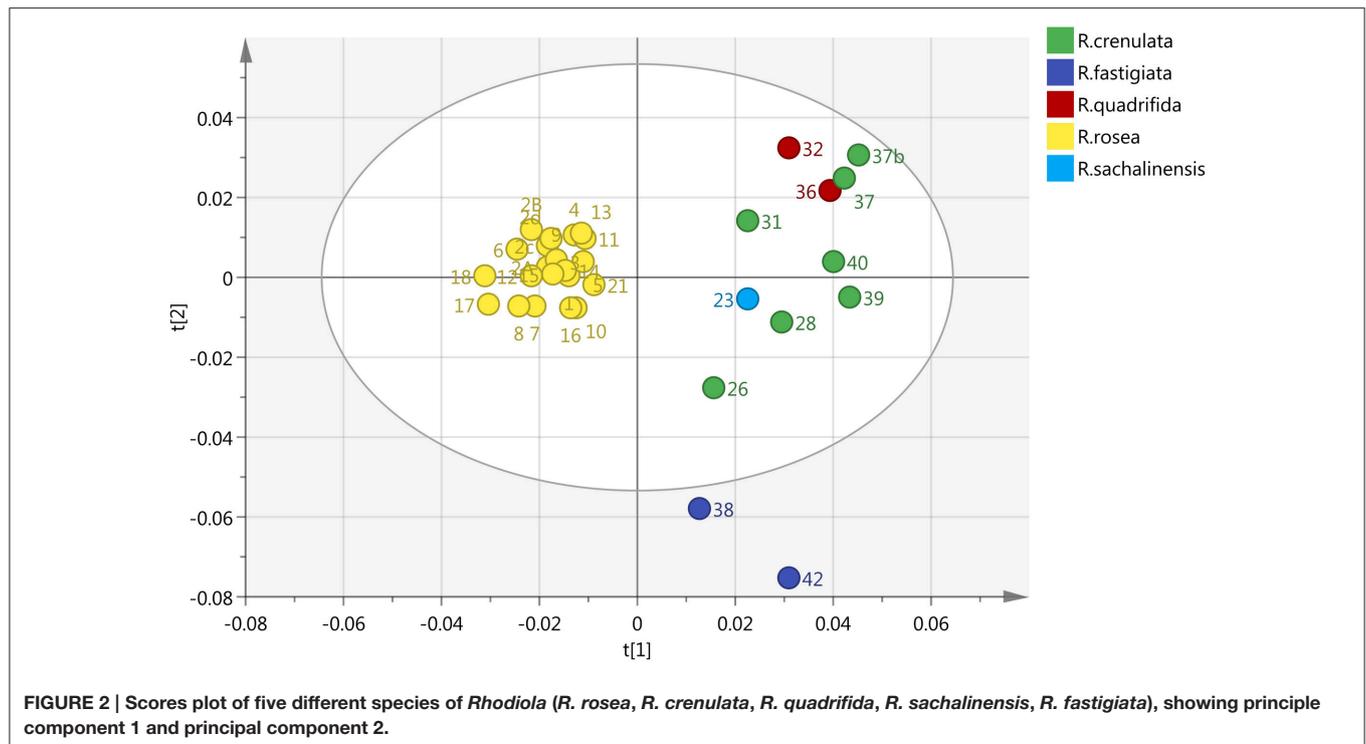
According to the spectra of the species (Figure 3), the aromatic region (6–8 ppm) is dominated by the main marker compounds (rosavin and salidroside). Hence, this region was analyzed independently using Par scaling (Figure 4). Based on the scores plot produced, *Rhodiola* species were separated more clearly compared to the scores plot of the whole region.

R. crenulata and *R. quadrifida* were also separated from the rest of the species. However, in this model they were clustered together. This suggests that there is no crucial metabolomic difference between them in the aromatic region. At this point it was considered important to visually inspect the spectra of the BRM's and detect any differences that might be lost with the integration of the data. *R. crenulata* BRM has an additional quartet at 6 ppm not detected in the rest of the species. This quartet can also be found in all the other *R. crenulata* samples investigated (figures not shown).

Therefore, an effective separation between *R. crenulata* and *R. quadrifida* samples can be accomplished by combining the PCA results with the detection of the additional peaks on the ^1H -NMR spectra only present in *R. crenulata* samples between 5 and 6 ppm.

We also studied the group-pair comparisons in PCA model with Par scaling (Figure 5). The score plots showed that *Rhodiola* species separated well (A: *R. crenulata* with other *Rhodiola* species; B: *R. rosea* with other *Rhodiola* species; C: *R. crenulata* with *R. rosea*).

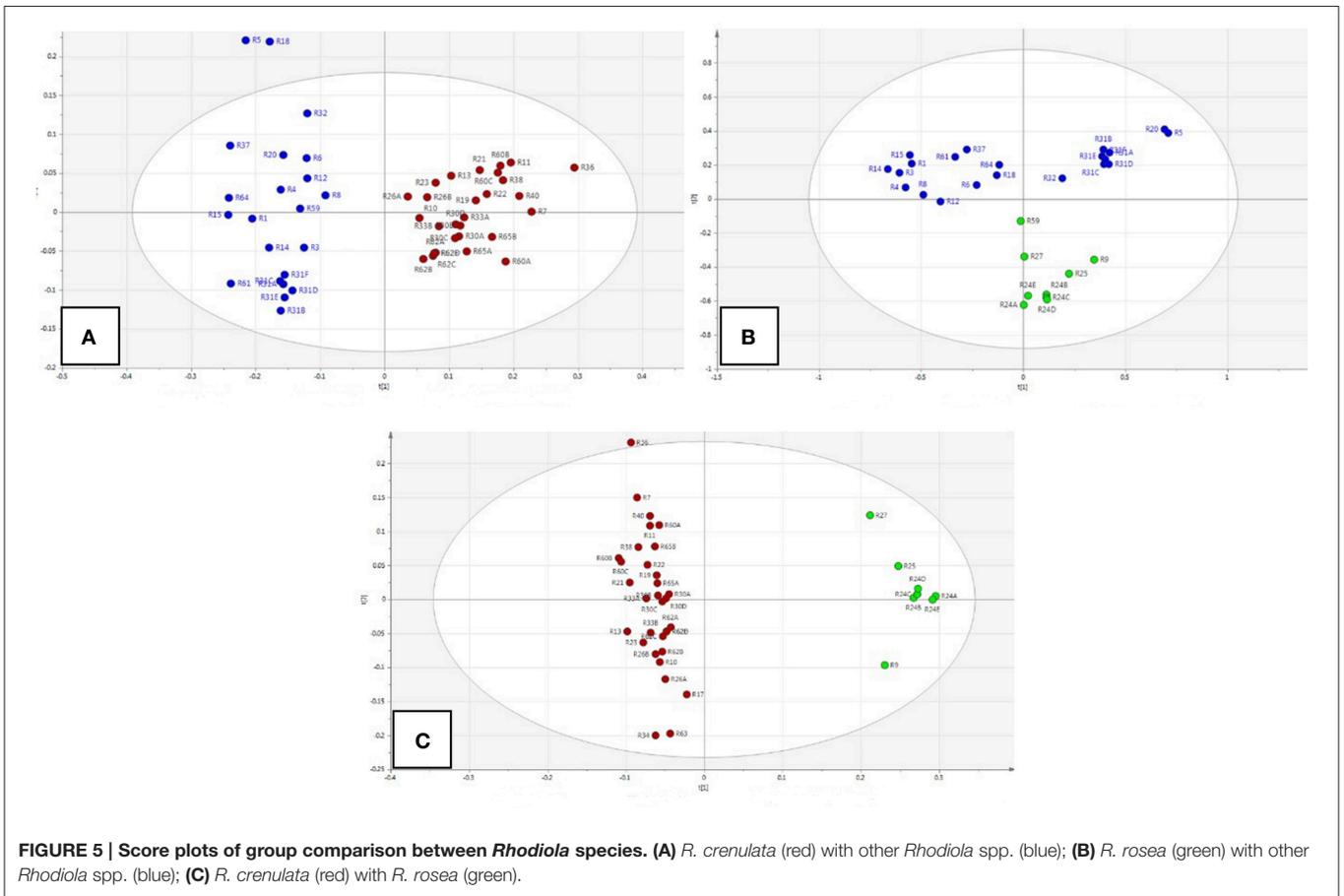
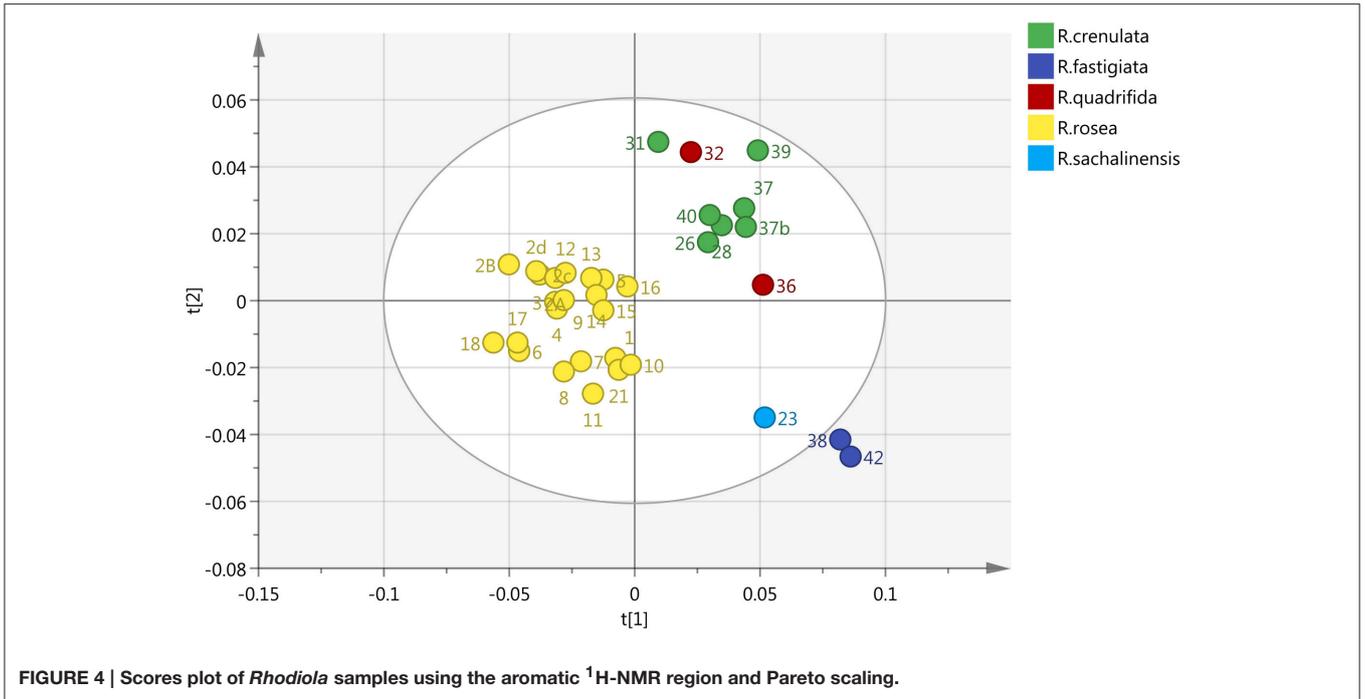
The main differences were between δ 7.5–7.3 ppm (PC1) and δ 7.0–6.8 ppm (PC2). The chemical shift of the main variable metabolites were mainly rosavin, rosarin, and cinnamyl alcohol derivatives.



The metabolites detected were elucidated by the analyses of the $^1\text{H-NMR}$ spectra as well as the comparison with the reference compounds, together with the in-house NMR chemical shift database (Mudge et al., 2013; Luo et al., 2015). The summary of the assignment of $^1\text{H-NMR}$ spectral peaks obtained from the *R. rosea*, *R. crenulata*, and *R. sachalinensis* BRM extracts are provided in Supplement (S3).

HPTLC Analysis

The band position and visibility of the standards rosavin, rosarin, and salidoside (Figure 6) appear with characteristic colors and increasing retention factors (Rf) 0.19, 0.26, and 0.31, respectively. Under UV light 254 nm, salidoside is not visible. Under 366 nm, after derivitisation with sulfuric acid, rosavin and rosarin appear as pale pink bands and salidoside as a green one.



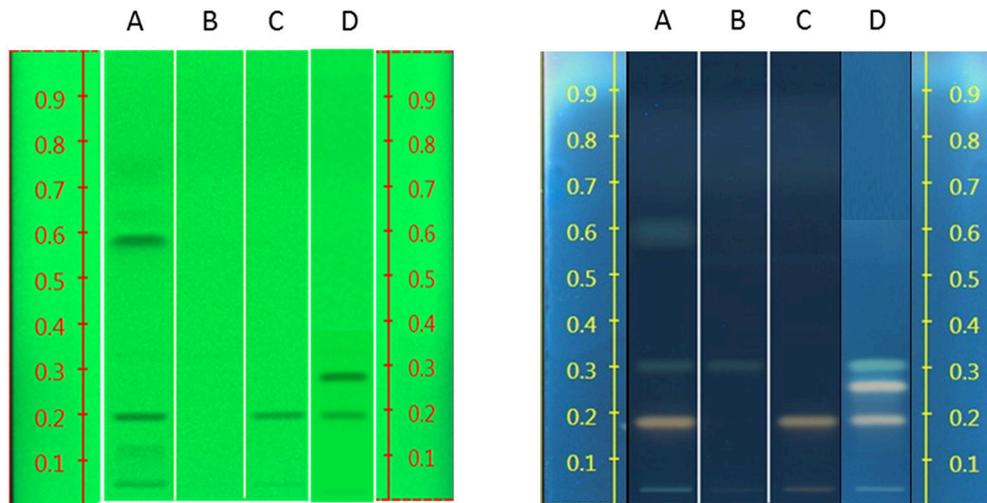


FIGURE 6 | Left: HPTLC results of standard compounds under UV 254 nm (rosavin Rf = 0.19, rosarin Rf = 0.26, gallic acid Rf = 0.58); Right: HPTLC results of standard compounds under UV 366 nm, after derivatisation with sulfuric acid (rosavin Rf = 0.19, rosarin Rf = 0.26, salidroside Rf = 0.31, gallic acid Rf = 0.58, tyrosol Rf = 0.76).

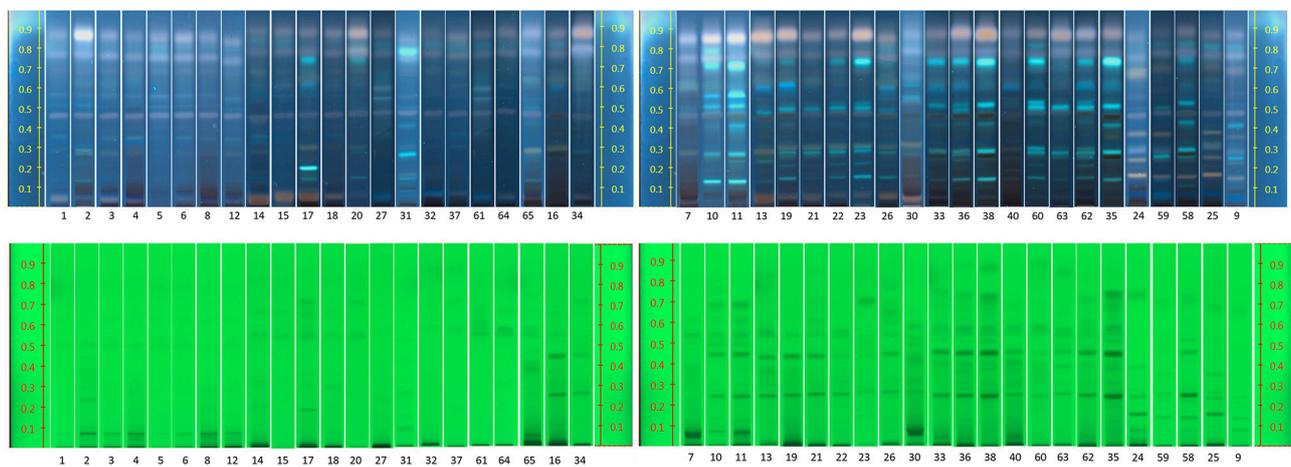


FIGURE 7 | HPTLC results for all *Rhodiola* market samples, mobile phase [Ethylacetate, methanol, water, formic acid (77:13:10:2)].

Gallic acid shows good visibility under UV 254 nm, while it is not easily detected under UV 366 nm at a dark blue back-ground. Tyrosol is visible in 254 nm but less clear in 366 nm.

The raw plant material obtained from the market was also studied by our HPTLC method (list of samples in Supplement S4). Under UV 254 nm (Figure 7), there were two obvious bands among these samples (Rf = 0.27 and 0.48). However, due to lack of reference standards, their identity remains unknown. Further studies need to be conducted using NMR and LC-MS. The majority of the samples investigated contained concentrations of tyrosol similar to the standard raw material used (R24, R30, and R31). Samples R1–R6 contained lower levels of this compound possibly due to their longer storage time. Therefore, tyrosol could be considered as a marker to study duration of *Rhodiola* storage.

It was also found that only five samples (R9, R25, R58, R59, and R24) contained high levels of rosavin, which turned out to be the ones from *R. rosea*. Moreover, this result can also be verified by the NMR results (Figure 5). However, it is not evident whether there is adulteration of *R. sachalinensis* in *R. rosea* since their metabolites are similar.

Under UV 366 nm after derivatisation eight samples (R1, R5, R6, R15, R27, R32, R61, and R64) had a low concentration of salidroside (Rf = 0.31). These samples could have been kept for a long time after collection and the salidroside content could have decreased due to lack of a good storage environment.

Combining the results of HPTLC and ¹H-NMR multivariate statistical analysis, we also analyzed the adulteration rate among all the market samples (Supplement, S4).

Thirty percent of the *Rhodiola* samples collected from the market were not, as declared on the label, i.e., either *R. rosea* or *R. crenulata*. Some *R. rosea* samples were also being sold as *R. crenulata*. 47.7% of raw material samples were not labeled properly and their species information were not clearly illustrated to customers. This highlights a clear lack of proper local government policies and good quality control strategies.

According to our study, different *Rhodiola* species (including *R. rosea* and *R. crenulata*) can be found in the Chinese market. However, they are neither sold separately nor well-identified. Therefore, there is a high potential of adulteration and substitution among these species.

Qualitative and Quantitative Analysis of Mixtures

Since in the value chain, mixing of batches and, therefore, potentially also of species, is of major concern, the possibility of qualitatively and quantitatively detecting plant mixtures was also investigated. The additional species chosen for this study was *R. crenulata* which is considered to be the most common adulterant of *R. rosea*. The selected BRMs were weighed individually in different proportions and then added together in an Eppendorf reaction tube. The rest of the sample preparation was identical to the methodology for the $^1\text{H-NMR}$ spectroscopy. The samples were renamed as seen in Table 4. After the acquisition of the spectra, they were baseline and phase corrected and zeroed to the TMS peak in Topspin 3.2.

TABLE 4 | $^1\text{H-NMR}$ -based detection of plant mixtures by.

Sample name	Mg of <i>R. rosea</i> BRM	Mg of <i>R. crenulata</i> BRM
RR100	100	00
RR80RC20	80	20
RR60RC40	60	40
RR40RC60	40	60
RR20RC80	20	80
RC100	00	100

In all samples, the salidroside peak intensity remains almost the same since this constituent is present in both species. The peaks of rosavin are gradually decreasing with the addition of *R. crenulata*, whereas the characteristic quartet at 6 ppm due to the presence of an unknown compound is increasing with the addition of *R. crenulata* and it is not detected in *R. rosea* at all (Figure 8).

The acquired spectra were bucketed using Amix and only focused on this region (6 ppm). When the whole quartet was

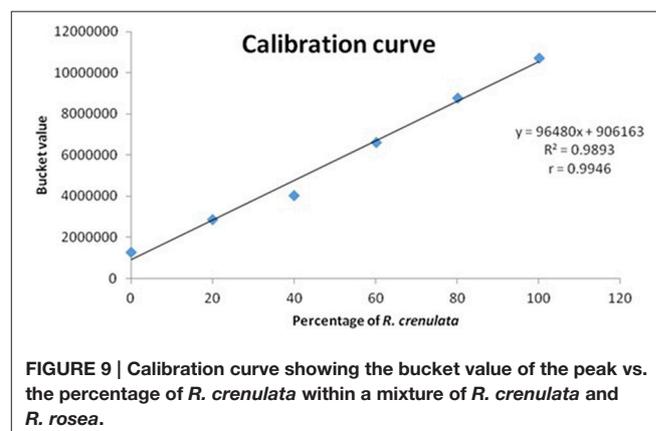


FIGURE 9 | Calibration curve showing the bucket value of the peak vs. the percentage of *R. crenulata* within a mixture of *R. crenulata* and *R. rosea*.

TABLE 5 | Sample preparation for the detection of plant mixtures by HPTLC.

<i>R. rosea</i> 100%	RR100	<i>R. rosea</i> BRM	5 μL
<i>R. rosea</i> 80% and <i>R. crenulata</i> 20%	RR80	<i>R. rosea</i> BRM	4 μL
		<i>R. crenulata</i> BRM	1 μL
<i>R. rosea</i> 60% and <i>R. crenulata</i> 40%	RR60	<i>R. rosea</i> BRM	3 μL
		<i>R. crenulata</i> BRM	2 μL
<i>R. rosea</i> 40% and <i>R. crenulata</i> 60%	RR40	<i>R. rosea</i> BRM	2 μL
		<i>R. crenulata</i> BRM	3 μL
<i>R. rosea</i> 20% and <i>R. crenulata</i> 80%	RR20	<i>R. rosea</i> BRM	1 μL
		<i>R. crenulata</i> BRM	4 μL
<i>R. crenulata</i> 100%	RC100	<i>R. crenulata</i> BRM	5 μL

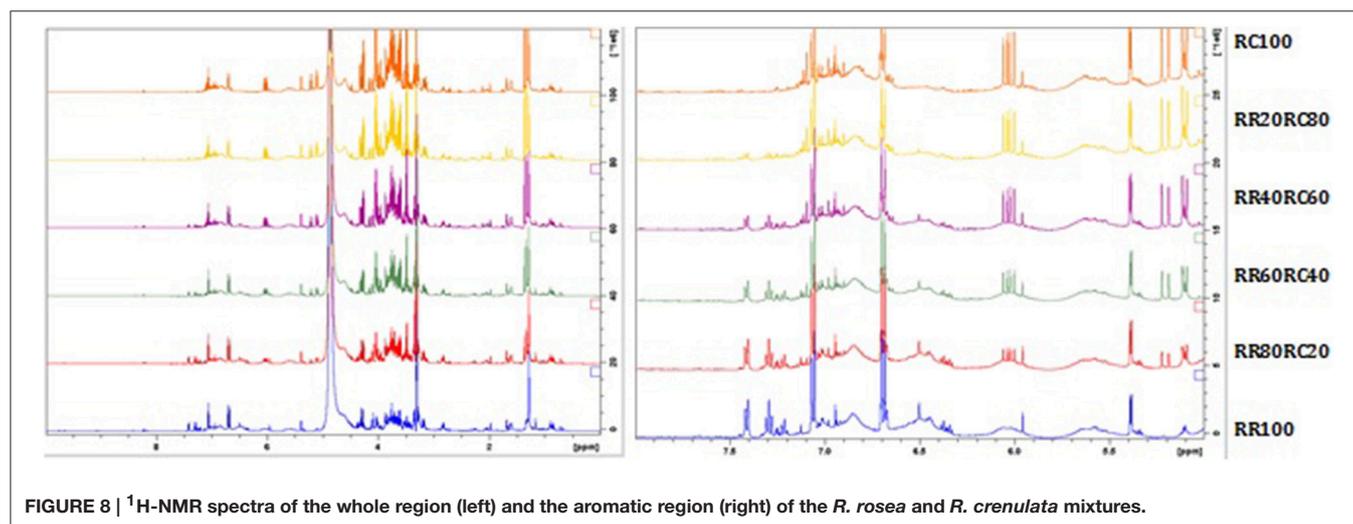
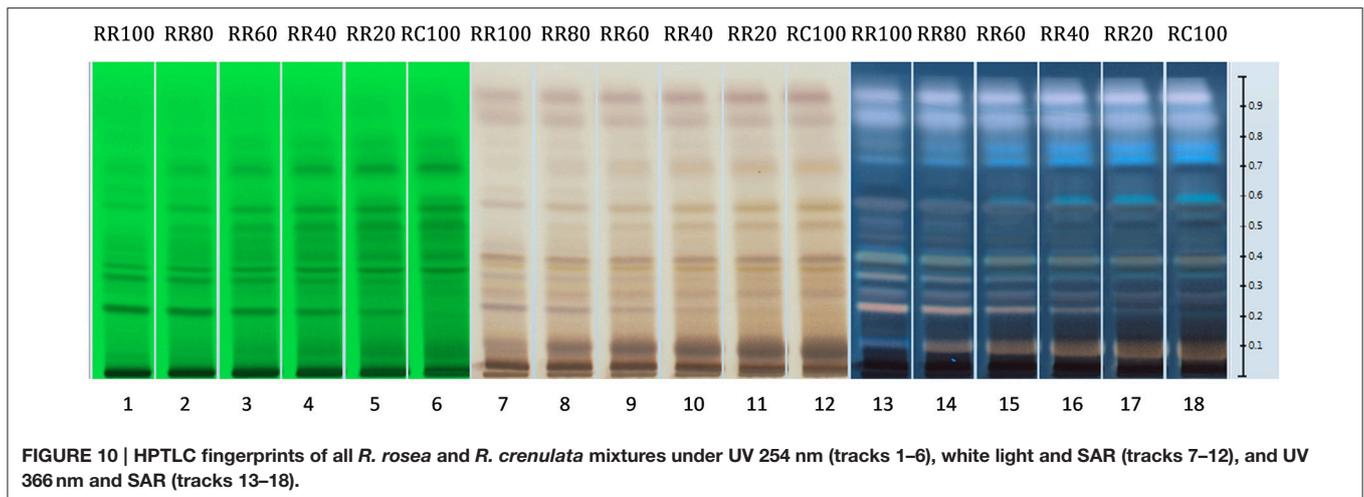


FIGURE 8 | $^1\text{H-NMR}$ spectra of the whole region (left) and the aromatic region (right) of the *R. rosea* and *R. crenulata* mixtures.



integrated into a single bucket, the observed increase of its intensity was not adequately represented. Therefore, the bucket size used changed to 0.002 ppm and only incorporated the first peak of the quartet (6.0028–6.0048 ppm). The buckets obtained from Amix were transferred into Excel, where the relationship between the bucket value and the percentage of *R. crenulata* in the mixture was expressed graphically as a calibration curve. The bucket value of the respective peak is increasing in a linear mode (Figure 9).

Similar results can also be obtained with HPTLC analysis. The HPTLC fingerprints produced consist of the over-spotted BRM extracts in different volumes as seen in Table 5. The final volume applied was 5 μ l.

As seen in Figure 10, when the loading volume of the *R. rosea* decreases, the representative markers of this species (rosavin and rosarin) decrease as well. However, the band for salidroside, (since it occurs in both species) remains almost the same.

By gradually increasing the *R. crenulata* proportion, several bands gradually appear above salidroside that could potentially be used as markers for the qualitative and semi-quantitative HPTLC analysis of mixtures of these two *Rhodiola* species. Further work needs to be carried out to determine the identity and species-specificity of these compounds.

CONCLUSIONS

This study provided a method for distinguishing five different species of *Rhodiola* and suggests possible methods for quantifying different species within mixtures. The metabolomic and phytochemical differences between these different species has been demonstrated through NMR spectroscopy and HPTLC analysis. Species represented with only a small number of samples will need further investigation in order to accurately define their chemical characteristics.

There is a need to study the links between producers and consumers especially when in trans-national trade and re-enforce the hypothesis that poor quality and adulterated products can be products of poorly governed value chains, particularly at the early stages of supply. Moreover, through the establishment of well-controlled and well-managed value chains it is possible to better prevent accidental or deliberate contamination and adulteration from occurring.

AUTHOR CONTRIBUTIONS

AB, main author and collector of samples in China, assisted with multivariate analysis of NMR data, responsible for contribution toward discussions and conclusions. CG, Responsible for HPTLC analysis and writing the methods, results, and part of the discussion for HPTLC. LZ, Responsible for NMR analysis and writing of the methods, results and part of the discussion relating to NMR. SL, Responsible for authentication of Chinese *Rhodiola* specimens and DNA analysis shown in supplementary data. MH, Principle investigator and overall director of the project, played a major role in the writing of the introduction and conclusions. All authors proof read manuscript and made contributions to the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fphar.2016.00254>

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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