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A comparative study of *Salvia miltiorrhiza* Radix & Rhizoma raw material and granule products using chromatographic analysis and antioxidant activity

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Abstract— One common medicinal preparation used in traditional Chinese medicine is granules of botanical extracts. But there is no assessment of their quality or effectiveness. Dan Shen (Salvia miltiorrhiza Radix & Rhizoma) granule extracts and their herbal counterparts were the subjects of this comparative investigation on antioxidant activity and purity. Methods: A comparison was made between the herb's water extracts and 12 granule extracts using a chromatographic technique to ascertain the concentration of seven marker chemicals. Principal component analysis (PCA) and agglomerative hierarchical clustering (AHC) were used to differentiate the herbal and granule extracts according to the marker chemical content. This study used DPPH, ferric ion reducing antioxidant power (FRAP), and 2, 20-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) tests to assess the antioxidant properties of herbal and granule extract The results revealed that salvianolic acid B, sodium danshensu, and cryptotanshinone levels were much greater in the herbal extracts group than in the granule group. The increased ABTS, DPPH, and FRAP activities of the herbal extracts were correlated with this ($P < .05$). Salvianolic acid B concentration was the primary differentiator between granule extracts and other herbal extracts in the AHC and PCA analyses.

In order to better educate healthcare practitioners and customers about the quality and effectiveness of granule products, the findings reinforce the necessity for their evaluation.

Introduction

To make a therapeutic herbal decoction, practitioners of traditional Chinese medicine (TCM) choose certain raw plant components, dry them, and then either grind them into powder or chop them into smaller pieces. Inconsistent herbal ingredients, a bad aftertaste, and a lengthy preparation procedure are just a few of the numerous drawbacks of this method.1 Herbal extracts that are both concentrated and standardized, with excipients in a granular form, are thought to have reliability and effectiveness.2 Over time, the granule form of herbal extracts surpassed the herbal decoction as the preferred method of giving herbal medications in clinical settings.3 On the other hand, some people are worried about the granule products' effectiveness, safety, and quality.There was a positive correlation between the amount of marker compounds and the extraction yields, marker compounds, and antioxidant capacities of the herb/decoction pieces

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compared to the granule products in a previous study that compared the raw and granular preparations of two popular medicinal plants (4,5).6,7 Our results are in line with those of a previous research by Liang et al., which found that the chemical profiles, antioxidant and anti-inflammatory bioactivities of baical skullcap root (Scutellariae Radix) granules and traditional decoctions are only partially identical.8 A clustering study driven by bioactive markers

1. taken 34 out of 39 samples of granules from the conventional decoction batches. In relation to the chemical fingerprint, 61.5% of the granule samples had anti-inflammatory activity that was bioequivalent to traditional decoction, while none of the granule samples had antioxidant activity that was bioequivalent to traditional decoction. Crucially, there is still no clear evidence that granules are safer and more effective than the traditional decoction in clinical practice.2 The significance and need of evaluating the biology and quality of granule products before they are used by both practitioners and consumers is highlighted by these findings. One of the most popular herbs in Asian therapeutic treatment, Dan Shen (Salvia miltiorrhiza Radix & Rhizoma) is a component in several formulas, including Danshen pian, Danshen injection, and compound Danshen dripping tablet.9, 10 S. miltiorrhiza is a versatile and important therapeutic plant, as shown by the 841 Chinese herbal remedies that include it, according to the Chinese National Medical remedies Administration database.11 While S. miltiorrhiza cultivation is common in most Chinese provinces, the provinces of Shanxi, Sichuan, Hebei, Henan, and Shandong have long been known as the main production locations for high-quality S. miltiorrhiza.12 Its chemical components include

2. As early as the 1930s, S. miltiorrhiza was isolated. More than 70 structural components have been found and categorized according to whether they are hydrophilic or lipophilic.13 Tanshinone I (TI), crypto-tanshinone (CT), dihydrotanshinone I (DT), and tanshinone IIA (TIIA) are among the over 30 lipophilic chemicals that have been isolated and identified. These molecules are mostly diterpene quinones. For example, phenolic acids, protocatechuic acid, sodium danshensu (DSS), and protocatechuic aldehyde are all hydrophilic substances. With more than 1% and 3%e5% of total dry weight, respectively, DSS and salvianolic acid B (SB) are the most abundant components.14, 15 Reference standards for S. miltiorrhiza in the People's Republic of China Pharmacopoeia (PPRC) are SB and TIIA at now. To represent the efficacy and purity of S. miltiorrhiza, the Korean Pharmacopoeia relied on SB, the only marker chemical.16, 17 The antioxi-dant, anti-inflammatory, and angiogenesis characteristics of S. miltiorrhiza products make them a popular choice for the therapeutic treatment of cardiovascular illnesses, acute ischemic stroke, hyperlipidaemia, and cerebrovascular disorders in Asian nations.18e20

The granule products of S. miltiorrhiza have not been evaluated for quality or efficacy, despite the fact that they are rather popular.13 As part of their standard operating procedure for ensuring the highest quality of their Chinese herb products, the PPRC often employs thin layer chromatography (TLC), an analytical technique for the separation and identification of bioactive components in herbal combinations.16 Rapid and cost-effective chemical quantification via TLC has also been achieved using the automated CAMAG Linomat system.21e23 Liquid chromatography methods, including HPLC and UPLC, are extensively used because they allow for the exact and accurate quantitative detection of biomarkers in Chinese herbs.24 It is unusual, nevertheless, to see herbal granules quality-controlled utilizing TLC and UPLC quantification in accordance with their original herbal decoction. Granule product efficacy is unclear, despite substantial in vitro

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and in vivo investigation of S. miltiorrhiza antioxidant properties.15 In line with the general agreement that multiple methods should be used to determine the efficacy of herbal remedies, this study intends to compare the raw (unprocessed herbal material/decoction pieces) and manufactured granule samples of S. miltiorrhiza with respect to antioxidant activity, chemometrics, and TLC/UPLC chromatography. In addition to using statistical clustering techniques like agglomerative hierarchical clustering (AHC) and principal component analysis (PCA), this study also included a Pearson correlation of the chemical markers to antioxidant activity, which allowed for a thorough examination of the variations in product quality and ability.

Part 2: Resources and Procedures

Part 2.1: Chemicals and botanicals

Thermo Fisher Scientific of Scoresby, Australia, supplied the 85% phosphoric acid, 85% methanol, and HPLC grade acetonitrile. Ajax Finechem of Cheltenham, Australia, supplied the analytical grade ethyl acetate, toluene, and formic acid. Millipore, located in Burlington, MA, used its Milli-Q Reagent Water System to get the water. Sigma (Kemps Creek, Australia) supplied the 2, 20-azino-bis (3-ethyl-benz-thiazoline-6-sulfonic acid) (ABTS) working solution, DPPH, Trolox, sodium acetate trihydrate, glacial acetic acid, 2, 4, 6-tripyridyl-s-triazine (TPTZ), hydrochloric acid (HCl), ferric chloride hexahydrate, potassium persulfate, and hydrochloric acid (HCl) were all acquired from Sigma.
Sources in China and Australia were consulted for the raw herbal materials of S. miltiorrhiza, which included both crude herbal materials (R2, R4eR6) and decoction components (R1, R3) (Supplemental Fig. 1). In accordance with the Hong Kong Materia Medica Standards and PPRC, all of the raw materials were certified by the Department of Applied Biology and Chemical Technology, Hong Kong Polytechnic University (Hong Kong, China) (China, 2015). The NICM Health Research Institute at Western Sydney University received a voucher specimen from every sample. The lack of permission for disclosure has resulted in the omission of product commercial names.
To make it seem like a typical water decoction, the S. miltiorrhiza raw plant ingredients were soaked. The first step was to reflux 1 gram of R1–R6 powdered S. miltiorrhiza herbal material in 30 milliliters of boiling water. The liquid samples were then spun in a centrifuge at 672×g for 5 minutes and dried using a rotary evaporator. To get rid of the water-soluble excipients, the granule products (1 g of G1-G12) and the water-based herbal extract were extracted three times with 10 mL of methanol. Before being centrifuged at 672×g for 5 minutes, the samples were sonicated for 30 minutes. After that, the liquid above the solid was collected and cooled using a rotary evaporator until it was completely dry. For immediate analysis, the dry residue was weighed and redissolved in methanol at a concentration of 10 mg/mL. It was then kept at 4◦C, or at e20◦C, until it was needed again.
Using the Herbal Chemical Marker Ranking System (Herb MaRS), the primary chemical components of S. miltiorrhiza were chosen for this investigation.Twenty-five marker chemicals were chosen for bioassay testing and content measurement; all seven of them scored higher than 1 on this scale. Salvianolic acid A (SA), DSS, SB, DT, CT, TI, and TIIA are the chosen compounds. Chengdu Biopurify Phytochemicals Ltd of Chengdu, China, supplied the reference standards (with a purity level of >98%), and we used liquid chromatography-mass spectrometry (LC-MS) to confirm their accuracy.
The reference compounds' standard stock solutions were made in methanol at a concentration of 2 mg/mL and kept at 4◦C for quick analysis or at —20◦C for later usage. For the UPLC calibration curve, a range of concentrations was produced, ranging from 0.1 to 2000 mg/mL for DSS, DT, CT, and TIIA, and from 1 to 1500 mg/mL for SA, SB, and TI.

Instrumentation and chromatographic conditions

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2.1.1. Thin layer liquid chromatography

3. Preparation for usage included cutting the silica gel 60 F254 TLC plates (20 x 20 cm) from Merck KGaA in Darmstadt, Germany, into 10 × 10 cm squares. Camag Chemie-Linomat 5 automated applicator was used to apply the S. miltiorrhiza samples and standards (6 mL) on the plate. (Erzeugnisse & Adsorptionstechnik AG, Muttenz, Switzerland) using 100 mL syringes that were adjusted as follows: With an 8-millimeter band width, 2-millimeter track spacing, and 8 tracks per plate. Ten millimeters below the TLC plate's bottom border was where the application was made. After that, the prepared mobile phase solution consisting of ethyl acetate, toluene, formic acid, and methanol (15:20:10:10:1) was added to a stainless steel lid on top of a CAMAG Twin Trough chamber (10 × 10 cm) that held around 6 mL of the plate.16 For a minimum of 30 minutes at 20 ◦C, the mobile phase was applied vertically to the plate, starting from the bottom border and continuing up to 80 mm. The plate was allowed to air dry for 10 minutes after development prior to imaging. The TLC plates were examined using a Canon PSG × digital camera and a CAMAG Scanner 3 with 366 nm light from CAMAG in Muttenz, Switzerland. Applying the winCATs ver.1.3.0 system (CAMAG, Muttenz, Switzerland), the data and images were analyzed.

3.1.1. Ultra-performance liquid chromatography

4. The analysis was carried out utilizing a Waters ACQUITY UPLC system, manufactured by Waters in Milford, MA, which stands for ultra-performance liquid chromatography combined with photodiode array. UPLC separations were carried out with the use of a pre-column (2.1 × 5 mm, 1.7 mm) from Waters in Milford, MA, connected to an ACQUITY UPLC BEH C18 column (150 × 2.1 mm, 1.7 mm). Temperatures of 20 ◦C and 4 ◦C were maintained for the column and sample, correspondingly. S. miltiorrhiza was cultured in mobile phase solutions that included 0.1 percent phosphoric acid.

mixture of (A)-acetonitrile and (B). The gradient elution conditions were derived from a previously disclosed approach with the following modifications: 0–5 minutes, A 90%–75%; 5–10 minutes, A 75%–50%; 10–16 minutes, A 50%–20%; and 16–18 minutes, A 20%–90%.26 For a further two minutes, the column was isocratically reconditioned with 90% A. A volume of 10 mL was injected at a flow rate of 0.3 mL/min. A wavelength of 280 nm was used for detection. Using linearity and repeatability, the UPLC techniques were partly validated in accordance with the International Guidelines for Single Laboratory Validation of Chemical techniques for Dietary Supplements and Botanicals (AOAC) as outlined in the aforementioned publication.27 For the purpose of validating the standard curve calibration, we created over four concentrations of each standard component and evaluated the compound in triplicate at each concentration. The calibration regression was determined by plugging in the values of the reference samples' concentration (x) and the peak area (y), which were y ¼ ax + b. Using the formulas as follows: LOD = 3.33 × (standard deviation [SD] of y-intercept/mean of slope) and LOQ = 10 × (SD of y intercept/mean of slope) from the repeated analyses, the UPLC techniques' limit of detection (LOD) and limit of quantification (LOQ) were determined.27 One way to quantify consistency was by looking at the relative standard deviation-RSD. Each marker compound's intra-day accuracy was tested by measuring four different concentrations three times in a single day, while the inter-day repeatability was tested over the course of three days in a row.

4.1. Antioxidant activity assays

4.1.1. ABTS antioxidant assay

5. We used a standard protocol to determine the ABTS radical scavenging capabilities of the

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raw and granule extracts of S. miltiorrhiza.28 After 7 mmol/L of ABTS and 2.45 mmol/L of potassium persulfate were mixed in equal volumes, they were let to sit at room temperature for 12–16 hours in the dark to create the ABTS radical working solution. An initial absorbance value of 0.4 at 410 nm was achieved by diluting the stock solution with phosphate-buffered saline (PBS, pH 7.4) prior to analysis. After that, 20 mL of either the sample or the Trolox standard (0.045e0.330 mmol/L) was mixed with 200 mL of diluted ABTS. Five minutes after mixing, the absorbance was measured at 410 nm using a microplate reader (BMG).

6. Victoria, Australia is home to CLARIOstar. Measured in milligrams per gram of dry weight (mg/g of DW), the ABTS antioxidant activity was calculated.29

6.1.1. DPPH antioxidant assay

7. The DPPH test followed the protocol that had been previously detailed.30 Combine each sample with the same volume of DPPH radical solution (0.24 mg/mL DPPH in methanol), incubate for 30 minutes in the dark, and then measure absorbance at 515 nm. The experiment was conducted on a 96-well plate. As for the calibration curve, Trolox was used. Following the same procedure as for ABTS, we were able to acquire the data and the standard curve.

7.1.1. FRAP antioxidant assay

8. The FRAP (ferric ion reducing antioxidant power) test was carried out according to the methods previously detailed.30 The FRAP working solution was made by combining 10 volumes of 300 mM acetate buffer (pH 3.6), 1 volume of 20 mM ferric chloride (FeCl3$6H2O), and 1 volume of 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl. After that, every sample was combined with the pre-heated FRAP solution on a 96-well plate and let to sit at 37 ◦C for half an hour. Microplate readers (BMG CLARIOstar, Victoria, Australia) were used to measure the absorbance at 595 nm. The methodology used to generate the data and standard curve was same to that which was detailed for ABTS.

8.1. Statistical analyses

To compare the granule samples to the original herbal material, we divided their values by their concentrated ratio. This included yield, TLC and UPLC quantification, and antioxidant capacities. For example, a ratio of 1:5 would mean that 1 g of granule is equivalent to 5 g of the original raw material (refer to Supplemental Table 1), allowing us to draw comparisons.

We used non-parametric tests in GraphPad Prism 8 (GraphPad, San Diego, CA) or SPSS 20.0 (IBM Corp., Armonk, NY) to look for statistically significant differences in the data between the raw herbs and the granules. For AHC and PCA, the compounds that demonstrated significant differences were designated as variables. Ward's approach and Euclidean distances formed the basis of AHC analysis, which yielded data shown as dendrograms. The degree of similarity between analytes was indicated by the length of the branches. The original variables, which consisted of seven marker compounds, were transformed into a new set of linearly uncorrelated factors (PCs) that matched the original variables' maximum potential variance via the use of principal component analysis (PCA) in XLSTAT (Addinsoft, New York, NY). The PCA-generated biplot (scoring plot and loading plot) displayed the sample distribution according to the variables' and PCs' correlations.31

Antioxidant capabilities vary significantly, and their Graph-Pad Prism 8 was used for non-parametric analysis, whereas Pearson correlation was used for content correlation analysis with the marker molecules. The strength of the connection was shown by the Pearson correlation coefficients

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(r), and the statistical significance was suggested by the P-value, which was fixed at P <.05.

9. Results

9.1. Quantification of the marker compounds

The seven marker chemicals were identified and quantified using TLC and UPLC-PDA on both the raw and granule samples of S. miltiorrhiza. The seven marker compounds' molecular blueprints are shown forth in Fig. 1. The raw herb extract (R6) and granules of S. miltiorrhiza are shown in Supplemental Figs. 1e3, which exhibit representative TLC and UPLC fingerprints. Validation of the TLC and UPLC methods using linear regression equations, R2, LOQ, and LOD

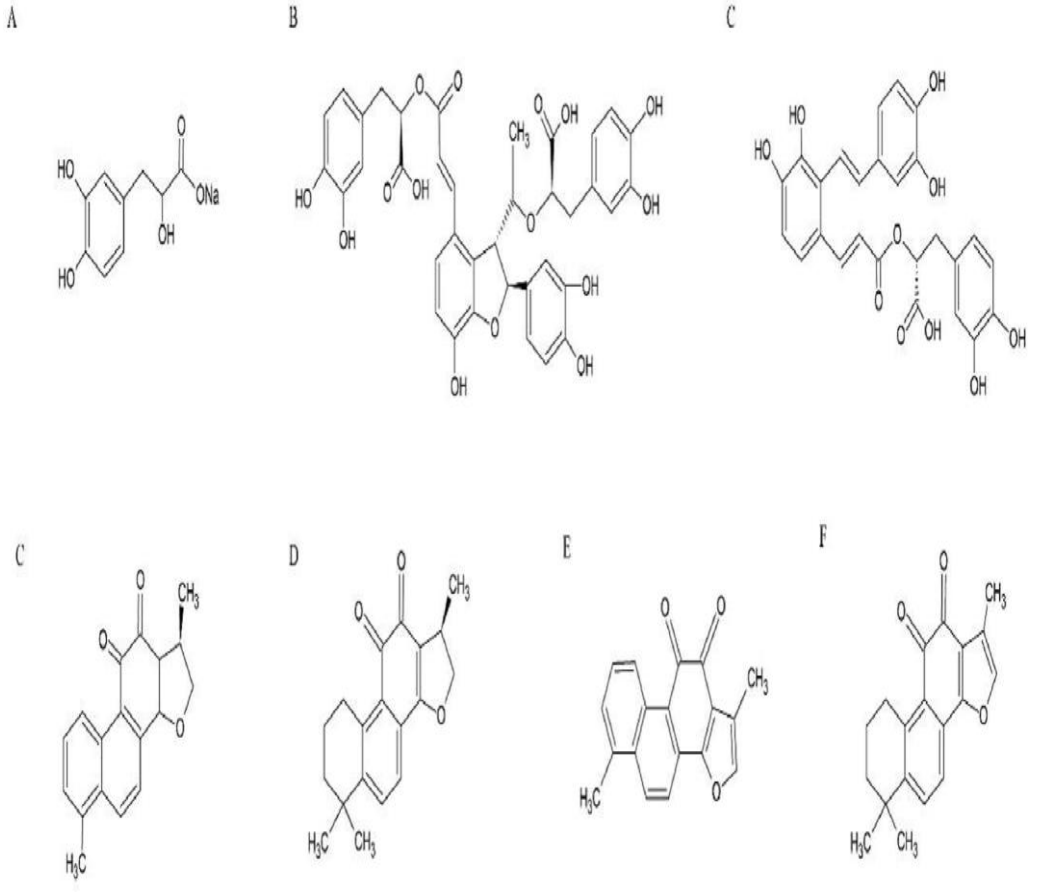

Fig. 1. Chemical structures of seven standard compounds in *S. miltiorrhiza* extracts using ACD/ChemSketch (Canada).
Notes: The hydrophilic compounds include (A) DSS, (B) SB and (C) SA. Lipophilic compounds include (D) DT, (E) CT, (F) TI, (G) TIIA. Their contents in the *S. miltiorrhiza* raw herb and granule samples were determined by TLC and UPLC analysis. In particular, SB and TIIA were used as marker compounds for the quality control of *S. miltiorrhiza* raw herb extract in the PPRC. DSS:sodium danshensu; SB: salvianolic acid B; SA: salvianolic acid; DT: dihydrotanshinone I; CT: cryptotanshinone; TI: tanshinone I; TIIA: tanshinone IIA.

Table 1 displays them. Good linearity of the experimental data for the analytical procedures was shown by R2 values better than 0.988 (TLC) and 0.994 (UPLC) for all analytes. In terms of linear optical density (LOD), the seven marker chemicals were detected using UPLC with a range of 0.001–0.215 mg/mL and TLC with a range of 0.001–0.019 mg/mL. The 7 marker compounds have LOQs ranging from 0.003 to 0.647 mg/mL for TLC and 0.002 to 0.056

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mg/mL for UPLC. When compared to TLC, UPLC often had lower LOD and LOQ values, indicating that UPLC was more sensitive when it came to identifying and quantifying chemicals. The UPLC technique and instruments both produced very accurate results. The intra-day precision of TLC varied between 4.656% and 21.251% and that of UPLC between 1.334% and 3.732%, as indicated in Table 1. For TLC, the RSD for inter-day accuracy was 7.257% to 27.536%, whereas for UPLC, it was 3.040% to 7.065%. Accordingly, when compared with TLC, the UPLC technique and apparatus seem to have superior reproducibility for

S. miltiorrhiza chemical analysis.

Table 2 displays the contents of the marker chemicals that were found in the samples using TLC. Marker chemicals DSS and tanshinones (CT, DT, TI, and TIIA) were not adequately quantified independently for certain samples due to insufficient TLC resolution (Supplemental Fig. 1). The amounts of SA and SB in the granules were much lower than what was found in the samples of raw herbs. SB and SA were the two most abundant compounds in the raw and granule samples, respectively. The raw herb samples often showed negligible or non-existent levels of tanshinones, including CT, DT, TI, and TIIA. The amounts of all the chemicals examined did not differ substantially between the raw and granule samples, according to the non-parametric t-test (P >.05). The raw sample group had a considerably greater SB than the granule sample group (P <.0001). See Table 2 for a list of the marker chemicals found in the UPLC-analyzed raw and granule samples. Repeatability in the quantification was shown by the low standard deviations of the seven standards in the raw herbs and granules. The S. miltiorrhiza samples showed that SB and SA were the two most abundant compounds, in agreement with the TLC quantification findings. The amounts of all the chemicals examined did not differ substantially between the raw and granule samples, according to the non-parametric t-test (P >.05). Still, a non-parametric independent t-test revealed that, when comparing the two groups, the raw samples included considerably more DSS, SB, and CT than the granule samples (P =.010, 0.000, and 0.000, respectively).

8.2 AHC and PCA multivariate analysis

The quantification findings from UPLC were submitted to AHC utilizing all 7 marker chemicals as variables as UPLC demonstrated improved sensitivity and capacity to quantify the marker compounds in S. miltiorrhiza samples. The most striking contrast, as illustrated in Figure 2A, was found between the cluster containing all twelve granules and two raw herbs (R2 and R4) and the cluster containing four raw samples of S. miltiorrhiza [R1, R3 (both decoction pieces), R5 and R6]. This is because the four raw samples contained a substantially higher concentration of the seven marker compounds when contrasted with the twelve granule samples. The granule samples clustered with R2 and R4 because their concentrations of the seven chemicals were similar. From most to least, the clusters might be further subdivided into several groups based on

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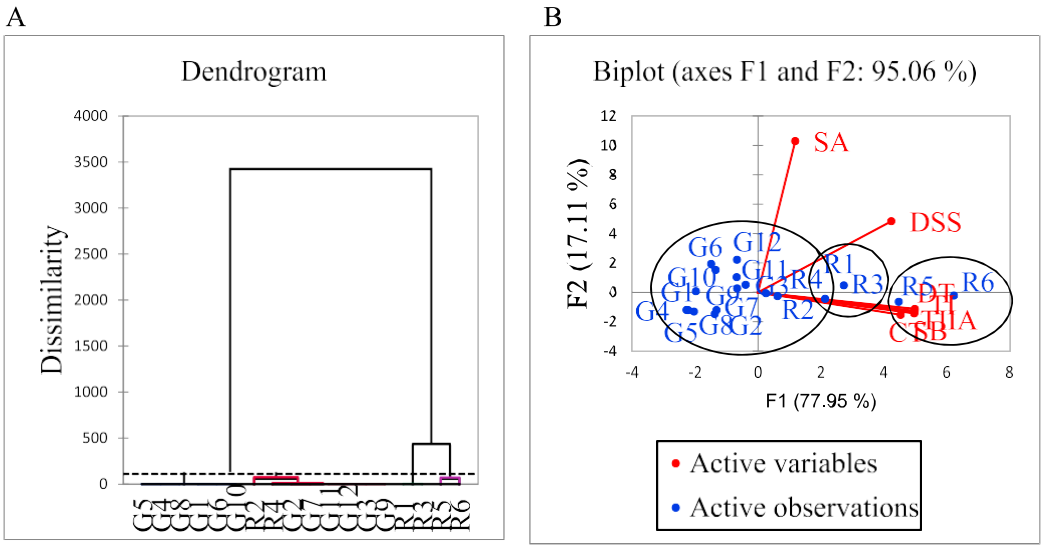

Fig. 2. AHC dendrograms and PCA biplot of *S. miltiorrhiza* extracts analyzed by UPLC.
Notes: A: AHC dendrograms for the UPLC results divided the raw herb and granule extracts into 2 main clusters, with raw herb samples R1, R3, R5 and R6 classified into one cluster, whereas R2 and R4 were grouped with the granules. B: PCA biplot (loading and score plot) of *S. miltiorrhiza* raw herb and granule extracts as analyzed by UPLC. From the UPLC biplot, raw samples (R5 and R6) demonstrated relatively higher amount of DT, TI, TIIA, SB, CT and DSS (cluster 1), especially R6 (highest amount). The content of these compounds in R1 and R3 were slightly lower. In contrast, all the granule samples plus R2 and R4 were closely distributed due to lower amount of these marker compounds (cluster 2). F1 represents 77.95% of the total variance from the 7 marker compounds and F2 (17.11%) cumulatively explains up to 95.06% of total variance. AHC: agglomerative hierarchical clustering; PCA: principal component analysis; UPLC: ultra-performance liquid chromatography.

57.74 mg/g DW, which were much higher than that of granule samples (4.37e9.57 mg/g DW). R6 exhibited the highest antioxi- dant activity among all the samples (87.33 and 57.74 mg/g DW) in both the ABTS and DPPH assays. In the FRAP assay (Fig. 3C), the optical density (O.D.) at 590 nm of the raw herbs/decoction pieces and granules ranged from 1.06 to 1.69 and 0.08 to 0.20, respectively. The non-parametric analysis in Fig. 3D showed that the antioxidant capacities of the raw herbs (as one group) were significantly higher than that of the granule group ($P < .0001$) in all 3 assays.

Pearson correlation examined the relationship between the content of the marker compounds and radical scavenging capac- ities. There were significant correlations between the UPLC results for the marker contents of DSS, SB and CT to the 3 antioxidant ac- tivities (Table 3). R6 possessed the highest amount of these marker compounds and thus showed the highest ABTS and DPPH scav-
enging capacities. In contrast, the amount of DT was negatively correlated with ABTS, DPPH and FRAP assays (—0.343, —0.283 and —0.293) without any significance ($P > .05$). The results showed
that the compounds SA, TI and TIIA did not contribute to the antioxidant activities of the raw and granule samples.

10. Discussion

There have been many attempts worldwide at developing rapid techniques and methods for analyzing the chemical variability of herbal granule products using TLC, high-performance liquid chro- matography, UPLC, and using new techniques such as FT-NIR spectroscopy.[32e34] However, it is important to note that industry requires simple, accessible and inexpensive methods to examine the quality control of their manufactured products. TLC has been used extensively as an initial tool for the identification and semi- quantitative analysis of herbal products due to its simple and inexpensive set up.[35] In the PPRC and herbal monographs, TLC analysis is highly recommended for the identification and quality control of *S. miltiorrhiza* samples.[16,36] HPLC is the standard chro-matography methods used by industry for the quality control ofherbal products. However, UPLC is preferred over HPLC for the accurate quantification of marker compounds in herbal products due to its higher sensitivity and resolution.[35] In our previous studies, we have shown that the content of the marker compounds and antioxidant capacities using UPLC and/or TLC were generally lower in the granule form compared to that of the raw herbs in Sanchi (*Notoginseng* Radix et Rhizoma) and Chinese Angelica (*An- gelica Sinensis* Radix) which raises concern for the quality control and efficacy of herbal granule products.[6,7] In 2009, Song et al compared the amount of 8 major components in *S. miltiorrhiza* granules with raw extracts using a HPLC system, and their results showed that the average content of the total components were similar to that in aqueous extracts of *S. miltiorrhiza.*[37] However, the equivalent ratio of granule was not taken into account for the calculation, and the bioactivities were not conducted. The present study assesses the quality and efficacy of granule formulations of another valuable Chinese herbal medicine, *S. miltiorrhiza*. By using advanced UPLC system, antioxidant assays and multi-variant analysis, our data suggests that granules presented with a lower quality and antioxidant activity compared with their herbal coun- terparts when compared with the dried weight of the

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herb.

Twelve *S. miltiorrhiza.* granules and 6 raw samples were collected from various sources and examined in this study. The raw samples were decocted with water (to reflect traditional con- sumption) and then extracted with methanol, whilst the granule samples (which were manufactured extracts) were extracted with methanol only to remove the water-soluble excipients. This is because herbal granule products are assumed to be formed from a concentrated water decoction. Based on the standard manufacturing process, granules are marketed as the concentrated herbal extract with added excipients. The labelled ratio (e.g. 1 g of the granule is equivalent to 5 g of the crude material etc.) is essential for correct dosing. Thus, the results generated from granule samples were divided by their concentration ratio as indicated on the product label so that the granule was compared with the raw sample at the same baseline. By comparing the

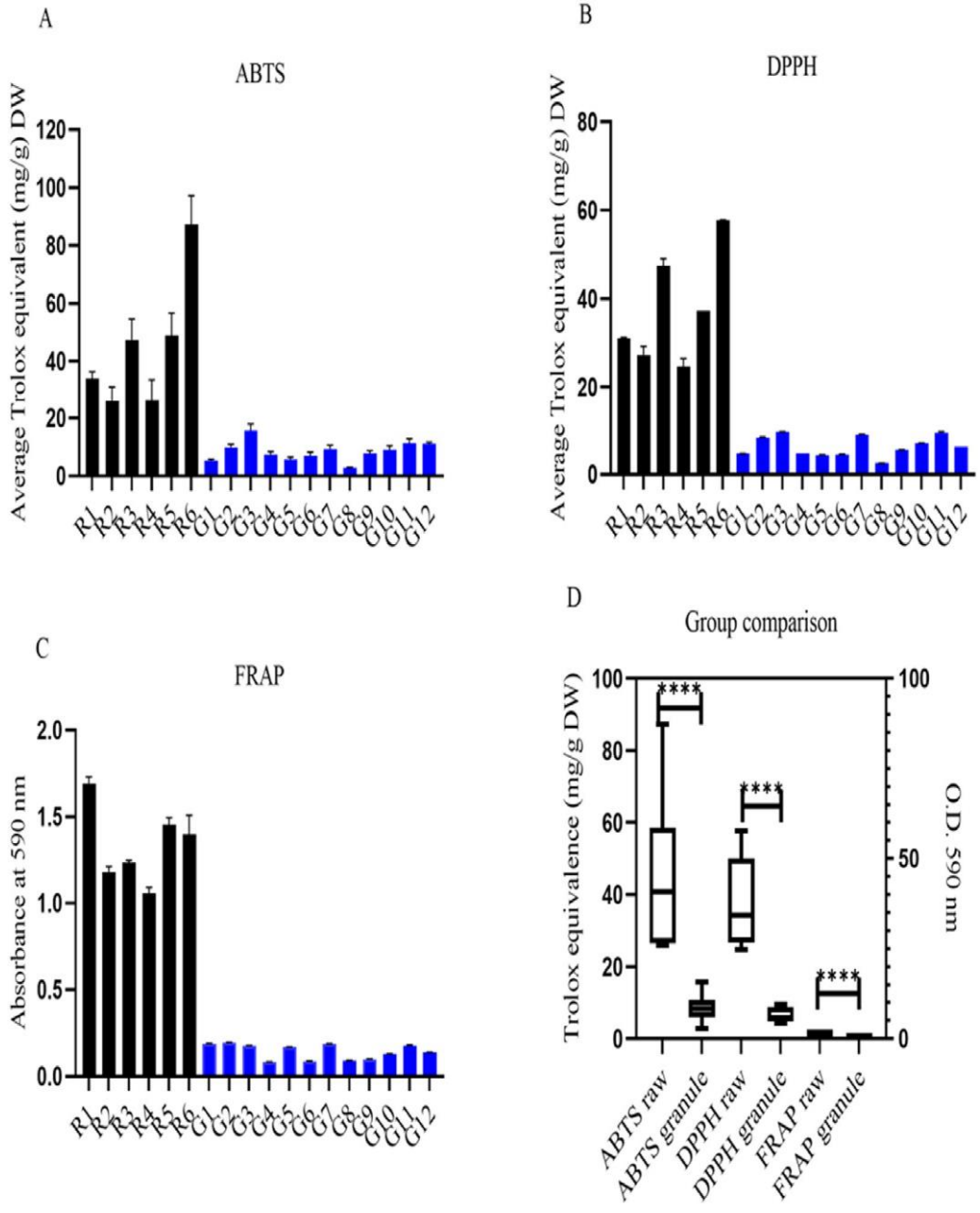

Fig. 3. ABTS, DPPH and FRAP activities of *S. miltiorrhiza* raw herb and granule extracts.

Notes: A: Raw herb samples (25.95e87.33 mg/g DW) showed a higher ABTS radical scavenging activity compared with granule samples (5.38e15.67 mg/g DW). R6 exhibited the highest ABTS antioxidant activity among all the samples (87.33 ± 9.97 mg/g DW). B: Raw herb samples (24.71e57.74 mg/g DW) showed a higher DPPH radical scavenging activity compared with granule samples (4.37e9.57 mg/g DW). R6 exhibited the highest DPPH antioxidant activity among all the samples (57.74 ± 0.09 mg/g DW). C: At 590 nm, raw herb samples (1.06e1.69) showed a higher FRAP radical scavenging activity than granule samples (0.08e0.20). R1 exhibited the highest FRAP antioxidant activity among all the samples (1.69 ± 0.04). D: Group comparison between raw and granule samples in ABTS, DPPH and FRAP assays. ****$P < .0001$ *vs.* raw group. $n = 3$. ABTS: 2,2'-azino-bis (3- ethylbenzothiazoline-6-sulfonic acid; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric ion reducing antioxidant power.

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extraction yield between groups, the raw herbs were generally higher than the granules group. The different extraction yield in the granule products could be caused by the different manufacturing processes used to produce the granules. The excipients added to the granules during manufacturing may be soluble in methanol and this may affect the final calculation of the content of the granule product. Upon a closer inspection, granulation was generally inconsistent for each product examined and thus, smaller particles

Table 3
Pearson correlation between the 3 chemical markers and antioxidant activities.

Assay	Markers						
	DSS	SB	SA	DT	CT	TI	TIIA
ABTS	0.774**	0.965**	0.202	−0.343	0.886**	0.340	0.231
DPPH	0.730**	0.934**	0.184	−0.283	0.936**	0.347	0.272
FRAP	0.665**	0.844**	0.083	−0.293	0.890**	0.320	0.267

Notes: The r value (between −1 and +1) as analyzed by the Pearson correlation represents the correlation coefficient, where an r value < 0 refers to a negative correlation, and an r value > 0 refers to a positive correlation. $^{**}P < .01$ refers to a significant correlation (either positive or negative). ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric ion reducing antioxidant power; DSS: sodium danshensu; SB: salvianolic acid B; SA: salvianolic acid; DT: dihydrotanshinone I; CT: cryptotanshinone; TI: tan- shinone I; TIIA: tanshinone IIA.

(which have greater surface area for extraction) may find them- selves at the bottom of the container. Manufacturing differences such as extraction with solvents other than water, temperature/ pressure conditions, the type and quantity of excipients used and adulteration can affect the quality of the finished product.

It is well-recognized that the quality control of medicinal plant products is the foundation for their development and acceptance in integrative medicine.[38] The complex nature of plants, environ- mental influences and poor-quality control processes bring great challenge for the quality control of the herbal products.

S. miltiorrhiza has a complex chemical composition which includes 2 major groups of chemicals: salvianolic acids (hydrophilic) and tanshinones (hydrophobic). Based on the TLC method documented in the PPRC, we have further optimized the condition and mobile phase, and identified 6 compounds presented in the *S. miltiorrhiza* raw and granule samples. Unfortunately, DSS was not detected due to its extremely hydrophilic property and thus, could not move with the mobile phase. This may limit the single TLC method (as used in this study) for establishing the herb's complete profile.[39] In addition, the optimized TLC method was partially validated in terms of linearity, LOD, LOQ and precision and compared to UPLC. In agreement with our previous studies, the relatively large RSD value for precision reflected the drawbacks of using TLC for quantification purposes as the resolution of TLC is easily affected by the temperature, humidity, mobile phase condition, etc.[6,7] Moreover, we noticed that TLC failed to quantify the lipid-soluble compounds of tanshonines which may be due to their minor amounts in the extracts and the sensitivity limit of the TLC method. Here, a vali- dated UPLC method was employed to quantify *S. miltiorrhiza* marker compounds. The significantly lower values of LOD, LOQ and RSD suggested good instrumental and method precision for UPLC compared with TLC. In agreement with literature, SB was found to be the most abundant compound in *S. miltiorrhiza* raw and granule samples.[40] The amounts of SB were significantly higher in the raw samples compared to that of the granule sample. However, the PPRC marker compound, TIIA, did not show significant difference between the 2 main sample groups which is consistent with its hydrophobicity in water. Although TLC and UPLC had differing quantification results, the trend was similar, with SB significantly higher in the raw herb groups compared with that of the granule group. Thus, our results showed that TLC is not as accurate and efficient as UPLC for quantitative purposes. However, it can be used as a qualitative tool for a quick scan of the bioactive compounds in the herbal extract.

We then examined the similarities of the products using multivariate analysis. Using the 7 marker compounds as variables, AHC analysis showed that most of the raw samples were grouped in the same cluster, except for R2 and R4 (raw herb samples) which were classified into the granule cluster due to its lower amount of marker compounds. This reflects the lower content of components in the starting raw material which may influence the granule quality.[41,42] This could be a primary reason for *S. miltiorrhiza*'s inconsistency which would subsequently affect the quality of the manufactured granule, especially if the raw materials are collected from different sources (i.e not the recommended growing areas as per PPRC) or not at the optimal time of the year. These results are in line with our previous comparative studies on *Notoginseng* and *Angelica Sinensis* which showed comparable amounts of marker compounds in the raw herbal samples, whereas the contents of marker compounds in the granule samples were significantly lower and possessed large variance.[6,7] It is interesting to note that the *S. miltiorrhiza* decoction pieces (R1 and R3) showed comparable amounts of all the marker compounds as in most raw herb mate- rials. This corresponded to our previous study where the *Angelica Sinensis* decoction pieces were consistent in composition and showed comparable amounts of the marker compounds to the raw herbs.[7] Decoction pieces are processed raw materials (i.e., washed, fried, cut, dried as per TCM practice), and can be applied directly to clinical treatment. Thus, they should present a similar quality as for the raw material. However, it is reiterated that the quality man- agement of decoction pieces needs to be strengthened, as the processing mechanism remains unclear and the quality control not standardized.[43]

The AHC analysis showed that SB and TIIA are the key com- pounds that differentiate the raw and granule samples which support the PPRC. Moreover, SB, the most abundant compound, played a predominant role amongst the tested compounds. How- ever, PCA analysis suggested that the other 3 tanshinones also contributed to the differentiation of raw and granule samples. Moreover, the AHC display of samples using individual

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compounds showed distinct dendrogram (excluding SB to TIIA), suggesting that more compounds (i.e. CT, DT and TI) may need to be considered for the quality control of *S. miltiorrhiza* raw and granule samples.

Although TIIA and SB are listed as the key marker compound for the quality control of *S. miltiorrhiza* in the PPRC, it is noted that the quantity of SB and TIIA in granules were all below the standard in PPRC.[16] For *S. miltiorrhiza*, methanol is used as the solvent in the PPRC and this would favor higher extraction yields of the
compounds. However, it is suggested that the quality control standard for the herbal water decoction should be considered for inclusion in the PPRC or relevant regulation standard to honor the tradition method of preparing herbs for medicinal use.

SB is the most abundant and bioactive compound found in
S. miltiorrhiza and is known for its potent antioxidative and reactive oxygen species scavenging activity which is attributed to its poly- phenolic structure.[40] Thus, *S. miltiorrhiza* has been extensively used clinically as a principal herb in TCM for cardiovascular diseases.[44] Tanshinones are a hydrophobic group of compounds isolated from *S. miltiorrhiza,* and emerging experimental and clinical in- vestigations have supported their pharmacological activities in preventing or slowing the progression of a wide variety of diseases due to their potent antioxidant, anti-inflammatory and anti-cancer activities.[45] Our study assessed the antioxidant activity of the
S. miltiorrhiza raw and granule products and correlated their ac- tivity to the content of the key bioactive compounds. The results show that the significantly lower amounts of SB, DSS and CT (relatively more polar compounds) in the granule samples not only contributed to differentiating the granule samples from raw sam- ples in the PCA analysis, but also lead to significantly lower ABTS, DPPH and FRAP scavenging activity. This was evidenced by the positive and significant correlation. It was observed that marker compound TIIA also played a minor role in discerning the antioxi- dant activities of the *S. miltiorrhiza* raw and granule samples but was not significantly correlated to the overall antioxidant activity of the extract ($P > .05$). Isolated TIIA has been recognized as a pharmacological active compound with various promising health ben- efits including antioxidant, anti-inflammatory and anti-cancer. Here, TIIA did not contribute to the antioxidant activity which was attributed to its minimal presence in the *S. miltiorrhiza* water extract.[46] The different antioxidant activities in the raw and granule samples can also be attributed to other minor bioactives that were not included in this study such as rosmarinic acid, rosmarinic acid methyl ester and rosmarinic acid ethyl ester.[47]

11. Conclusions

The present study assessed the quality and bioactivity differ- ences between *S. miltiorrhiza* raw and granule products using TLC and UPLC coupled with multivariate analysis, and antioxidant as- says. UPLC proved to be a better differentiator of the marker com- pounds between samples. It was revealed that the content of the marker compounds (SB, DSS, CT) was significantly lower in the granule samples compared with that of the raw samples which led to the lower antioxidant activity of the granule. It has been recog- nized that the inconsistent amount of bioactive compounds in herbal products is likely to induce fluctuated levels of therapeutic effects, which represent one of the major concerns for the clinical effectiveness of herbal products, including granule formulations.[1] With the gaining popularity of herbal medicinal granules around the world, this study provides important scientific evidence for standardization committees, industry, practitioners and consumers on the quality control and efficacy assessment of herbs and its related granule products. It is vital for healthcare professionals to be aware of granule quality differences and know the correct dos- ages to match the contents of traditional decoctions. We believe this study provides a rational argument for the continued investi- gation of the quality and efficacy assessment of the granular form of medicinal plants. More rigorous pharmacological, toxicological and clinical studies using granules compared to herbal extract water decoctions are warranted to confirm these findings and to advocate high-standard, safe and efficacious herbal preparations to the consumers.

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CRediT authorship contribution statement

Xian Zhou: Methodology, validation, formal analysis, investi- gation, resources, writing – original draft, writing – review & editing. Valentina Razmovski-Naumovski: Conceptualization, supervision, methodology, validation, resources, writing – original draft, writing – review & editing, project administration. Raynold Mendoza: Methodology, investigation. John Truong: Methodology, formal analysis. Kelvin Chan: Conceptualization, supervision, methodology, resources, writing – review & editing, project administration.

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