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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

Objective: Granules of herbal extracts are a popular medicinal preparation consumed in traditional Chinese medicine clinical practice. However, their quality and efficacy evaluation are lacking. This study aimed to compare the quality and anti-oxidant activity of Dan Shen (*Salvia miltiorrhiza* Radix & Rhizoma) granule extracts with their herbal extracts.

Methods: Chromatographic method was used to determine the content of 7 marker compounds in the water extracts of the herb compared to that of 12 granule extracts. Agglomerative hierarchical clustering (AHC) and principal component analysis (PCA) distinguished the herbal and granule extracts based on the content of the marker compounds. The antioxidant activities of herbal and granule extracts were evaluated by 2, 2′-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS), organic chemical compound 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric ion reducing antioxidant power (FRAP) assays.

Results: The herbal extracts group showed significantly higher contents of salvianolic acid B, sodium danshensu and cryptotanshinone compared with that of the granule group. This corresponded to significantly higher ABTS, DPPH and FRAP ($P < .05$) activities of the herbal extracts. The AHC and PCA analysis distinguished granule extracts from most herbal extracts predominantly by the content of salvianolic acid B.

Conclusion: The results confirm the need for the assessment of granule products so that healthcare practitioners and consumers are better informed of their quality and efficacy.

1. Introduction

In traditional Chinese medicine (TCM), specific parts of the raw herbs are harvested, dried and ground into powder or cut into pieces before being soaked and boiled as a herbal decoction for medicinal use. This preparation process has many disadvantages including being time-consuming, yielding inconsistent herbal ingredients and having an unpleasant taste.[1] In comparison, concentrated and standardized herbal extract with excipients in the granular form are easier to consume and are assumed to have consistent quality and efficacy.[2] Clinically, the granule form of herbal extracts has gradually replaced the herbal decoction and has since become the most popular way of administering herbal medicines.[3] However, concerns have been raised regarding the quality, safety and efficacy of granule products on the market.[4,5] Previously, we compared the raw and granular preparations of 2 popular medicinal plants and showed that the herb/decoction pieces had comparatively higher extraction yields, significantly higher marker compounds and antioxidant capacities than the granule products which was positively correlated to the amount of marker compounds.[6,7] In agreement to our findings, a recent study from Liang et al showed only a partial similarity between granule and traditional decoction of baical skullcap root *(Scutellariae* Radix) based on the chemical profiles, antioxidant and anti-inflammatory bioactivities.[8] The bioactive marker-guided clustering analysis

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separated granule samples (34 out of 39) from the batches of traditional decoction samples. Correlated to the chemical fingerprint, 61.5% of the granule samples were bioequivalent to traditional decoction in terms of anti-inflammatory activity, whilst none of granule samples were bioequivalent to the traditional decoction in antioxidant activity. Most importantly, the effectiveness and safety of granules in clinical practice in comparison to the traditional decoction remain inconclusive.[2] These finding reflect the importance and the need for the quality and biological assessment of granule products for widespread practitioner and consumer use.

Dan Shen (*Salvia miltiorrhiza* Radix & Rhizoma) is one the most widely used herbs in clinical practice in Asia and is included in many formulae such as compound Danshen dripping pill, Danshen pian and Danshen injection.[9,10] According to the Chinese National Medical Products Administration database, around 841 Chinese herbal products containing *S. miltiorrhiza* are produced in China which shows its versatility and importance as a medicinal herb.[11] The cultivation of *S. miltiorrhiza* is widespread across most provinces of China, Shanxi, Sichuan, Hebei, Henan and Shandong provinces are recognized as traditional primary production areas yielding high quality *S. miltiorrhiza*.[12] The chemical constituents of *S. miltiorrhiza* were isolated as early as the 1930s, and there are more than 70 components structurally identified and classified according to their lipophilic or hydrophilic nature.[13] More than 30 lipophilic compounds, mainly diterpene quinone compounds, have been separated and identified, including tanshinone I (TI), cryptotanshinone (CT), dihydrotanshinone I (DT) and tanshinone IIA (TIIA). Hydrophilic compounds include sodium danshensu (DSS), protocatechuic aldehyde, protocatechuic acid and phenolic acids. Among them, DSS and salvianolic acid B (SB) have the highest content among all components, accounting for over 1% and 3%–5% of total dried weight, respectively.[14,15] In *the Pharmacopoeia of the People's Republic of China* (PPRC), SB and TIIA are currently used as reference standards for *S. miltiorrhiza*. SB was selected as the only marker compound in the *Korean Pharmacopoeia* to reflect the quality and active pharmacological activities of *S. miltiorrhiza*.[16,17] In Asian countries, *S. miltiorrhiza* products are used clinically for treating cardiovascular diseases, acute ischaemic stroke, hyperlipidaemia and cerebrovascular diseases attributed to its antioxidant, anti-inflammatory and angiogenesis properties.[18–20]

Despite *S. miltiorrhiza's* popularity, there is no quality and efficacy assessment of its granule products.[13] Thin layer chromatography (TLC) is a widely used analytical method for the separation and identification of bioactive compounds in herbal mixtures and is routinely used in the PPRC for the quality control of Chinese herbs.[16] With the automatic CAMAG Linomat system, TLC has also been used to quantify compounds for the quick and economical purposes.[21–23] Moreover, liquid chromatography techniques such as high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) are widely used and provide accurate and precise quantitative determination of biomarkers in the Chinese herbs.[24] However, the quality control of herbal granules per their original herbal decoction using TLC and UPLC quantification remains quite rare. Although the antioxidant effects of *S. miltiorrhiza* have been investigated extensively in *in vitro* and *in vivo* studies, the activity of the respective granule products remains unknown.[15] With the consensus of using a multi-method approach in assessing the quality of herbal products, the present study aims to evaluate the differences between the *S. miltiorrhiza* raw (crude herbal material/decoction pieces) and manufactured granule samples using both TLC and UPLC chromatography, chemometrics and antioxidant activity. Coupled with statistical clustering analysis including agglomerative hierarchical clustering (AHC) and principal component analysis (PCA), and Pearson correlation of the chemical markers to antioxidant activity, this work provided a comprehensive study of the quality and efficacy differences between the products.

2. Material and methods

2.1. Chemicals and plant materials

HPLC grade acetonitrile, methanol and phosphoric acid (85%) were purchased from Thermo Fisher Scientific (Scoresby, Australia). Ethyl acetate, toluene and formic acid (analytical grade) were purchased from Ajax Finechem (Cheltenham, Australia). Water was obtained from a Milli-Q Reagent Water System (Millipore, Burlington, MA). For the antioxidant assays, 2, 2′-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) working solution, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), sodium acetate trihydrate, glacial acetic acid, 2, 4, 6-tripyridyl-s-triazine (TPTZ), hydrochloric acid (HCl) and ferric chloride hexahydrate, potassium persulfate were purchased from Sigma (Kemps Creek, Australia).

Raw herbal materials of *S. miltiorrhiza,* including crude herbal materials (R2, R4–R6) and decoction pieces (R1, R3), were collected from various sources in China and Australia (Supplemental Fig. 1). All the raw materials were authenticated by the Department of Applied Biology and Chemical Technology, Hong Kong Polytechnic University (Hong Kong, China) according to the Hong Kong Materia Medica Standards and PPRC (China, 2015). A voucher specimen of each sample was deposited at the NICM Health Research Institute, Western Sydney University. Product commercial names have been omitted due to the absence of consent for disclosure.

The raw herb materials of *S. miltiorrhiza* were subjected to an aqueous extraction to mimic traditional water decoction. Firstly, the ground powder of the *S. miltiorrhiza* herbal material (1 g of R1-R6) was refluxed in 30 mL of boiling water. The aqueous extracts were then centrifuged at 672×*g* for 5 min and evaporated to dryness under the rotary evaporator. The aqueous extract of the raw herbal materials and the granule products (1 g of G1-G12) were subjected to methanol extraction (10 mL, 3 times) to remove the water-soluble excipients. The samples were sonicated for 30 min, followed by the centrifugation at 672×*g* for 5 min. The supernatants were then collected and evaporated to dryness at 60°C under the rotary evaporator. The dry residue was weighed and re-dissolved in methanol at 10 mg/mL and stored at 4°C for immediate analysis or at −20°C until further use.

The selection of the major chemical compounds from *S. miltiorrhiza* for this study was based on the Herbal Chemical Marker Ranking System (Herb MaRS).[25] Seven marker compounds, which were all ranked above 1 on this scale, were selected for content quantification and bioassay testing. The selected compounds include DSS, salvianolic acid A (SA), SB, DT, CT, TI, TIIA. The reference standards were purchased from Chengdu Biopurify Phytochemicals Ltd (Chengdu, China; purity >98%) and verified in-house with liquid chromatography-mass spectrometry (LC-MS). The standard stock solutions of the reference compounds were prepared in methanol at 2 mg/mL and stored at 4°C for immediate analysis or at −20°C until further use. A series of concentrations (0.1–2000 μg/mL for DSS, DT, CT and TIIA, 1–1500 μg/mL for SA, SB and TI) were prepared for the UPLC calibration curve.

2.2. Instrumentation and chromatographic conditions

2.2.1. Thin layer liquid chromatography

The silica gel 60 F254 TLC plates (20 × 20 cm) (Merck KGaA, Darmstadt, Germany) were cut into 10 × 10 cm squares before use. The *S. miltiorrhiza* samples and standards (6 μL) were applied to the plate by a Linomat 5 automatic applicator (CAMAG Chemie-

Erzeugnisse & Adsorptionstechnik AG, Muttenz, Switzerland) with 100-μL syringes according to the following settings: 8-mm bandwidth, 2-mm space between tracks and 8 tracks on each plate. The application position was 10 mm from the lower edge of the TLC plate. The plate was then placed in a CAMAG Twin Trough chamber (10 × 10 cm) with a stainless steel lid containing approximately 6 mL of the optimised mobile phase solution: ethyl acetate: toluene: formic acid: methanol (15:20:10:10:1).[16] The plate was developed with the mobile phase vertically from the lower edge to 80 mm for at least 30 min at 20 °C. After development, the plate was air-dried for 10 min before imaging. TLC plates were evaluated using a CAMAG Scanner 3 (CAMAG, Muttenz, Switzerland) with 366 nm light, and a camera (Canon PSG × digital camera). The image and the data analysis were conducted using the winCATs ver.1.3.0 system (CAMAG, Muttenz, Switzerland).

2.2.2. Ultra-performance liquid chromatography

Ultra-performance liquid chromatography coupled with photodiode array (UPLC-PDA) was performed using a Waters ACQUITY UPLC system (Waters, Milford, MA). UPLC separations were conducted using an ACQUITY UPLC BEH C18 column (150 × 2.1 mm, 1.7 μm) with an attached pre-column (2.1 × 5 mm, 1.7 μm) (Waters, Milford, MA). The column and sample temperature were kept at 20 °C and 4 °C, respectively. The mobile phase systems used for *S. miltiorrhiza* consisted of 0.1% phosphoric acid (A)-acetonitrile (B). Gradient conditions were based on a modification of a method described previously, with a gradient elution of 0–5 min, A 90%–75%; 5–10 min, A 75%–50%; 10–16 min, A 50%–20%; 16–18 min, A 20%–90%.[26] The column was reconditioned isocratically with 90% A for another 2 min. The flow rate was 0.3 mL/min and the injection volume was 10 μL. The detection wavelength was 280 nm.

The UPLC methods were partially validated using linearity and repeatability according to the Association of Official Agricultural Chemists (AOAC) International Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals.[27] To validate the calibration of the standard curves, more than 4 concentrations for each standard compound were prepared, and the compound at each concentration was analyzed in triplicate. The regression of the calibration was calculated as $y = ax + b$, where x and y were the concentration of the reference samples and the peak area, respectively. The limit of detection (LOD) and limit of quantification (LOQ) of the UPLC methods were calculated according to the equations as: LOD = 3.33 × (standard deviation [SD] of y-intercept/mean of slope) and LOQ = 10 × (SD of y intercept/mean of slope) from the replicate analyses.[27] The relative standard deviation (RSD) was used as a measure of repeatability. The intra-day precision was evaluated by analyzing 4 concentrations of each marker compound 3 times within a day, and the inter-day reproducibility was examined on 3 consecutive days.

2.3. Antioxidant activity assays

2.3.1. ABTS antioxidant assay

The ABTS radical scavenging capacities of the *S. miltiorrhiza* raw and granule extracts were conducted following an established procedure.[28] The ABTS radical working solution was prepared by mixing equal volumes of 7 mmol/L ABTS and 2.45 mmol/L potassium persulfate solution in the dark for 12–16 h at room temperature. Before the analysis, the stock solution was diluted with phosphate-buffered saline (PBS, pH 7.4) until an initial absorbance value of 0.4 at 410 nm was reached. Diluted ABTS (200 μL) was then mixed with 20 μL of the sample or Trolox standard (0.045–0.330 mmol/L), and the absorbance was measured 5 min after the mixing at 410 nm using a microplate reader (BMG CLARIOstar, Victoria, Australia). The ABTS antioxidant activity was expressed as averaged Trolox equivalents per dry weight (DW) of the sample (mg/g of DW).[29]

2.3.2. DPPH antioxidant assay

The DPPH assay was performed as previously described.[30] The equivalent amount of DPPH radical solution (0.24 mg/mL DPPH in methanol) was mixed with each sample in a 96-well plate, incubated for 30 min in the dark and tested for absorbance at 515 nm. Trolox was used for the calibration curve. The standard curve and the results were obtained by the same approach as for ABTS.

2.3.3. FRAP antioxidant assay

The ferric ion reducing antioxidant power (FRAP) assay was performed as previously described.[30] The FRAP working solution was prepared by mixing 1 volume of 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl, 10 volumes of 300 mM acetate buffer (pH 3.6) and 1 volume of 20 mM ferric chloride ($FeCl3 \cdot 6H2O$). The pre-warmed FRAP solution was then mixed with each sample in a 96-well plate at 37 °C for 30 min. The absorbance was measured at 595 nm using a microplate reader (BMG CLARIOstar, Victoria, Australia). The standard curve and the results were obtained by the same approach as described for ABTS.

2.4. Statistical analyses

The values for the granule samples including yield, TLC and UPLC quantification and antioxidant capacities were divided by their concentrated ratio (e.g. the ratio of 1:5 represents that 1 g of granule is equivalent to 5 g of the original raw material, see Supplemental Table 1) so that the comparison to the original herbal material could be made.

The significant difference of the data between the raw herbs and granules was analyzed by the non-parametric tests by GraphPad Prism 8 (GraphPad, San Diego, CA) or SPSS 20.0 (IBM Corp., Armonk, NY). The compound(s) that showed significant differences were assigned as variables for AHC and PCA. AHC analysis was based on Ward's method and Euclidean distances and the results were expressed as dendrograms where the length of the branches between analytes reflected the degree of similarity. PCA was conducted by XLSTAT (Addinsoft, New York, NY) which converted the original variables (7 marker compounds) into a new set of linearly uncorrelated factors (PCs) which corresponded to the largest possible variance of the original variables. The biplot figure (score plot and loading plot) generated by PCA showed the distribution of the samples based on the correlation to the variables and PCs.[31]

The significant difference in the antioxidant capacities and their correlation to the content of the marker compounds were analyzed by non-parametric analysis and Pearson correlation using GraphPad Prism 8, respectively. Pearson correlation coefficients (r) indicated the strength of the correlation, and P-value suggested the statistical significance, which was set to $P < .05$.

3. Results

3.1. Quantification of the marker compounds

The raw and granule samples of *S. miltiorrhiza* underwent TLC and UPLC-PDA identification and quantification of the 7 marker compounds. The chemical structures of the 7 marker compounds are shown in Fig. 1. Representative TLC and UPLC fingerprints of *S. miltiorrhiza* raw herb extract (R6) and granules are shown in Supplemental Figs. 1–3.

The linear regression equations, coefficient of determination (R^2), LOQ and the LOD for the method validation of TLC and UPLC

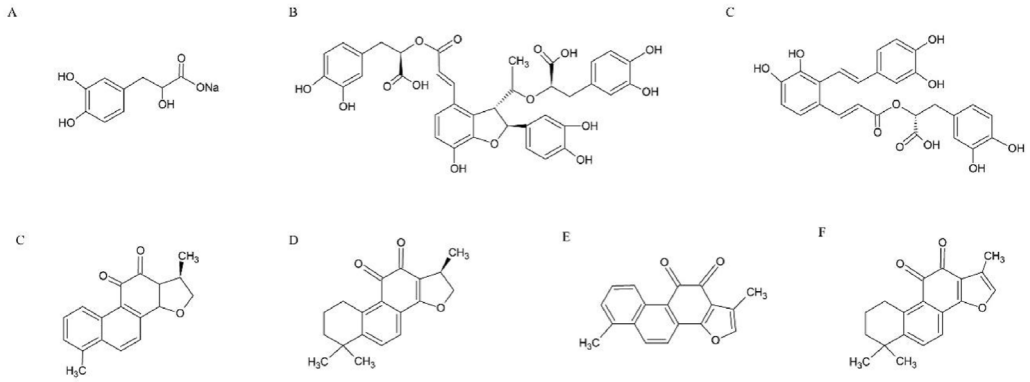

Fig. 1. Chemical structures of seven standard compounds in *S. miltiorrhiza* extracts using ACD/ChemSketch (Canada).
Notes: The hydrophilic compounds include (A) DSS, (B) SB and (C) SA. Lipophilic compounds include (D) DT, (E) CT, (F) TI, (G) TIIA. Their contents in the *S. miltiorrhiza* raw herb and 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.

are shown in Table 1. The R^2 values were greater than 0.988 (TLC) and 0.994 (UPLC) for all analytes, showing good linearity of the experimental data for the analytical methods. The LODs of the 7 marker compounds from TLC and UPLC ranged from 0.001 – 0.215 μg/mL and 0.001–0.019 μg/mL, respectively. The LOQs for TLC and UPLC of the 7 marker compounds ranged from 0.003 – 0.647 μg/mL and 0.002–0.056 μg/mL, respectively. The LOD and LOQ values of UPLC were generally lower than that of TLC showing better sensitivity of detecting and quantitating compounds using UPLC. Furthermore, good instrumental and method precision was obtained for UPLC. As shown in Table 1, the RSD (%) for the intra-day precision of TLC and UPLC ranged from 4.656% to 21.251% and 1.334%–3.732%, respectively. The RSD for the inter-day precision of TLC and UPLC ranged from 7.257% to 27.536% and 3.040%–7.065%, respectively. This suggests the better repeatability for the UPLC method and instrument compared with that of TLC for *S. miltiorrhiza* chemical analysis.

The contents for the marker compounds detected in the samples using TLC are shown in Table 2. The TLC resolution for the marker compounds DSS and tanshinones including CT, DT, TI and TIIA for some samples were not sufficient to quantify separately (Supplemental Fig. 1). The contents of SA and SB in the granules were substantially lower than those in the raw herb samples. SB was the most dominant compound in both raw and granule samples followed by SA. Tanshinones (including CT, DT, TI and TIIA) were generally not detected or very low in the raw herb samples. Non-parametric *t*-test revealed that the amount of all the tested compounds did not vary significantly within the raw or granule samples ($P > .05$). However, SB was significantly higher in the raw sample group compared with that of the granule sample group ($P < .0001$).

The contents of the marker compounds in the raw and granule samples analyzed by UPLC are shown in Table 2. The low standard deviations of the 7 standards in the raw herbs and granules showed a good repeatability for the quantification. Similar to the TLC quantification results, SB was the most dominant compound in the *S. miltiorrhiza* samples followed by SA. Non-parametric *t*-test showed that the amount of all the tested compounds did not vary significantly within the raw or granule samples ($P > .05$). However, when compared between groups, non-parametric independent *t*-test showed that the amount of DSS, SB and CT were significantly higher in the raw samples compared with that of the granule samples ($P = .010$, 0.000, 0.000, respectively).

3.2. Multivariate analysis using AHC and PCA

Since UPLC showed greater sensitivity and ability to quantitate the marker compounds in *S. miltiorrhiza* samples, the quantification results from UPLC were subjected to AHC using all 7 marker compounds as variables. As shown in Fig. 2A, the largest difference was noticed between the cluster of all 12 granules plus 2 raw herbs (R2 and R4) and the cluster of 4 raw samples [R1, R3 (both decoction pieces), R5 and R6], highlighting the significantly higher amount of the 7 marker compounds in 4 *S. miltiorrhiza* raw samples compared with all the granule samples. R2 and R4 were added to the granule samples cluster due to the comparable amount of the 7 compounds to that of the granule samples. The clusters could be further divided into different sub-clusters from the highest to the lowest amount of

Table 1
Validation parameters of TLC and UPLC analysis.

Compounds	Regression equation		R^2		LOD (μg/mL)		LOQ (μg/mL)		Intra-day RSD (%) ($n > 3$)		Inter-day RSD (%) ($n > 3$)	
	TLC	UPLC	TLC	UPLC	TLC	UPLC	TLC	UPLC	TLC	UPLC	TLC	UPLC
DSS	NA	y = 3E+06x-30113a	NA	0.998	NA	0.015	NA	0.044	NA	1.334	NA	3.635
SA	y = 8465.6x+850.66	y = 9E+06x-16212a	0.996	0.999	0.215	0.003	0.647	0.010	21.251	2.850	24.766	6.294
SB	y = 4191.7x+937.77	y = 3E+06x+79925a	0.993	0.994	0.089	0.009	0.267	0.026	11.737	1.738	27.536	3.040
CT	y = 85639x+357.3	y = 1E+07x-63831a	0.991	0.999	0.001	0.005	0.003	0.014	4.656	3.732	7.257	7.065
DT	y = 34356x+362.99	y = 8E+06x-11388a	0.996	0.999	0.162	0.019	0.487	0.056	10.790	2.865	16.553	4.951
TI	y = 12004x+394.48	y = 6E+06x-6694.3a	0.995	0.998	0.061	0.001	0.182	0.002	4.989	3.211	11.468	5.112
TIIA	y = 17100x+2467.3	Y = 1E+07x-27826a	0.988	0.999	0.140	0.004	0.422	0.013	9.105	3.253	26.517	3.271

Notes: NA: not available; LOD: limit of detection; LOQ: limit of quantification; RSD: relative standard deviation; TLC: thin layer liquid chromatography; UPLC: ultra-performance liquid chromatography; DSS: sodium danshensu; SB: salvianolic acid B; SA: salvianolic acid; DT: dihydrotanshinone I; CT: cryptotanshinone; TI: tanshinone I; TIIA: tanshinone IIA.

Table 2
Contents of the 7 compounds in *S. miltiorrhiza* raw and granules by TLC and UPLC.

Sample	DSS (mg/g)		SA (mg/g)		SB (mg/g)		DT (mg/g)		CT (mg/g)		TI (mg/g)		TIIA (mg/g)	
	TLC	UPLC	TLC	UPLC	TLC	UPLC	TLC	UPLC	TLC	UPLC	TLC	UPLC	TLC	UPLC
R1	ND	1.34(0.06)	1.35(0.23)	0.56(0.01)	22.71(0.01)	25.56(0.30)	ND	0.00(0.00)	ND	0.19(0.01)	ND	0.06(0.01)	ND	0.09(0.00)
R2	ND	1.01(0.05)	0.52(0.23)	0.60(0.01)	12.35(0.78)	14.52(0.05)	0.10(0.07)	0.02(0.00)	ND	0.22(0.00)	0.51(0.05)	0.10(0.00)	ND	0.12(0.00)
R3	ND	1.01(0.01)	ND	0.94(0.00)	21.63(0.79)	23.63(0.18)	ND	0.01(0.00)	0.26(0.07)	0.33(0.00)	ND	0.09((0.01)	ND	0.16(0.00)
R4	ND	1.53(0.01)	3.98(1.54)	0.44(0.01)	13.17(0.54)	9.27(0.10)	ND	<LOQ	0.30(0.04)	0.22(0.00)	ND	0.10(0.00)	ND	0.14(0.00)
R5	ND	2.08(0.01)	1.11(0.40)	0.46(0.00)	23.81(0.46)	37.34(0.21)	ND	0.04(0.00)	ND	0.28(0.00)	1.74(1.51)	0.09(0.01)	ND	0.14(0.00)
R6	ND	2.25(0.06)	8.30(0.94)	0.77(0.02)	34.34(0.33)	47.52(1.70)	ND	<LOQ	ND	0.34(0.00)	ND	0.10(0.01)	ND	0.17(0.00)
G1	ND	0.77(0.04)	1.61(0.16)	0.53(0.02)	3.80(0.56)	1.49(0.09)	0.08(0.00)	0.01(0.00)	0.03(0.00)	0.03(0.00)	0.25(0.07)	0.02(0.00)	ND	0.02(0.00)
G2	ND	0.36(0.02)	2.69(0.17)	0.10(0.01)	6.32(0.37)	6.52(0.38)	0.18(0.04)	0.06(0.00)	0.17(0.01)	0.12(0.00)	0.79(0.23)	0.12(0.00)	0.68(0.25)	0.34(0.00)
G3	ND	1.41(0.05)	0.97(0.12)	0.65(0.01)	4.16(0.12)	5.80(0.10)	0.05(0.04)	0.04(0.00)	0.29(0.03)	0.17(0.00)	1.10(0.32)	0.11(0.00)	0.87(0.14)	0.25(0.00)
G4	ND	0.38(0.02)	0.72(0.09)	0.10(0.00)	3.48(0.16)	2.46(0.11)	ND	0.00(0.00)	0.01(0.00)	0.03(0.00)	0.22(0.07)	0.02(0.00)	ND	0.02(0.00)
G5	ND	0.42(0.03)	0.81(0.04)	0.09(0.00)	2.78(0.12)	2.09(0.03)	ND	0.00(0.00)	0.00(0.00)	0.03(0.00)	0.16(0.09)	0.01(0.00)	ND	0.02(0.00)
G6	ND	1.03(0.04)	1.46(0.24)	1.27(0.04)	3.34(0.21)	2.20(0.10)	ND	0.00(0.00)	ND	0.03(0.00)	0.19(0.15)	0.01(0.00)	ND	0.01(0.00)
G7	ND	0.49(0.02)	0.37(0.17)	0.17(0.01)	1.03(0.10)	6.43(0.39)	0.11(0.07)	0.04(0.00)	0.17(0.03)	0.11(0.00)	1.34(0.23)	0.12(0.01)	0.44(0.16)	0.18(0.00)
G8	ND	0.49(0.04)	0.17(0.01)	0.04(0.00)	2.98(0.08)	25.56(0.30)	ND	0.02(0.01)	0.06(0.01)	0.06(0.00)	0.73(0.06)	0.08(0.00)	0.20(0.05)	0.12(0.01)
G9	ND	1.29(0.05)	1.01(0.25)	0.56(0.04)	0.22(0.06)	14.52(0.05)	0.08(0.04)	0.06(0.00)	0.05(0.05)	0.13(0.04)	0.79(0.29)	0.13(0.06)	0.41(0.23)	0.16(0.00)
G10	ND	1.35(0.02)	2.45(0.16)	0.99(0.03)	1.87(0.14)	23.63(0.18)	ND	0.00(0.00)	0.05(0.01)	0.05(0.00)	ND	0.02(0.00)	ND	0.03(0.06)
G11	ND	1.18(0.02)	1.74(0.65)	0.93(0.04)	3.28(0.32)	9.27(0.10)	0.04(0.01)	0.03(0.00)	ND	0.09(0.00)	0.19(0.02)	0.06(0.00)	ND	0.05(0.00)
G12	ND	1.48(0.05)	2.60(0.13)	1.31(0.05)	2.47(0.32)	37.34(0.21)	ND	0.01(0.00)	0.05(0.01)	0.04(0.00)	0.08(0.04)	0.02(0.00)	ND	0.03(0.00)

Notes: Data are expressed as mean (standard deviation). $n = 3$. ND: Not detected. TLC: thin layer liquid chromatography; UPLC: ultra-performance liquid chromatography; DSS:sodium danshensu; SB: salvianolic acid B; SA: salvianolic acid; DT: dihydrotanshinone I; CT: cryptotanshinone; TI: tanshinone I; TIIA: tanshinone IIA.

the 7 compounds: cluster 1 (R5 and R6) with the highest amount of all compounds, followed by cluster 2 (R1 and R3). R2 and R4 were similar to G2, G7, G11, G12, G3 and G9 (cluster 3), followed by G1, G4, G5, G6, G8 and G10 (cluster 4). Interestingly, when SB or TIIA was used as the single variable in the AHC analysis, the dendrogram display was identical to using all the 7 compounds (Supplemental Fig. 4).

PCA was also used to differentiate the samples and displayed each sample in a biplot map based on the contents of the 7 marker compounds. In the PCA biplot, each blue point refers to an individual sample and the red line represents the contribution of each marker compound contributing to the score of 2 major principal components (F1 and F2 as shown in Fig. 2B). From the UPLC quantification results, F1 represented 77.95% of the total variance from the 7 marker compounds and F2 (17.11%) cumulatively explains up to 95.06% of total variance. The distribution of the samples based on F1 was predominantly due to the variance of DSS, DT, SB, CT, TI and TIIA, while the separation of the samples by F2 was mostly based on the contents of DSS and SA. The UPLC biplot differentiated the raw herbs and granules into 3 major clusters primarily due to the variance of F1. Similar to AHC, R5 and R6 showed higher contents of SB, DT, TI, CT and TIIA than the other samples and were grouped in cluster 1, which was followed by R1 and R3 (cluster 2). Noticeably, R6 had the highest contents of these compounds. Due to the relatively lower amount of F1 compounds, R2 and R4 were grouped together with the granules (cluster 3). PCA showed a similar cluster display to the AHC.

3.3. Current pharmacopoeia standards for S. miltiorrhiza

The PPRC 2015 provides the standard protocol for the quality control of raw materials of Chinese medicinal plants. In terms of the quality control of *S. miltiorrhiza*, SB and TIIA are recommended for the quality assessment of the raw herb, and the amount should not be less than 3% and 0.2% of the total amount of starting material, respectively.[16] From the TLC calculations (Table 2), only raw herb sample R6 met this minimum requirement of SB (content at 3.43%), however, none of the raw samples have met the standards of TIIA. The amounts of SB and TIIA in all the granule samples were below the PPRC standard. From the UPLC data (Table 2), the amounts of SB in the raw herb samples (R5 and R6) met the minimum PPRC requirement, with SB at 3.73 and 4.75%, respectively. However, none of the raw samples met the PPRC standard for TIIA. The amounts of SB and TIIA in all the granule samples were below the PPRC standard.

For comparative purposes, AHC was conducted using the contents of the 2 marker compounds (SB and TIIA) as specified by the PPRC.[16] From the UPLC results, AHC produced clusters were identical to those derived when using the contents of all 7 marker compounds studied (Supplemental Fig. 4). Interestingly, the AHC dendrograms using individual marker compound (excluding TIIA) all looked different from each other, but the AHC dendrograms looked identical once SB was added.

3.4. Antioxidant activity

The antioxidant activities of *S. miltiorrhiza* raw and granule samples were analyzed by ABTS, DPPH and FRAP assays. The antioxidant capacities equivalent to Trolox at the absorbance at 590 nm are shown in Fig. 3. All 3 assays showed a similar trend: the antioxidant activities of the raw samples were higher than that of granules. In ABTS, the Trolox equivalent values of raw herb samples ranged from 25.95 to 87.33 mg/g DW, whereas granule samples ranged from 5.38 to 15.67 mg/g DW (Fig. 3A). In DPPH (Fig. 3B), the Trolox equivalent values of raw herb samples ranged from 24.71 to

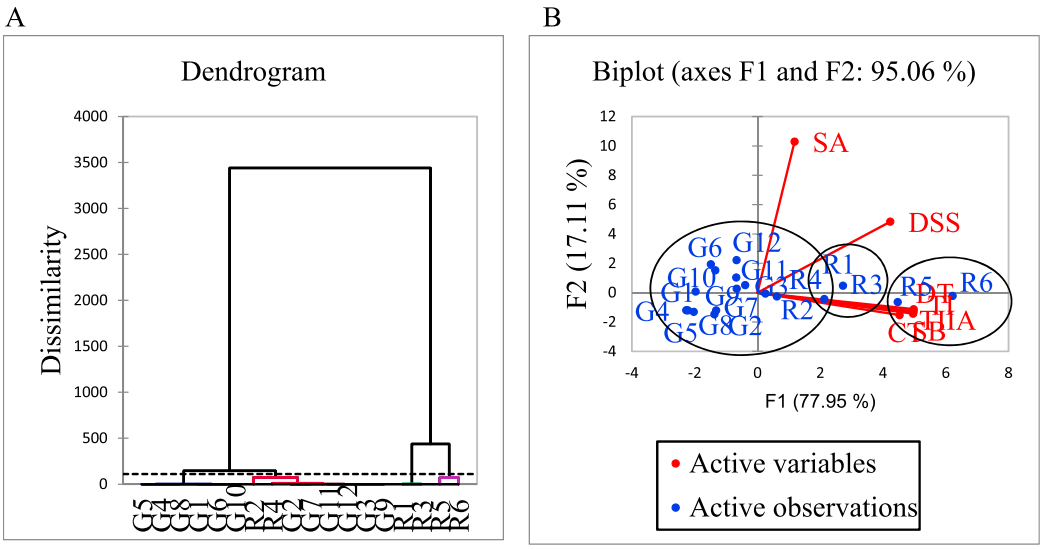

Fig. 2. AHC dendrograms and PCA biplot of *S. miltiorrhiza* extracts analyzed by UPLC.
Notes: A: AHC dendrograms for the UPLC results divided the raw herb and granule extracts into 2 main clusters, with raw herb samples R1, R3, R5 and R6 classified into one cluster, whereas R2 and R4 were grouped with 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 (4.37–9.57 mg/g DW). R6 exhibited the highest antioxidant 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 capacities. There were significant correlations between the UPLC results for the marker contents of DSS, SB and CT to the 3 antioxidant activities (Table 3). R6 possessed the highest amount of these marker compounds and thus showed the highest ABTS and DPPH scavenging 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.

4. 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 chromatography, UPLC, and using new techniques such as FT-NIR spectroscopy.[32–34] 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 chromatography methods used by industry for the quality control of herbal 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 (*Angelica 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 counterparts when compared with the dried weight of the 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 consumption) 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

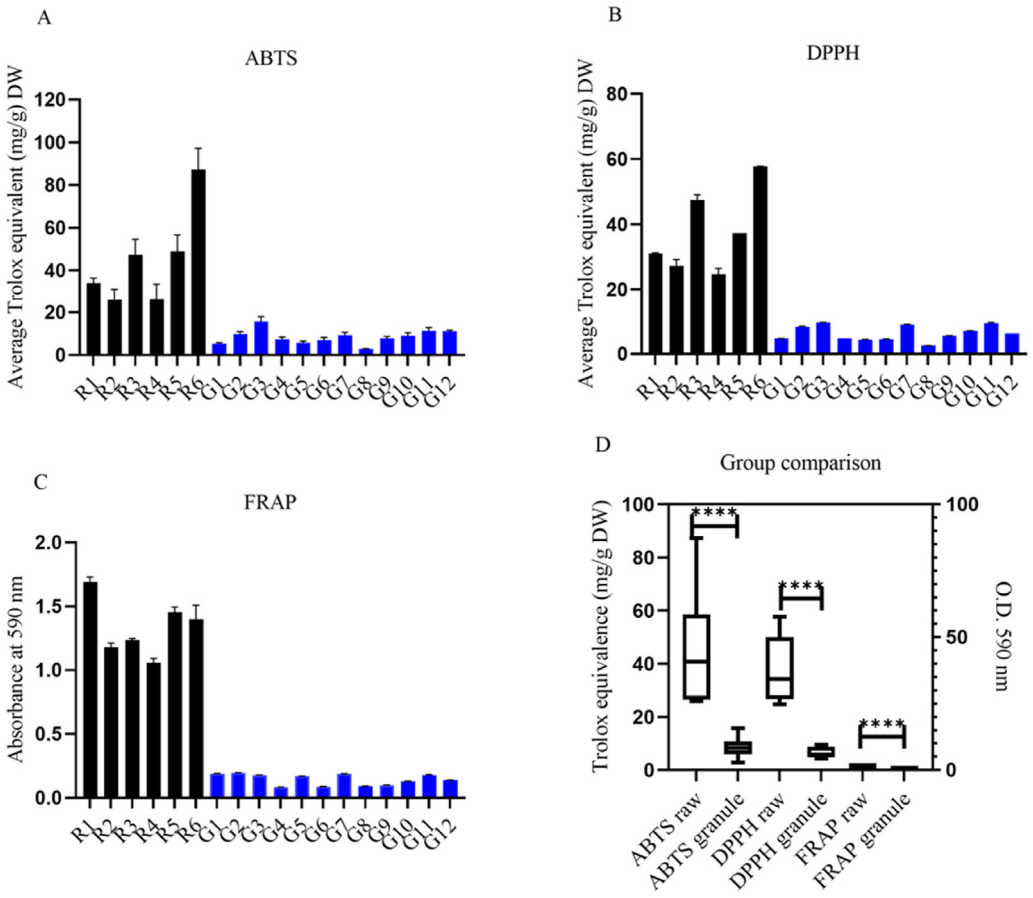

Fig. 3. ABTS, DPPH and FRAP activities of *S. miltiorrhiza* raw herb and granule extracts.
Notes: A: Raw herb samples (25.95–87.33 mg/g DW) showed a higher ABTS radical scavenging activity compared with granule samples (5.38–15.67 mg/g DW). R6 exhibited the highest ABTS antioxidant activity among all the samples (87.33 ± 9.97 mg/g DW). B: Raw herb samples (24.71–57.74 mg/g DW) showed a higher DPPH radical scavenging activity compared with granule samples (4.37–9.57 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 (1.06–1.69) showed a higher FRAP radical scavenging activity than granule samples (0.08–0.20). 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.

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 (which have greater surface area for extraction) may find themselves 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.

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: tanshinone I; TIIA: tanshinone IIA.

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, environmental 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 validated 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 materials. 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 management 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 compounds 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. However, 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 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 polyphenolic 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 investigations 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 activity 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 samples 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 antioxidant 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 benefits 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]

5. Conclusions

The present study assessed the quality and bioactivity differences between *S. miltiorrhiza* raw and granule products using TLC and UPLC coupled with multivariate analysis, and antioxidant assays. UPLC proved to be a better differentiator of the marker compounds 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 recognized 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 dosages to match the contents of traditional decoctions. We believe this study provides a rational argument for the continued investigation 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, investigation, 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.

Declaration of competing interest

There is no conflict of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcms.2022.06.004.

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