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Herbal products' quality control using ^{1}H-NMR metabolomics approach – An example from Danshen (*Salvia miltiorrhiza* Bunge.)

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I. Abstract

Radix *Salviae miltiorrhizae* is the only source of Danshen in pharmacopoeias. For thousands of years, it has been used in China for vascular and pain-related diseases. The current major chemical standards for Danshen products are tanshinone IIA and salvianolic acid B (Zhou, Zuo and Chow, 2005). However, in some regions, Danshen substitutes have been used. Such local substitutes are often erroneously called Danshen (Li *et al.*, 2008, 2013). About ten thousand tonnes of Danshen, *Salvia miltiorrhiza* Bunge, achieved a total revenue of around £54 million in 2015 (*Translated from Chinese: Chinese Danshen market research and investment prospect from 2017 to 2023 forecast report*, 2017).

The global rise of Danshen-related products in different supply chains puts into question the consistency of product quality and the accuracy of information provided by suppliers. This project aims at using a metabolomics approach to understand the standard of Danshen products in the global market and establish a novel strategy to define the quality of herbal medicinal products.

This study project included three disciplines, which were chemistry, pharmacology and value chain analysis. The data obtained from these three disciplines were linked by principal component analysis by the correlation of its chemical compositions and pharmacological effects and its related supply chain.

Taiwan local farmers struggled to sell their organic Danshen (≈ £23/kg) to pharmaceutical companies. Taiwanese pharmaceutical company representatives highlighted that Danshen has always been in high demand, but they would source from China (≈ £1.6 to7.8/kg) because of the lower price. Some farmers would follow the traditional processing after harvesting which allows the roots to ferment for several days

before sun-drying. With this processing, the products had less tanshinones, but it did not show any effects on salvianolic acids in High Performance Thin Layer Chromatography and Nuclear Magnetic Resonance.

With sixty-two samples collected from different countries and sources, there were distinctive chemical differences between the samples from Vietnam and China as well as authenticated samples. In HPTLC and NMR, it showed the contents of salvianolic acids and tanshinones vary. In trace metals analysis, two out of fifteen samples (0.3 and 0.67 mg/kg) from Chinese online stores exceeded acceptable cadmium levels according to Chinese pharmacopoeia.

For the pharmacology variation, the evaluation of the inhibition of H_2O_2 induced apoptosis and the inhibition of LPS induced NO production assays were used. In LPS induced NO production, only seven dried root samples (two authenticated and five from Vietnamese market) out of sixty-two samples exhibited inhibition of NO production at 100 ug/ml without cytotoxicity (10.28%~26.17%). Three samples from Chinese online stores had a protective effect on H_2O_2 induced apoptosis.

II. Impact statement

This is the first interdisciplinary study, coupled with NMR, HPTLC, trace metals analysis, pharmacological assay and market study together, in quality of herbal medicine. The result suggests that the variations of chemical compositions and processing of Danshen does have a significant influence on the biological activity. The inconsistency of TCM products would decrease the safety of the products and damage customer trust. The result also suggests there is a need for the improvement of E-commerce channel monitoring in herbal products.

The study summarises the problem of Danshen products in the market and the pharmacopoeia problem. It raises the potential of using metabolomics to monitor the quality of Danshen. It also suggests using blockchain system to monitor the supply chain in order to resolve overharvesting, inconsistent quality and contamination problem. This approach is not limited to TCM but it can also apply in other herbal medicines.

I. Declaration

‘I, Ka Yui Kum, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.'

Signature:

Date:

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V. Abbreviations

°C = degree Celsius; ^{1}H-NMR = proton nuclear magnetic resonance; As = arsenic; ATCM = Association of Traditional Chinese Medicine and Acupuncture UK; Aβ = amyloid beta peptide; Bcl/xL = B-cell lymphoma-extra large; BP = British Pharmacopoeia; Cd = cadmium; Cdk5 = Cyclin-dependent kinase 5; CE = capillary electrophoresis; CFDA = China Food and Drug Administration; $CHCl_3$ = chloroform; Chloroform $_d$ = deuterated chloroform; ChP = Pharmacopoeia of the People's Republic of China; CNS = central nervous system; COSY = two dimensional correlated proton nuclear magnetic resonance; Cu = copper; D_2O = deuterium oxide; D-MEM = Dulbecco's modified Eagle's medium; DSS = sodium trimethylsilylpropanesulfonate; EtOAc = ethyl Acetate; FA = formid acid; FBS = fetal bovine serum; FDA = Food and Drug Administration; GC = gas chromatography; HCA = Hierarchical cluster analysis; HKCMMS = Hong Kong Chinese Materia Medica Standards; HPLC = high performance liquid chromatography; HPTLC = high performance thin layer chromatography; IL = interleukin; ISO = International Organization for Standardization; JP = Japanese Pharmacopoeia; K_2HPO_4 = potassium dihydrogen phosphate; KH_2PO_4 = potassium dihydrogen orthophosphate; LC = liquid chromatography; LPS = lipopolysaccharide ; MA = market authorisation; MeOD = deuterated methanol; MeOH = methanol; MePh = toluene; MHRA = Medicines and Healthcare products Regulatory Agency; MMP = matrix metalloproteinase; MS = mass spectrometry; MTT = Thiazolyl Blue Tetrazolium Bromide; NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells; NIFDC = National Institute of Food and Drug Centre; NMPA = National Medical Products Administration; NO = nitric oxide; Par = Pareto scaling; Pb = lead; PBS = phosphate buffer saline; PCA = principal component analysis; pCm = proprietary Chinese medicines; Ph. Eur = European Pharmacopoeia; PLS-DA = partial least squares discriminant analysis; Q2 = the estimate of goodness of prediction; R2 = the estimate of goodness of fit; RCHM = Register of Chinese Herbal Medicine; R_f = retardation factor; rpm = round per minute; Sample no. = sample number; SEM = standard error of mean; SFDA = State Food and Drug Administration; TCM = traditional Chinese medicine; THP = Taiwan Herbal Pharmacopeia; THR = traditional herbal registration; TLC = thin layer chromatography; TMS = tetramethylsilane; TNF-α = tumour necrosis factor alpha; U.K. = United Kingdom; U.S.A. = United State of America; USP = United States Pharmacopeia; UV = univariate scaling; VIP = variable importance; WHO = World Health Organisation

VI. Contents

Chapter 1. Danshen and herbal medicine

1.1 The safety issues of using herbal products

Problems in TCM materials

Cultivation and processing stages are considered key to the quality of herbal medicine in the supply chain. However, numbers of cases of contamination and adulteration in herbal medicine were reported. Different countries have set the limitation level of heavy metals, mycotoxins and pesticides. The presence of contamination in medicinal plants depends on the soil, water and air (M.J McLaughlina D.R Parkerb, 1999) and the contamination resulting from industrial wastes, mining, use of synthetic pesticides and fertilisers and inappropriate or unhygienic storage (Abou-Arab *et al.*, 1999; Annan *et al.*, 2010). In the study Harris *et al.*, (2011), out of a the total of 334 TCM samples in 126 species, all of them contained at least one type of heavy metals including arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb) or mercury (Hg) and over one-third (34%) contained all of these. Two hundred ninety-four samples of the total were examined for pesticide level, and more than one-third (36.7%) contained one or more different kinds of pesticides. Greenpeace, a global NGO campaigning for environmental conservation, assessed herbal materials from China including chrysanthemum, wolfberry, honeysuckle, dried lily bulb, san qi, Chinese date, and rosebud, and found that they were mostly contaminated, and these were exported internationally to countries such as Canada, the U.K., France, Germany, Italy, Netherlands, and the U.S.A. (Greenpeace 2017).

Year	Total sample size	Qualified samples	Pass rate
2013	45,297	28,960	63.93%
2014	44,137	30,074	68.14%
2015	61,742	46,233	74.88%
2016	54,276	41,967	77.32%
2017	64,102	53,783	83.90%

Table 1 Chinese national herbal medicinal materials inspection from 2013 – 2017 (Ping *et al.*, 2018; Zhang *et al.*, 2018)

NMPA (formerly called SFDA), the Chinese governmental regulatory body for food, drugs, medical devices and cosmetics, has been undertaking food and drug quality sampling inspections on a regular basis in each province since 2013 (Ping *et al.*, 2018; Zhang *et al.*, 2018). The results showed several common quality problems in decoction pieces including species adulteration, adding of industrial dyes, and mixing of additives or unwanted herbal parts to increase weight or quantity, treating with sulfur fumigation and low quality of cultivars and variants.

Although the satisfaction rate of sampling inspection has been gradually increasing, the summary also illustrated several problems that need to be solved. One of the issues is that cultivars and substitutes of some herbs are circulating in the market as the official materials. Also, in order to boost production, growth agents are commonly used, and farmers harvest the herbs before maturity. Hence, numerous samples have failed in the evaluation of the macroscopic features.

In the reports of Ping *et al.*, (2018) and Zhang *et al.*, (2018), the unsatisfactory rate for most of the TCM materials have decreased, and some of the TCM materials have had a significant improvement in quality. For instance, 39.7% of Danshen (Radix *Salvia miltiorrhiza*) failed in macroscopic characteristics and extract, water and biomarker content in 2015, and compared to a fail rate of under 15% in 2017 which was out of the top list of poor quality TCM materials in the report. Another example is that 50.8% of

Bupleuri radix failed the test because of impurities, biomarker, extract and water content in 2014, but the situation improved in 2017 and the fail rate was down to 17.61%.

In converse, some TCM materials still maintain a high unsatisfactory rate. For instance, *Sessileflower acanthopanax* bark had a 74% unsatisfactory rate in 2013, which dropped to 57.3% in 2017. *Fritillariae cirrhosae* bulbus had 30% to 44% unsatisfaction rate during 2014 to 2017 including failing in macroscopic characteristic, SO_2 level, extract content, biomarker content, and PCR tests.

Regulation of herbal medicinal products

The construction of regulation is not simply based on science, but also on the market, the policymakers and the government. It is the reason why the norms and standards of food safety differ from countries and cultures nowadays (Augustin-Jean, 2016). However, safety should be the main consideration of constructing the regulation in the healthcare industry. Customers have a certain expectation to herbal medicinal products giving them better health. Science is the foundation of the evidence of food safety, and standardising herbal medicinal products on a scientific basis will increase food safety and security.

One of the strategic objectives in the WHO Traditional Medicine Strategy 2014–2023 is "To promote universal health coverage by integrating TCM services into health care service delivery and self-health care". In 2019, the WHO released a traditional and complementary medicine report. It gathered the information of the member states regarding regulation framework, product regulation, practices and practitioners, the challenges faced by countries, and, the country profiles. In the global survey conducted by the WHO, one hundred member states acknowledged the use of traditional herbal medicine, but only eighteen of them have regulations for providers. Ninety-nine out of the 133 respondent member states mentioned the lack of research data as their top

challenge. Furthermore, general technical guidance for research and evaluation related to safety, quality and efficacy, information sharing on regulatory issues and provision of research databases are the main needs ranked by the member states (WHO, 2019). These results indicate the need for scientific research on quality and an information exchange platform for a regulation basis.

TCM practitioners' issue

Regarding TCM practitioner regulation, in China, TCM pharmacists and doctors need to register and be examined through the National Health Professional Technical Qualification Examination according to Issue 114 of Notification of Enhanced Health Professional Technical Qualification Identification from the Ministry of Personnel. This ensures the pharmacists have specific knowledge about the TCM application and communication with other healthcare professionals. In the U.K., even though TCM practitioners may offer individual patients TCM herbal products under Regulation 3 of the Human Medicine Regulation, there are no associated regulations about TCM practitioner registration around European countries. Consequently, the way to be a TCM practitioner is varied. In the U.K., two non-governmental organisations: Association of TCM and acupuncture (ATCM) and the Register of Chinese Herbal Medicine (RCHM) title "herbalist" to TCM practitioners. ATCM requires member to obtain the code of ethics and practice, while RCHM requires members to get a relevant degree or experience on TCM and insurance. However, these memberships are voluntary, and anyone is able to carry the title "herbalist" (Sammons *et al.*, 2016).

1.2 The ethnopharmacology and the diversity of Danshen

The origin of Danshen

Danshen has been used as a traditional Chinese medicine for more than two thousand years. The first recorded use is found in “Shen Nong’s Classic of the Materia Medica” (Shennong Ben Cao Jing (Shen Nong’s Classic of the Materia Medica (Shennong Ben Cao Jing), BC 200 - 250). It classified 365 herbs into three classes: Upper, middle and lower; Danshen was one of the 120 upper-class herbs. The name “Dan-shen” also originated from this Chinese medicine classic (Yan, 2014; Deng et al., 2016). The character “Dan” in Chinese means the colour red as in cinnabar and “shen” means thick roots which promote good health in humans. In the Bencao Tujing (Song, 1061), Danshen was named as “Chi-shen” (red roots) according to the five elements philosophical theory in Taoism. Red, in this theory, represents the heart, and Danshen is matched with this category; the other four “shen”s including Renshen (ginseng) are matched with other colours.

The ethnopharmacology of Danshen

According to TCM classics, Danshen was recommended for different medical uses. Miscellaneous Records of Famous Physicians (Mingyi Bielu, (Tao, 450AD), stated that Danshen nourishes “blood”, eliminates heart and chronic abdominal diseases and removes stagnation of “Qi” (air/energy flow). It could also be used to relieve numbness of feet. WuPu’s Materia Medica (Wu Pu Bencao, (Wu, 589)) said it could be used for chest and abdominal pain. The Compendium of Materia Medica (Bencao gangmu, (Li, 1596)) said it helps to promote blood circulation and also helps to clear the pericardium channel network in the TCM theory.

Currently, according to the TCM theory, Chinese pharmacopoeia contends that the main functions of Danshen are removing blood stasis and promoting blood circulation;

clearing menstruation and relieving pain; eliminating the heat from the heart and relieving restlessness; cooling blood down and dispelling swelling. The medical indications for Danshen are menstrual disorders, abdominal masses, thoracic obstruction, chest pain and abdominal pain, sores swelling pain, restlessness and insomnia (the Pharmacopoeia Commission of the Ministry of Health of the People's Republic of China, 2015).

The diversity of Danshen

The official source of Danshen in pharmacopeia is *Salvia miltiorrhiza* Bunge, which is a perennial plant in the family *Lamiaceae*. However,

more than 40 various sibling species and cultivars can be found on the market. This is due to the fact that quite a number of sibling species are morphologically similar, hence the description of the materials in the historical text is not sufficient enough to identify the species. Some regions use other species similar to the description in the historical text as the source of Danshen according to their local traditions and local availability. Sibling species include *Salvia bowleyana* Dunn, *Salvia przewalskii* Maxim, *Salvia yunnanensis* C. H. Wright, *Salvia sinica* Migo, *Salvia digitaloides* Diels, *Salvia trijuga* Diels, *Salvia honania* L.H. Bailey, and more (Li *et al.*, 2008; Yan, 2014; Deng *et al.*, 2016). Since the genetic engineering technique has been improving, more and more major variants or cultivars have been cultivated, but there are two cultivars documented in the flora of China including *Salvia miltiorriza* var. *miltiorrhiza* and var. *charbommellii* under the taxa of *Salvia miltiorrhiza* (Li and Ian, 2015). Currently, apart from *Salvia miltiorrhiza*, there are two other species commonly found on the market including *Salvia bowleyana* and *przewalskii*. For *Salvia bowleyana*, the cultivation can be found in the South East of China in places including Zhejiang, Hunan, Jiangxi, Fujian, Guangdong and Guangxi. For *Salvia przewalskii*, the cultivation can be found in

the West of China including Yunnan, Sichuan, Gansu and Tibet. The altitude for *Salvia przewalskii* cultivation is around 2,100 to 4,040 metres which is much higher than *Salvia miltiorrhiza* (120 to 1,300 m) or *Salvia bowleyana* (30 to 960 m). *Salvia miltiorrhiza* is cultivated all over China, with the exception of the Southernmost province of China (Hainan), three northern provinces (Inner Mongolia, Jilin and Heilongjiang) and two provinces in the far west (Tibet and Xinjiang). Most of the Danshen acquired is from cultivation instead of wild harvesting. *Salvia miltiorrhiza* can also be found in Japan as well. The provinces considered ideal for cultivating Danshen are Sichuan, Henan, Hubei, and Anhui (Yan, 2014). These areas all belong to humid subtropical inlands which have a warm climate with high humidity. Currently, most of the materials on the market are cultivated while before the 1950s, there were only a few Danshen cultivation sites in Sichuan (Deng *et al.*, 2016).

The description of Danshen in TCM classics

Although nowadays *Salvia miltiorrhiza* Bunge has been considered the only official source of Danshen, the TCM classics do not specify *Salvia miltiorrhiza* as the only source. The descriptions of Danshen materials vary among the classics.

Figure 1 The sketch of Danshen in Essentials of Materia Medica Distinctions (Bencao Pinhui Jingyao) – *A.D. 1505 sourced*

Shen Nong's Classic of the Materia Medica – the first record of Danshen – made no mention of its morphology, life cycle or habitats. The first record of the habitat of Danshen is in Miscellaneous Records of Famous Physicians (Tao, AD 220 - 450). It highlights that Danshen grew in the valleys of Tongbai and Taishan Mountain. These locations were verified to be in Nanyang, Henan by Collective Commentaries on the Classic of Materia Medica *(Bencao Jing Ji Zhu* (Tao, AD 220 - 450), which also mentioned that

Danshen grew in Taian, Shandong. However, it also suggested that Danshen was not only limited to Nanyang but easily found elsewhere.

Bencao Tujing described Danshen as follows: "it germinates in February [Lunar calendar], grows to more than one-inch tall [as in more than 23 cm nowadays at Song dynasty], with a square stem, with a grass-green hue. Leaves grow in pairs, look like mint (*Lamiaceae*), and it has villous surface. It flowers in March, with a purple-red colour like perilla (*Lamiaceae*). The roots are as thick as fingers and more than one-inch long; each plant has several roots. They are harvested in May and sundried.". This was verified in *Essentials of Materia Medica Distinctions* (*Bencao Pinhui Jingyao,* (Liu, 1505)) with a similar description, which also provided the sketch of the species as Figure 1. However, it did not look similar to *Salvia miltiorrhiza* but preferably other *Salvia* species like *Salvia przewalskii* or *Salvia honania* L. which have simple leaves. It raises doubt over whether *Salvia miltiorrhiza* is the species described in the historical text.

Figure 2 The sketch of Danshen in Compilation of plants with illustrations (Zhiwu Minashi Tukao) –

The Compendium of Materia Medica (Li, 1596) specified the leaves of Danshen as "one branch five leaves", which indicates it might be an odd-pinnate; it also said the flowers grew like wheat, which suggests that it was an inflorescence. In the past, herbalists might not have noticed the difference between spike and verticillate since they are similar. Similar descriptions are to be found in the herbal medicine classics published afterwards, such as *Bencao Huiyan* and *Bencao Xushu* (Deng *et al.*, 2016). *Compilation of plants with illustrations (Zhiwu Mingshi Tukao,* (Wu, 1848)) shows a draw

ing of the whole plant of Danshen which shares the same pattern of the leaves and flowers as in Figure 2. Although the descriptions were not very conclusive, at least from the historical text, we know Danshen should be of the *Salvia* genus and that it might vary from region to region.

Classics	**Published year**	**Location**	**Morphological description**
Shen Nong's Classic of the Materia Medica *(Shennong Ben Cao Jing)*	250 to 200 B.C.	Henan	/
Collective Commentaries on the Classic of Materia Medica *(Bencao Jing Ji Zhu)*	200 to 450 A.D	Shandong	/
Bencao Tujing	1061 A.D.	Shanxi, Shandong, Henan	germinates in February a-squared stem with a grass-green hue. Villous leaves grow in pairs, look like mint flowers in March, with a purple-red colour like perilla
Bencao Pinhui Jingyao	1505 A.D.		provided the sketch of the species
The Compendium of Materia Medica	1552 to 1578 A.D.		one branch five leaves
Compilation of plants with illustrations *(Zhiwu Mingshi Tukao)*	1848 A.D.	Everywhere	drawing of the whole plant

Table 2 The geographical location and morphological description of Danshen in a different era of TCM classics

Year	Nourishing the blood, Relieve pain, Invigorating and promoting qi	Eliminating numbness Strengthening bone	Relieving mental stress	Regulating menstruation Stopping metrorrhagia	Resolving toxin Evacuating pus
250 to 200 B.C.	√		√		
200 to 450 A.D	√	√			
1505 A.D.	√	√	√	√	√
1552 to 1578 A.D.	√	√	√	√	√
1848 A.D.	√	√	√	√	√

Table 3 The ethnopharmacological description in a different era of TCM theory based on the corresponding TCM classics

The morphology of Danshen species

According to the flora of China, Table 4 shows the morphological difference between *Salvia miltiorrhiza*, *Salvia bowleyana* and *Salvia przewalskii*. *Salvia przewalskii* is the easiest to distinguish from the others. The bases of the leaves in *Salvia przewalskii* are cordate to hastate while in *Salvia miltiorrhiza* and *Salvia bowleyana* they are ovate to circular in shape, and the apexes of the leaves are acuminate in form. Another distinction is that the verticillasters are 2 to 4 flowered, whereas the verticillasters of *Salvia miltiorrhiza* and other substitute have more flowers.

Salvia miltiorrhiza and *bowleyana* are similar, but it is still possible to distinguish them, especially in flowering season. The upper lip of the corolla in *Salvia miltiorrhiza*

is more extended than lower lip while in *Salvia bowleyana* it is the other way round. Another difference is pilose of the leaves in *Salvia miltiorrhiza* is denser than in *Salvia bowleyana*, but this can be affected by the environment and depends on the individual plant.

	Salvia miltiorrhiza	*Salvia bowleyana*	*Salvia przewalskii*
Plant	Perennial	Perennial	Perennial
Stem	Erect, 40 to 80 cm tall	Erect, around 100 cm	Erect, around 60 cm
Leaves	Simple to odd-pinnate	1- or 2-pinnate	Simple
	Blades or leaflets circular to broadly lanceolate, margin crenate, apex acute to acuminate	Terminal leaflet ovate-lanceolate, margin crenate-serrate or serrate, apex acuminate to caudate-acuminate	Blade triangular-hastate to oblong-lanceolate, rarely cordate-ovate, base cordate to hastate, margin crenate-dentate, apex acute
	Pilose, densely so abaxially	Glabrous, finely pilose on veins	Adaxially minutely hirsute
Verticillasters	6- to many flowered	8- to many flowered	2-4-flowered
Calyx	Campanulate, 1.1 cm	Tubular, 8-10 mm	Campanulate, 1.1 cm
	Lower lip slightly shorter than upper lip	Lower lip slightly shorter than upper lip	Lower lip longer than upper lip
Corolla	Upper lip 1.2-1.5 cm, falcate; lower lip shorter, middle lobe ca. 5 × to 10 mm, 2-lobulate	Upper lip slightly falcate, 0.8-1.2 cm × ca. 5 mm; lower lip oblong, ca. 1.1 × 1.2 cm; middle lobe obcordate, ca. 3 × 6 mm	Upper lip oblong, ca. 5 mm, margin entire, slightly concave, ciliate; lower lip ca. 7 × 11 mm; middle lobe obovate, apex subtruncate

Table 4 The morphological differences between Danshen related species including Salvia miltiorrhiza, bowleyana and przewalskii

1.3 Current resources of Danshen (geographical distribution, cultivation, demand and supply)

TCM has drawn international attention, and its increasing popularity is opening an innovative market and creating new business opportunities. In 2002, the market of Danshen was over US$120 million (Cheng, 2007). The net revenue of TCM industry in 2010 was around ¥30 billion (around £4.6 billion), and the annual merchandise exports on TCM products was around US$2 billion (around £1.76 billion)(*Translated: China Industry Research Report (Zhōngguó chǎnyè yán jiù bàogào wǎng)*, 2011). In 2014, the annual merchandise export increased to around US$3.5 billion and US$1.8 billion of it

(more than 50% of export) was contributed from TCM extracts (Wang, Van der Borght and Song, 2016). TCM entered into the European market in the 1970s, and according to (MHRA, 2009), 5% of patients had used TCM before, and 48% of those responders used within two years.

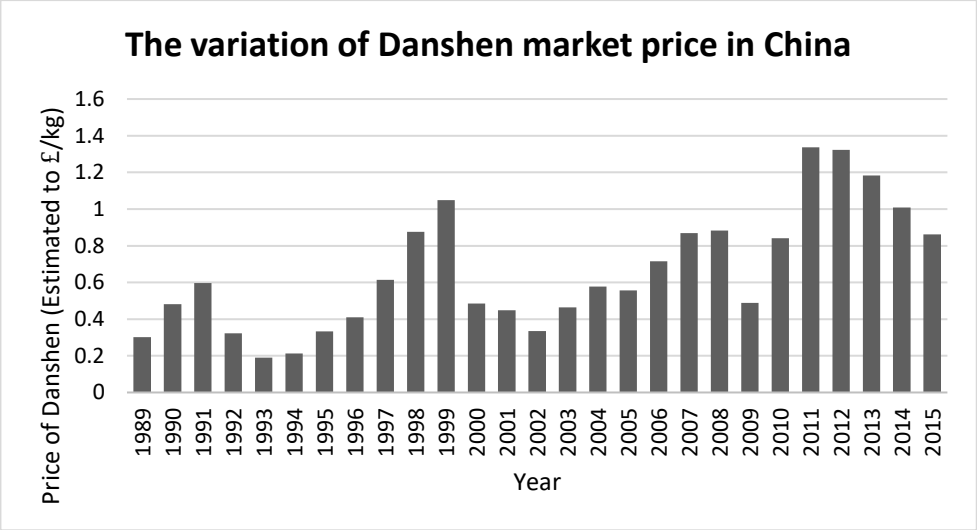


Figure 3 Danshen price variation from 1989 to 2015 in China

Shandong which occupied more than 37% of the market supply in China was the most prominent supply area (*Translated from Chinese: Chinese Danshen market research and investment prospect from 2017 to 2023 forecast report*, 2017) and it sold approximately ¥189 million (around £20 million) with 3,700 tons Danshen in 2015 according to China Association of Traditional Chinese Medicine. The price of Danshen in the early 90s was around £0.4 to 0.5 per kg, and recently, it increases to approximately £0.8 to 1.2 per kg. The price variation is shown in Figure 3. However, it is inexpensive compared to most of the TCM materials. With the economic growth and increase in cost, the price of Danshen is nearly comparable to its cost. In Taiwan, the annual import amount of Danshen dried roots is from 150 tons to 300 tons, and it costs pharmaceutical companies in Taiwan around £4.3 per kg (*Translated: Danshen Theme Pavilion*, 2014) (Council of Agriculture 2014). In the U.K., the price of herbal materials is even ten times more than the original dealer price in China which is around

£10 to 20 per kg (Booker, Johnston and Heinrich, 2012). The retail price in TCM pharmacy in central London would even increase to £100 per kg.

1.4 Quality problems of Danshen products

CFDA released the report of TCM materials quality summary and Danshen was listed as one of the drugs which was highly likely to fail from the quality inspection in 2015 (Ping *et al.*, 2018). Within the fail rate of 39.7% (223 out of 561), the most common problems included the extract content, macroscopic features and the water content. The problem usually found in cultivated Danshen was that tanshinone IIA content was lower than in the wild plant. The report also stated that some Danshen was dyed by artificial dye auramine O in order to make it look better as the "good quality" Danshen are those with a brownish-red skin color according to the traditional identification.

In a recent pesticide residue evaluation in *Salvia miltirrhiza* study, twenty out forty samples contained banned or restricted pesticides, and eight of them did not meet the requirement of ChP (Yan *et al.*, 2018). One study showed five out of thirteen Danshen samples exceeded the heavy metal limit of ChP. Four samples exceeded Cu limits; one sample exceeded As limit; one sample excceded Cd limit; and one sample exceeded Pb limit (Yan *et al.*, 2012).

1.5 Pharmacopoeia difference among countries and consequences

Pharmacopoeias differences

Regarding the regulation among different countries, *Salvia miltiorrhiza* is included in the pharmacopoeias of China, Japan, Korea, Taiwan, Europe, U.K. and the U.S (The Japanese pharmacopoeia 17th Edition, 2017; Chinese Pharmacopoeia 2015, 2015; Pharmacopoeia Vietnamica 4th Edition 2010; The Korean pharmacopoeia 11th Edition, 2014; British Pharmacopoeia 2015, 2015; Taiwan Herbal Pharmacopeia 2nd Edition English version, 2016; United States Pharmacopeia 39th Edition National Formulary

34, 2016; European pharmacopoeia, 2017). However, the quality standards of Danshen are not consistent, for example, in the ChP, it requires an HPLC assay to detect more than 2% tanshinone IIA and 3% salvianolic acid B while Korean and Vietnamese pharmacopoeia only require TLC detection for qualitative without quantitative analysis. Besides, the TLC developing solvent systems among all these countries are different. In the U.K., only one solvent system (Ethyl Acetate: Methanol: Water (v/v) = 10: 1.35: 1) is used in *Salvia miltiorrhiza* roots while the U.S. and China both are using two different solvent systems to analyse lipophilic and hydrophilic compounds separately. Also, the limitation of heavy metals, pesticides and mycotoxins vary from country to country. Chinese pharmacopoeia accepts not more than 10 ppm of lead in herbal materials while the United States pharmacopoeia only accepts less than 0.5 ppm in drug substance and excipients and less than 1 ppm in the dietary supplement (S.-H. Liu *et al.*, 2015).

	Water-soluble extractives	Alcohol-soluble extractives	Tanshinones	Tanshinone IIA	Salvianolic acid B	Rosmarinic acid	Loss of drying	Ash insoluble	Ash insoluble in HCl
ChP 2015	50%	46%	0.25%		3%		13%	10%	3%
THP 2th	35%	15%		0.20%			15%	10%	
USP 40th	35%	15%	0.20%	0.10%	3%		13%	10%	3%
JP 17th		42%							
Ph. Eur 9th				0.12%	3%		10%	10%	3%
BP 2015				0.12%	3%		10%	10%	3%
HKCMMS	57%	52%		0.12%	4%	0.17%	12%	8%	2%

Table 5 The comparison of Radix et Rhizoma Salvia miltiorrhiza criteria in different regulatory bodies

	Heavy metal (mg/kg)				
	Pb	Cd	As	Hg	Cu
ChP 2015	5	0.3	2	0.2	20
THP 2th	5	0.3	2	0.2	20
USP 40th	5	0.3	2	0.2	
JP 17th	10		5		
Ph. Eur 9th	5	0.2		0.1	
BP 2015	5	1	5	0.1	
HKCMMS	5	1	2	0.2	
WHO	10	0.3			
ISO	10	4	2	3	

Table 6 The comparison of the heavy metal limits of Radix et Rhizoma Salvia miltiorrhiza in different regulatory bodies or standard authorities

Danshen derived products registration

Apart from the differences of pharmacopoeia among nations and regions, Danshen products are in the grey area between herbal medicines and food supplements. In Hong Kong, more than 490 products containing *Salvia miltiorrhiza* are registered as herbal medicines, but any product that does not have claims for any medical uses or suggested dose, it can be considered as food (*Chinese Medicine Council of Hong Kong > Chinese Medicine Practitioners > Welcome Message*, no date). The condition in China is even more prevalent. Over 800 Danshen derived medicines have been registered. More than 40 of cosmetic products or food supplements which the name contains the word “Danshen” have been found while these products do not need to fit the criteria set in pharmacopoeia monograph of Danshen (*Translated: China Food and Drug Administration Data Search*, no date). This does not include all the products containing Danshen, but those that have a product name that contains the word

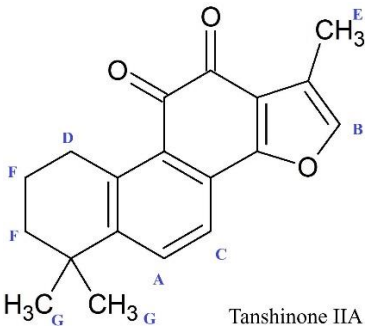
"Danshen". Besides, Danshen derived products are not necessary to register as a drug, but functional food as long as the product does not have specific health claims but CFDA reserves the right to the decision. Different types of Danshen derived products can be found in China such as extracts, tinctures, pills, tablets, blended powder and dried roots. The U.K. has strongly restricted large scale TCM products unless it has Traditional Herbal Registration (THR) license. The registration requires a company to comply UK specific quality and safety requirements, and the product must have been on the market of an EU member state for at least thirty years. Currently, within nearly 400 THR registration, none of them are related to *Salvia miltiorrhiza* (Sammons *et al.*, 2016).

1.6 Chemistry and pharmacology of Danshen (chemical types, skeletal structure and NMR spectrum relationship)

Pharmacological evidence

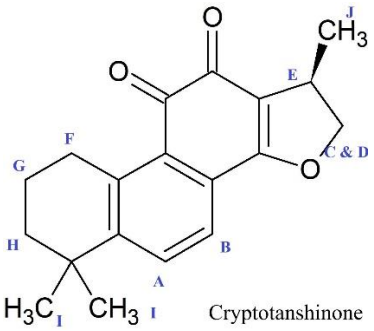
Current studies focus on tanshinones which are lipophilic diterpene quinones and salvianolic acids which are hydrophilic caffeic acid derivatives. More than 40 tanshinones and 50 caffeic acid derivatives in Danshen have been discovered and identified (Jiang *et al.*, 2005; Zhang *et al.*, 2012; Chen *et al.*, 2014).

a)



Tanshinone IIA

b)



Cryptotanshinone

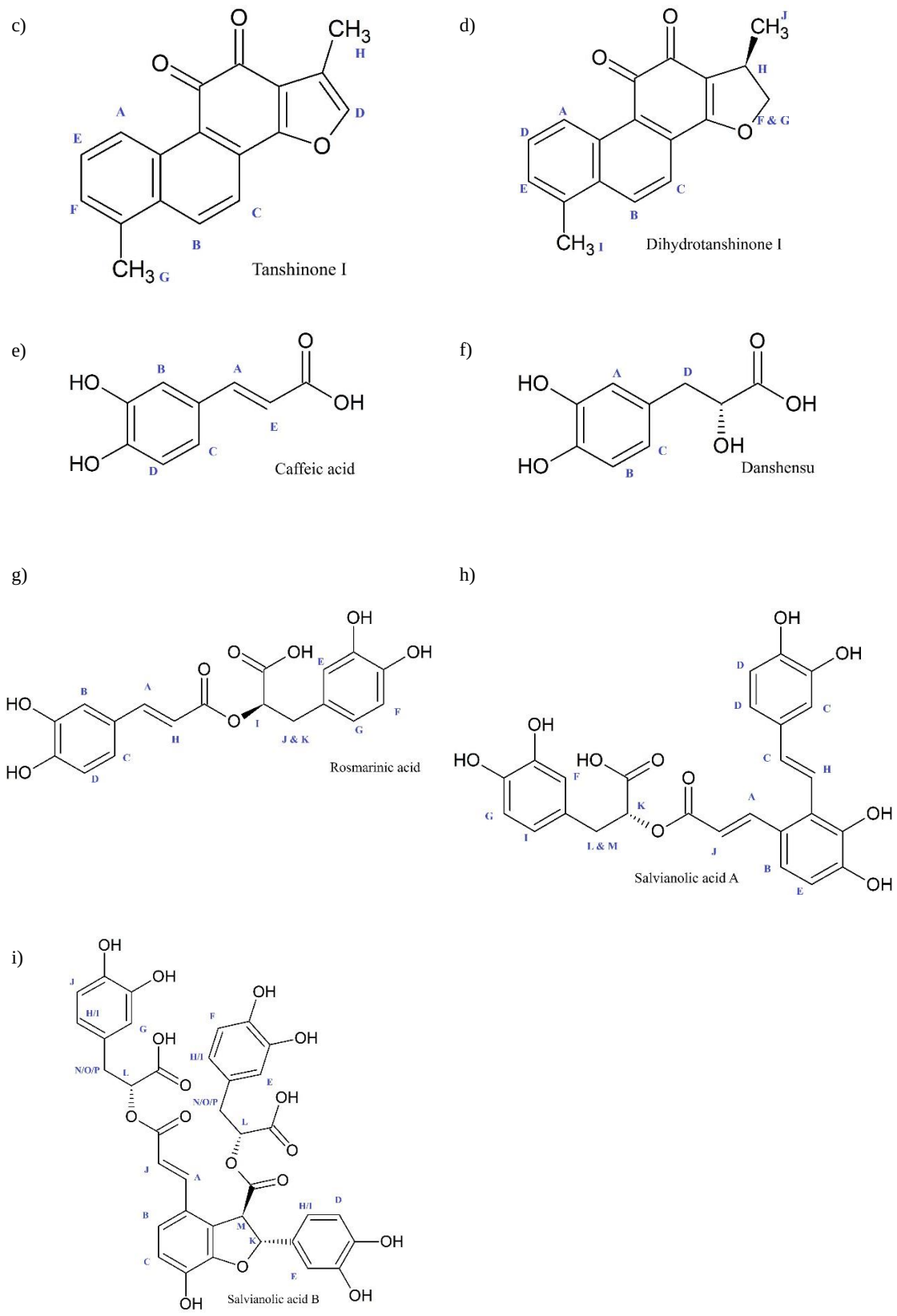


Figure 4 chemical structures of tanshinones and salvianolic acids

a) tanshinone IIA, b) cryptotanshinone, c) tanshinone I, d) dihydrotanshinone I, e) caffeic acid, f) danshensu, g) rosmarinic acid, h) salvianolic acid A, and i) salvianolic acid B

Tanshinones

The first tanshinone was isolated in 1930 by Nakao in *Salvia miltiorrhiza* (Dong, Morris-Natschke and Lee, 2011). It commonly consists of four rings, and the tanshinones with either 1,2 o-quinone furan rings or dihydrofuran rings usually are major constituents in *Salvia miltiorrhiza* (Yan, 2014). Tanshinone IIA, tanshinone I, cryptotanshinone and 15,16-dihydrotanshinone were commonly used as the biomarkers for *Salvia miltiorrhiza*, but they are not limited in *Salvia miltiorrhiza* only. Tanshinone IIA has been considered one of the representatives of tanshinones in *Salvia miltiorrhiza*.

Regarding pharmacological effects about the circulatory system, tanshinones have illustrated some evidence on treating atherosclerosis and cardiovascular diseases. It showed the inhibition of the low-density lipid oxidisation in RAW 264.7 macrophage (Chen *et al.*, 2007). Besides, it inhibited the oxidised low-density lipid uptake in macrophage through NF-κβ signalling (Jang *et al.*, 2006). The anti-inflammation activity may involve the inhibition of the expression of pro-inflammatory cytokines in NF-κβ pathway including IL-1, IL-6 and TNF-α (Jang *et al.*, 2003) as well as MAPKs and Nrf2 pathway including the regulation of IKK, ERK and JNK expression (Zhang and Wang, 2007; Li *et al.*, 2010; Bi *et al.*, 2012). It also inhibited the matrix metalloproteinase expression in human vascular endothelial cells (Bi *et al.*, 2012).

Salvianolic acids

Salvianolic acids are hydrophilic compounds in *Salvia* species. It is composed of caffeic acids and according to the number of caffeic acid units categorises as monomer, dimer, trimer and tetramer. The major salvianolic acids are danshensu (monomer), salvianolic acid A (trimer) and salvianolic acid B (tetramer) (Jiang *et al.*, 2005).

Salvianolic acids commonly act as a protective effect on the circulatory system. Salvianolic acid A and B inhibit the oxidisation of low-density lipid and have similar results in several studies (Zhao *et al.*, 2008; Ho and Hong, 2011; Yang *et al.*, 2012). Salvianolic acids also provide protective effects on vascular endothelial cells through multiple pathways including regulating VCAM-1, ICAM-1, TNF-α, etc. (Du, Qiu and Zhang, 1995; Chen *et al.*, 2001; Lay *et al.*, 2003; Pan *et al.*, 2011).

Chapter 2. Metabolomics

2.1 The rise of metabolomics and its terminology

Metabolomics

Terms	Definition
Metabolites (Dettmer et al. 2007)	Small molecules that participate in general metabolic reactions and that are required for the maintenance, growth and normal function of a cell.
Metabolome (Dettmer et al. 2007)	The whole set of metabolites in an organism.
Metabolomics (Dunn et al. 2005)	Non-biased identification and quantification of all metabolites in a biological system.
Metabonomics (Nicholson and Lindon 2008)	The measurement of the global, dynamic metabolic response of living systems to biological stimuli or genetic manipulation.
Metabolite target analysis (Dunn et al. 2005)	Quantitative determination of one or a few metabolites related to specific metabolic pathway after extensive sample preparation and separation from the sample matrix and employing chromatographic separation and sensitive detection.
Metabolic profiling (Dettmer et al. 2007)	Quantitative analysis of set of metabolites in a selected biochemical pathway or a specific class of compounds.
Metabolic fingerprinting (Dettmer et al. 2007)	Unbiased, global screening approach to classify samples based on metabolite patterns or ''fingerprints'' that change in response to disease, environmental or genetic perturbations with the ultimate goal to identify discriminating metabolites.

Table 7 The definitions of metabolomics related terms

The term "metabolome" was introduced in 1998 by (Oliver *et al.*, 1998; Schripsema, 2010). The study was about the relationship between metabolomes and genomes in

yeast. At that time, “omics”, which is the research strategy in a holistic approach, was used in the studies of proteins (proteomics) and genes (genomics). This raised the interest of scientists in combining the “omics” strategies to metabolomes, and that is the origin of the term “metabolomics”. The definition of metabolomics and its related terms were summarised from several reviews. The summary of the definitions of metabolomics and its related terms (Dunn and Ellis, 2005; Dettmer, Aronov and Hammock, 2007; Nicholson and Lindon, 2008).

In metabolomics research, diverse strategies can be found, and the choice of metabolomics platforms depends on the objective of the study and the availability of the platform. The potentials of metabolomics include pharmacological elucidation of mechanism, chemical profiling and sample classification. It has been widely used in various areas which involve high complexity of metabolites information including but not limiting food (Le Gall, Colquhoun and Defernez, 2004; Moco *et al.*, 2006), herbal medicine (Kim *et al.*, 2005; Lee *et al.*, 2009), pharmacology (Hounoum *et al.*, 2015), pathology (Viant, Rosenblum and Tjeerdema, 2003; Abaffy *et al.*, 2010; Waterman, Kian-Kai and Griffin, 2010), bioengineering (Park *et al.*, 2005; Kai *et al.*, 2014; Cai *et al.*, 2016; Diamond and Desgagne-Penix, 2016) and diagnosis (Pasikanti *et al.*, 2010; Sinclair *et al.*, 2010; Zhang *et al.*, 2011).

	Advantages	Disadvantages
Direct infusion MS	· High throughput · Rapid · Unbiased · Minimal sample preparation · Metabolite quantitative analysis available · Metabolite identification available	· Highly complex results · Destructive · Unequal weigh of metabolites · Isomers undefinable · Easily affected by salt concentration and matrix effect
1H-NMR	· High throughput · Rapid · Unbiased · Minimal sample preparation · Metabolite quantitative analysis available · Metabolite identification available · Reasonable resolution and reproducibility	· Complex results · Deuterated solvent needed · Rely on database or strong metabolite elucidation with solvent specificity
FT-IR	· High throughput · Rapid · Non-biased · Minimal sample preparation · Relatively good resolution and reproducibility	· Metabolite quantitative or qualitative analysis unavailable
Chromatography based MS/UV	· Very good sensitivity · Metabolite quantitative analysis available · Metabolite identification available · Relatively good resolution and reproducibility	· Relatively time consuming · Destructive · Unequal weigh of metabolites · Potential loss of metabolites from preparations · Rely on database or metabolite elucidation without solvent specificity

Table 8 Summary of advantages and disadvantages of metabolomics analytical technologies commonly used

Certainly, in the past, researchers focused on single compound activity in herbal medicine as a single variable which made easier to understand but limiting variables in the experiment restrict the level of understanding, hence a false conclusion might be drawn. With the characteristics of herbal medicine which are usually coming up with combinations of herbs and treating patients in a holistic approach, the idea of metabolomics is to comprehend the totality of metabolome changes. It helps to translate the traditional knowledge of herbal medicine into a modern science language, and eventually, it helps to discover new treatments. The practice of metabolomics commonly consists of qualifying and quantifying metabolome with statistics power. Ideally, it classifies samples by the comprehensive and unbiased information about the dynamics of metabolites analysed in biofluid or organs using a suitable statistical model.

Using metabolomics approach to study herbal medicine overcomes diverse of experimental methodological limitation in herbal medicine research of the past. ^{1}H-NMR spectrometry, direct infusion MS, chromatography based coupled with MS or UV, IR spectrometry and CE have been reported as analytical platform for metabolomics and ^{1}H-NMR and chromatography-based MS or UV have been the most common analytical platforms in plant metabolomics (Kim *et al.*, 2004; Hall, 2006; Kim, Choi and Verpoorte, 2010a; Schripsema, 2010). The summary of the advantages and limitations of different analytical technologies are included in this report. Comparatively, ^{1}H-NMR is a high throughput and rapid analytical technology for metabolomics which enable qualitative and quantitative analysis, whereas metabolomics needs to comprehend the dynamics of living organisms. Currently, even though given the limitation that none of the analytical platforms is available to fulfil all these desirable properties perfectly, MS or NMR metabolomics are still commonly used to cluster geographical origins and identify pathological difference, in practice, with great success.

Metabolomics linked with pharmacological evidence

As stated earlier, various studies have supported the pharmacological benefits of Danshen in vascular-related diseases. The research strategy has focused on anti-inflammation and protective effects provided by Danshen extracts or some Danshen chemical constituents (Zhou *et al.*, 2005; Lee *et al.*, 2012; Tung *et al.*, 2013; Chang *et al.*, 2014; Orgah *et al.*, 2015; Z. Liu *et al.*, 2015; Liu *et al.*, 2016; Ma *et al.*, 2016; Zhang *et al.*, 2018). However, as the chemistry of the plant is influenced by several factors including geographical location, cultivation method, harvesting time and processing technique, the pharmacological activity is likely to be different from sample to sample. However, while herbal medicines always emphasise holistic approach, there are currently no pharmacological studies that have full chemical profiles with the

variance of sampling in Danshen, which helps to comprehend the possible synergistic effects or mechanisms. In the study of J. L. Zhang *et al.*, (2005) and J. L, Zhang *et al.*, (2005b), ten batches of Danshen were collected and analysed using HPLC-UV, but it showed the 15 chemical constituents including Danshensu and salvianolic acid B greatly varied from batch to batch. The range of Danshensu was from 7.69% to 10.20% and of salvianolic acid B was from 6.71% to 19.78%. In the study of (J. Zhang *et al.*, 2005), the water-soluble part of Danshen was investigated and Zhang proposed its metabolic pathway, but it did not investigate pharmacological effects. Similar to the study of (Sun *et al.*, 2007), the rat bile was examined after the oral administration of lipid-soluble part but studied its metabolic pathway instead of pharmacological effects. Zhao *et al.*, (2015) investigated the metabolic pathway of Danshen methanolic extract instead of the partial composition of Danshen extract. However, no pharmacological effects were studied along with chemical profiles.

Inhibition of LPS induced NO production and H_2O_2 induced apoptosis were used in the evaluation of the pharmacological activity of Danshen. The increase of NO production is an indicator of inflammation activity caused by chemicals, physical or biological damage or bacteria. In vascular diseases, for example, in atherosclerosis, patients usually come up with inflammation in their blood vessels. Possible causes of atherosclerosis are not only physical damages or accumulation of LDL, but it also includes bacterial infection and chemical exposures. These cause a significant change of cytokines secretion, which initiates inflammation (Ambriz-Perez *et al.*, 2016; Chen *et al.*, 2016; Hu *et al.*, 2016).

2.2 The comparison of different metabolomics techniques and platforms

Although the industrial chromatography analytical methods for herbal medicine, including GC and HPLC, are the most popular analysis for quality control, TLC is still ubiquitous to differentiate herbal species. TLC requires a sheet, most commonly aluminium foil, coated with a thin layer of adsorbent materials like silica gel, aluminium oxide or cellulose as the stationary phase. By applying suitable solvent system as the mobile phase carried along by capillary force to the TLC sheet, the chemicals of the sample would be separated according to the affinities against the stationary phase and the mobile phase.

Unlike HPLC and NMR, HPTLC facilitates easily in the industry due to the economically friendly price and operator's knowledge requirement. The advance of the technology of HPTLC has overcome the drawbacks of TLC, including low consistency, sensitivity, reproducibility and resolution. HPTLC reduces the particle size of the stationary phase from 20 μm to 5 μm resulting in more compact bands and increased detection sensitivity and analysis speed.

Several pieces of Danshen related research used HPTLC as an approach to study the quality of different batches. (Luo and Ji, 1989; Li, He and Song, 1993; Hu *et al.*, 2005; Yang *et al.*, 2011; Azadniya and Morlock, 2018). Luo and Ji (1989) used six different solvent systems to differentiate twenty-eight different tanshinones from *Salvia miltiorrhiza* and *Salvia przewalskii*. However, some R_f values between compounds were close and using six solvent systems to identify tanshinones only was not efficient. (Li, He and Song, 1993) was the first one developed HPTLC systems to differentiate salvianolic acids. The solvents included chloroform, ethyl acetate, benzene, formic acid and methanol and it differentiated protocatechualdehyde, caffeic acid, methyl

rosmarinate, rosmarinic acid, salvianolic acid A, B and C. The technology at that time still hindered the result accuracy because the monitoring system of the environmental factors such as temperature and humidity was not comprehended. Hu *et al.* (2005) developed a solvent system with chloroform, ethyl acetate, toluene, formic acid and methanol for hydrophilic compounds while another solvent system with ether, ethyl acetate and cyclohexane was used for lipophilic compounds in Danshen. These two solvent systems have successfully identified the characteristic chemicals in Danshen, and most of the pharmacopoeias have been using its modified version in the monograph of *Salvia miltiorrhiza* (Chinese Pharmacopoeia 2015, 2015; British Pharmacopoeia 2015, 2015; Taiwan Herbal Pharmacopeia 2nd Edition English version, 2016; United States Pharmacopeia 39th Edition National Formulary 34, 2016; European pharmacopoeia, 2017). In recent years, Azadniya and Morlock (2018) combined HPTLC-HRMS and bioassays to identify the bioactive compounds in Danshen. It also provided a qualitative and quantitative analysis for Danshen chemical constituents.

Chapter 3. Hypothesis, objectives and goals

Plant derivative medicinal products have attracted a lot of public interest and it is undeniable that these products need more attention to their authenticity and quality assessment. However, there is lack of understanding as to their value chain. How can we best monitor them across all sectors of the supply chain? How do we understand plant derivative medicinal products in terms of chemistry and pharmacology, and link these into a better picture of quality?

Hypothesis

There will be variations in chemical composition and biological effects between Danshen samples. The correlation between processing, chemical composition and biological activities will be demonstrated using metabolomics.

Aims

This thesis aims to establish a method to evaluate the value chain of plant derivative medicinal products from cultivation to marketing. It also aims to understand the quality of plant derivative medicinal products using a metabolomic approach.

Objectives

Using Danshen as an example

- To identify the problem(s) of quality in the market regarding chemical composition and biological relevance variations as well as processing practice.

Goals

• To understand the cultivation and process of Danshen from direct observation and first-hand information.

- To interview different sectors involved Danshen products' value chain so to understand their perspectives.
- To identify the level of Danshen value chain knowledge from different key informants, including farmers', pharmacists', middlemen's and retailers' representatives.
- To collect Danshen derived products as well as authenticated samples of Danshen with voucher specimens from different selling channels for chemical and pharmacological analysis.
- To analyse the quality of Danshen using trace metals analysis, HPTLC and 1H-NMR analysis which is suitable for both product differentiation and each Danshen species.
- To develop relevant pharmacological assays that are linked with the Danshen metabolomics results.

Chapter 4. Methods

4.1 Materials and Apparatus

Solvents

Milli-Q water (purified by Elix® S water purification with Q-Gard® 1 Purification Cartridge, Merck, Germany), chloroform, methanol and ethanol (HPLC grade, Sigma-Aldrich, U.S.A.), ethyl acetate, toluene and glacial acetic acid (HPLC grade, Fisher Scientific, U.K.), formic acid (ACS reagent, 98%, BDH Chemicals Ltd., U.K.), $DMSO_{d6}$, ≥99.0% (Cambridge Isotope Laboratories, Inc, U.S.A), PBS buffer pH 7.4, D-MEM, penicillin-streptomycin 10,000U/ml, FBS and trypan blue stain 0.4% (Gibco, Stockholm, Sweden), MTT ≥ 97.5% HPLC grade, DMSO anhydrous ≥ 99.9%, DMSO Hybri-Max® (Sigma-Aldrich, U.S.A.), H_2O_2 (HPLC grade, Sigma-Aldrich, U.S.A./ analytical grade, Carl Roth, Germany), HNO_3 (analytical grade, Carl Roth, Germany), HCl (analytical grade, Carl Roth, Germany), phosphoric acid (≥98%, ACROS Organic, U.S.A), RotiStar ICP-standard matrix: 5% HNO3 (Carl Roth, Germany)

Cell line

RAW 264.7 (American Type Culture Collection, U.S.A)

Chemical standards

DSS (NMR grade, ≥99.9%, Sigma-Aldrich, U.S.A.), $KMnO_4$ (analytical grade, 99+%, ACROS Organic, U.S.A), sulfanilamide ≥99%, N-1-naphthylethelene (ACS reagent, ≥98%, Sigma Aldrich, U.S.A.), MTT ≥ 97.5% HPLC grade, LPS from *Escherichia coli* (Sigma Aldrich, U.S.A)

Instrument

Grinder (GT203840, Tefal, U.K.), Rotamixer (Hook & Tucker Instruments Ltd., U.K.), Grant XB22 ultrasonic bath (Grant Instruments, U.K.), Centrifugator (Centrifuge 5804

R, Eppendorf, Germany), electronic balance (Sartorius CP64, Sartorius AG, Germany), Freeze dryer (ModulyoD Freeze Dryer, Thermo Fisher Scientific, U.K.), NMR tube (VWR international Ltd., U.S.A.), Bruker Advance 500 MHz spectrometer (Bruker, Germany), HPTLC plates silica gel 60 F 254 (Merck, Germany), Linomat 5 (CAMAG, Switzerland) coupled with a 100µl syringe (CAMAG, Switzerland) and compressed air with 60-90psi., Automatic Developing Chamber ADC 2 (CAMAG, Switzerland), TLC Visualizer (CAMAG, Switzerland) Microwell plate Nunclon 96 well (Thermo Scientific Nunc, U.K.), GalaxyB CO2 incubator (Scientific Laboratory Supplier Ltd., U.K.), water bath (LAUDA Aqualine AL 12, Germany), microscope (Olympus CK40 microscope, Japan), plate shaker (MS3 basic, IKA ®, Germany) microplate reader (Infinite M200, Tecan, Switzerland), Multiwave Go (Anton Paar, Graz, Austria), ICP-OES SPECTROBLUE T1(SPECTRO Analytical Instruments, Kleve, Germany)

Software

MestreNova 12.0.1-20560 (Mestrelab Research S.L., Spain), SIMCA 14.1 (MKS Umetric AB, Switzerland), Excel 365 (Microsoft, U.S.A), VisionCATS (CAMAG, Switzerland), Smart Analyser Vision 5.02.0937 (SPECTRO, Germany)

4.2 Case study: Background to semi-structured interview & fieldwork

A case study research is a research approach to obtain an in-depth understanding of subject by analysing its practical dimension instead of making theoretical hypothesis merely based on the grounds of statistics or observation. Cross-cases analysis would help to understand the interactions and boundaries between firms. Identifying the interrelationships of the sectors involved is vital to move the industry forward by tackling problems and upgrading the competitiveness. Given by the literature review, only a few fieldwork studies of Danshen have been done on.

The research was conducted with semi-structured qualitative interviews with different sectors in the supply chains of Danshen products. The information was later compared with literature and open-source data and on-site fieldwork. The semi-structured interview enables the key informant to openly discuss a specific theme to illustrate an overview of the reality. Moreover, it encouraged the interaction between the researcher and the key informants. Thus, easing the transmission of information. However, the interview was time-consuming, and the sample size was limited due to participants' availability and willingness. On the other hand, on-site non-participant observation alleviated the researcher's subjectivity to observe the phenomenon of a subject compared to first-person experience.

The research aimed at understanding the supply chain systems, plus any associated parties involved in, of Danshen products including cultivation, primary and secondary processing, packaging, and storage. It helped to develop practical strategies of the supply chain study and laboratory experiment planning in the chemistry and pharmacological study.

The interview framework has been provided in the supplementary document. It included six parts: 1) management, 2) cultivation and processing, 3) secondary processing, 4) packaging and storage, 5) transport and distribution, and 6) marketing. During the interview, interviewees were encouraged to provide extra information as long as they were comfortable; but if they were not comfortable with questions or even did not want to be recorded, the interviewee could discontinue at any time.

The selection of interviewees was based on the importance of the herbal product value chains; therefore, the pharmaceutical company, farmers, national government organisations and distributors/ retailers were the targets of the selection. The interviewees also had to be involved in the supply chain of Danshen or its derived

product for more than five years. All the participants, including myself, the interviewer, had signed the consent form and all the data was used in the research purpose only before the interview started. The interviews were conducted in Mandarin or Cantonese because all the parties involved were Chinese and the contents were translated and summarised in English afterwards.

The fieldwork was conducted during August to September in 2016. The choice of time was due to the harvesting and processing time of Danshen as well as the availability of the farmers. The on-site fieldwork was undertaken in Fuli Township, Hualien County, Taiwan 983 (Degrees/Minutes/Seconds: 23°16'32.9"N 121°19'13.0"E). The site chosen is an organic farm producing Chinese medicine materials including *Salvia miltiorrhiza* Bunge. (Danshen), *Bletilla striata* Rchb.f. (Baiji) and *Angelica acutiloba* Sieb. et Zucc. (Japanese Angelica/ Dongdanggui) Kitagawa. The on-site fieldwork focuses on the cultivation and processing practice of Danshen, as well as its market channel, and non-participant observation was used as the strategy. Video recording and photos were taken to be the objective information of the entire process. All the records and the whole process were consented and permitted by the farmers.

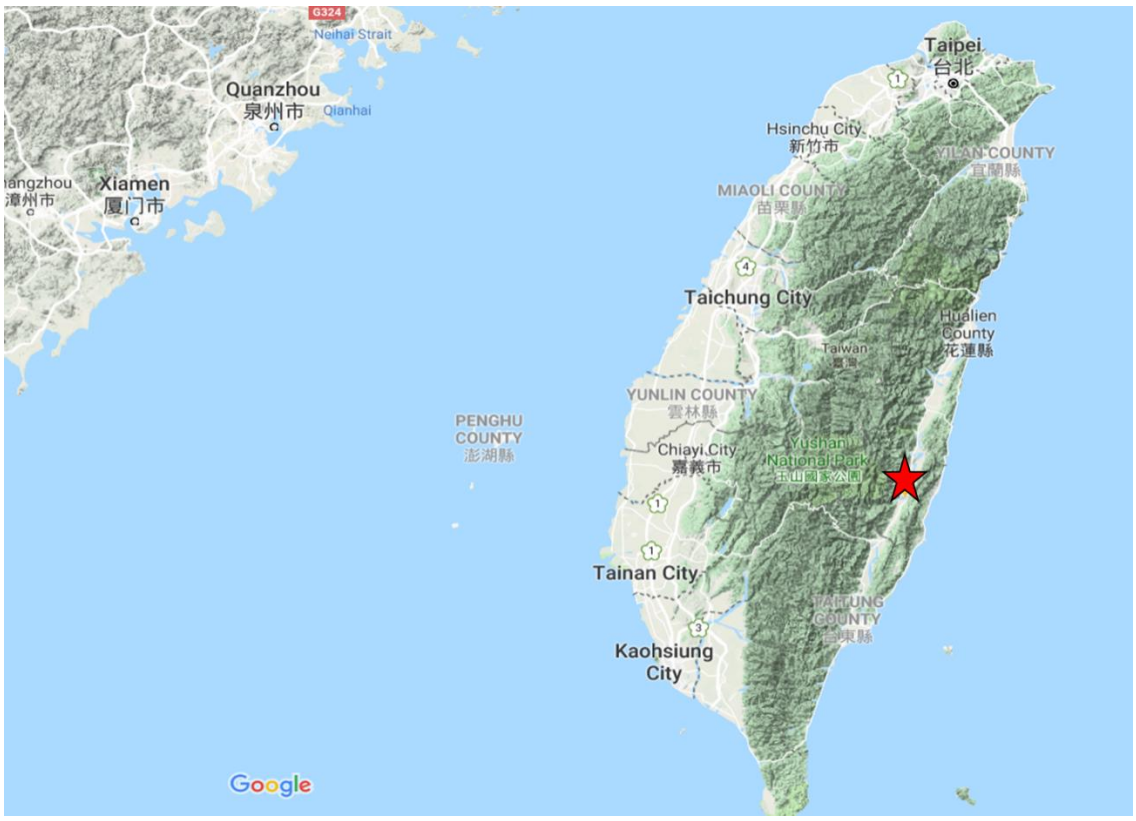


Figure 5 The site location of the fieldwork conducted in Taiwan (sited and edited from Google maps)

4.3 Sample Collection

Criteria inclusion

Products which fulfilled the following parameters were eligible for the study: 1) the label, description or promotional materials of the products claimed to contain *Salvia miltiorrhiza* or Danshen, 2) the product was available in the market and reproducible and 3) the product was a single herb preparation which included mostly Danshen. All the product forms, for example, raw materials, concentrated extracts, capsules, blended powder etc. were accepted as study samples. Multi-herb preparations were excluded from this study since this study focused on the chemistry and pharmacological evidence of Danshen.

Market samples collection strategy

Market Danshen products were collected via different sales channels, such as online stores, pharmaceuticals, pharmacies and herbal markets. To look for Danshen products

from online stores, “Salvia miltiorrhiza”, “danshen” and “red sage”, were the keywords searched in Amazon and eBay UK from November 2014 to January 2015. Also, during May 2017, “丹参” (the simplified Chinese word of Danshen), and “丹参粉” (Danshen powder) were the keywords searched in Taobao which is a Chinese e-commerce platform containing accredited online stores and typical online sellers,

To look for commercial Danshen products, three major herbal markets from different countries or regions were visited.

1) Qingping market in China is the only legitimate Chinese herbal medicine trading place in Guangzhou province. It began in 1979 when there were just around 50 stores. Nowadays, it increased to nearly 2000 stores. It is a composite market selling a wide range of TCM materials or medicinal food, as well as some agricultural products. (*Qingping Market, Guangzhou China*, no date; *Translate from Chinese: Qingping Market, w\hich belongs to Guangzhou, the herbal scent - Guangzhou Allusion - Guangzhou Tourism Bureau. Information Channel*, no date).

2) Ko Sing Street, Hong Kong which is recognized as TCM street over 100 years. Its location which is adjacent to the pier benefits the facilitation of loading and unloading goods, hence, has attracted TCM wholesalers. Most of the practitioners or herbalists there have been working on TCM materials for years, and they have earned a high reputation in TCM knowledge. (*Ko Shing Street (Chinese Medicine) | Hong Kong Tourism Board*, no date)

3) Phố Lãn Ông in Vietnam. According to traditional medicine wholesalers in Vietnam, Vietnamese farmers are cultivating *Salvia miltiorrhiza* as well. Pauline Ling who conducted the Vietnamese herbal market sample collection said that there were a number of local herbal stores selling TCM raw materials. The reason of selecting this location is that the traceability is low and the quality of herbal materials in Vietnam was

not well regulated which would be different from Hong Kong and China or other countries where herbal medicine is monitored by the governmental department of health like Taiwan or the United States.

4) This project also included the Danshen products from local TCM pharmacies; most of these samples were collected in London. London TCM pharmacies are regulated so that TCM materials should be prescribed by TCM practitioners. It is not easy to get Danshen products straight forward, therefore a declaration of research purpose was needed during the sample collection.

For TCM pharmaceuticals, this project included the products from the most common brands in Taiwan, People's Republic of China and Hong Kong. The information was given by SunTen pharmaceuticals representatives, TCM practitioners and pharmacists as well as personal experience. There were some individual samples from Germany and Switzerland. The U.K., Chinese, Vietnamese, samples are listed in Table 14, Table 15, Table 16 and Table 17.

Authenticated *Salvia przewalskii* Maxim, *Salvia bowleyana* Dunn and *Salvia miltiorrhiza* Bunge samples were obtained from NIFDC, Kew Gardens, America Herbal Pharmacopoeia, Brion, Lfl and YuFu biotek. The details are listed in Table 13. .

4.4 Sample preparation

The method of sample preparation was developed from (Kim, Choi and Verpoorte, 2010b). Three types of samples were collected: 1) raw dried roots or rhizomes, 2) dried root or rhizome powder and 3) concentrated extracts. For the raw roots or rhizomes, samples were ground into fine powder by a mechanic grinder and passed through a sieve, resulting in particle of less than 1 mm size. All samples were stored with silica gel packs to avoid the influence of humidity in the environment.

All samples were accurately weighed with less than 0.5% error and extracted in 75% methanol with 1 g: 20 ml drug and solvent ratio followed by 30 seconds of vortexing and 30 minutes of ultra-sonication. The extracted samples were centrifuged under 1400 rpm at 15°C for 10 minutes. The methanol content of the supernatant was evaporated at 60°C dry heat plate under the fume hood for 1.5 hours and dried by freeze dry. The yield of each sample was recorded and attached in Table 19. All extracts were stored in -80°C and defrosted if it is needed.

4.5 High Performance Thin Layer Chromatography

The method of HPTLC was developed from *Hong Kong Material Medica - Radix Salviae Miltiorrhizae Monograph* (2005) and Booker *et al.*, (2014). The experiment aimed at understanding the chemical differences between samples using a rapid screening technique. 2 µl 75% methanol extract of each sample was loaded on the HPTLC plate, as well as chemical standards. The HPTLC was started after the developing solvent system (MePh: $CHCl_3$: EtOAc: MeOH: FA = 2: 3: 4: 0.2: 2) saturated for 20 minutes and pre-conditioned for 5 minutes. The condition of the tank was held at the humidity of 33% and a temperature of 23°C. The process was stopped once the developing solvent reached the plate to 80 mm migration distance. After 5 minutes plate drying, the plates were visualised under white light, 254 nm and 366 nm wavelength. VisionCATS, the HPTLC workflow and analytical software, was used in plate analysis and graphic editing.

4.6 Nuclear Magnetic Resonance and its analysis

The method of NMR was developed from Li *et al.* (2015). This experiment aimed at understanding the chemical variation of market samples an differentiate the samples using multivariate analysis. 275 ul 200 mg/ml of each sample dissolved in $DMSO_{d6}$ was mixed with 275 ul of 0.2% DSS dissolved in $DMSO_{d6}$. All the samples were analysed

by 500MHz ^{1}H-NMR spectrometry with 256 scans which required 20 minutes approximately. The parameters of ^{1}H-NMR spectra were listed below:

Spectral width = 10,330.578 Hz, 0.1576 Hz per point, pulse width = 13.9 usec and relaxation delay (RD) = 1.0 sec. Free induction decays (FIDs) were Fourier transformed with a line broadening (LB) of 0.3 Hz. All the sample were repeated twice at least individually.

The NMR spectra were processed by the analytical chemistry software, MestreNova. All the spectra were stacked and optimised with the interactive phase correction in global metabolomics mode, and the baseline correction with Bernstein polynomial fit order = 3. The chemical shift of DSS, set at 0 ppm, was used as the internal standard and normalised all the spectra. The range from δ 9.00 ppm to δ 0.66 ppm of the spectra were selected and the following areas were manually cut: δ 5.5 to δ 3.15 ppm, δ 2.6 to δ 2.4 ppm, δ 2.02 to δ 1.99 ppm, δ 1.70 to δ 1.50 ppm and δ 1.25 to δ 1.16 ppm. The spectra were binned into 0.03 ppm by the method of the average sum. All the data were analysed by the multivariate analysis software, SIMCA.

4.7 Trace metals Analysis

Calibration of metals

The method of metal analysis was developed from (Olesik, Kinzer and Olesik, 1995; Cooper *et al.*, 2007). 300 mg of sample powder was weighed accurately in the microwave digestion tube. To each sample was added 1 ml of H_2O_2 for 10 minutes, 2 ml of 65% HNO_3 for an hour and another 2 ml of 65% HNO_3 for an hour, and 1.5 ml of 35% HCl for 18 hours in order. Before microwave digestion started, 7.5 ml of 35% HCl was added to samples. The tubes were sealed and placed in the microwave digestion system. The microwave digestion was programmed as Table 9. After digestion, the digestates were diluted to 50 ml with milli-Q water and the samples were analysed by

ICP-OES. The metals of the sample would be measured due to the wavelength variation under ionizations caused by extremely high temperature in plasma status. The operating system was listed in Table 10 and the limitation of detection was listed in Table 11

Time (min)	Temperature (°C)
0 → 10	room temperature → 175
10 → 45	175
45 → 45	175 → 185
45 → 50	185
50 → 60	185 → room temperature

Table 9 The microwave digestion system program for Danshen samples

Parameters	
Nebulizer	Concentric
Spray chamber	Cyclonic
Radio frequency power	1.45 kW
Plasma gas flow rate	13 L/min
Auxiliary gas flow rate	1.2 L/min
Nebulizer gas flow rate	0.75 L/min
Replicates per sample	3

Table 10 The parameters of the operation system of ICP-OES

Element	Wavelength [nm]	Limit min. [ppm]	Limit min. [mg/kg]	Limit max. [ppm]	Limit max. [mg/kg]
Ag	328.07	0.0010	0.1634	1.208	203.37
Ag	338.29	0.0010	0.1634	1.208	203.37
Al	396.15	0.0001	0.0178	59.86	10077.44
As	189.04	0.0001	0.0178	1.208	203.37
As	228.81	0.0001	0.0178	1.208	203.37
B	249.77	0.0001	0.0178	23.96	4033.67
Ba	233.53	0.0001	0.0178	12	2020.20
Ba	455.40	0.0001	0.0178	12	2020.20
Be	313.04	0.0001	0.0178	1.208	203.37
Bi	223.06	0.0001	0.0178	12	2020.20
Ca	317.93	0.0001	0.0178	59.86	10077.44
Cd	214.44	0.0001	0.0178	1.208	203.37
Cd	226.50	0.0001	0.0178	1.208	203.37
Co	228.62	0.0001	0.0178	1.208	203.37
Co	230.79	0.0001	0.0178	1.208	203.37

Cr	205.62	0.0001	0.0178	12	2020.20
Cr	267.72	0.0001	0.0178	1.208	203.37
Cu	224.70	0.0001	0.0178	12	2020.20
Cu	324.75	0.0001	0.0178	23.96	4033.67
Fe	238.20	0.0001	0.0178	59.86	10077.44
Fe	259.94	0.0001	0.0178	24	4040.40
K	766.49	0.0001	0.0178	59.86	10077.44
K	769.90	0.1000	16.5017	59.86	10077.44
Li	670.78	0.0001	0.0178	12	2020.20
Mg	279.55	0.0001	0.0178	1.294	217.85
Mg	285.21	0.0001	0.0178	59.86	10077.44
Mn	257.61	0.0001	0.0178	59.86	10077.44
Mn	403.08	0.0001	0.0178	59.86	10077.44
Mo	202.10	0.0001	0.0178	1.208	203.37
Na	589.59	0.0010	0.1634	23.96	4033.67
Ni	231.60	0.0001	0.0178	12	2020.20
Pb	220.35	0.0001	0.0178	12	2020.20
Pb	405.78	0.0001	0.0178	12	2020.20
Sb	206.83	0.0001	0.0178	1.208	203.37
Sb	217.58	0.0001	0.0178	1.208	203.37
Se	196.09	0.0001	0.0178	1.208	203.37
Sr	407.77	0.0001	0.0178	12	2020.20
Sr	460.73	0.0001	0.0178	12	2020.20
Ti	307.86	0.0001	0.0178	12	2020.20
Ti	334.94	0.0001	0.0178	12	2020.20
Tl	190.86	0.0001	0.0178	1.208	203.37
Tl	276.79	0.0001	0.0178	1.208	203.37
V	292.46	0.0001	0.0178	12	2020.20
V	311.07	0.0001	0.0178	1.208	203.37
Zn	206.20	0.0001	0.0178	12	2020.20
Zn	213.86	0.0001	0.0178	59.86	10077.44

Table 11 Limitation of detection for each element in ICP-OES analysis

4.8 Evaluation of the extract to RAW 264.7

Maintenance of RAW 264.7 cell line

The RAW 264.7 cell line, a macrophage transformed by Abelson murine leukaemia virus, was cultured in D-MEM supplemented with 10% heat-inactivated FBS (Hyclone, Utah, Logan, USA), penicillin-streptomycin (100 IU/mL and 100 µg/mL). The cells were incubated in a humidified atmosphere at 37°C with 5% CO_2 supplied. Only passages from 5 to 11 of RAW 264.7 were used.

Evaluation of the cell viability

MTT assay was used as the method for evaluating cell viability developed from Funk *et al.* (1993). It aimed at understanding the biological effect of extracts on macrophage cells. 200 ul of RAW 264.7 was seeded at a density of 3×10^4 in 96-well plate. The cells were incubated at 37°C and 5% CO_2 supplied. After 24 hours, the cells were treated with the sample at the concentrations of 100, 50 and 25 ug/ml, as well as the blank and negative control, for 24 hours. All the treatments were adjusted to the final concentration of 0.05% DMSO. After treatment, the cells were incubated with 100 ul of the MTT solution, which was 5 mg/ml of MTT dissolved in PBS mixed with DMSO at the ratio of 1:10, for 4 hours. After the removal of the MTT solution, 100 µl of DMSO was added into each well, and the plate was shaken horizontally for 1 minute. Cell viability was measured at 570 nm against 630 nm wavelength by the microplate reader.

Evaluation of H_2O_2 induced apoptosis

MTT assay was used as the method of evaluation developed from (Jin *et al.*, 2009; Seo *et al.*, 2010; Cho, Hunt and Hussain, 2017). It aimed at understanding the variation of the protection from reactive oxygen species damage and anti-inflammation effect among Danshen products. Similar to the evaluation of the cell viability, after the

treatment of extract, 1 mM H_2O_2 was added into each well for 2 hours and followed with MTT to measure the cell viability. The concentration of H_2O_2 was determined by the method of redox titration. The determination of H_2O_2 concentration experiment is listed in the supplementary experiment (Determination of H2O2 concentration). 15 ul/mg of baicalein was used as the positive control in this experiment. The concentration of H_2O_2 used in this experiment was evaluated and recorded in the supplementary experiment (**Error! Reference source not found.**).

$$5\,H_2O_2\ +\ 6\,H^+ +\ 2\,KMnO_4\ \rightarrow 5O_2\ +\ 2\,Mn^{2+} +\ +\ 8H_2O\ +\ 2\,K^+$$

The measure of the protection to H_2O_2 induced apoptosis from the extract was listed as follows:

$$Protection\ to\ H_2O_2\ induced\ apoptosis = \frac{V_{sample} - V_{H_3O_2}}{V_{blank\ medium} - V_{H_3O_2}}$$

Whereas, V_{sample} = the viability of sample + H_2O_2; $V_{H_3O_2}$= the viability of H_2O_2; $V_{blank\ medium}$ = the viability of blank medium.

Evaluation of LPS induced NO production

The Griess assay was used as the method of evaluation developed from Jin *et al.*, 2009. It aimed at understanding the anti-inflammatory activity of different Danshen products. 200 ul of RAW 264.7 was seeded at the density of 2.5 X 10^5 in the 96-well plate. The cells were incubated with 37°C and 5% CO_2 supplied. After 24 hours, the cells were treated with the sample at the concentrations of 100, 50 and 25 ug/ml, as well as the blank, negative control and positive control which was 20 ul indomethacin. All the treatments were adjusted to the final concentration of 0.5% DMSO. After the treatment, the cells were incubated with 50 ng/ml of LPS for 24 hours. Griess solution A and B (solution A: 4% sulphanilamide in 10% phosphoric acid; solution B: 0.4% N-1-napthylethylenediamine) were freshly prepared within an hour before the next step.

100 ul of cell culture medium was mixed with 25 ul of solution B, and 25 ul of solution A. 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 uM $NaNO_2$ and non-phenol red D-MEM were used as the reference standard of NO_2^-. The reaction was undertaken under light-protection, and the plate was shaken horizontally for 1 minute after. The absorbance was measured at 550 nm wavelength in the microplate reader. The calculation of nitrate/ nitrite concentration listed:

$$\text{Nitrate/nitrite concentration} = \frac{[\text{Ab} - \text{Ab}_{blank}]}{\text{Slope of NaN}O_2\text{ standard curve}}$$

Whereas, Ab = the absorbance of the well with cells with different treatment, Ab_{blank} = absorbance of the well with complete medium without seeding cells

Statistical analysis

Each sample was run in three replicates in a plate three repeats (n = 9). All results were expressed as with means ± SDE. Data were analysed using student's t-test using Excel 365. $P<0.05$ was considered significant.

4.9 Multivariate analysis

All the data such as product information, NMR binned spectra, cytotoxicity MTT, H_2O_2 induced apoptosis, and LPS induced NO inhibition, was input to the dataset of SIMCA software. NMR binned spectra data was set as X variables whereas other data was set as Y variables. The data was scaled by Pareto scaling. PCA-X was performed to evaluate the chemical variation level between samples. The PLS-DA was performed to evaluate the interrelationship between the chemistry and other information of the samples including the pharmacological evidence. The R^2 and Q^2 were used to understand the goodness of fit and predictability of the model. The first two components were used, and loading plot and VIP were used to evaluate the importance of the X variables to the model and sample distribution. The outliers among the samples were

identified using the hotelling T_2 range. HCA was used in categorising samples with similar pattern.

Chapter 5. Results and discussion

5.1 Market Study

In order to understand the supply chain of Danshen products from different perspectives, anonymous semi-structured interview, and fieldwork using non-participant observation and interviews were conducted to obtain personal experience and expert opinion on Danshen processing. Seven key informants within the supply chains of Danshen products, including a farmer, TCM pharmaceutical representatives, a quality-control assessor, a governmental department representative, a supplier representative, and a TCM pharmacist and doctor, were included. The basic background of the interviewees (Table 22) excludes personal information for the sake of protecting interviewees. The fieldwork was also conducted in a non-invasive, non-participant observational method in order to gain trust between the farmers or practitioners and the conductor. In order to obtain a better overview of the supply chain, some literature reviews and governmental reports have been included in this section.

The practice of farmer in the Danshen supply chain – a case study in Taiwan

A case study was made on a Taiwan organic TCM materials company with thirty staff working. The business of the company includes cultivation and primary processing of organic TCM materials, and TCM related organic food supplements or food. The cultivation of TCM materials on the site is done through contract farming. The main crops are Japanese angelica (*Angelica acutiloba* (S. *et* Z.) Kitag.) and Danshen. Japanese angelica is considered as a kind of TCM material in Taiwan because it was used to substitute Chinese angelica. Other TCM materials are in small production, for instance, baizhi (*Angelica dahurica* Benth. et Hook), baiji (*Bletilla striata* (Thunb.) Reichb.f.) and huangqin (*Scutellaria baicalnsis* Geprgi). Due to organic farming, all the materials used during the cultivation, including soil, pesticides and fertilisers, need to be

verified by the Council of Agriculture and obtain organic certificates to make sure they are not contaminated and organic.

The company is supported by the governmental department – the Council of Agriculture. The collaboration was initiated because of the promotion of an organic TCM industry of Taiwan. The research department of the Council of Agriculture was responsible for the selection and verification of TCM species-specific for Taiwanese soil while the farmers were responsible for providing the land and trying to cultivate organic TCM. Danshen was one of the selected species. The first batch of the seeds was introduced from Shandong, China and was authenticated by an experienced botanist professor in morphology, chemistry and DNA barcoding. Danshen was successfully cultivated in Taiwan highland, and the contents of the biomarkers including tanshinone IIA and salvianolic acid B were high. The project was considered very successful in the first cultivation, and the farmers decided to develop the project into a business and continue the collaboration with the government. The council is now responsible for the research, education, promotion and development of herbal materials cultivation whereas the company cultivates organic other herbal materials and demonstrates to other farmers. All the services provided by the council are free to the farmers. They also collaborate with universities researching the pharmacology and chemical quality of Danshen.

The cycle of the business was confirmed through the interviews and first-person non-participant observation. The start of the cultivation of Danshen is preparation and fertilisation of the land. It usually starts in August after harvesting the crops. The soil in the land is overturned at least two times in two weeks. All the plants on the land are removed mechanically, and the land is covered by black thick nonwoven fabric during the process. The land is sprayed with the organic fertiliser evenly, and the farmers mix with the soil by overturning. The soil is then harrowed to make the clods and other plant

residues finer. The field is prepared with fertile sandy soil and proper water drainage. After flattening the land, the farmers level up the soil into one-meter width and thirty-centimetre height to prepare the seedling position.

The Council of Agriculture prepares the seedlings for the farmers in October. The seeds normally germinate a week after seeding, and the seedlings are ready to transplant after the plant has grown four to six leaves. The farmers cut open the fabric covering the land and transplant the seedlings in order to control the weeds. Additional fertiliser is applied four months after the transplant of seedlings. The pest problem of Danshen in Taiwan does not affect the production much; therefore, they do not need to apply any pesticides, but they have guidelines for a different potential treatment for different pests. The fertilisers and pesticides are sourced from organic certificated companies. While Danshen is growing, the farmers also need to be aware of the status of weeds, plant health and moisture of the soil. From March to June, the staff from the council collects the seeds from the field. This seed picking has to be done continuously and manually due to the indeterminate inflorescence flowering of Danshen. The collected seeds are stored in 40 ± 10 % humidity under 4 ± 2 °C

The best harvesting time would be between nine to twelve months after seeding. In August, farmers use plant excavators to harvest and remove the soil from the roots manually. They do not wait until October because the quality of the roots would worsen after flowering. The harvested plants are separated into three parts: Aerial parts, rhizomes and roots. The aerial parts are processed to make fertilisers. First, the rhizomes and roots are briefly washed, then dried and cut into slices for selling as food supplement directly. The roots are graded by the size of the main roots, lateral roots and fibrous roots, and sold to restaurants and biotechnology companies. At the time of the study, the price was 550 NT per 600 grams for the dried materials and 550 NT per 3 kg for the fresh materials, and the total cost of production would be half of the price

approximately. The drying method depends on the weather and demand. If the weather is not suitable for drying or supplier requests it urgently, oven drying will be selected; otherwise, it is usually dried under shade, which usually takes half a day for the fresh roots.

In order to maximise the profit margin, the oversupply of Danshen is mixed with other self-cultivated TCM materials to derive different types of food supplements, like TCM tea bags, decoction piece and chicken essence, sold to individual customers directly. The quality of Danshen roots is monitored by the research teams of the university, the council and the organic certification body. After processing all Danshen roots are stored under 4 °C before selling to the market. Fresh roots will be stored up to from three days to a week. The transportation of products will be outsourced to other home delivery companies.

The role of TCM pharmaceutical in the company supply chain

The main business for TCM pharmaceutical companies is the concentrated extracts. Its local target customers are hospitals and some distributors, and only a small portion of customers are individual clinics, but usually, the local clinics would purchase their products through a distributor. The reason is that the acceptance of the concentrated extracts in China, or Taiwan is not adequate in the local TCM clinics. Most of the pharmacists prefer the traditional decoctions directly derived from dried herbs. In contrast, international target customers are mainly distributors who would be around 80%. It is because the import of raw dried herbal materials is restricted in the U.K., and the TCM concentrated extract is the only possible alternative. Although Danshen is considered to be the top ten of the most demanding TCM single herb extracts, there are usually more than four hundred kinds of extracts in a typical TCM pharmaceutical company. Danshen products only seize a tiny portion of their entire business.

Some companies would also provide herbal materials to the TCM distributors because they have already had some partnerships or contracts with specific farmers and TCM suppliers in China for the demand of herbal materials for the extracts. The contracts or partnerships include cultivation and wild harvesting, though the supplier has direct management to the farmers. Therefore, the supply of herbal materials is relatively stable, and the usual storage time for Danshen herbal materials is from six months to a year. Providing herbal material supply service to the distributors will increase the quantity of the order, so it is beneficial for them to increase bargaining power with the suppliers. The collaborative relationship with the supplier would be stronger, and the cost of manufacturing TCM concentrated extracts would be lower. Also, TCM pharmaceutical companies usually have spacious warehouses to store extra materials. Selling herbal materials to the distributors also maintain a consistent flow of the stock and guarantee that the materials they have or use are not “expired”. On the other hand, due to the series of quality assessments and quality control during the process of herbal material selection from the pharmaceutical companies including chemical tests, TCM distributors benefit from obtaining verified “good quality” materials by the pharmaceutical companies.

The materials from China usually need further selection and processing, including cutting and grinding before extraction and concentration. The herbal materials with poor quality would be sent back to the corresponding supplier for each batch. The extraction and concentration methods vary according to materials, but the common drying method used in the pharmaceutical industry is fluid bed drying method. During this process, apart from adding corresponding extract(s) and starch and fibres to combine as crude granules, some of the TCM pharmaceutical companies would add herbal pieces as one of the excipients in some specific TCM extracts, for example, Danshen, for its consistency and better chemical composition. The crude drug granules are screened into

suitable size, and the quality is assessed. The quality criteria typically follow the ChP, but some herbal materials would have additional criteria in some pharmaceutical companies, for instance, the glycoside content of Danshen might be added. The process of manufacturing TCM extract was restricted by law in Taiwan that it cannot be subcontracted or outsourced; therefore, all the pharmaceutical companies have to control every part of the procedures even though there are differences between the quality control for the international market and local market. Most of the staff would obtain a higher educational degree in these quality assessments and quality control. After the process above, the product would be packaged and have a final quality assessment before it goes to the customer.

The variety of Danshen products in the market

The sample collection was started in December 2014 and ended in December 2017. One hundred twenty-nine commercial samples and 18 authenticated samples related to Danshen were collected. Commercial samples included purchased samples from retailers and suppliers and donated samples. The product information of purchased samples, including price, quantity, originality, market channel and market place were recorded as much as possible according to the sample provider.

Eighteen authenticated samples were obtained from NIFDC (China), Kew Gardens (U.K), Bavarian State Research Center for Agriculture (LfL) (Germany), American herbal pharmacopoeia (U.S.A), Brion (Taiwan) and YuFu (Taiwan). All the samples were dried straight after harvesting. Each included a corresponding voucher specimen and was labelled with the area and date of harvesting. Each dried sample then was kept in an individual sealed plastic bag. A7 was the sample provided by Brion which has been genetically authenticated. It is also the source of Sun Ten pharmaceutical's Danshen concentrated extract. A8 to A11 were the organically cultivated samples

collected in Taiwan during the fieldwork study with YuFu. All of them were harvested at the same time and at the same place but processed in different practices. These processing conditions were meant to imitate the practice in the real world.

Seventeen samples were obtained from the Chinese online store platform – TaoBao/ Tmall. C1 to C7 and C12 to C14 were the samples sold by Taobao registered online stores from Tmall. C8 to C11 and C15 to C16 were the samples sold by individual traders from TaoBao. C17 was the sample from the entrepreneur trader who registered with Taobao but not Tmall. Although TaoBao and Tmall are both operated by Alibaba group, the difference between these two types of sellers was that Tmall is a business to customer platform and the sellers in Tmall needs to be brand owners or authorised distributors, while TaoBao is a customer to customer platform where anyone can sell their products.

Two types of Danshen products were found in TaoBao/Tmall. One was dried root and the other one was dried root powder. The prices of the samples from individual traders ranged from £0.5 to £1.20 per 100 g. It was significantly lower than the price from Tmall sellers, ranging from £1.31 to £5.03 per 100 g. C17 cost £5.28 per 100 g, which was the highest price among all dried root pieces or dried root powder. The price of C1, cost £34.2 per 100 g, was remarkably higher than all the samples. The cell walls of the Danshen root were claimed to be broken by jet mill so the chemicals in Danshen would be easily released. The general cost of products also will increase if the product is in powder form.

Fifteen samples were obtained from Vietnam herbal market. All of them are dried root samples, and no other type of Danshen related products was found. The price of the dried root samples ranged from £0.53 to £1.06 per 100 g. Three samples (V2, V9 and V12) were claimed to be cultivated in Vietnam. No relationships between the price and

the originality of Danshen was found. Only one TCM pharmacy in Vietnam was able to provide more specific details regarding the originality of Danshen. We also see that the herbalists in Vietnam would also regularly sun drying the Danshen roots which were different from other market samples

Twenty-three non-extract market samples were obtained from the U.K. market or U.K. based online stores. One finished Danshen product was found in U.K. TCM pharmacies. All the dried root powder form was found in the capsule only. On the London high street, the price of the dried root samples varied with the range from £5 to £20 per 100 g. One capsule form was found, and it cost £133.33 per 100 g. In the U.K. based online stores, though there were not many products, more forms of Danshen related products, like tinctures, oil or capsules, were found. The price of dried root samples ranged from £1.6 to £12.5, which was lower than local TCM pharmacies. The capsules sold online costed around £40 which were three times lower than the one from London high street.

Although the price of the samples from Ko Sing Street (Hong Kong) and Qingping herbal market (China) was not recorded, the expenditure of the total of thirty-four dried root samples was £20, and each sample was 50 g. The average price of the samples from Hong Kong and China herbal market was £1.18.

Among all the dried root samples, different processing methods of Danshen roots can be found in different markets. All the samples were bevelled cut. The colour of the epidermis was brownish-red whereas the cortex was pale yellow. It also seems to be pressed and flattened. The samples from the U.K. looked similar to the samples from Vietnam herbal market, but they were crosscut and did not get flattened or pressed. The samples from Hong Kong and China showed a variety of processing. Both crosscut and bevelled cut could be found, but it is more common to have bevelled cut pressed

Danshen in Hong Kong, but crosscut and non-pressed in China. Another phenomenon is that the colours of the epidermis and cortex of some of the samples were darkened. It is potentially related to the traditional processing “fahan”, also called “sweating”. The definition of “sweating” is to pile up freshly harvested herbal materials stuffily for several days to create a humid and hot environment. This process would darken the herbal substances.

The extract collection was based on the interview from Taiwan pharmaceutical representatives and local TCM pharmacists, then purchased from the suppliers, local TCM pharmacies or online stores. The cost of some of the products could not be obtained because the suppliers asked that should not to be revealed. The suppliers also revealed that the price of the products depended on customers. The lowest price of concentrated extracts collected was £8 per 100 g from the U.K. supplier while the highest price went to £150 per 100 g from the U.K. local TCM pharmacy. All the extracts claimed to be 5 to 1 raw material against the product.

Overall, the price of dried root samples from Hong Kong, China and Vietnam were much more stable than the samples from the U.K. As the price of the samples from Vietnam were lower than China and Hong Kong, the cost of Danshen dried root should not be the major factor of the price. The supply chains of the samples from the U.K. were more complicated since all the sources of Danshen were from China. Noted that the U.K. law also increased the difficulty of importing dried root herbal materials, the cost of transport and storage would shift to customers. The price of similar Danshen products can go up to 10 times more in the U.K. The extract, in general, was ten times at least more expensive than raw dried materials whereas the claims of concentrate extracts were usually equivalent to 5 times the cost of the dried roots.

	Pharmacy	Pharmaceutical companies	Herbal market	Online store	Supplier	Total
Hong Kong	18	3	/	/	1	22
Vietnam	15	/	/	/	/	15
China	/	/	15	17	5	37
United Kingdom	13	/	/	12	15	40
Switzerland	2	/	/	/	/	2
Germany	/	/	/	1	/	1
Taiwan	/	12	/	/	/	12
	48	15	15	29	16	**129**

Table 12 Commercial sample collection statistic

Sample No.	Name	Form	Originality	Collector	Collection time	Other information
A1	*Salvia miltiorrhiza*	Root	Pilot, VA, U.S.A	American herbal pharmacopeia	30/08/2016	Voucher specimen: #4331
A2	*Salvia miltiorrhiza*	Root	Williams, OR, U.S.A	American herbal pharmacopeia	18/08/2016	Voucher specimen: #4326,
A3	*Salvia miltiorrhiza*	Root	Williams, OR, U.S.A	American herbal pharmacopeia	27/03/2009	Voucher specimen: #2592,
A4	*Salvia miltiorrhiza*	Root	Jacksonville, OR, U.S.A	American herbal pharmacopeia	25/08/2016	Voucher specimen: #4320
A5	*Salvia miltiorrhiza*	Root	Petaluma, CA, U.S.A	American herbal pharmacopeia	2007	Voucher specimen: #2550
A6	*Salvia przewalskii*	Root	Petaluma, CA, U.S.A	American herbal pharmacopeia	30/08/2016	Voucher specimen: #4332
A7	*Salvia miltiorrhiza*	Root	Shandong, China	Brion	23/09/2014	Geno-verified Botanical Reference Material
A8	*Salvia miltiorrhiza*	Root	Taiwan	YuFu biotek	01/09/2016	Voucher specimen: KKY 20160901, Oven 100 ℃ 30 min, 120 ℃ 30 min

A9	*Salvia miltiorrhiza*	Root	Taiwan	YuFu biotek	01/09/2016	Voucher specimen: KKY 20160901, Oven 30 ℃ 48 hours
A10	*Salvia miltiorrhiza*	Root	Taiwan	YuFu biotek	01/09/2016	Voucher specimen: KKY 20160901, Freeze dry 24 hours
A11	*Salvia miltiorrhiza*	Root	Taiwan	YuFu biotek	01/09/2016	Voucher Specimen: KKY 20160901 Oven 30 ℃ uncut 48 hours
A12	*Salvia miltiorrhiza*	Root	Taiwan	YuFu biotek	01/09/2016	Voucher specimen: KKY 20160901, Air condition dry shade 48 hours
A13	*Salvia miltiorrhiza*	Root	Germany	LfL experimental station Baumannshof	10/2016	Loamy sand (planting of seedlings gained from seeds, planting date April 2016). Dried at 42 °C with high air ventilation in a hurdle drier
A14	*Salvia miltiorrhiza*	Root	Germany	LfL experimental station Baumannshof	2014	Planted in 2014
A15	*Salvia miltiorrhiza*	Root	China	NIFDC	Unknown	Governmental department raw material standard
A16	*Salvia bowleyana*	Root	Jiangxi, China	Kew Gardens	09-11/2001	Voucher specimen: TCMK 226, EBC no.: 81195
A17	*Salvia przewalskii*	Root	China	Kew Gardens	02/2018	Voucher specimen: TCMK 790, EBC no.: 82956
A18	*Salvia miltiorrhiza* (wild)	Root	China	Kew Gardens	11/2004	Voucher specimen: TCMK391, EBC no.: 82956

Table 13 Authenticated sample (A1 - A18) list

Sample No.	Commercial Name	Form	Originality	Quantity (g)	Retailer	Manufacturer	Cost in RMB	Cost in pound *	Cost per 100 g
C1	Danshen (broken cell wall)	Granule	Shandong	20 * 1 g	Jinkang Pharmacy Flagship Store 金康大药房旗舰店	ZEUS 中智	¥ 60.00	£ 6.84	£ 34.20
C2	Danshen powder	Powder	Shandong	2 * 150 g	Qiancaotang 千草堂	Qiancaotang 千草堂	¥ 59.00	£ 6.73	£ 2.24
C3	Danshen powder	Powder	Anhui	88 g	Kangmei 康美	Kangmei Pharmaceutical Co., Ltd.	¥ 38.80	£ 4.42	£ 5.03
C4	Danshen	Dried root	Shandong	200 g	Beijing Tongrentang	Beijing Tongrentang	¥ 32.00	£ 3.65	£ 1.82

C5	Danshen powder	Powder	Yunnan	90 g	Qidan flagship store 七丹旗舰店	Qidan 七丹	¥ 35.00	£ 3.99	£ 4.43
C6	Danshen powder	Powder	Juxian, Shandong	100 g	Jinhao flagship store 金貅旗舰店	Jinhao 金貅	¥ 15.80	£ 1.80	£ 1.80
C7	Purple Danshen powder	Powder	Diandong, Yunnan	150 g	Noland Health Products Specialstores 诺兰德保健品专营店	Zunrentang 尊仁堂	¥ 28.00	£ 3.19	£ 2.13
C8	Yimeng mountain special grade wild Danshen	Dried root	Yimeng Mountain, Shandong	500 g	80s farmer store 八零后农民小店	/	¥ 21.90	£ 2.50	£ 0.50
C9	Danshen powder	Powder	Qiubei County, Wenshan Prefecture, Yunnan	250 g	Wenshan University Student Entrepreneurship Dream Shop 文山大学生创业梦之店	/	¥ 26.00	£ 2.96	£ 1.19
C10	Danshen powder	Powder	Yunnan	180 g	Xiyi herbs 希夷药材	/	¥ 19.00	£ 2.17	£ 1.20
C11	Danshen	Dried root	Sichuan Zhongjiang	500 g	Mr. Shiso 佰草氏	/	¥ 25.00	£ 2.85	£ 0.57
C12	Danshen	Dried root	Sichuan	120 g	Leiyun Upper West District Pharmacy Flagship Store 雷允上西区大药房旗舰店	Shanghai Kangqiao Chinese Medicine Co., Ltd. 上海康桥中药饮片有限公司	¥ 20.00	£ 2.28	£ 1.90
C13	Danshen	Dried root	Shandong	250 g	Bohaixiang 晟海祥	Jiangsu Bohaixiang Pharmaceutic	¥ 28.80	£ 3.28	£ 1.31

						al Co., Ltd. 江苏晟海祥药业有限公司			
C14	Danshen	Dried root	Jiangsu	100 g	Li Liangji Flagship Store 李良济旗舰店	Suzhou Tianling Chinese Medicine Co., Ltd. 苏州市天灵中药饮片有限公司	¥ 17.00	£ 1.94	£ 1.94
C15	Danshen	Dried root	Sichuan Zhongjiang	500 g	Yichitang TCM Pharmacy 一致堂中药材行	/	¥ 23.00	£ 2.62	£ 0.52
C16	Danshen	Dried root	Sichuan Zhongjiang	250 g	山男经方缘中药材保健养生精	/	¥ 25.00	£ 2.85	£ 1.14
C17	Danshen powder	Powder	Yunnan	220 g	Shengshi Hanfang 盛世汉方	Yunnan Manlei Trading Co., Ltd. 云南曼雷商贸有限公司	¥ 102.00	£ 11.63	£ 5.28

* 1st June, 2017, Chinese Yuan exchange rate to British pound = 0.11399 according to www.xe.com

*All the Chinese was translated to English by pinyin or the meaning of the words

Table 14 Chinese online store sample (C1 - C17) obtained via Taobao in 1st June 2017

Sample No.	Commercial Name	Form	Originality	Quantity (g)	Market Channel	Retailer	Cost VND	Cost in pound	Cost per 100g
V1	Đan Sâm	Dried root	China	100 g	TCM pharmacy/store	Phố Lãn Ông 32	VND 15,000.00	£0.53	£0.53
V2	Đan Sâm	Dried root	Viet Nam	100 g	TCM pharmacy/store	Phố Lãn Ông 69A	VND 20,000.00	£0.70	£0.70

V3	Đan Sâm	Dried root	China (imported through Vietnamese Lạng Sơn province)	100 g	TCM pharmacy/store	Phố Lãn Ông 28B	VND 15,000.00	£0.53	£0.53
V4	Đan Sâm	Dried root	N.A.	100 g	TCM pharmacy/store	Phố Lãn Ông 2	VND 30,000.00	£1.06	£1.06
V5	Đan Sâm	Dried root	China	100 g	TCM pharmacy/store	Phố Lãn Ông 36	VND 20,000.00	£0.70	£0.70
V6	Đan Sâm	Dried root	N.A.	100 g	TCM pharmacy/store	Phố Lãn Ông 30	VND 15,000.00	£0.53	£0.53
V7	Đan Sâm	Dried root	China	100 g	TCM pharmacy/store	Phố Lãn Ông 24	VND 15,000.00	£0.53	£0.53
V8	Đan Sâm	Dried root	China	100 g	TCM pharmacy/store	Phố Lãn Ông 8	VND 20,000.00	£0.70	£0.70
V9	Đan Sâm	Dried root	Viet Nam	100 g	TCM pharmacy/store	Phố Lãn Ông 38	VND 25,000.00	£0.88	£0.88
V10	Đan Sâm	Dried root	China	100 g	TCM pharmacy/store	Phố Lãn Ông 48	VND 20,000.00	£0.70	£0.70
V11	Đan Sâm	Dried root	China	100 g	TCM pharmacy/store	Phố Lãn Ông 33	VND 20,000.00	£0.70	£0.70
V12	Đan Sâm	Dried root	Viet Nam (highland areas such as Tam Đảo and plateau land, e.g. Ha Noi)	100 g	TCM pharmacy/store	Purchased through the internet (caythuocnam.com.vn)	VND 25,000.00	£0.88	£0.88
V13	Đan Sâm	Dried root	China	100 g	TCM pharmacy/store	P310 nhà 7, Tập thể ĐH Thủy Lợi, F. Trung Liệt, Q. Đống Đa	VND 30,000.00	£1.06	£1.06

V14	Đan Sâm	Dried root	N.A.	100 g	TCM pharmacy/store	N.A.	N.A	N.A	N.A
V15	Đan Sâm	Dried root	N.A.	100 g	TCM pharmacy/store	N.A.	N.A	N.A	N.A

* 27th July, 2016, Vietnamese Dong exchange rate to British pound = 0.0000339 according to www.xe.com

Table 15 Vietnamese sample (V1 - V15) list

Extract No.	Commercial Name	Originality	Quantity (g)	Market	Market Channel	Retailer	Other information	Manufacturer	Cost in pound	Cost per 100 g
E1	Danshen Sheng Foong Extract Pulveres	Taiwan	100 g	Hong Kong	Supplier	Sheng Foong Co., Ltd	GMP, contain starch and herb powder	Sheng Foong Co., Ltd	N.A.	N.A.
E2	Chuan Danshen Extract	Taiwan	100 g	Hong Kong	Supplier	Kaiser Pharmaceutical Co., Ltd	GMP	Kaiser Pharmaceutical Co., Ltd	N.A.	N.A.
E3	Chuang Song Zong Dan Tsan Granula Subtilae	Taiwan	100 g	Hong Kong	Supplier	Chuang Song-Zong Pharmaceutical Factory	GMP, contain starch and herb powder	Chuang Song-Zong Pharmaceutical Factory	N.A.	N.A.
E4	Salvia miltiorrhiza Extract powder	Taiwan	100 g	Hong Kong	Supplier	Sheng Chang Pharmaceutical Factory	GMP, contain starch and herb powder	Sheng Chang Pharmaceutical Factory	N.A.	N.A.
E5	Tan shen Herbal extract	Taiwan	100 g	Hong Kong	Supplier	Sun Ten Pharmaceutical Co., Ltd	GMP, contain starch and herb powder	Sun Ten Pharmaceutical Co., Ltd	N.A.	N.A.
E6	Fu Fang Danshen Pian Herbal extract	Taiwan	100 g	Hong Kong	Supplier	Sun Ten Pharmaceutical Co., Ltd	GMP, contain starch and herb powder	Sun Ten Pharmaceutical Co., Ltd	N.A.	N.A.

E7	MinTong Danshen	Taiwan	100 g	The U.K.	Internet	Vitamin World	GMP, contain starch and herb powder	Min Tong Pharmaceutical Co., Ltd	£ 24.19	£ 24.19
E8	Danshen extract	China	50 g	The U.K.	TCM pharmacy/ store	養元堂	GMP	Yangyuantang - 養元堂	£ 20.00	£ 40.00
E9	100 g concentrated powder	China	100 g	The U.K.	Internet	Jiangyin Tianjiang Pharmaceutical Co., Ltd.	GMP, contain starch	Jiangyin Tianjiang Pharmaceutical Co., Ltd.	£ 13.97	£ 13.97
E10	Danshen, Concentrated Powder	China	100 g	The U.K.	supplier	Mayway	N.A.	Unknown	£ 8.00	£ 8.00
E11	Salvia	China	100 g	Hong Kong	TCM pharmacy/ store	Skylight Pharmaceutical Co., Ltd	N.A.	Skylight Pharmaceutical Co., Ltd	N.A.	NA.
E12	Herbal powder (Qin dynasty)	N.A.	10 g	The U.K.	TCM pharmacy/ store	Natural Health Acupuncture Massage Herbs	N.A.	Unknown	£ 15.00	£ 150.00
E13	Danshen granule	China	100 g	The U.K.	Supplier	Donica	GMP, Drug: Extract= 1:10	Beijing Temages Pharmaceutical Co., Ltd	£ 14.42	£ 14.42
E14	Danshen Concentrated Extract	Taiwan	50 g	The U.K.	Supplier	Shizhen TCM UK Ltd.	GMP	Koda Pharmaceutical Co., Ltd	£ 20.00	£ 40.00
E15	*Salvia miltiorrhiza* extract	Taiwan	100 g	Hong Kong	TCM pharmacy/ store	Han-Fang Chinese Medicine Co. Ltd.	GMP, contain starch	Han-Fang Chinese Medicine Co. Ltd.	£ 18.93	£ 18.93
E16	*Salvia miltiorrhiza* extract	China	100 g	Hong Kong	TCM pharmacy/ store	Premier Concentrated Chinese Herbs (Hong Kong)	N.A.	Premier Concentrated Chinese Herbs (Hong Kong)	£ 10.60	£ 10.60
E17	*Salvia miltiorrhiza* extract	Shandong, China	200 * 2 g	Hong Kong	TCM pharmacy/ store	Sanjiu Medical &	N.A.	Sanjiu Medical &	£ 37.86	£ 9.47

						Pharmaceutical Co., Ltd		Pharmaceutical Co., Ltd		
E18	*Salvia miltiorrhiza* extract	China	200 g	Hong Kong	TCM pharmacy/ store	PuraPharm International (H.K.) Ltd	GMP	PuraPharm International (H.K.) Ltd	£ 32.56	£ 16.28
E19	Dan Shen Salvia extract	Taiwan	100 g	The U.K.	Internet	dullmeat	GMP, contain starch and herb powder	Sun Ten Pharmaceutical Co., Ltd	£ 12.50	£ 12.50

Table 16 Concentrated extract sample (E1 - E19) list

Sample No.	Commercial Name	Form	Originality	Quantity	Market Channel	Retailer	Cost	Cost per 100 g
UK1	Danshen pian	Tablet	China	80 tab	TCM pharmacy/store	Double-Crane Co. Ltd	£ 8.00	N.A.
UK2	Red sage tea	Aerial part	sourced from Albania	30 cups	Internet	Mountain Fresh	£ 10.99	£ 10.99
UK3	Red sage capsule	Capsule	Albania	100 * 250 mg	Internet	Mountain Fresh	£ 10.99	£ 43.96
UK4	Red sage non alcoholic tincture	Concentrated extract liquid	sourced from Albania	100 ml	Internet	Mountain Fresh	£ 11.99	N.A.
UK5	Red sage oil infusion	Oil	sourced from Albania	50 ml	Internet	Mountain Fresh	£ 9.99	N.A.
UK6	Red sage tincture	Concentrated extract liquid	sourced from Albania	100 ml	Internet	Mountain Fresh	£ 9.99	N.A.
UK7	Red sage tea	Aerial part	Albania	5* 50 g	Internet	Just Ingredients	£ 8.11	£ 4.24
UK8	Danshen	Dried root	Henan	100 g	TCM pharmacy/store	Everwell Chinese Medical Center - London high street	£ 10.00	£ 10.00
UK9	Danshen	Dried root	Henan	60 g	TCM pharmacy/store	Everwell Chinese Medical Center - London high street	£ 10.00	£ 16.67
UK10	100 g red sage	Dried root	N.A.	100 g	Internet	Purecapsules.co.uk	£ 4.97	£ 4.97

UK11	Herb Tea	Dried root	N.A.	100 g	Internet	Guangyingxiadelan	£ 8.50	£ 8.50
UK12	Wild *Salvia Miltiorrhiza* Tea	Dried root	N.A.	250 g	Internet	Zhang-specialty-shop	£ 17.99	£ 7.20
UK13	Salvia Miltiorrhiza Root	Powder	N.A.	100 g	Internet	dullmeat	£ 12.50	£ 12.50
UK14	*Salvia* Danshen	Capsule	N.A.	60 * 250 mg	Supplier - Internet	Purehealth	£ 6.00	£ 40.00
UK15	Danshen Fen, Raw Herb Powder	Powder	N.A.	500 g	Supplier - Internet	mayway	£ 8.00	£ 1.60
UK16	Danshen, Whole Herbs	Dried root	N.A.	500 g	Supplier - Internet	Mayway	£ 8.00	£ 1.60
UK17	*Salvia miltiorrhiza* Root WH Decocted 1:3 TR	Concentrated extract liquid	N.A.	500 ml	Supplier - Internet	Avicenna	£ 9.75	N.A.
UK18	Danshen	Dried root	N.A.	50 g	TCM pharmacy/store	GinSen	£ 10.00	£ 20.00
UK19	Danshen	Dried root	N.A.	50 g	TCM pharmacy/store	Chiltern Nature Health	£ 10.00	£ 20.00
UK20	Danshen	Dried root	N.A.	50 g	TCM pharmacy/store	San Ling	£ 2.50	£ 5.00
UK21	Danshen	Dried root	N.A.	/	TCM pharmacy/store	London high street	£ 10.00	£ 10.00
UK22	Danshen capsule	Capsule	N.A.	60 * 250 mg	TCM pharmacy/store	AcuMedic	£ 20.00	£ 133.33
UK23	Danshen	Dried root	N.A.	100 g	TCM pharmacy/store	London high street	£ 10.00	£ 10.00
UK24	Danshen	Dried root	N.A.	100 g	TCM pharmacy/store	London high street	£ 12.00	£ 12.00

Table 17 UK sample (UK1 - UK23) list

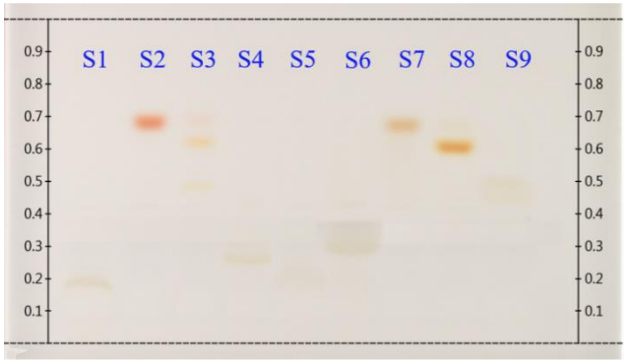
5.2 The chemical variations of Danshen products

HPTLC analysis of Danshen secondary metabolites

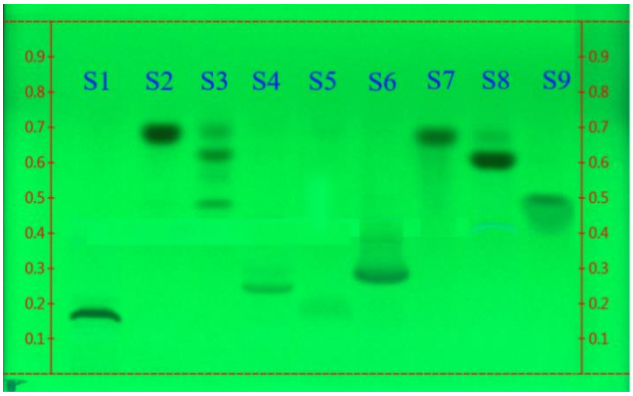
Chemical standards and their physical properties are shown in Figure 6. The structures of the chemical standards are shown in Figure 4. Regarding the water-soluble chemical standards, though not all showed any colour under white light, S1, S4, S6 and S9 showed clear bands in 254 and fluorescent bands in 366 nm wavelength. S5 did not show clear bands in 254 or 366 nm, but there were some traces in 254 and 366 nm wavelength. Overall, water-soluble chemicals were distinguishable, and most of them had high resolution.

Regarding lipid-soluble chemical standards, all S2, S3, S7 and S8 showed red or orange colour bands under white light and clear bands in 254 and 366 nm wavelength. The R_f of S2 (0.69) and S7 (0.68) were close but can be differentiated by the colour of the band. The R_f of S3 (0.615) and S8 (0.61) were almost the same and showed similar colours under white light. Reference is required to distinguish these two compounds due to their similarity in this solvent system. In general, this solvent system might not be ideal to differentiate all the tanshinones, but it is possible to quantify the totality of tanshinones. All the standards R_f value match with the results from the literature (Luo and Ji, 1989; Shuai Sun, 2014; Azadniya and Morlock, 2018).

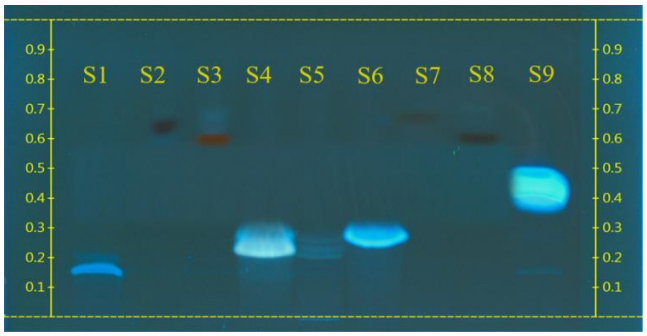
a)



b)



c)



d)

	Chemical Name	Molecular formula	CAS No.	Color in DMSO	R_f	Solubility in DMSO (mg/ml)	Molecular Weight (g/mol)
S1	Salvianolic acid B	$C_{36}H_{30}O_{16}$	121521-90-2	Colorless	0.19	100	718.61
S2	Tanshinone IIA	$C_{19}H_{18}O_3$	568-72-9	Red	0.69	25	294.34
S3	Cryptotanshinone	$C_{19}H_{20}O_3$	35825-57-1	Orange	0.61	12	296.36

S4	Salvianolic acid A	$C_{26}H_{22}O_{10}$	96574-01-5	Pale yellow	0.24	125	494.5
S5	Danshensu	$C_9H_{10}O_5$	76822-21-4	Colorless	0.21	44	198.174
S6	Rosmarinic Acid	$C_{18}H_{16}O_8$	20283-92-5	Colorless	0.26	25	360.318
S7	Tanshinone I	$C_{18}H_{12}O_3$	568-73-0	Red	0.68	23	276.291
S8	Dihydrotanshinone	$C_{18}H_{14}O_3$	87205-99-0	Orange	0.61	5	278.3
S9	Caffeic acid	$C_9H_8O_4$	331-39-5	Colorless	0.49	7	180.16

* The solubility was based on the datasheet from the corresponding chemical supplier

** The chemical standard certificates were attached in supplementary

Figure 6 Chemical standards related to Danshen metabolites (S1 – S9) HPTLC results under the wavelength a) white light and b) 254 and c) 366 nm developed with solvent system (toluene: chloroform: ethyl acetate: methanol: formic acid (v/v) = 2: 3: 4: 0.2: 2) d) the physical properties of chemical standards (S1-S9)

The HPTLC results of authenticated Danshen samples showed high content levels of salvianolic acids and tanshinones, especially compared to the market samples. A6 and A17 (*Salvia przewalskii* Maxim. samples), even though they were cultivated from different countries (the U.S.A. and China), showed tanshinones including tanshinone IIA and crytotanshinone. Only trace amounts of salvianolic acid B and rosmarinic acid were found, and both contained characteristic bands from R_f 0.4 to 0.65 under 254 or 366 nm wavelength. These characteristics could be principal features of *Salvia przewalskii* Maxim., but further detailed and structured research is needed.

A16 (*Salvia bowleyana* Dunn.) showed a comparable amount of salvianolic acid B and rosmarinic acid, but the content of tanshinone IIA and crytotanshinone were relatively lower than other *Salvia miltiorrhiza* samples. However, no characteristic bands were found to differentiate *Salvia miltiorrhiza* and *bowleyana* under white light, 254 nm or 366 nm wavelength. Hence, due to the chemical similarity between these two

species, it was difficult to acknowledge the substitution of *Salvia bowleyana* in the drug forms other than dried roots under HPTLC.

A1 – A5 were the authenticated cultivated in different states of the U.S.A. The chemical profiles were similar, and no distinctive bands were found. A4 had slightly higher contents of tanshinones, but it is not significant. A3 and A5 were harvested in 2007 and 2009 respectively, but no significant differences in salvianolic acid B nor tanshinone IIA were found compared to A1, A2 and A4 which were harvested in recent year, 2016. Also, the level of caffeic acid and its derivatives, for instance, danshensu and rosmarinic acid, were similar. It indicated that the time of storage might not be an essential factor for Danshen chemical constituents' deterioration.

A8 – A12 samples were the same source of material but processed differently from organic farming. A11 and A12 were prepared as imitating farmers traditional practice, "sweating" (fahan). A11 was uncut and put into the oven for 30 °C to simulate "sweating" during summertime and A9 was the control because it was cut and spread evenly. Unfortunately, the simulation of autumn time, which was shade dried uncut failed due to fungal infection, but A12 was cut and air-conditioned dried under shade. A8 was put into the oven at 100°C first 30 minutes and 120°C for 30 minutes afterwards. This was the standard Danshen root drying method. A10 was a freeze-dried sample. Among all the samples, A9. A10 and A12 had the highest level of tanshinone IIA and crytotanshinone. This might be due to organic cultivation or processing method, but more systematic investigation is needed before drawing conclusion.

A11 had significantly lower contents in tanshinones but no significant difference in salvianolic acid B compared to A9. A8, A9, A10 and A12 were similar to each other, but A9 and A10 showed slightly higher content of tanshinone IIA and crytotanshinone.

A13 and A14 were genetically identical and cultivated and harvested in the same method but planted in a different year. Although most of the bands were identical and showed a similar level of content, A14 had a characteristic band in R_f value = 0.6 under 366 nm wavelength. It highlighted that genetic identity, cultivation and processing methods would not hundred per cent secure the quality of herbal materials.

A7 was the source material of Danshen extract sample which was identified as *Salvia miltiorrhiza* by DNA barcoding from a TCM pharmaceutical. Clear bands of tanshinone IIA crytotanshinone, rosmarinic acid and salvianolic acid B were found but the amounts of the chemicals were not as much as other authenticated *Salvia miltiorrhiza* samples, for instance, A15 which was the reference materials of Danshen provided from NIFDC, China.

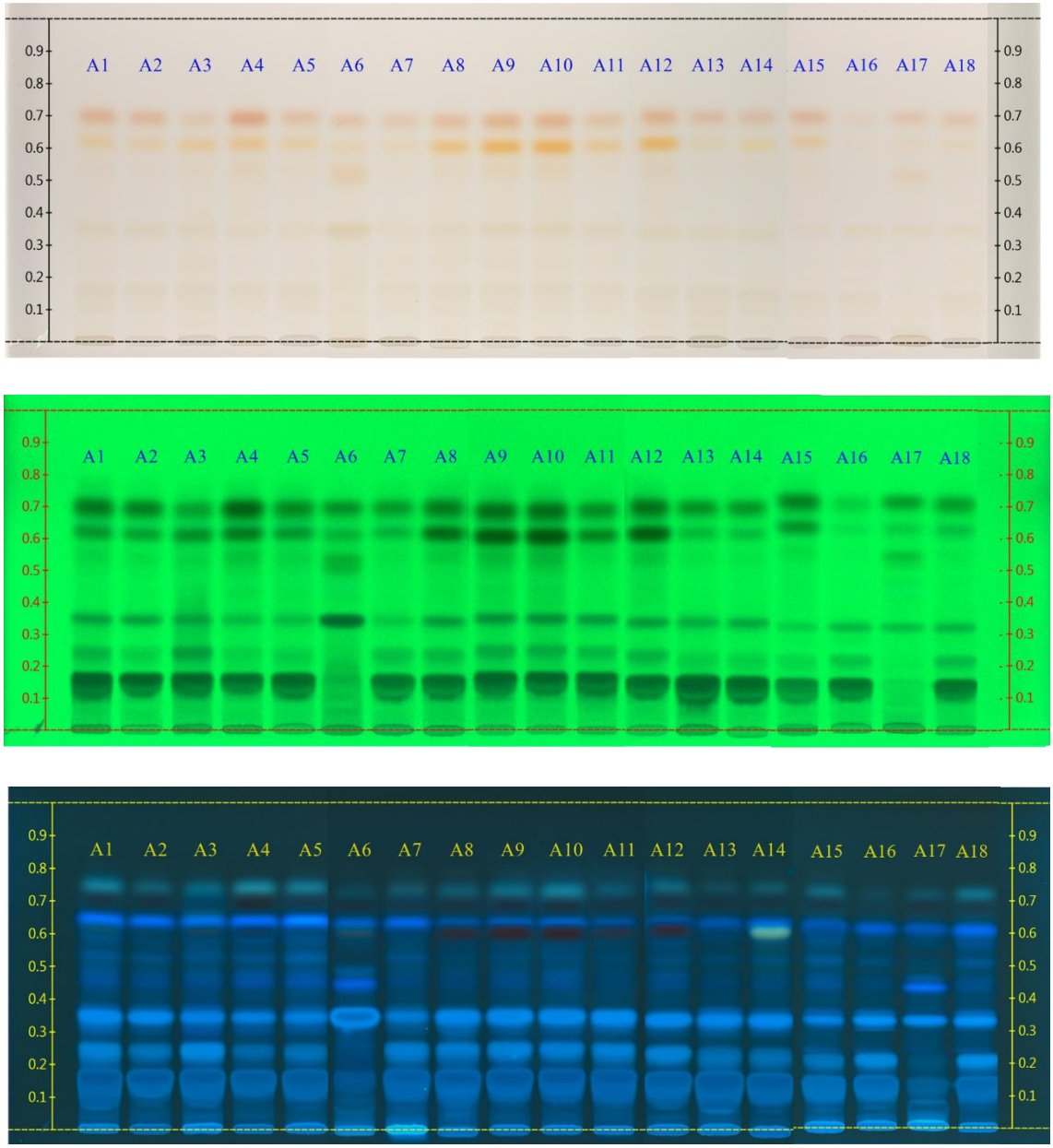


Figure 7 Authenticated Danshen samples (A1 – A18) including Salvia miltiorrhiza Bunge. *Salvia bowleyana* Dunn. *and Salvia przewalskii* Maxim. *HPTLC results under the wavelength a) normal white light and b) 254 and c) 366 nm developed with solvent system (toluene: chloroform: ethyl acetate: methanol: formic acid (v/v) = 2: 3: 4: 0.2: 2)*

Figure 8 shows the HPTLC profiles of nineteen Danshen concentrate extracts samples from the pharmaceutical companies. In the result, E12 was very highly likely to be an adulterant. It was suspected to be prepared with the absence of Danshen root materials due to the lack of characteristic caffeic acid derivatives and tanshinones. On the other hand, nine out of nineteen samples (E1, E7, E8, E9, E10, E11, E16, E17 and E18) showed undetectable levels of tanshinone contents including tanshinone IIA and crytotanshinone; five samples (E1, E7, E8, E9 and E16) showed low levels of

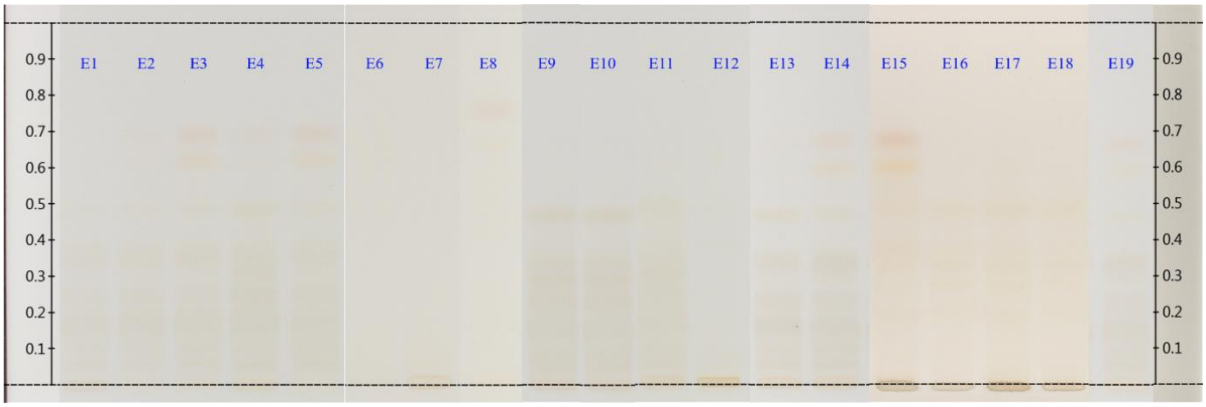
danshensu. All the samples apart from E12 contained salvianolic acid B and rosmarinic acid, but levels were varied. Only E13 might contain salvianolic acid A. In other words, all the samples from Taiwan apart from E1 and E7 (eight out of ten samples) contained both tanshinones and salvianolic acids; the quality of the samples from China or unknown source was not consistent, and most of them (eight out of nine samples) did not contain lipid-soluble metabolites of Danshen or in a low content. It also indicated that the quality of products from TCM pharmaceutical companies was varied.

Similar compositions between different samples indicated they might have a similar source of materials and processing. E2, E3, E5 and E6 showed similar chemical compositions in HPTLC. These four were manufactured in Taiwan, and according to supplier information, they all contained root powder and starch as the excipient and stabiliser. Starch is a common excipient used in herbal extract during the drying status. It usually provides higher stability and consistency with low moisture content and hygroscopic drugs, but the bioavailability and bioequivalence might be influenced depending on the chemical constituents of the plant extract (Bernal, Aragón and Baena, 2016; Santana *et al.*, 2018). Only some of the samples added Danshen root powder as an additional ingredient. One of the advantages is to reduce the waste of materials generated from the extraction status, and the whole process becomes more environmentally friendly. It also reduces the amount of excipient, in this case, starch and minimises its influence on bioavailability and bioequivalence. Moreover, tanshinones are lipid-soluble and not easy to extract through aqueous extraction. It might help to preserve the lipophilic content of Danshen, resulting in the higher content of tanshinones in the samples added Danshen root powder.

E16, E17 and E18 were also quite similar in HPTLC. These samples had a distinctive band in R_f 0.3 under 254 and 366 nm wavelength. The unknown compound is potentially hydrophilic due to the low R_f value and the R_f value between rosmarinic acid

and caffeic acid, and rosmarinic acid is a caffeic acid dimer, respectively. Therefore, it could be one of the characteristic caffeic acid dimers or monomers in *Salvia miltiorrhiza* due to its affinity preference towards stationary phase. Although E15 also had the same band, it had a higher content of tanshinones and salvianolic acid B. Hence, the supply chain of sample E15 should be different from samples E16, E17 and E18.

a)



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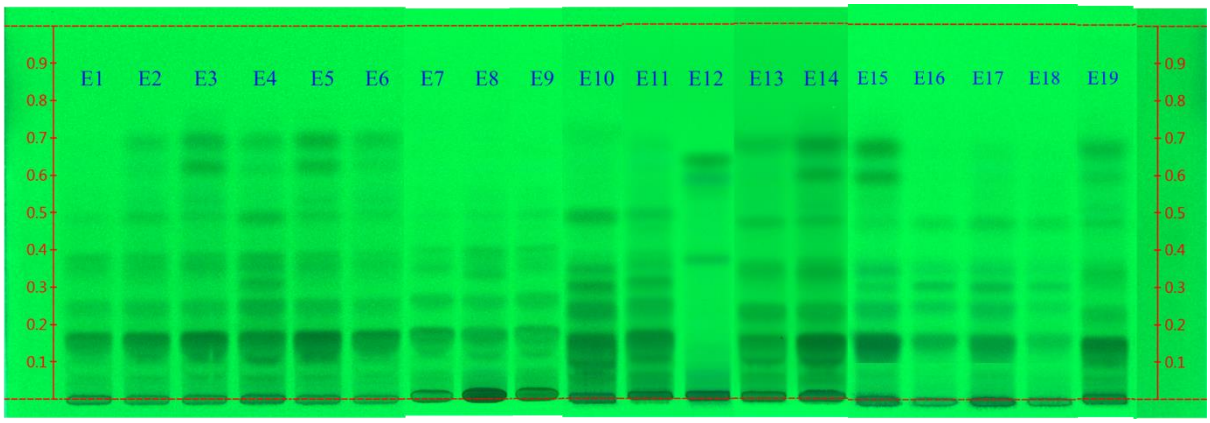

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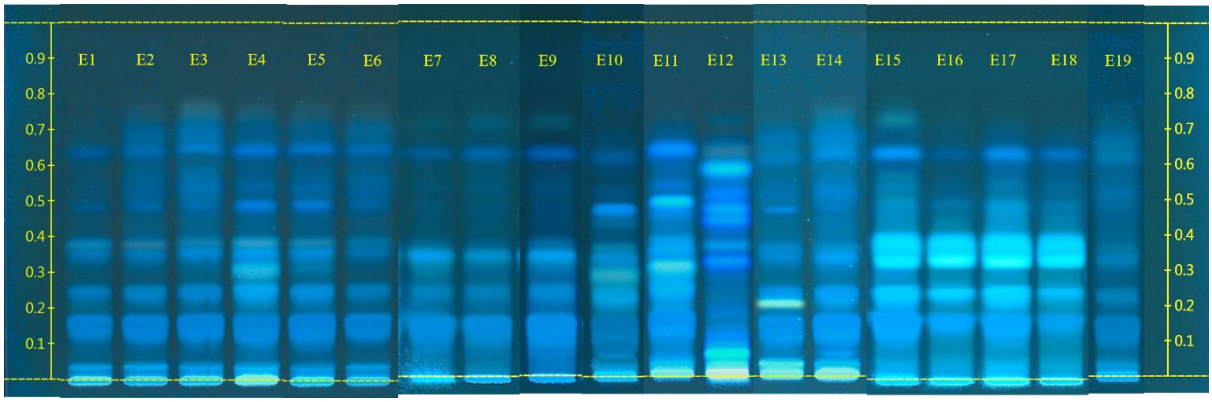


Figure 8 Danshen-derived extract samples (E1 – E19) HPTLC results under the wavelength a) normal white light and b) 254 and c) 366 nm developed with solvent system (toluene: chloroform: ethyl acetate: methanol: formic acid (v/v) = 2: 3: 4: 0.2: 2)

Figure 9, Figure 10 and Figure *11* illustrate the HPTLC chemical profiles of the samples obtained from Vietnam herbal market and Chinese online stores and the UK retailers. In the results, the samples from Vietnam and the samples from Chinese online stores showed high chemical constituent similarities respectively, while the samples from the UK retailers showed high inconsistency. Vietnamese samples showed four to five clear bands under 254 nm and similar chemical profile under 366 nm. All the samples showed a high level of salvianolic acid B, and V1, V8, V9, V10 and V12 showed slightly higher levels of tanshinone contents, including tanshinone IIA.

The samples from Chinese online stores showed more apparent bands in tanshinone area than Vietnam herbal market samples, especially the samples in powder form. It was also found that the quality of the raw dried roots products in Chinese online store market was more inconsistent than the ready-blended powder form in tanshinones or salvianolic acids. One of the challenges in the quality of herbal medicine is adulteration, and it is easier to add foreign materials or substitutes in powder form due to impractical or inefficient taxonomic measurement (Liang, Xie and Chan, 2004; Pan *et al.*, 2013;

Augustin-Jean, 2016). However, the result of the samples from Chinese online stores did not show the problem above.

5.3 C2, C3, C5 and C6 showed the highest amount of tanshinones and salvianolic acids contents among the samples in powder form; and C8 and C13 were the samples contained the highest amount of tanshinones and salvianolic acids content among the raw dried root form. According to 5.1VI.5.1 Market Study

In order to understand the supply chain of Danshen products from different perspectives, anonymous semi-structured interview, and fieldwork using non-participant observation and interviews were conducted to obtain personal experience and expert opinion on Danshen processing. Seven key informants within the supply chains of Danshen products, including a farmer, TCM pharmaceutical representatives, a quality-control assessor, a governmental department representative, a supplier representative, and a TCM pharmacist and doctor, were included. The basic background of the interviewees (Table 22) excludes personal information for the sake of protecting interviewees. The fieldwork was also conducted in a non-invasive, non-participant observational method in order to gain trust between the farmers or practitioners and the conductor. In order to obtain a better overview of the supply chain, some literature reviews and governmental reports have been included in this section.

The practice of farmer in the Danshen supply chain – a case study in Taiwan

A case study was made on a Taiwan organic TCM materials company with thirty staff working. The business of the company includes cultivation and primary processing of organic TCM materials, and TCM related organic food supplements or food. The cultivation of TCM materials on the site is done through contract farming. The main

crops are Japanese angelica (*Angelica acutiloba* (S. *et* Z.) Kitag.) and Danshen. Japanese angelica is considered as a kind of TCM material in Taiwan because it was used to substitute Chinese angelica. Other TCM materials are in small production, for instance, baizhi (*Angelica dahurica* Benth. et Hook), baiji (*Bletilla striata* (Thunb.) Reichb.f.) and huangqin (*Scutellaria baicalnsis* Geprgi). Due to organic farming, all the materials used during the cultivation, including soil, pesticides and fertilisers, need to be verified by the Council of Agriculture and obtain organic certificates to make sure they are not contaminated and organic.

The company is supported by the governmental department – the Council of Agriculture. The collaboration was initiated because of the promotion of an organic TCM industry of Taiwan. The research department of the Council of Agriculture was responsible for the selection and verification of TCM species-specific for Taiwanese soil while the farmers were responsible for providing the land and trying to cultivate organic TCM. Danshen was one of the selected species. The first batch of the seeds was introduced from Shandong, China and was authenticated by an experienced botanist professor in morphology, chemistry and DNA barcoding. Danshen was successfully cultivated in Taiwan highland, and the contents of the biomarkers including tanshinone IIA and salvianolic acid B were high. The project was considered very successful in the first cultivation, and the farmers decided to develop the project into a business and continue the collaboration with the government. The council is now responsible for the research, education, promotion and development of herbal materials cultivation whereas the company cultivates organic other herbal materials and demonstrates to other farmers. All the services provided by the council are free to the farmers. They also collaborate with universities researching the pharmacology and chemical quality of Danshen.

The cycle of the business was confirmed through the interviews and first-person non-participant observation. The start of the cultivation of Danshen is preparation and fertilisation of the land. It usually starts in August after harvesting the crops. The soil in the land is overturned at least two times in two weeks. All the plants on the land are removed mechanically, and the land is covered by black thick nonwoven fabric during the process. The land is sprayed with the organic fertiliser evenly, and the farmers mix with the soil by overturning. The soil is then harrowed to make the clods and other plant residues finer. The field is prepared with fertile sandy soil and proper water drainage. After flattening the land, the farmers level up the soil into one-meter width and thirty-centimetre height to prepare the seedling position.

The Council of Agriculture prepares the seedlings for the farmers in October. The seeds normally germinate a week after seeding, and the seedlings are ready to transplant after the plant has grown four to six leaves. The farmers cut open the fabric covering the land and transplant the seedlings in order to control the weeds. Additional fertiliser is applied four months after the transplant of seedlings. The pest problem of Danshen in Taiwan does not affect the production much; therefore, they do not need to apply any pesticides, but they have guidelines for a different potential treatment for different pests. The fertilisers and pesticides are sourced from organic certificated companies. While Danshen is growing, the farmers also need to be aware of the status of weeds, plant health and moisture of the soil. From March to June, the staff from the council collects the seeds from the field. This seed picking has to be done continuously and manually due to the indeterminate inflorescence flowering of Danshen. The collected seeds are stored in 40 ± 10 % humidity under 4 ± 2 °C

The best harvesting time would be between nine to twelve months after seeding. In August, farmers use plant excavators to harvest and remove the soil from the roots manually. They do not wait until October because the quality of the roots would worsen

after flowering. The harvested plants are separated into three parts: Aerial parts, rhizomes and roots. The aerial parts are processed to make fertilisers. First, the rhizomes and roots are briefly washed, then dried and cut into slices for selling as food supplement directly. The roots are graded by the size of the main roots, lateral roots and fibrous roots, and sold to restaurants and biotechnology companies. At the time of the study, the price was 550 NT per 600 grams for the dried materials and 550 NT per 3 kg for the fresh materials, and the total cost of production would be half of the price approximately. The drying method depends on the weather and demand. If the weather is not suitable for drying or supplier requests it urgently, oven drying will be selected; otherwise, it is usually dried under shade, which usually takes half a day for the fresh roots.

In order to maximise the profit margin, the oversupply of Danshen is mixed with other self-cultivated TCM materials to derive different types of food supplements, like TCM tea bags, decoction piece and chicken essence, sold to individual customers directly. The quality of Danshen roots is monitored by the research teams of the university, the council and the organic certification body. After processing all Danshen roots are stored under 4 °C before selling to the market. Fresh roots will be stored up to from three days to a week. The transportation of products will be outsourced to other home delivery companies.

The role of TCM pharmaceutical in the company supply chain

The main business for TCM pharmaceutical companies is the concentrated extracts. Its local target customers are hospitals and some distributors, and only a small portion of customers are individual clinics, but usually, the local clinics would purchase their products through a distributor. The reason is that the acceptance of the concentrated extracts in China, or Taiwan is not adequate in the local TCM clinics. Most of the

pharmacists prefer the traditional decoctions directly derived from dried herbs. In contrast, international target customers are mainly distributors who would be around 80%. It is because the import of raw dried herbal materials is restricted in the U.K., and the TCM concentrated extract is the only possible alternative. Although Danshen is considered to be the top ten of the most demanding TCM single herb extracts, there are usually more than four hundred kinds of extracts in a typical TCM pharmaceutical company. Danshen products only seize a tiny portion of their entire business.

Some companies would also provide herbal materials to the TCM distributors because they have already had some partnerships or contracts with specific farmers and TCM suppliers in China for the demand of herbal materials for the extracts. The contracts or partnerships include cultivation and wild harvesting, though the supplier has direct management to the farmers. Therefore, the supply of herbal materials is relatively stable, and the usual storage time for Danshen herbal materials is from six months to a year. Providing herbal material supply service to the distributors will increase the quantity of the order, so it is beneficial for them to increase bargaining power with the suppliers. The collaborative relationship with the supplier would be stronger, and the cost of manufacturing TCM concentrated extracts would be lower. Also, TCM pharmaceutical companies usually have spacious warehouses to store extra materials. Selling herbal materials to the distributors also maintain a consistent flow of the stock and guarantee that the materials they have or use are not “expired”. On the other hand, due to the series of quality assessments and quality control during the process of herbal material selection from the pharmaceutical companies including chemical tests, TCM distributors benefit from obtaining verified “good quality” materials by the pharmaceutical companies.

The materials from China usually need further selection and processing, including cutting and grinding before extraction and concentration. The herbal materials with poor

quality would be sent back to the corresponding supplier for each batch. The extraction and concentration methods vary according to materials, but the common drying method used in the pharmaceutical industry is fluid bed drying method. During this process, apart from adding corresponding extract(s) and starch and fibres to combine as crude granules, some of the TCM pharmaceutical companies would add herbal pieces as one of the excipients in some specific TCM extracts, for example, Danshen, for its consistency and better chemical composition. The crude drug granules are screened into suitable size, and the quality is assessed. The quality criteria typically follow the ChP, but some herbal materials would have additional criteria in some pharmaceutical companies, for instance, the glycoside content of Danshen might be added. The process of manufacturing TCM extract was restricted by law in Taiwan that it cannot be subcontracted or outsourced; therefore, all the pharmaceutical companies have to control every part of the procedures even though there are differences between the quality control for the international market and local market. Most of the staff would obtain a higher educational degree in these quality assessments and quality control. After the process above, the product would be packaged and have a final quality assessment before it goes to the customer.

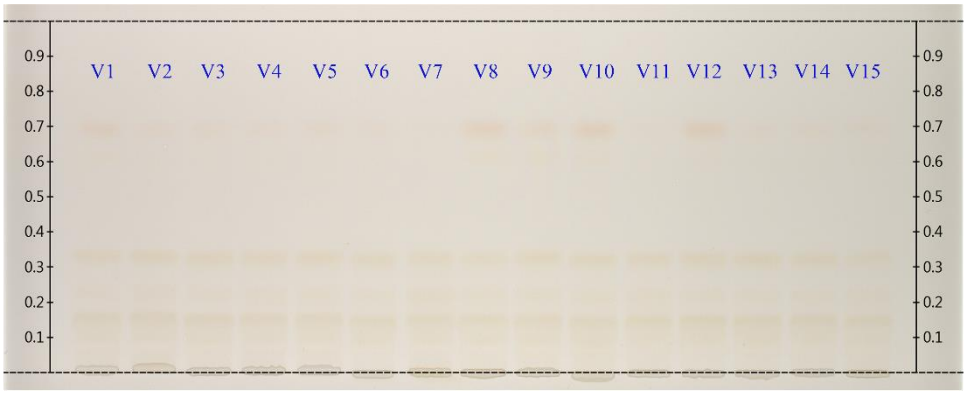
The variety of Danshen products in the market, in other words, one out of seven samples obtained from individual traders was of relatively better quality while five out of ten samples collected from registered traders was of better quality. One of the explanations is that those registered traders were larger companies which potentially acquire quality control technicians who have better herbal knowledge or quality analysis for the selection of herbal supply (Booker, Johnston and Heinrich, 2012).

The twenty-four samples obtained from the U.K. showed a diverse quality of Danshen products. Fourteen of these samples were in different product forms purchased from eight different UK online retailers or suppliers. And of these, four samples (UK3,

UK4, UK6 and UK7) were highly likely to be adulterated due to entirely different chemical profiles from Danshen and lack of its characteristic chemicals. One sample (UK5) showed absolutely nothing under white light, 254 nm or 366 nm wavelength. All of these adulterated samples were obtained from two different companies and purchased via the internet. The result from the UK online market was different from the Chinese online market.

Overall, there was a significant difference in tanshinones between the authenticated samples and market samples. Only four out of seventy-five samples, including concentrated extracts and dried roots, were comparable in tanshinones with authenticated samples.

a)



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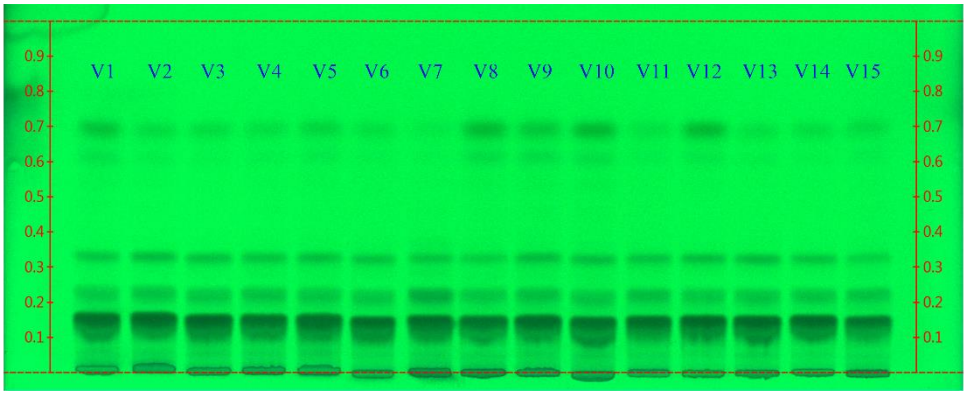

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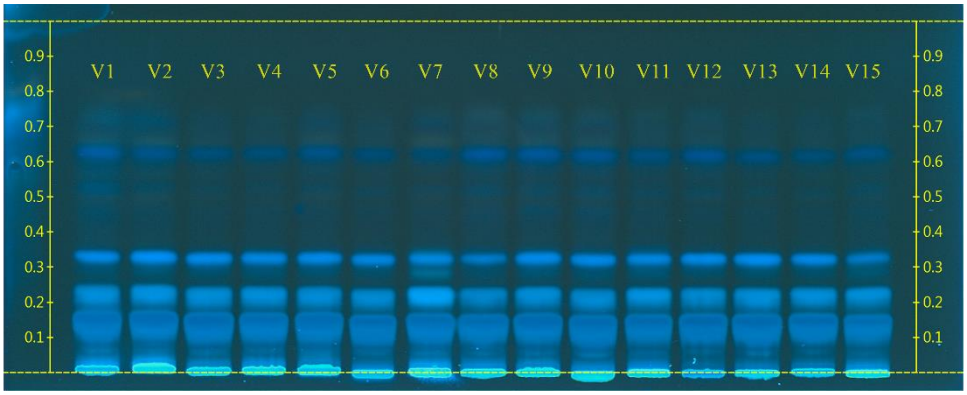
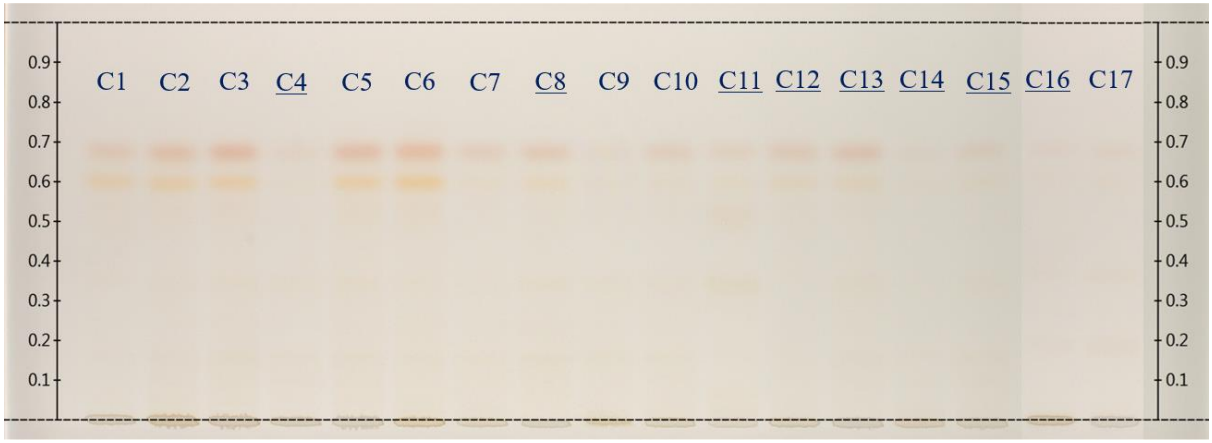


Figure 9 Danshen-derived extract samples (V1 – V15) HPTLC results under the wavelength a) normal white light and b) 254 and c) 366 nm developed with solvent system (toluene: chloroform: ethyl acetate: methanol: formic acid (v/v) = 2: 3: 4: 0.2: 2)

a)



b)

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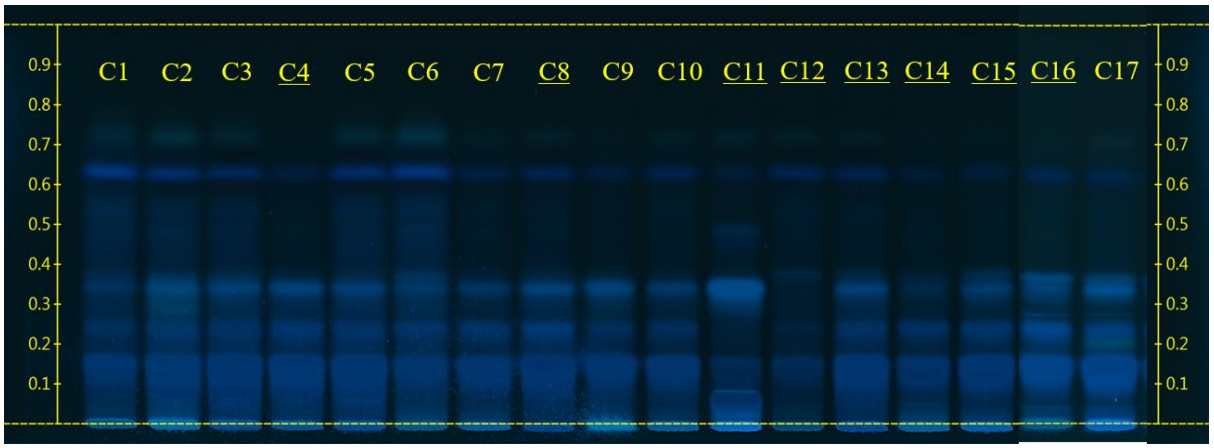
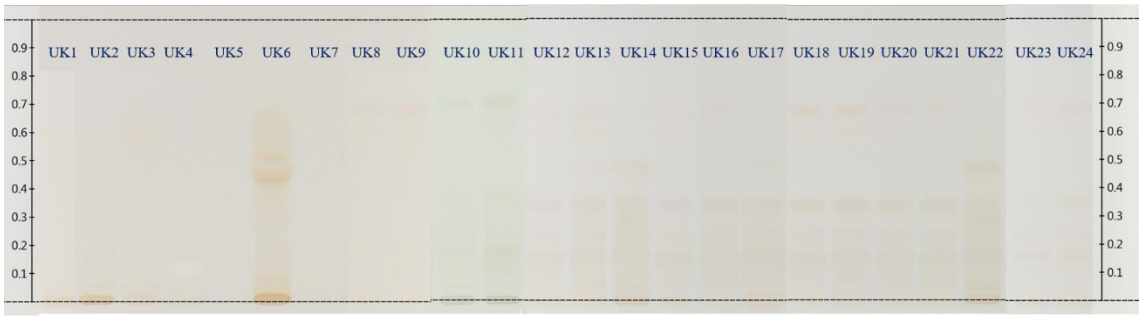


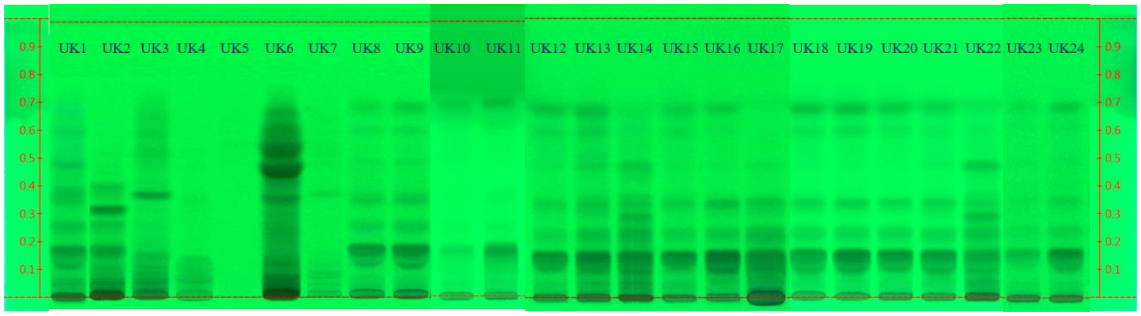
* the underlined samples represent it sold as raw material roots as form by the retailer or supplier; others were sold as ground form.

Figure 10 Danshen-derived extract samples (C1 – C19) HPTLC results under the wavelength a) normal white light and b) 254 and c) 366 nm developed with solvent system (toluene: chloroform: ethyl acetate: methanol: formic acid (v/v) = 2: 3: 4: 0.2: 2)

a)



b)



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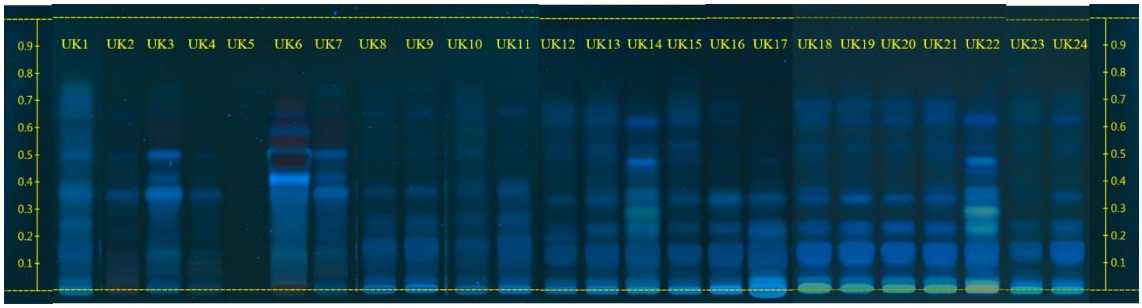


Figure 11 *Danshen-derived extract samples (UK1 – UK24) HPTLC results under the wavelength a) normal white light and b) 254 and c) 366 nm developed with solvent system (toluene: chloroform: ethyl acetate: methanol: formic acid (v/v) = 2: 3: 4: 0.2: 2)*

Trace metals analysis

In order to evaluate the level of the heavy metal elements in the Danshen samples, microwave digestion and ICP-OES were used to analyse the elemental difference between the samples. The results of the trace metals analysis (Figure 12, Figure 13, Figure 14 and Figure 15) showed a higher chance of getting contamination from the Chinese online store samples and concentrated extracts. Due to feasibility, the sample size was limited to sixty-one samples, including thirteen authenticated samples, fifteen Vietnamese samples, seventeen Chinese online store samples, and sixteen concentrated extracts. A7 was the raw material of E5 the finished concentrated extract, whereas E6 was the multi herbs concentrated extract contained Danshen, and E19 was as same as E5 but from a different supplier only. Therefore, E6 and E19 were not included in the

trace metals analysis. E12 was excluded because the acid digestion during the analysis was over-reactive.

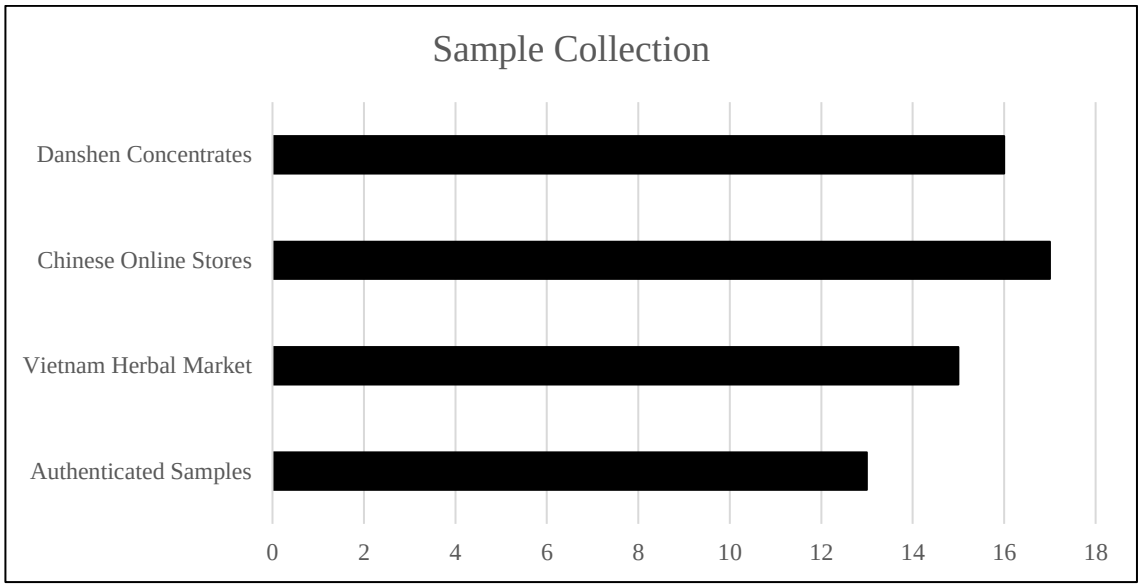


Table 18 Sample statistic of trace metals analysis

Inorganic arsenic can be easily found in groundwater; therefore, herbal materials might be contaminated via washing after harvesting and watering during farming. Overdose of inorganic arsenic leads to gastrointestinal disorders such as severe vomiting, and disturbance of blood circulation and central nervous system. Chronic intake excessive arsenic also increases the risk of lung, bladder, kidney, and skin cancer (Järup, 2003; Mahurpawar Govt, 2015; Yang *et al.*, 2018). From Figure 12, although all the samples were under the limits from ChP or other pharmacopoeias (less than 2 mg/kg), only six samples from authenticated, Vietnam and individual samples obtain detectable arsenic content and most of them were lower than 0.2 mg/kg, whereas only three samples from Chinese online store samples and concentrated extracts had undetectable As. The highest As content was from E17, which had 1.33 mg/kg.

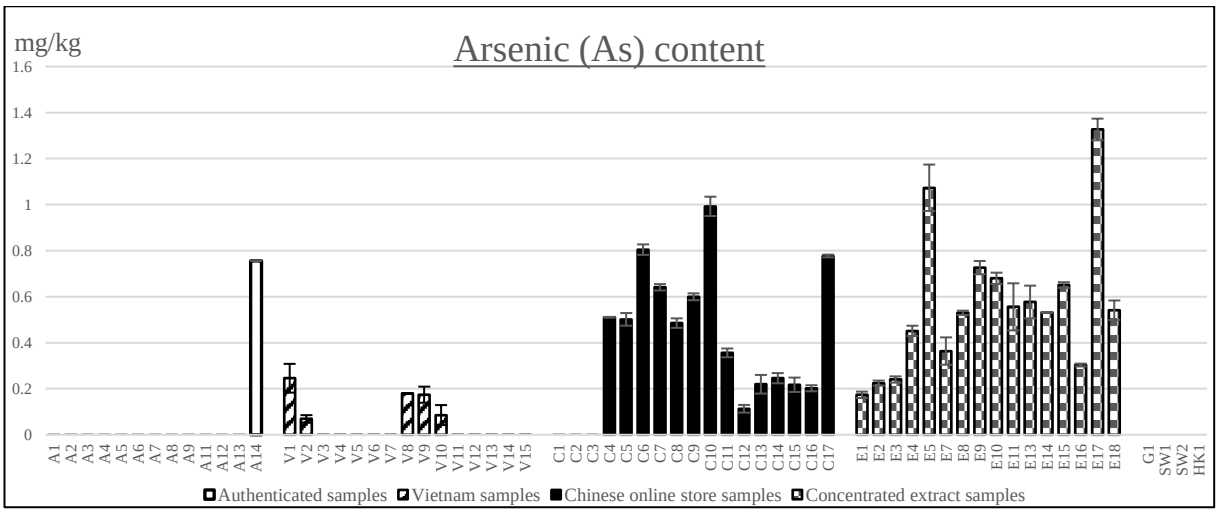


Figure 12 Arsenic content of the samples of authenticated (A1-A14), Vietnam (V1-V15), Chinese online stores (C1-C17) and concentrated extracts (E1-E5, E7-E11 and E13-E18) as well as some individual samples (G1, SW1, SW2 and HK1)

Cadmium can be found in household and industrial wastes as well as fertilisers on agriculture. Hence, sewage sludge to farmland may contaminate the soils and the medicinal crops grown for human consumption. Cadmium content is unlikely to be reduced by degradation in the environment; therefore, it accumulates via the food chain. Intaking cadmium may lead to irreversible kidney damage or more serious problems like renal failure. Chronic cadmium exposure, even at a low level, may lead to bone diseases like osteomalacia and osteoporosis (Järup, 2003; Mahurpawar Govt, 2015; Yang *et al.*, 2018). From Figure 13, six out of sixty-one samples were over the limits of ChP or USP, 0.3 mg/kg; and of which one sample was over the limits of BP or Ph. Eur, 1 mg/kg. All the authenticated and Vietnamese samples were under detectable levels.

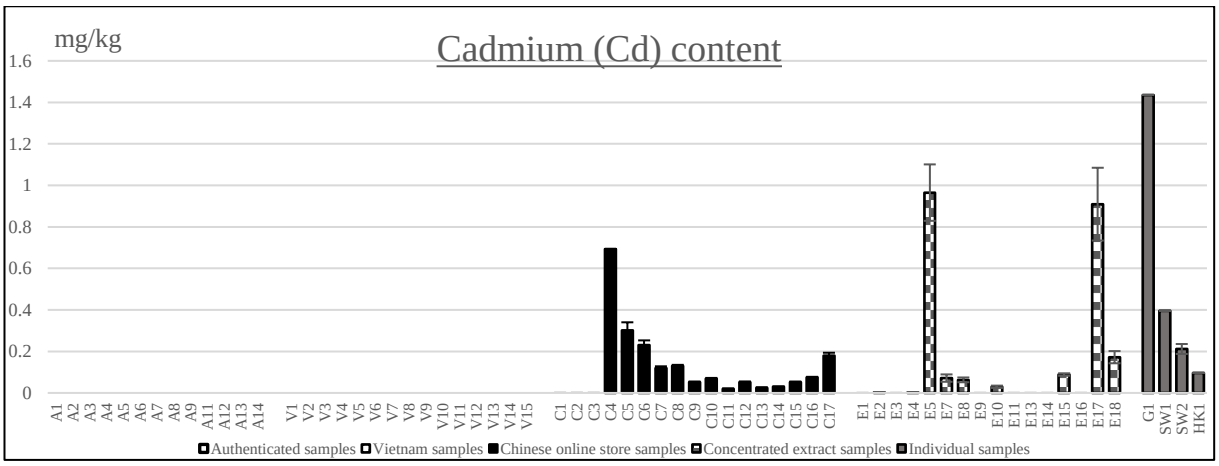


Figure 13 Cadmium content of the samples of authenticated (A1-A14), Vietnam (V1-V15), Chinese online stores (C1-C17) and concentrated extracts (E1-E5, E7-E11 and E13-E18) as well as some individual samples (G1, SW1, SW2 and HK1)

Copper is one of the heavy metals that commonly occurs in our living environment. The most common occurrence includes mining and smelting of copper, wires and pipes production, and fossil fuel combustion. A high level of copper in water can be caused by leaching of copper from pipes or agriculture use for the treatments of plant diseases and water pollution. Intaking copper is necessary for humans, and it maintains the normal metabolic functions and enzymatic activities. Deficiency of copper in humans can lead to anaemia, a decrease of white blood cells, osteoporosis in infants and children as well as skeletal problems. However, excessive copper intake also leads to temporary gastrointestinal distress, liver toxicity and anaemia due to red blood cell destruction. Chronic high-level exposure also leads to liver and kidney damage (Järup, 2003; Mahurpawar Govt, 2015; Yang *et al.*, 2018). Currently, only ChP limits the copper content of *Salvia miltiorrhiza* to 20 mg/kg. From Figure 14, all the samples were within the criteria of ChP, and the highest copper content was C11 with 19 mg/kg. The average copper content of the raw material and the powdered material samples (9.64 mg/kg) were at least eight times higher than of the concentrated extracts (1.12 mg/kg). Potentially, the extraction process of *Salvia miltiorrhiza* would not dissolve the copper in the materials, but the heavy metal analysis extraction did.

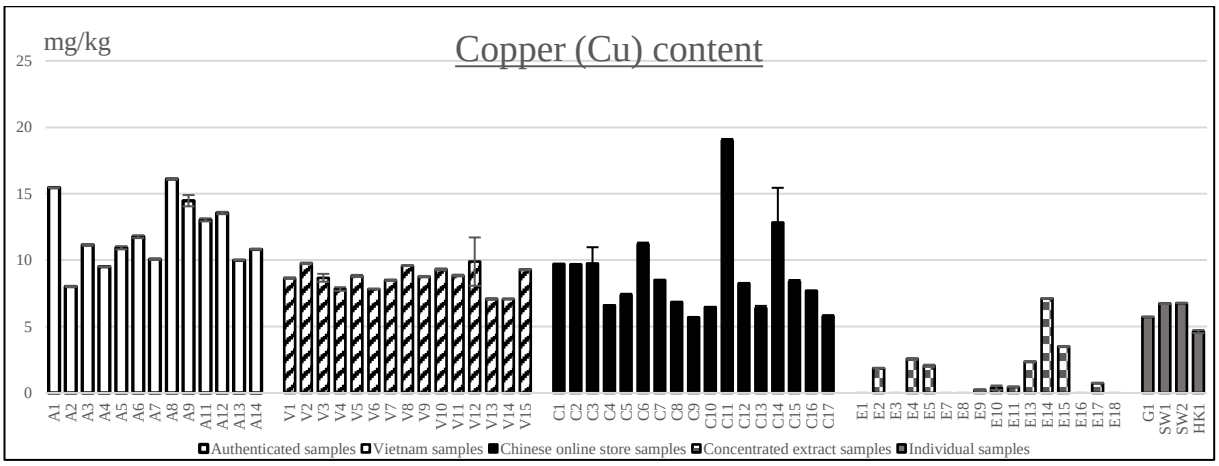


Figure 14 Copper content of the samples of authenticated (A1-A14), Vietnam (V1-V15), Chinese online stores (C1-C17) and concentrated extracts (E1-E5, E7-E11 and E13-E18) as well as some individual samples (G1, SW1, SW2 and HK1)

Lead contamination in herbal materials may come from polluted water or leached storage materials, hence cultivation and processing both equally essential to prevent the contamination. More than half of the lead emission comes from petrol combustion, and occupational exposure may come from mining and smelting. Although lead emission would not directly contaminate herbs or other food, airborne lead may deposit on soil and water, so reach herbal materials. Overdose of lead may lead to peripheral and central nervous system damage. In severe cases, patients can have acute psychosis, confusion and decreased consciousness. Memory loss, prolonged reaction time and decrease of comprehension ability may occur in long term lead exposure. Children are easily affected by lead because inorganic lead would be able to penetrate the blood-brain barrier of children due to its underdevelopment (Järup, 2003; Mahurpawar Govt, 2015; Yang *et al.*, 2018). From Figure 15, few authenticated samples have detectable lead content. No significant difference between raw material samples but more concentrated extract samples has a low or undetectable level of lead. The lowest limit of lead among the pharmacopoeia is 5 mg/kg, and all the samples were under 1.5 mg/kg.

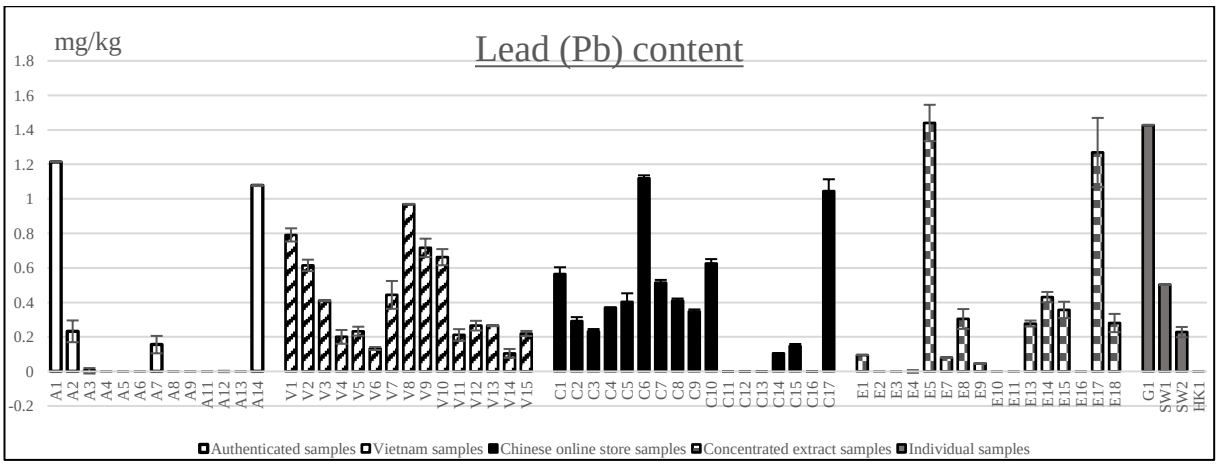


Figure 15 Lead content of the samples of authenticated (A1-A14), Vietnam (V1-V15), Chinese online stores (C1-C17) and concentrated extracts (E1-E5, E7-E11 and E13-E18) as well as some individual samples (G1, SW1, SW2 and HK1)

Sample No.	Average Yield (%, g/g)	SD.
A1	29.55%	0.018
A2	24.22%	0.004
A3	24.21%	0.007
A4	10.37%	0.013
A5	40.95%	0.010
A6	20.38%	0.005
A7	28.89%	0.006
A8	22.13%	0.003
A9	24.09%	0.009
A10	38.32%	0.004
A11	26.02%	0.010
A12	19.55%	0.002
A13	40.03%	0.012
A14	43.16%	0.002
A15	56.71%	0.025
A16	42.88%	0.022
A17	45.48%	0.015
A18	40.74%	0.006

Sample No.	Average Yield (%, g/g)	SD.
V1	35.25%	0.011
V2	33.84%	0.016
V3	32.65%	0.009
V4	38.31%	0.008
V5	38.13%	0.006
V6	38.94%	0.010
V7	29.79%	0.007
V8	41.31%	0.011
V9	36.00%	0.004
V10	35.45%	0.006
V11	43.13%	0.009
V12	32.73%	0.007
V13	37.14%	0.018
V14	33.36%	0.006
V15	27.38%	0.007

Sample No.	Average Yield (%, g/g)	SD.
C1	28.16%	0.004
C2	42.68%	0.026
C3	48.83%	0.020
C4	39.55%	0.006
C5	47.66%	0.010
C6	27.92%	0.012
C7	32.21%	0.005
C8	48.95%	0.008
C9	51.19%	0.007
C10	33.00%	0.009
C11	22.65%	0.008
C12	30.24%	0.007
C13	49.76%	0.014
C14	30.47%	0.019

Table 19 The yield of authenticated samples and market samples from Vietnam herbal market and Chinese online stores

Chemical shift assignment of secondary metabolites in Danshen in ^{1}H-NMR

All the chemical standards were analysed in one-dimensional ^{1}H-NMR spectrometry for at least five concentration points with the range from 0.39 to 12.5 μM. The chemical

shift assignments and NMR spectra of the chemical standards are shown in Figure 16Table 20 The chemical shift assignments of the chemical standards and its selected chemical shift linearity in the ^{1}H NMR spectra and Table 20, respectively. To select the best characteristic chemical shifts for the semi-quantitative analysis of the samples' chemical content, the chemical shifts should: 1) have relatively small magnitude of J's or be a singlet, 2) be in high resolution 3) not stack with other chemical shifts from other chemical standards and 4) not be shifted easily due to concentration difference or any other environment factors. The selected characteristic chemical shifts were highlighted, and its linearity and R^2 of the chemical shift was calculated. Danshensu was unable to be quantified in this analysis due to its low solubility in DMSO and low sensitivity in NMR. Rosmarinic acid did not have characteristic chemical shifts in *Salvia miltiorrhiza* extract NMR fingerprint due to chemical shift overlap with salvianolic acid B, salvianolic acid A and caffeic acid. All the protons in the aromatic ring of caffeic acid, danshensu, rosmarinic acid, salvianolic acid A and B were shown in the range from δ 7.0 ppm to 6 ppm. The chemical shifts from δ eight ppm to 7 ppm in the spectra of caffeic acid derivatives represented the proton in the β position of the unsaturated ester chain linked with the aromatic ring; hence, it appeared in downfield due to the deshielding effect. The chemical shift of the α position of the ester chain in the non-monomers of caffeic acid derivatives was situated in the range of δ 6.5 ppm to 6.25 ppm, which can be used to semi-quantify the amount of salvianolic acids. Salvianolic acid B and rosmarinic acid both had severe chemical shifting in the extracts and it was proved by spike solution, and it was matched with the literature (Jiang *et al.*, 2014; Zhao *et al.*, 2016).

Tanshinones behaved similarly in NMR. The proton in the aromatic ring situated at downfield in the range of δ 9.5 to 7.5 ppm, whereas the methyl group or proton in the cyclic ring situated at upfield in the range of δ 5.0 to 1.0 ppm. The chemical shifts of

tanshinones did not shift in the Danshen extract, but the concentration may slightly change the chemical shifts in the downfield, which did not affect the quantification of tanshinones. The chemical shift of tanshinone IIA, cryptotanshinone and dihydrotanshinone I at δ 1.31 ppm, was in high resolution, but it was not a characteristic peak for tanshinone. It can be used to semi-quantify the content of a specific structure of tanshinones. Similarly, the chemical shift at δ 2.71 ppm of tanshinone I and dihydrotanshinone can be useful in metabolomics. Although the NMR spectra between tanshinones, there were tiny differences in the downfield of the spectra. The drawback of using these chemical shifts as the quantitative analysis was the area of these shifts were usually small due to its low solubility.

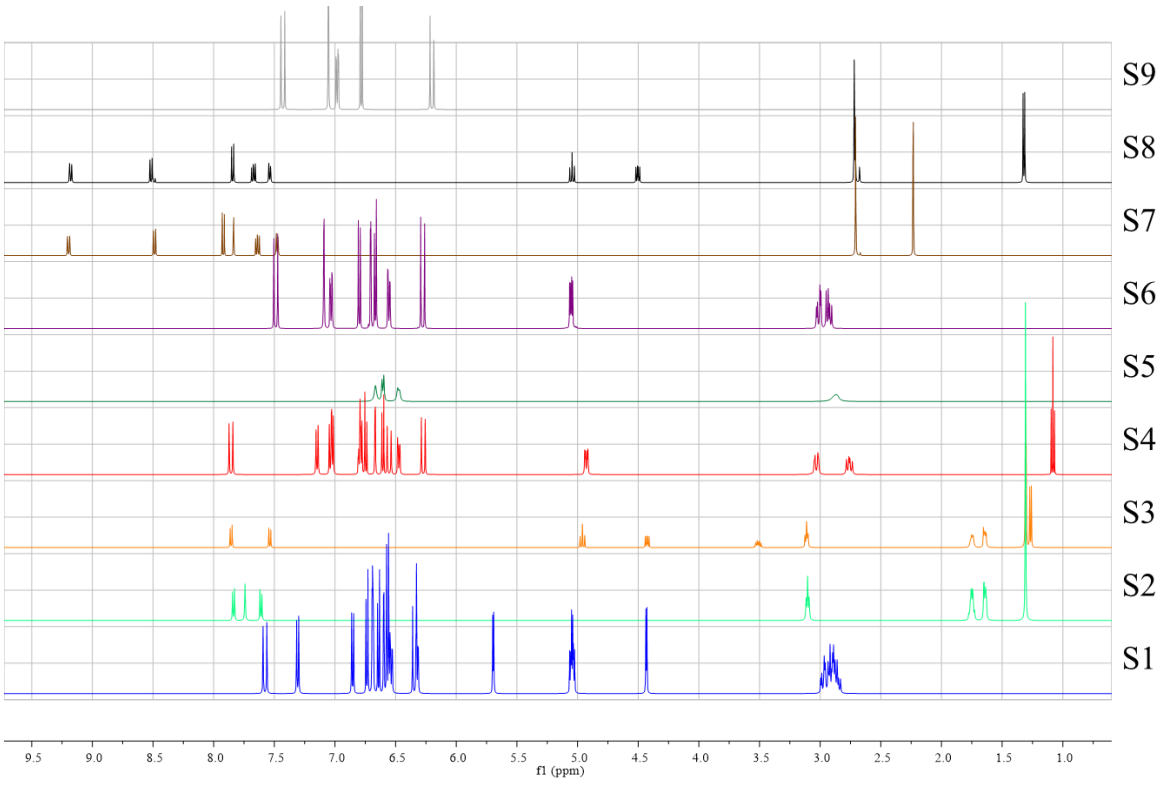


Figure 16 ^{1}H-NMR spectra of the chemical standards (S1-S9) from 10 ppm to 0.5 ppm

All the chemicals standards (S1 = salvianolic acid B, S2 = tanshinone IIA, S3 = cryptotanshinone, S4 = salvianolic acid A, S5 = danshensu, S6 = rosmarinic acid, S7 = tanshinone I, S8 = dihydrotanshinone I and S9 = caffeic acid) were dissolved in $DMSO_{d6}$ with 0.1% DSS as the internal standard reference and analysed in 500M Hz ^{1}H-NMR spectrometry. The spectra were modified to remove the noise, impurities and the solvent peaks

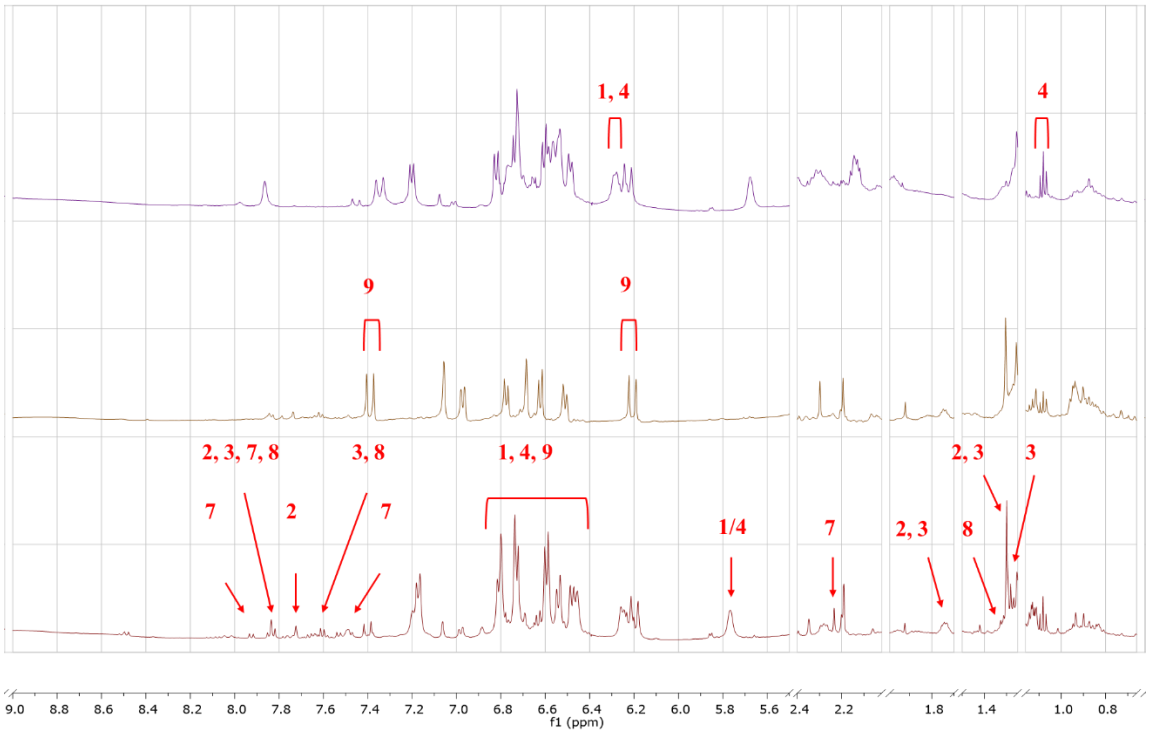


Figure 17 Chemical shift assignment of the chemical standards in the ^{1}H-NMR spectra of Danshen representative samples

Chemical name		Type	Shift	Integral	J's
Salvianolic acid B	A	d	7.58	1	15.81
	B	d	7.31	1	8.51
	C	d	6.85	1	8.47
	D	d	6.74	1	8.12
	E	dd	6.69	2	2.22, 2.08
	F	d	6.64	1	7.99
	G	d	6.6	1	2.07
	H	dd	6.56	2	2.13, 8.13
	I	dd	6.54	1	2.07, 8.07
	J	m	6.33	2	
	K	d	5.7	1	4.11
	L	td	5.05	2	4.58, 7.84, 7.72
	M	d	4.43	1	4.21
	N	dd	2.98	1	4.54, 14.38
	O	m	2.91	2	
	P	dd	2.85	1	8.30, 14.41
Tanshinone IIA	A	d	7.84	1	8.16
	B	s	7.74	1	
	C	d	7.61	1	8.14
	D	t	3.1	2	6.31, 6.31
	E	m	1.75	3	
	F	m	1.64	4	
	G	s	1.31	6	
Cryptotanshinone	A	d	7.86	1	8.15
	B	d	7.54	1	8.1

	C	t	4.96	1	9.54, 9.54
	D	dd	4.43	1	6.14, 9.45
	E	dq	3.52	1	6.60, 6.60, 6.52, 9.69
	F	t	3.11	2	6.38, 6.38
	G	m	1.75	2	
	H	m	1.64	2	
	I	d	1.31	6	2.89
	J	d	1.27	3	6.82
Salvianolic acid A	A	d	7.86	1	15.73
	B	d	7.15	1	8.43
	C	m	7.03	2	
	D	m	6.79	2	
	E	d	6.75	1	8.06
	F	d	6.67	1	2.09
	G	d	6.61	1	8.02
	H	d	6.55	1	16.29
	I	dd	6.47	1	2.08, 8.06
	J	d	6.27	1	15.74
	K	dd	4.93	1	3.25, 9.84
	L	dd	3.03	1	3.25, 14.50
	M	dd	2.76	1	9.92, 14.33
Danshensu	A	s	6.67	1	
	B	d	6.61	1	7.65
	C	d	6.48	1	7.88
	D	d	2.87	1	11.14
Rosmarinic acid	A	d	7.49	1	15.84
	B	d	7.09	1	2.1
	C	dd	7.03	1	2.12, 8.21
	D	d	6.8	1	8.13
	E	d	6.71	1	2.07
	F	d	6.67	1	8.01
	G	dd	6.56	1	2.07, 8.10
	H	d	6.28	1	15.87
	I	dd	5.05	1	4.26, 8.50
	J	dd	3.01	1	4.24, 14.36
	K	dd	2.93	1	8.49, 14.38
Tanshinone I	A	d	9.2	1	8.79
	B	dd	8.49	1	0.91, 8.72
	C	d	7.92	1	8.72
	D	d	7.84	1	1.27
	E	dd	7.64	1	6.93, 8.86
	F	d	7.48	1	6.95
	G	s	2.71	3	
	H	d	2.23	3	1.44
Dihydrotanshinone I	A	d	9.18	1	8.75
	B	dd	8.5	1	8.76, 12.46
	C	d	7.84	1	8.71
	D	dd	7.67	1	6.91, 8.89
	E	d	7.54	1	6.96
	F	t	5.05	1	9.59, 9.59

	G	dd	4.5	1	6.41, 9.41
	H	s	2.72	3	
	I	d	2.69	1	17.17
	J	d	1.32	3	6.83
Caffeic acid	A	d	7.43	1	15.84
	B	d	7.05	1	2.12
	C	dd	6.98	1	2.11, 8.19
	D	d	6.78	1	8.15
	E	d	6.2	1	15.84

Table 20 The chemical shift assignments of the chemical standards and its selected chemical shift linearity in the ^{1}H NMR spectra

All the chemicals standards were dissolved in $DMSO_{d6}$ with 0.1% DSS as the internal standard reference and analysed in 500M Hz ^{1}H-NMR spectrometry. The structure ecluidation was based on the shape of the chemical shifts, the coupling of J's magnitude in the ^{1}H-NMR and literatures

^{1}H-NMR analysis of Danshen samples

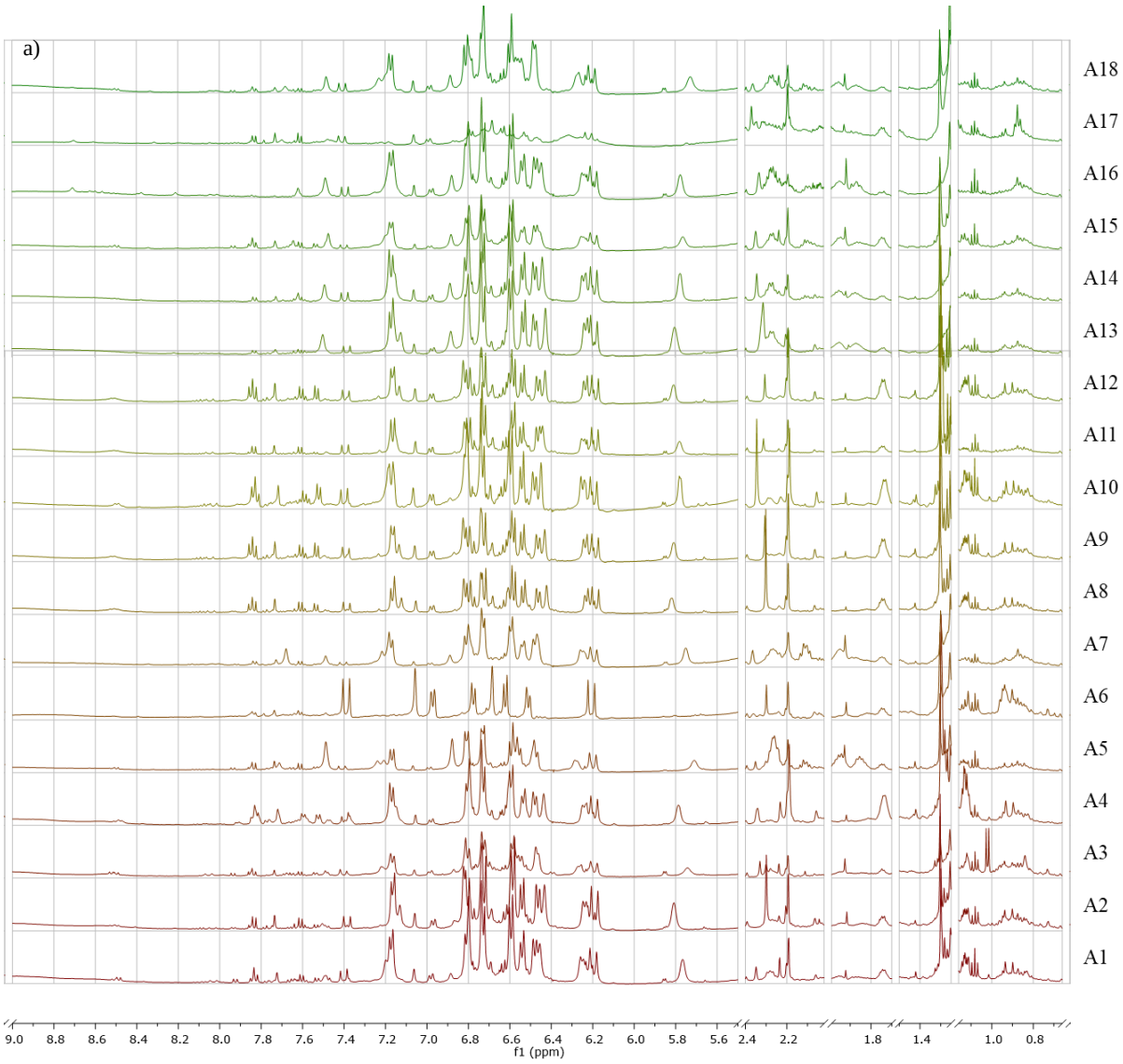
Figure 18 showed the chemical variation of the Danshen samples obtained from different areas and collected in different methods. Caffeic acid, rosmarinic acid, salvianolic acid B, salvianolic acid A, cryptotanshinone, tanshinone IIA, tanshinone I, and dihydrotanshinone I can be found in most of the authenticated *Salvia miltiorrhiza* samples. Most of the samples had similar levels of caffeic acid (δ 7.45 - 7.35 ppm) or its derivatives content (δ 6.8 – 6.5 ppm) apart from the fact that A6 had extremely high level of caffeic acid, and A6 and A17 had relatively low level of salvianolic acid B in the spectra which both were *Salvia przewalskii*. A1 – A5 and A8 – A12 had higher levels and a wider variety of tanshinones compared to others. All these samples were collected and processed by herbalists instead of farmers. Comparing the difference between A8 – A12, which were in the same batch but processed differently, A10, was dried by freeze-drying, had the highest content of salvianolic acids and tanshinones; also it had much higher unknown chemical shifts in around δ 2.35 – 2.3 ppm. A16, *Salvia bowleyana*, did not have any significant difference in salvianolic acids but much lower level tanshinones, especially in tanshinone IIA.

In the market samples, Vietnam and Chinese online store samples had distinctive differences in NMR spectra. Vietnamese samples all have similar fingerprints in ^{1}H-NMR analysis and contained high levels of salvianolic acid A, caffeic acid, especially salvianolic acid B, and low or undetectable tanshinone IIA, cryptotanshinone, tanshinone I and dihydrotanshinone. Also, all of the Vietnam market samples had an unknown singlet chemical shift in δ 7.85 – 7.8 ppm. Although they had a different chemical pattern with the authenticated *Salvia miltiorrhiza* samples, in general, the samples from the Vietnam herbal market were chemically qualitatively consistent in the ^{1}H-NMR analysis.

In contrast, the chemical compositions of the samples obtained from the Chinese online store were very inconsistent. Only C5 and C6 were qualitatively similar to the authenticated *Salvia miltiorrhiza* sample. C12, C14, and C16 had similar chemical shift pattern in the range of δ 2.40 – 1.30 ppm to other Danshen samples, but had low levels of characteristic secondary metabolites of *Salvia miltiorrhiza;* C1 and C11 both had low level of salvianolic acid B. Hence, these samples were highly likely to be "poor quality Danshen" in the quality test. C12 also had two unknown chemical shifts quartets in the range of δ 1.05 – 0.90 ppm, which could not find in any other Danshen samples. All the samples had low or undetectable levels of rosmarinic acid or salvianolic acid B. The geographical difference of the source did not seem to be the primary reason for the results. Although C11, C12, C15, and C16 were claimed to be from Sichuan province and three of them were considered poor quality in chemistry, C15 seems to be the best quality among all the Chinese online store samples collected according to the current pharmacopoeia standard. C1, C2, C3, and C5 were similar in chemical composition; whereas C9 and C10 were also similar to each other, but C1, 2, 4, 6, 8, 13 were claimed to be from Shandong; C3 was claimed to be from Anhui; and C5, C7, C9, C10, and C17

were claimed to be from Yunnan. No specific patterns were found in Chinese online store samples.

For the individual samples and the Danshen concentrated extracts, G1, which was the sample from Germany, had a similar chemical composition pattern with Vietnamese samples. SW1 and SW2, which were the samples collected from Switzerland pharmacies, were chemically similar. E16 and E18 were similar to each other, which indicated they had a similar source of materials and processing. E15 was different from other concentrated extracts, but it was similar to one of the samples from the Hong Kong TCM supplier (HK1). It indicated that E15 may have used the same source of HK1 as the primary ingredient of the product.


a)
A18
A17
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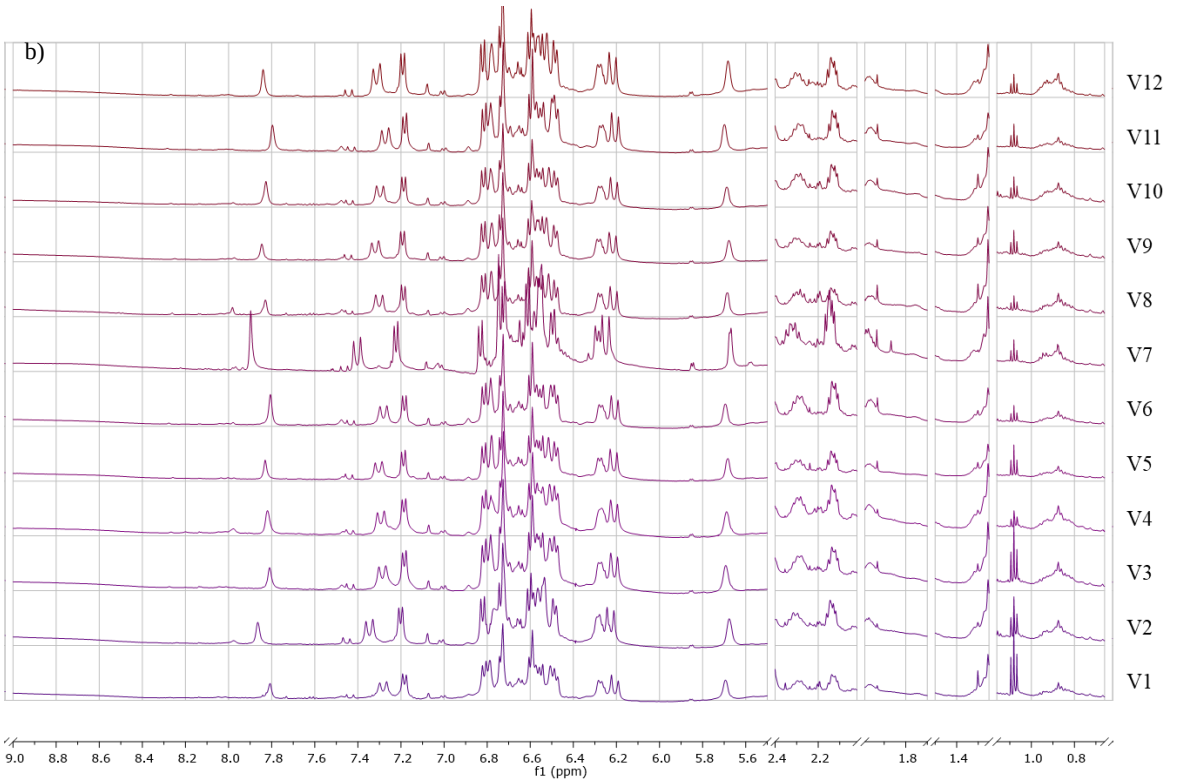
9.0 8.8 8.6 8.4 8.2 8.0 7.8 7.6 7.4 7.2 7.0 6.8 6.6 6.4 6.2 6.0 5.8 5.6 2.4 2.2 1.8 1.4 1.0 0.8
f1 (ppm)


b)
V12
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V4
V3
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V1
9.0 8.8 8.6 8.4 8.2 8.0 7.8 7.6 7.4 7.2 7.0 6.8 6.6 6.4 6.2 6.0 5.8 5.6 2.4 2.2 1.8 1.4 1.0 0.8
f1 (ppm)

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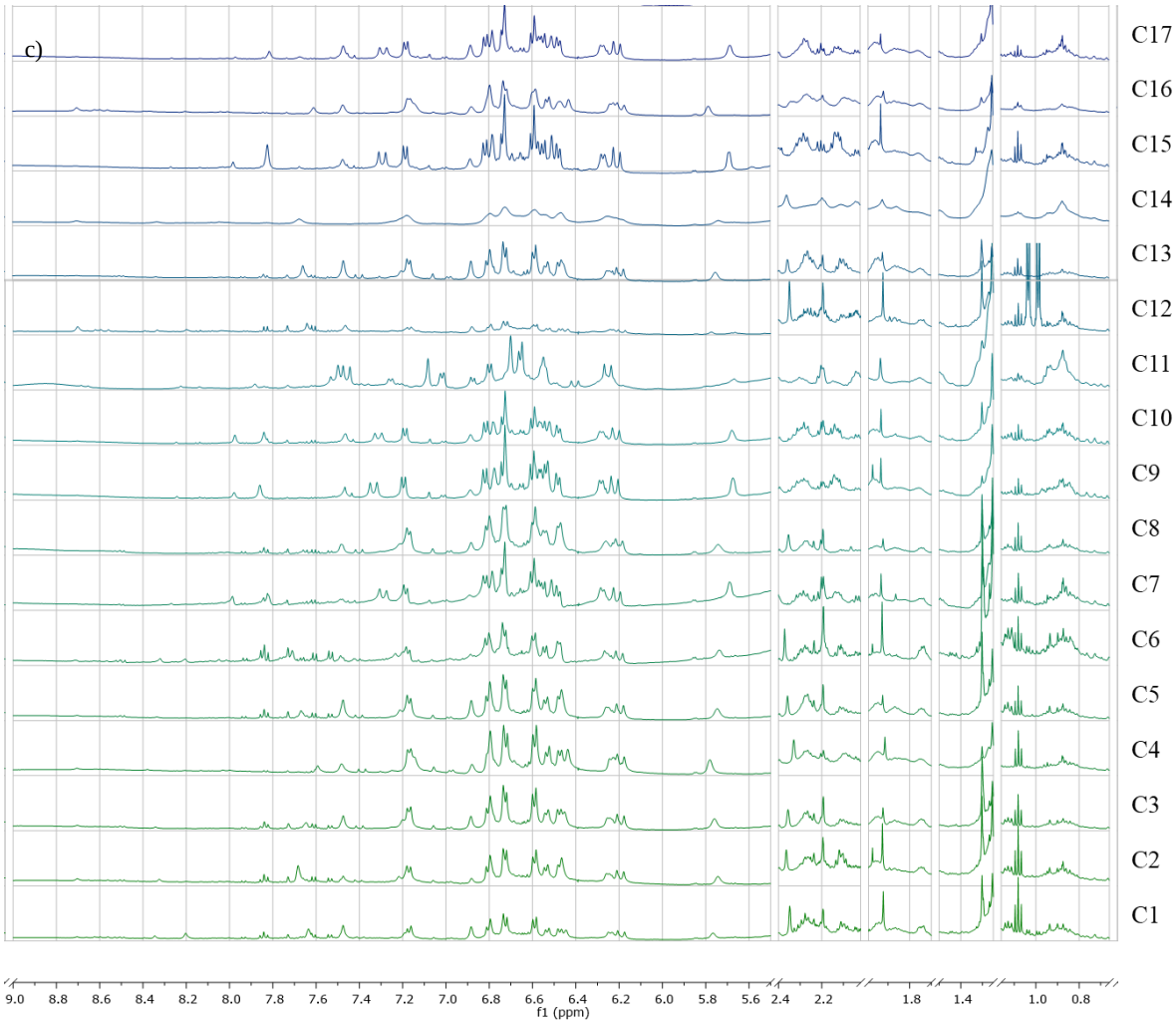
c)
C17
C16
C15
C14
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C11
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C9
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C5
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C2
C1
9.0 8.8 8.6 8.4 8.2 8.0 7.8 7.6 7.4 7.2 7.0 6.8 6.6 6.4 6.2 6.0 5.8 5.6 2.4 2.2 1.8 1.4 1.0 0.8
f1 (ppm)

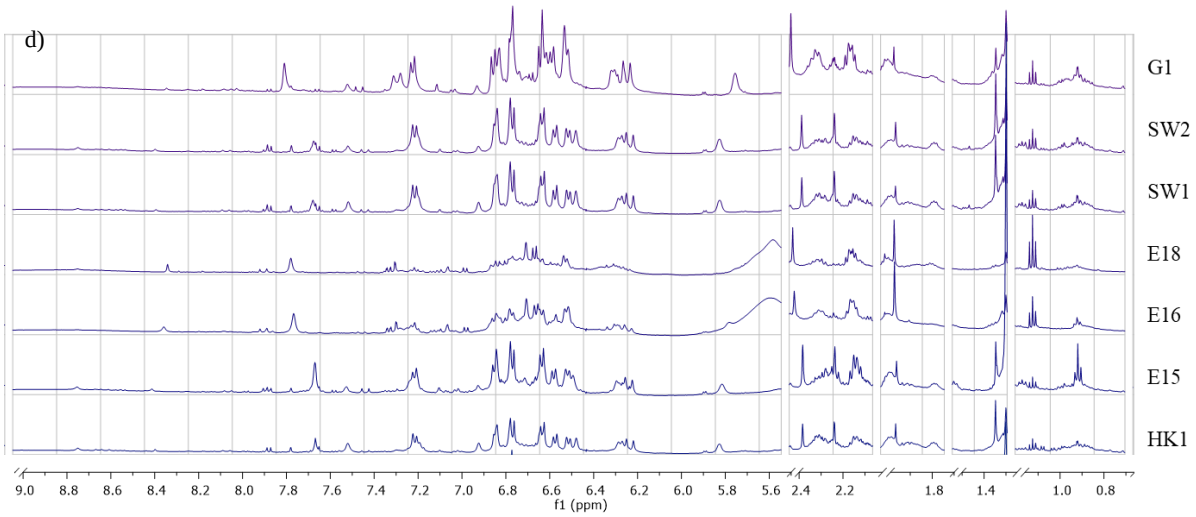


Figure 18 The [1]H-NMR spectra of the Danshen samples

All the samples were performed the extraction and processing at the same time. 0.8 g samples were exracted by 75% methanol in sonication for 30 minutes and the extract was dried under 1.5 hour 60°C heating then freeze drying. The dried extract was redissolved in $DMSO_{d6}$. The ^{1}H-NMR spectra was analysed by MestreNova 12.1. a) authenticated samples (A1 – A18) b) Vietnamese samples (V1 – V12) c) Chinese online store samples (C1 – C17) and d) individual samples and extracts (G1, SW1, SW2, HK1, and E15, E16 and E18)

5.4 The bioactivity variation of Danshen products

Several *in vitro* models with the murine RAW264.7 macrophage cell line were used to understand the influence of the biological activities from the chemical variations of Danshen products.

The influence of the Danshen samples to RAW 264.7

In order to evaluate the influence of Danshen samples to macrophage cells, MTT assay in the cell line of RAW 264.7 was used. Only three concentrations of 100, 50 and 25 μg/ml Danshen samples were tested in this experiment due to time and feasibility. Figure 19**Error! Reference source not found.** showed the biological activity variation of Danshen products to the RAW 264.7 macrophage cells. Twenty-one out of fifty-six samples showed significant differences to the control after applying Danshen sample extract at the concentration of 100 μg/ml. All the effects of these samples on the cells

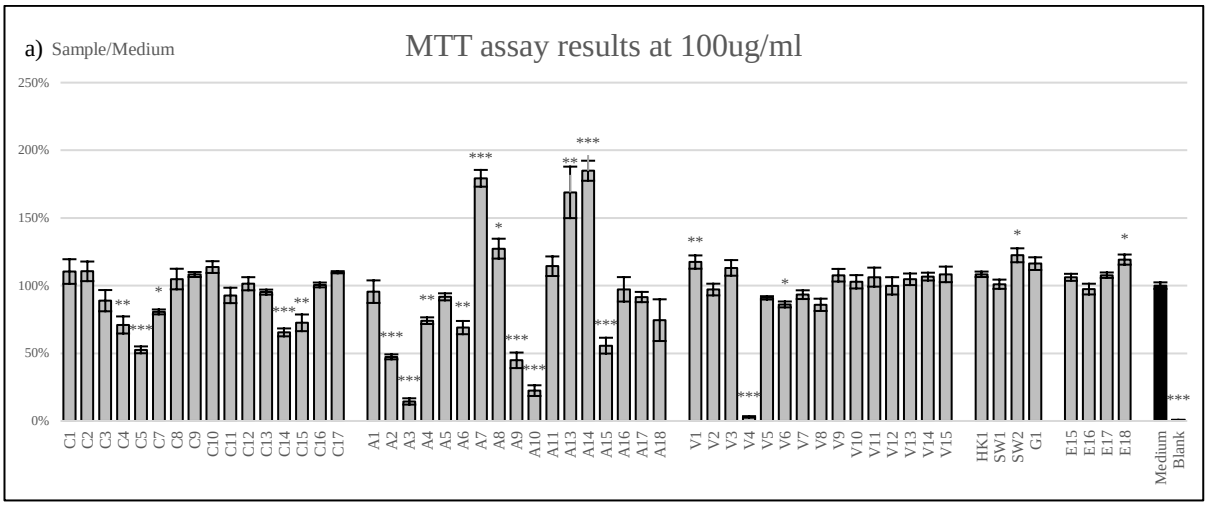
showed dose-response relationships, except C4, yet all the concentration tested in C4 showed cytotoxicity effects.

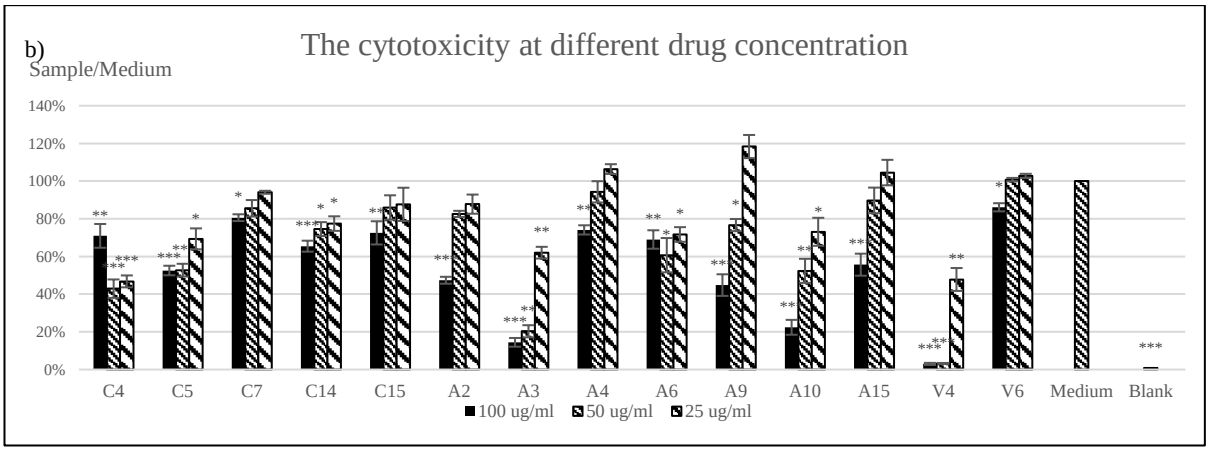
Fourteen samples showed cytotoxicity effect on the cells, including seven authenticated samples, five Chinese online store samples, and two Vietnamese samples. Of these, A3, A10, and V4 had the highest cytotoxicity effect of which the cell viability was down to less than one fourth compared to the control. Also, A3 and A10 had the highest level of salvianolic acid B and tanshinone IIA. They also contained a wider variety of tanshinones according to the ^{1}H-NMR result. It indicates that using the content of tanshinone IIA and salvianolic acid B, which were the chemical standards in most of the pharmacopoeias in *Salvia miltiorrhiza* would not be enough to evaluate biological activities of the product and to certain extent the quality as well.

A9 and A11 were the paired group, which both were self-collected from the same farm and the same batch. A9 was cut before the 30 °C oven-dry for 48 hours, and A11 was dried in the same condition without cutting. The result showed the extreme difference between these two samples, which A9 showed the cytotoxicity while A11 showed the increase of cell viability. It indicated that even having the same genes and the same cultivation and harvesting method, it may lead to diverse biological activities with different processing.

On another hand, four samples showed increases of cell viability, including four authenticated samples, one Vietnamese sample, one Switzerland sample, and one Danshen concentrated extract. Of these, A7, A13, and A14 had increased more than 50% compared to the control. A13 and A14 were cultivated on the same farm with the same method and processed in the same way but a different year. The time difference here did not significantly change the biological activities of the product, but further

study needs to be done, for instance, more systematic sample collection and recording the weather change and soil condition.





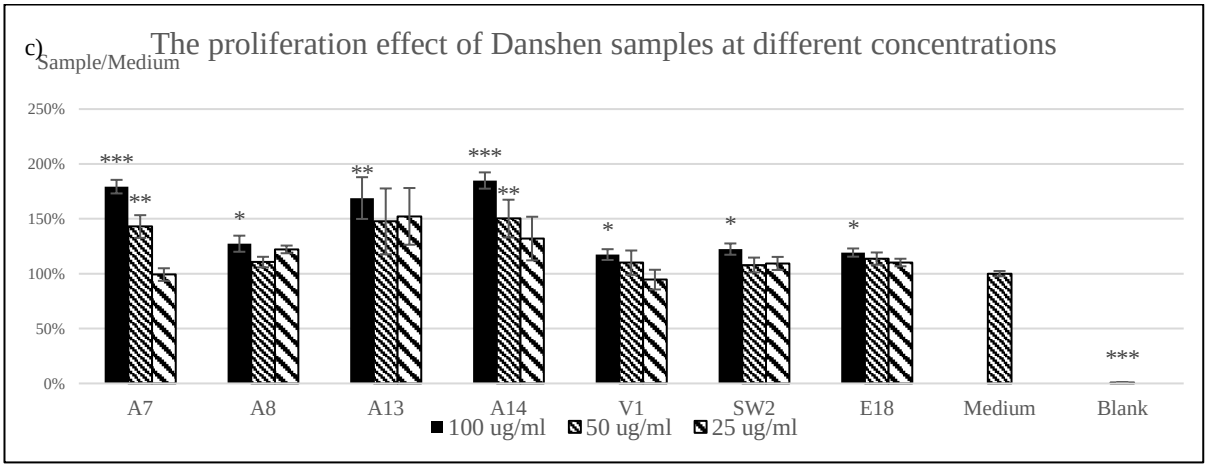


Figure 19 The cell viability results of Danshen sample extracts in RAW 264.7

200 μl of RAW 264.7 with the density of 3X10^4 was treated with 100, 50 and 25 μg/ml of sample for 24 hours. a) the results of all the samples in 100 μg/ml. b) the results of the samples that showed the significant cytotoxic effect to RAW 264.7 c) the results of the samples that showed a proliferative effect in RAW 264.7. The cell viability percentage was the mean absorbance of the sample absorbance divided by

*the medium absorbance ± SEM of three triplicated independent experiments. Statistical significance of the sample is shown as * = P <0.05, ** = P <0.01, and *** = P <0.001 compared with the medium control.*

The inhibitory effect of LPS induced NO production in RAW 264.7

In order to evaluate the biological variation of the inhibitory effect, LPS induced NO production on the Griess assay was used. 20 μg/ml of indomethacin, as a NASID drug, showed significant inhibition of LPS induced NO production which indicated this model was valid for the investigation. The samples which had cytotoxic effects on the cells were excluded in the test because a lower density of the living cells will produce less NO signal. Hence, negative-positive results would be found. Within the non-toxic Danshen samples, only seven samples, including two authenticated samples and five Vietnamese samples, showed significant inhibitory NO production effects on the RAW 264.7. All the samples with inhibition activity showed dose-response relationships. The low proportion of samples having potential anti-inflammatory effects showed that the chemical variation in the market samples significantly affects the biological activities of herbal products. V2, V9, and V12 were claimed to be cultivated in Vietnam, and two of them showed inhibition effects while all the samples from Chinese online stores did not show any inhibitory effects. This illustrated that obtaining products from the traditionally authenticated origin does not necessarily relate to its quality. Also, it shows it is worth investigating the possibility of cultivating herbal materials globally instead of focusing on cultivating in the “traditionally authenticated” herbal materials region. The high ratio of getting positive results in Vietnamese samples may also link to the storage practice of Danshen materials among the Vietnam TCM herbal pharmacies, which was mentioned on page **Error! Bookmark not defined.**.

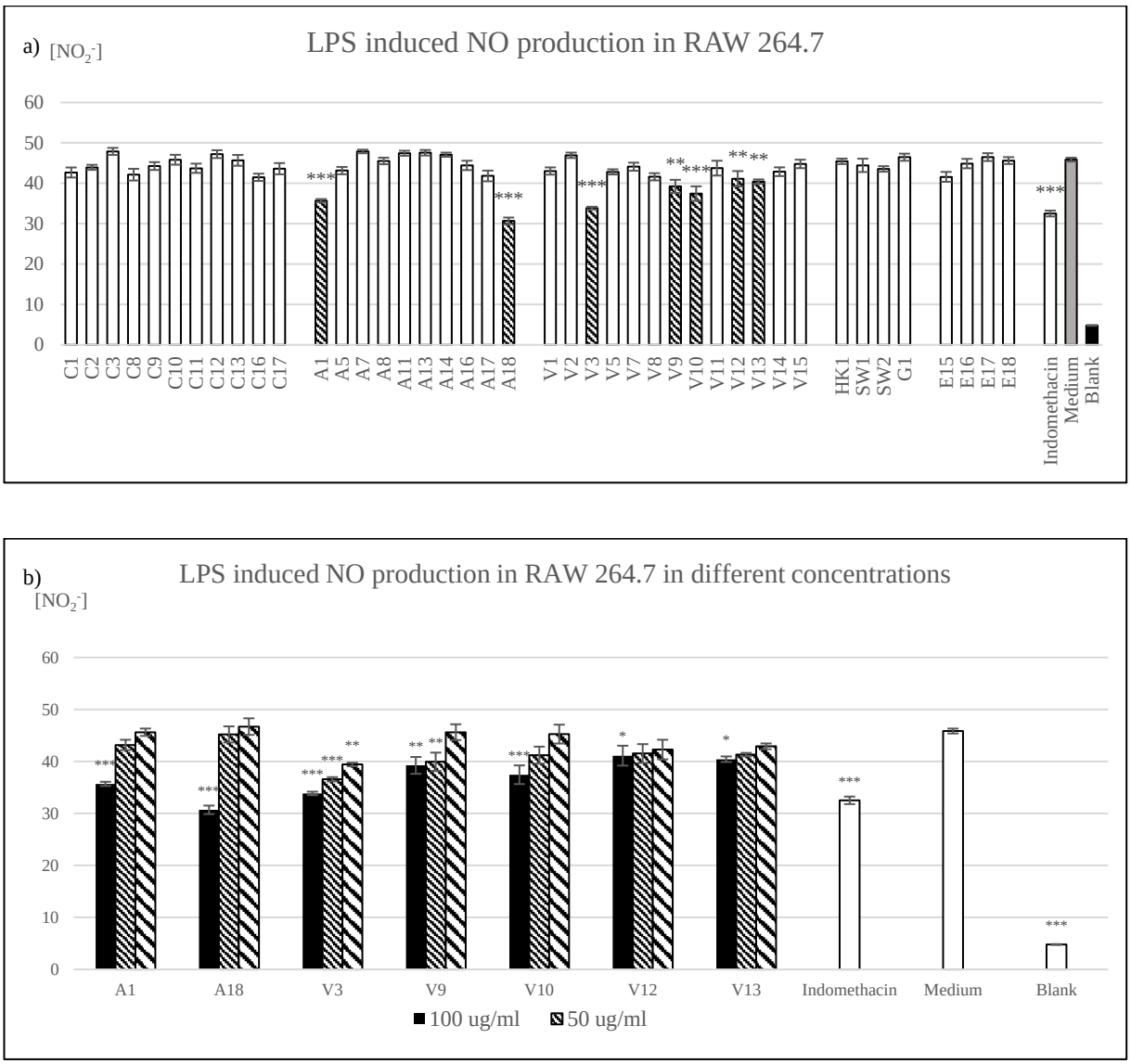


Figure 20 The effect of Danshen extracts on LPS induced NO production in RAW 264.7

200 µl of the RAW 264.7 cells with the density of $2.5X10^5$ was treated with 100, 50 and 25 µg/ml of sample for 24 hours. 20 µg/ml of indomethacin was used as the positive control. a) the results of all the samples in 100 µg/ml. b) the results of the samples that showed significant inhibition on LPS induced NO production on the RAW 264.7 The NO concentration was based on the standard curve of $NaNO_2$ ± SEM of three triplicated independent experiments. Statistical significance of the sample is shown as * = P <0.05, ** = P <0.01, and *** = P <0.001 compared with the medium control.

The protection of Danshen product on H_2O_2 induced apoptosis

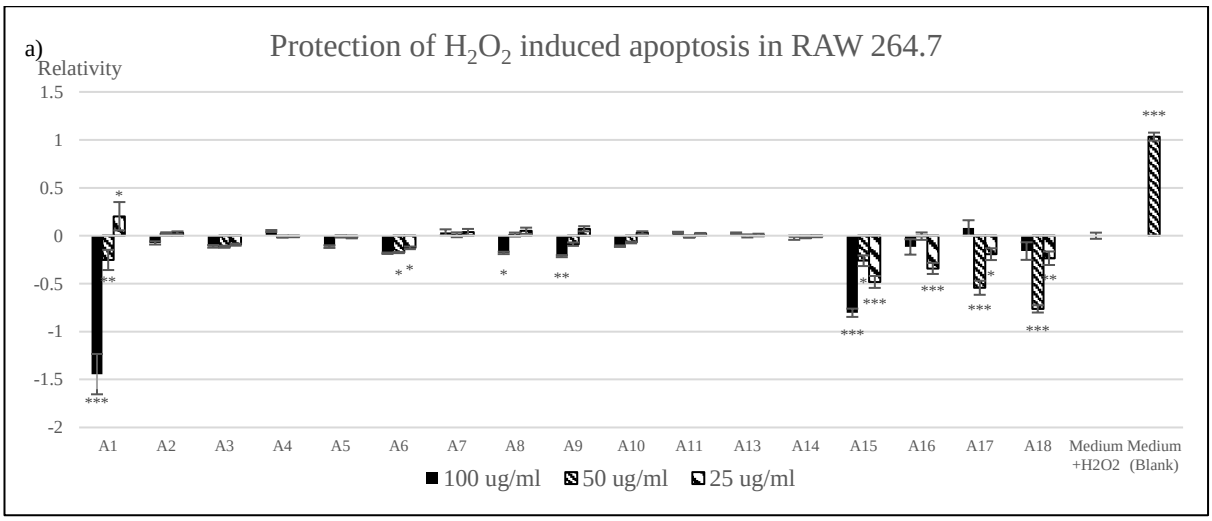
In order to see the protection from Danshen products on H_2O_2 induced apoptosis protective effect, H_2O_2 was acted as the oxidative stress triggering the cell apoptosis from the initiation of the NF-κB pathway. Similar to the LPS induced NO production, the samples which had cytotoxicity effect on the cells were excluded in the test. The

results in Figure 21 indicate that only one sample (C15) showed dose-related protection on H_2O_2 induced apoptosis from Danshen products. C14 showed a low protection effect from H_2O_2 in a high concentration of drug and high protection in low concentration of the drug. C8, C10, and C16 showed different protection effects in different concentrations of the drug. Nine samples showed synergistic effects to enhance the H_2O_2 apoptosis. A1 also had a strong dose-response on this effect.

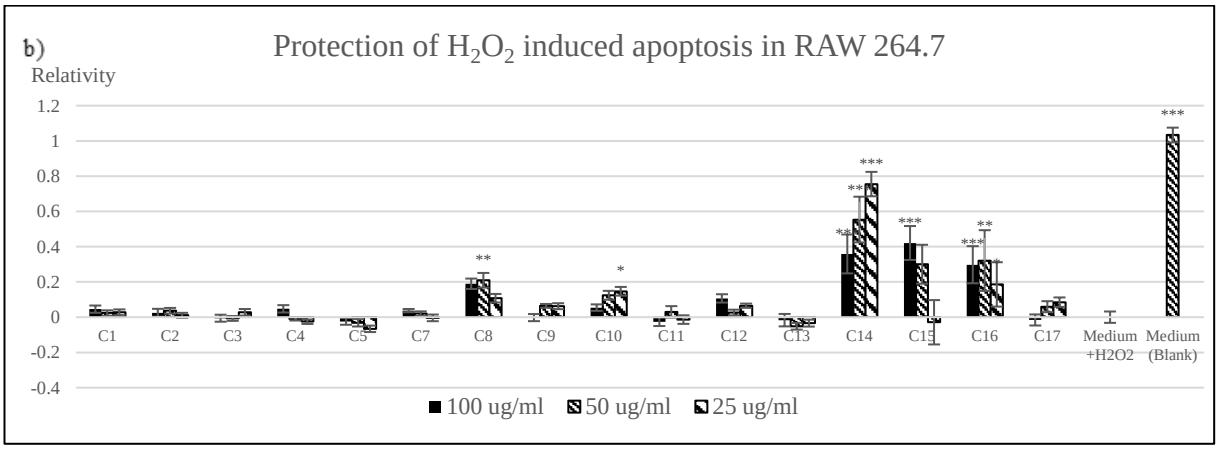
All the samples that showed the increase of cell viability mentioned above (Figure 19) did not induce any significant change of cell viability after H_2O_2. One of the explanations is that the positive result in the MTT assay mentioned could be caused by the increase of mitochondrial activity instead of cell proliferation, and the H_2O_2 led to cell apoptosis and also inhibited the activation of the mitochondrial activity from the drug.

However, this experiment has several limitations that require more extensive research before drawing a specific conclusion. One of the limitations is that the apoptosis led by H_2O_2 was highly dependent on the passage number of RAW 264.7. During the experiment, the effect of H_2O_2 on RAW 264.7 decreased when the passage number increased. A similar situation was reported in PC12, HepG2, U937, and C2C12 cell lines (Mazziotti and Perlmutter, 1998; Pronsato *et al.*, 2013). In this research, the resistance was found in passage 13, and this experiment was limited from passage 5 to 11 only.

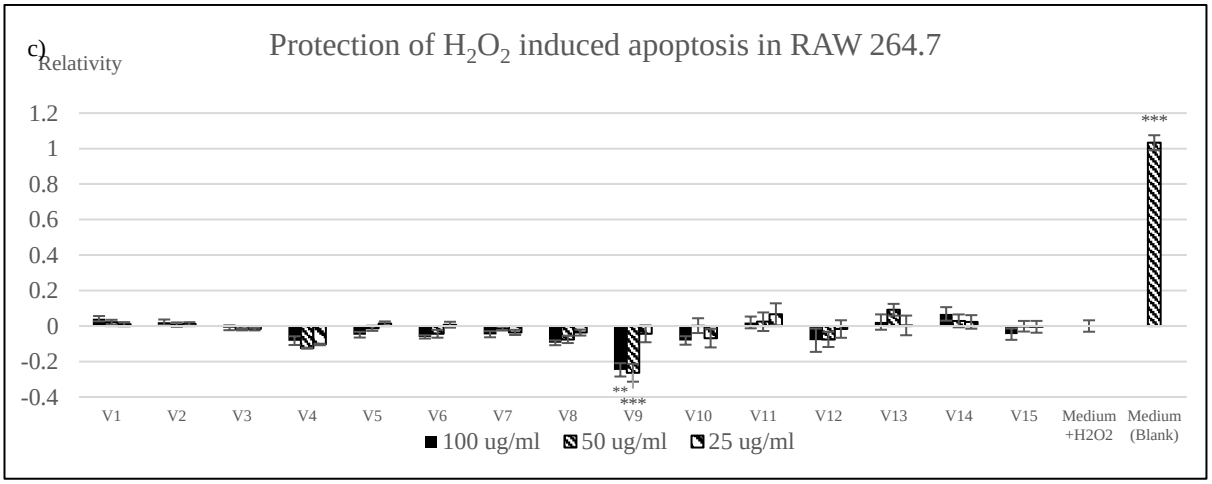
a)


Protection of H_2O_2 induced apoptosis in RAW 264.7
Relativity
1.5
1
0.5
0
-0.5
-1
-1.5
-2
A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 A13 A14 A15 A16 A17 A18 Medium +H2O2 Medium (Blank)
100 ug/ml 50 ug/ml 25 ug/ml

b)


Protection of H_2O_2 induced apoptosis in RAW 264.7
Relativity
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
C1 C2 C3 C4 C5 C7 C8 C9 C10 C11 C12 C13 C14 C15 C16 C17 Medium +H2O2 Medium (Blank)
100 ug/ml 50 ug/ml 25 ug/ml

c)


Protection of H_2O_2 induced apoptosis in RAW 264.7
Relativity
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
V1 V2 V3 V4 V5 V6 V7 V8 V9 V10 V11 V12 V13 V14 V15 Medium +H2O2 Medium (Blank)
100 ug/ml 50 ug/ml 25 ug/ml

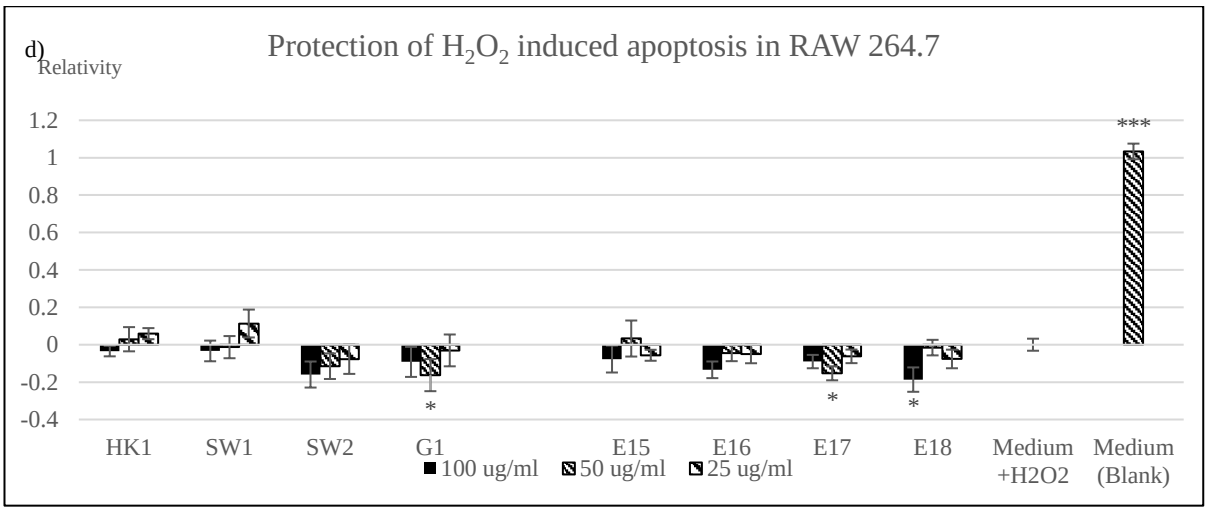


Figure 21 The results H_2O_2 induced apoptosis of Danshen sample extracts in RAW 264.7

200 µl of the RAW 264.7 cells with the density of $3X10^4$ was treated with 100, 50 and 25 µg/ml of sample for 24 hours. a) the results of the authenticated samples. b) of the Chinese online store samples c) of Vietnamese samples and d) of the individual samples and Danshen concentrated extracts in H_2O_2 induced apoptosis. The protection value was calculated by the equation below. The result < 0 = anti-protection effect and the result > 0 = protection effect. SEM showed the variation of the results from the three triplicated independent experiments. Statistical significance of the sample is shown as * = P <0.05, ** = P <0.01, and *** = P <0.001 compared with the medium control.

$$Protection\ of\ H_2O_2\ induced\ apoptosis = \frac{V_{sample} - V_{H_2O_2}}{V_{blank\ medium} - V_{H_2O_2}}$$

V = viability measured by MTT assay

5.5 The relationship between NMR results and other information

General chemical composition variation

In order to investigate the relationship between the chemical variation of samples and other information such as LPS induced NO production, cytotoxicity, supplier information, PCA, and PLS-DA were used to evaluate the potential correlation. Figure 22 illustrates the similarity between samples using PCA-X according to NMR results and the results showed the best correlation with the chemical variation was the supplier sales channel. The R^2 and Q^2 were 0.56 and 0.491 respectively. According to (Broadhurst, Broadhurst and Kell, 2006; Worley and Powers, 2013), the R^2 and Q^2 of an acceptable biological metabolomics model should be more than 0.4, but there are no fixed definitions of a good model. Generally, Q^2 has to be smaller than R^2 to avoid over prediction. In this case, the model was full of different types of Danshen species from different channels and forms. R^2 and Q^2 were expected to be smaller than other metabolomics models. Also, this model had several outliers which were identified by

the hotelling T^2 range (in Figure 22b) which would lower the fitness and prediction of the model. However, these samples should be part of the model because they were considered to be *Salvia miltiorrhiza* and it showed the chemical diversity of Danshen.

The loading plot and the loading column plot showed the contribution of X variables to the component. In other words, it is the reason for the sample situated at the PCA-X. The PCA-X loading column for component 1 was mainly composed of the NMR binned area δ 3.10 – 2.50 ppm, which was mainly primary metabolites according to (Jiang *et al.*, 2014). Although some secondary metabolites such as salvianolic acid B and tanshinone IIA would have chemical shifts in the region, it is difficult to identify the contribution of the metabolites because primary metabolites would have higher intensities and there were too many overlaps of chemical shifts at that region. Most of the Vietnamese samples had a high intensity of that region, and it would be interesting to understand what the composition of that region was in these samples.

All the authenticated *Salvia miltiorrhiza* samples and Vietnamese samples were situated at the positive part of component 2, which was comprised high contribution from caffeic acid, salvianolic acid B and rosmarinic acid binned NMR region. In contrast, most of the samples from Chinese online stores were situated in the negative part of component 2 which indicated that these samples had a lower content of those metabolites. It was also the reason why Vietnamese samples were different from authenticated samples. To further investigate the chemical difference of authenticated samples and market samples, PLS-DA was used, and the results showed in Figure 23 and Figure 24.

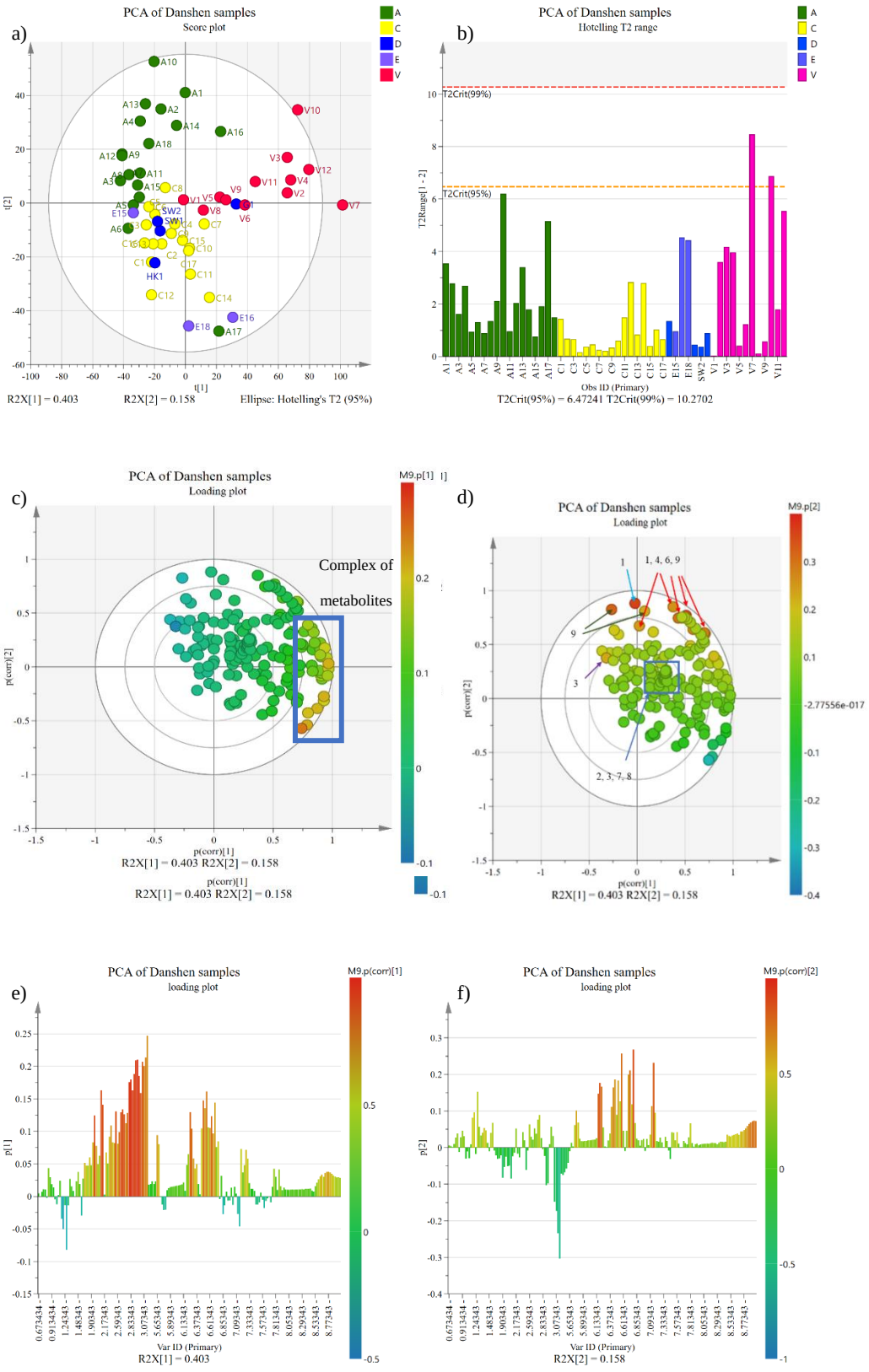


Figure 22 PCA of all Danshen samples

The PCA-X corresponded to the binned regions of all ^{1}H-NMR spectra of Danshen samples as X variables. All X variables were scaled by Pareto scaling. In a) PCA scatter score plot and b) Hotelling T^2 range plot, yellow = Chinese online store samples; pink = Vietnamese samples; green = authenticated samples; blue = individual samples and purple = Danshen concentrated extracts. The colors of the loading plots were referring the p value of the X variable contribute to principal component 1 in figure c) and component 2 in figure d).

Figure 23 showed the PLS-DA of the NMR results of Chinese online store samples against Vietnamese samples. The cumulative R^2X, cumulative R^2Y and Q^2Y were 0.652, 0.86 and 0.78 respectively, and the permutation test showed huge distinction between the random and actual model. These showed this model was valid and good in fitness and prediction. Only one sample (V7) was the outlier of this model which mean this sample was independent, and no samples were similar to it. Figure 23c), d) and e) showed that Chinese online store samples was higher in the contribution from tanshinone IIA, cryptotanshinone, tanshinone I and dihydrotanshinone I regions (δ 1.30 – 1.27 ppm and δ 7.6 – 7.0 ppm) while Vietnamese samples had higher contribution from salvianolic acids region (δ 6.8 – 6.0 ppm) and the primary metabolite region (δ 3.1 – 2.5 ppm) to the model.

Similarly, Figure 24 showed the PLS-DA of the NMR results of authenticated samples against Vietnamese samples. The cumulative R^2X, cumulative R^2Y and Q^2Y were 0.699, 0.903 and 0.867 respectively, and the permutation test showed a huge distinction between the random and actual model. No samples were the outlier according to the hotelling T2 range in Figure 24b). Figure 24c), d) and e) showed that Chinese online store samples was higher in the contribution from tanshinone IIA, cryptotanshinone, tanshinone I and dihydrotanshinone I regions (δ 1.30 – 1.27 ppm and δ 7.6 – 7.0 ppm) and salvianolic acids region (δ 6.8 – 6.0 ppm) while Vietnamese samples had higher contribution from and the primary metabolite region (δ 3.1 – 2.5 ppm) to the model.

In general, these three models concluded that Vietnamese samples had higher salvianolic acids than authenticated samples than Chinese online store samples. Authenticated samples had higher tanshinones than Chinese online store samples than Vietnamese samples. Vietnamese samples also had significantly stronger signals in the primary metabolite region (δ 3.1 – 2.5 ppm).

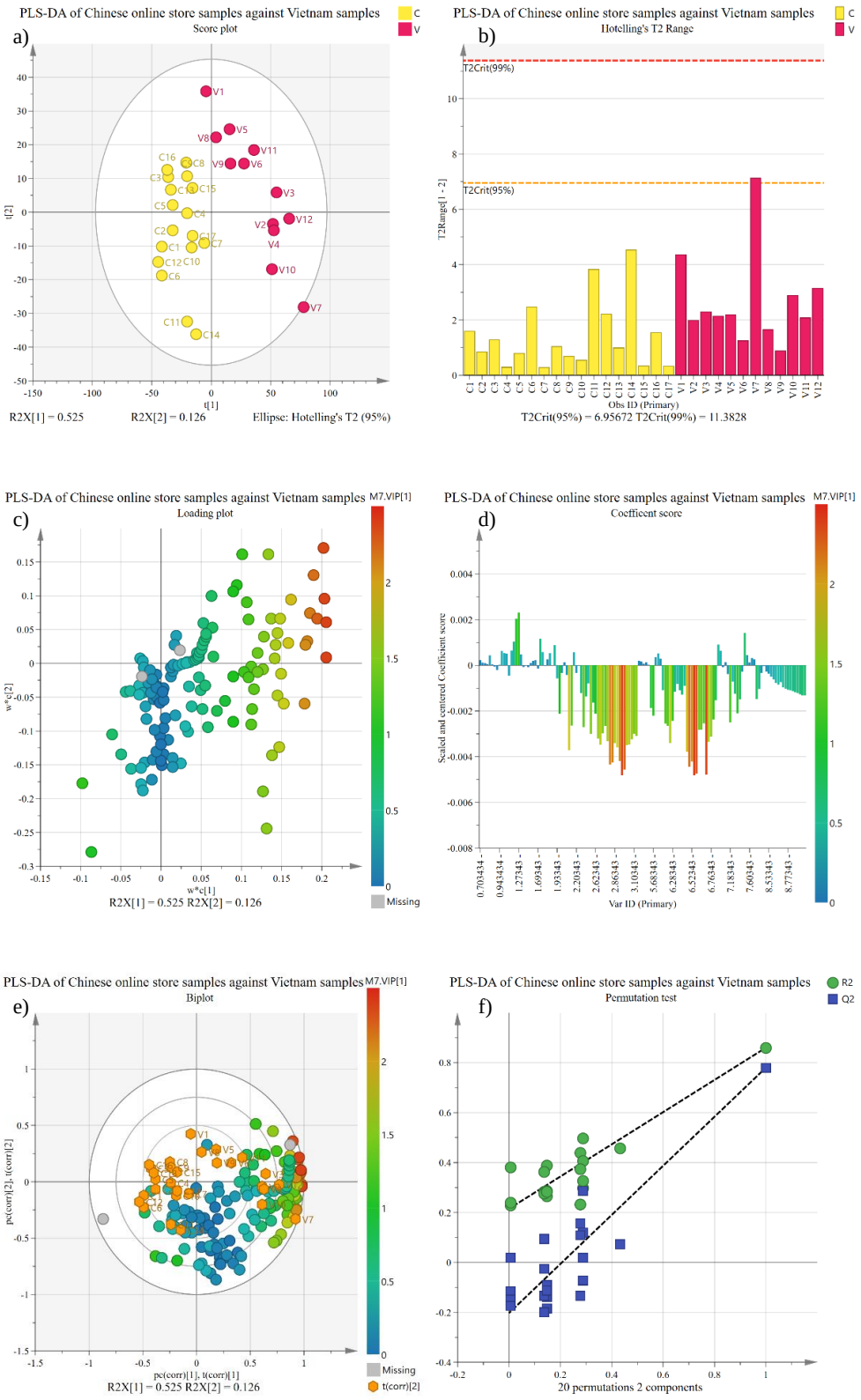


Figure 23 PLS-DA of Chinese online store samples against Vietnamese samples

The PLS-DA corresponded to the binned regions of ^{1}H-NMR spectra of Chinese online store samples (C1 – C17) and Vietnamese samples (V1 – V12) as X variables and the sales channel of samples as the Y variable. All X variables were scaled by Pareto scaling. The yellow colour in a) PLS-DA scatter score plot and b) Hotelling T^2 range plot represented the Chinese online store samples and the pink represented Vietnamese samples. The colours of the samples in figure c) loading plot, d) coefficient plot, and e) biplot were referring the VIP value of the X variable contribute to principal component 1. e) permutation test showed the intercepts of R^2 = (0.0, 0.224), and Q^2 = (0.0, -0.27)

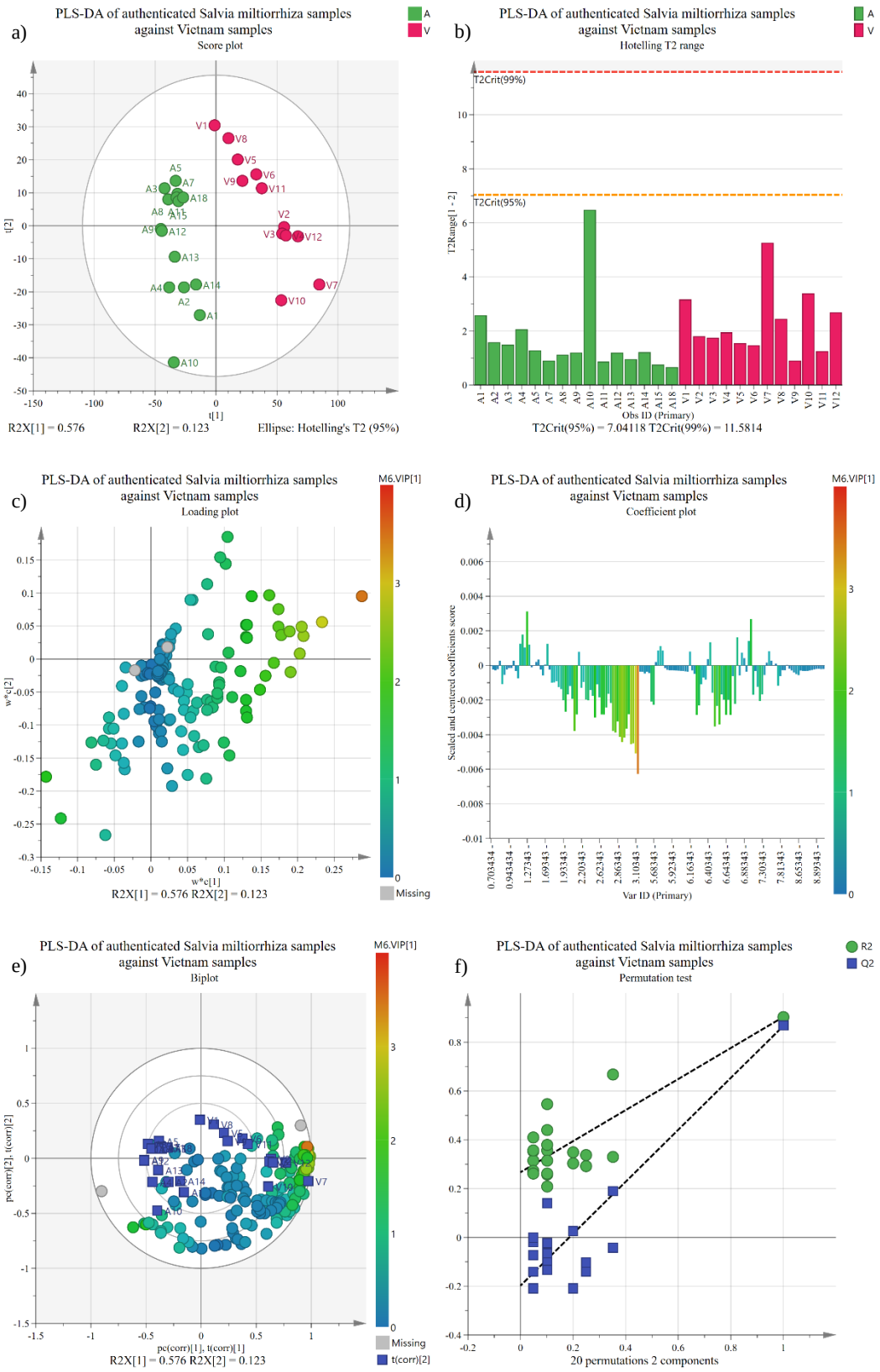


Figure 24 PLS-DA of authenticated Salvia miltiorrhiza samples against Vietnamese samples

The PLS-DA corresponded to the binned regions of ^{1}H-NMR spectra of authenticated *Salvia miltiorrhiza* samples (A1 – A18, except A6, A16 and A17) and Vietnamese samples (V1 – V12) as X variables and the sales channel of samples as the Y variable. All X variables were scaled by Pareto scaling. The green colour in a) PLS-DA scatter score plot and b) Hotelling T^2 range plot represented the authenticated *Salvia miltiorrhiza* samples and the pink represented Vietnamese samples. The colours of the samples in figure c) loading plot, d) coefficient plot, and e) biplot were referring the VIP value of the X variable contribute to principal component 1. e) permutation test showed the intercepts of R^2 = (0.0, 0.267), and Q^2 = (0.0, -0.197)

Chapter 6. General discussion, conclusion, and prospects

6.1 The Danshen quality issue and related challenges

Geographical origin and practice difference of Danshen

In our study, only the dried root samples from Chinese online store were labelled the geographical origin of the products. Neither the samples from Vietnamese herbal market nor Hong Kong's TCM pharmacies had any labels for this information; the retailers did not acknowledge the information at the time. It implied that the retailers do not pay adequate attention to product information, and showed a low traceability of Danshen raw materials.

Our study showed that the geographical origin is not the primary factor of chemical variation in Danshen. The PCA results did not show any relationships based on the origins of the products. However, several published studies found a significant chemical difference between Danshen roots of different origins. (Huang *et al.*, 1980; Huang, Yang and Hu, 1980) were one of the earliest investigations on the tanshinone constituents of different harvesting seasons, origins and species of Danshen using TLC-UV. The research showed the tanshinone IIA contents varied from 0.02% to 0.32% in different origins of Danshen. Seven out of ten samples contained under 0.12% tanshinone IIA, only the Danshen samples from Shandong (0.32%), Henan (0.23%) and Hubei (0.16%) had more than 0.12%.

A recent study also showed that the Danshen roots from Henan and Shandong have higher levels of biomarkers, such as salvianolic acid B and tanshinone IIA (Ni *et al.*, 2019). *Salvia miltiorrhiza* roots from Henan and Shandong had a two to three times higher content of tanshinone IIA, dihydrotanshinone I, cryptotanshinone and tanshinone I than the roots from Sichuan and Yunnan. The roots from Henan and Shandong

contained 6.31 mg/g and 5.70 mg/g tanshinone IIA on average while the roots from Sichuan and Yunan contained 2.18 mg/g and 1.41mg/g on average respectively.

The HPLC-MS^n metabolomics study by (Liang *et al.,* 2017) showed that it was not the geographical origin was not the primary factor of the quality variation of Danshen but the processing method was. As one would expect, samples from the same origin with different processing methods showed a significant difference in chemical composition, and the study successfully clustered the samples processed by sweating and sun curing (Cluster I), drying in shade (Cluster II) and oven drying (Cluster III). Cluster II contained higher common secondary metabolites than cluster III and cluster I. It also highlighted cryptotanshinone, trijuganone B, and 15,16-dihydrotanshinone I are the most significant variables that distinguish the differences between the processing methods in Danshen. In our study, the samples from Vietnam which were dried regularly under the sun had lower tanshinones including tanshinone I, tanshinone IIA, cryptotanshinone and dihydrotanshinone I in comparison to the authenticated samples dried in shade. It indicated that sun or high temperature may lower the content of tanshinones more than salvianolic acids.

Chen *et al.* (2019) investigated the different cultivation and processing methods in different geographical origins of Danshen in China including Shandong, Hebei, Henan, Shaanxi and Sichuan. The Danshen roots from Shandong, Henan, Shaanxi, Hebei are usually grown in sandy soil, but the roots from Sichaun are grown in clay. The study also found that the density of crops is higher in Shandong and Henan compared with other provinces on average. Moreover, the primary processing methods after harvesting are different between provinces. In China, too, the most common drying method is drying under the sun. The drying method used in Henan is oven drying while the method used in Sichuan is “sweating”. Only some of them use drying in shade. It shows that the primary processing of Danshen is inconsistent in China, and it might be one of

the reasons why the quality of Danshen varies greatly. It indicates that the processing method of Danshen mostly relies on the tradition of the region, and it lacks standardisation. It should be noted that some of the farmers have their own processing method for different reasons. For instance, contract farmer may have their standard processing method provided by their supplier. This, therefore, explains the diverse results regarding the relationship between the chemical composition and the geographical origin.

Genotype difference of Danshen

Huang *et al.* (1980), comparing different species of Danshen and its substitutes, found that *Salvia przewalskii* had a higher content of tanshinone IIA (0.38%) but lower content of tanshinone I (0.12%). *Salvia bowleyana* had lower content of tanshinone IIA (0.13%) and tanshinone I (0.01%). Also, *Salvia przewalskii* var. *mandarinorum* contained the highest content of tanshinone IIA (0.53%) but had a relatively low content of tanshinone I (0.038%).

Another study regarding salvianolic acids content in *Salvia* species in China showed that *Salvia bowleyana* has the highest content of salvianolic acid B (7.04%) and that *Salvia sinica* had the highest content of rosmarinic acid (1.41%) compared with *Salvia miltiorrhiza* and *Salvia yunnanensis*. (Li, He and Song, 1993)

Recent studies also showed that *Salvia przewalskii* had a high content of tanshinones, but a low content of salvianolic acids. The wild *Salvia przewalskii* from Gansu had fifteen times less in salvianolic acid B, two times less rosmarinic acid; but two times greater dihydrotanshinone I, five times greater tanshinone I, six times greater cryptotanshinone and twelve times greater than tanshinone IIA compared with the cultivated *Salvia miltiorrhiza* from Henan (Cheng *et al.*, 2011).

The chemical constituents of some cultivars of Danshen and some *Salvia* species in China were studied recently. Five out of six Danshen cultivars contained more than 3% salvianolic acid B, but all of them had less than 0.12% tanshinone IIA, which is unacceptable for most of the pharmacopoeia quality standard. The study also showed that the *Salvia* species in China had a low level of salvianolic acids. However, *S. trijuga* had 0.27% of tanshinone IIA (Wang *et al.*, 2014).

In our result, A6 and A17, both are *Salvia przewalskii*, showed low levels of salvianolic acid B and rosmarinic acid. They also showed high levels of tanshinone I, tanshinone IIA, cryptotanshinone and dihydrotanshinone I. The result is matched with the literature and is significantly different from *Salvia miltiorrhiza* in PCA. On the other hand, many studies were done regarding the chemical composition of *Salvia bowleyana*. In our result, *Salvia bowleyana* did not have a significant difference in salvianolic acids and tanshinones compared with *Salvia miltiorrhiza* in PCA. However, further investigation is needed and the sample size has to increase before a conclusion is made.

Heavy metal

In our study, none of the samples exceeded the limits of As, Cu or Pb, while six out of sixty samples exceeded the limits of Cd according to ChP or USP, including four market raw materials and two concentrated extracts. Additionally, none of these samples were sourced from Vietnam or authenticated samples. Our result also shows that the more processing of the materials the product had, the higher content of heavy metals it contained. The study from (Yan *et al.*, 2012) showed a similar result in Cd level, but it also stated the excessive Cu in *Salvia miltiorrhiza* should be common. Furthermore, the study showed that heavy metal content does not have a strong relationship with the soil. These two studies both showed that *Salvia miltiorrhiza* products do not have a serious excess of heavy metal, but certainly, that not all of them

are safe. However, not many heavy metal studies in *Salvia miltiorrhiza* products regarding the relationship between cultivation and processing methods have been carried out.

Apart from *Salvia miltiorrhiza* products, some pharmaceutical companies sell tanshinone IIA extracts as goods for the use in manufacturing for the final process of other pharmaceutical products. However, a study showed that the heavy metal contents of the market tanshinone IIA extracts are inconsistent. Different batches of product manufactured by the same pharmaceutical companies were highly likely sourced from different raw materials. Eleven out of fifteen samples exceeded the limits of heavy metal from ChP (Guo *et al.*, 2015).

Pharmacological activities

In our result, limited samples inhibited LPS-induced NO production. This assay aims at evaluating the level of anti-inflammation via NF-κB pathway. Fourteen out of fifty-six samples showed cytotoxicity effect on RAW 264.7 in the concentration of 100 ug/ml. No correlation was found regarding the NMR results and the activities above according to the PCA results. This can be caused by several factors. The most prominent reason is the power of the sample size of the positive results was too small and there were lots of variables (the chemical compositions of the samples) influencing the biological activities. Only six samples had a positive effect on LPS induced NO production, which makes the individuals no clustering. It suggests more samples with a positive result are required in order to acknowledge a correlation between variables statistically. Another explanation is that the ^{1}H-NMR results put into PCA cannot represent the totality of the chemical composition. Firstly, the sensitivity of 500Hz ^{1}H-NMR is about 30 nmol. The metabolites under this amount in the samples would be difficult to recognise, yet these metabolites might affect the biological activities. It can

be resolved by increasing the analyte quantity of sample and using a higher power of NMR to increase sensitivity and resolution. Secondly, some of the binned areas were removed due to the influence of water and impurity chemical shifts. It can be resolved by the change of the deuterated solvent. As different deuterated solvent has different effects on the chemical shift and the chemical composition is different from subjects, researchers need to investigate the best strategy of the solvent used to each subject.

However, the result indicates that the current metabolites detected by ^{1}H-NMR including salvianolic acid A, B, tanshinone IIA, cryptotanshinone are not the primary bioactive compounds in LPS induced NO production or cytotoxicity to RAW 264.7. It indicates that the current standard of *Salvia miltiorrhiza* from most pharmacopoeias does not represent its biological activities. To conclude, further adjustments of the method are needed for identifying the chemistry and biological activities before drawing a specific conclusion.

6.2 Supply chain and improvement

Supply chain

The quality of Danshen varies in the market, and the supply chain system of Danshen, as well as any other herbal medicine, often involves several stakeholders. The complexity of the supply chain of herbal medicine varies, and it influences the quality and the value of the products. The supply chain of herbal product often obtains multiple middlemen or processors, which makes the supply chain longer (Booker, Johnston and Heinrich, 2012). In China, which is vastly developing E-commerce, the herbal medicine which can be food supplement as well can be sold via an E-commerce platform. The supply chain of these herbal products does not have a common form.

In the report of a product information quality of St John's Wort structured research (Thakor *et al.*, 2011), 96% of the products from the internet showed poor quality of

information scoring one to two out of five as the maximum score. Although there are arguments that customers who purchase products from the pharmacy may not seek or receive appropriate medical or product advice. Consumers with a good therapeutic relationship with healthcare professionals tend to discuss the products (Batchelor and Ohya, 2009; Cramer *et al.*, 2010).

In our result of heavy metal and chemical composition variation analysis, the Danshen products from online stores either in the UK or China encountered quality problems. The content of tanshinones, especially tanshinone IIA, varied, and four out of fourteen samples from the UK online stores had an adulteration problem. Two out of seventeen samples from Chinese online stores exceeded heavy metal limits. All of them had higher heavy metal limits compared to the samples from Vietnamese herbal market and authenticated samples. These results imply that samples from the online store have a higher risk of safety and security than an actual store.

According to our market study, all the online herbal products in China labelled the source of materials. However, the reliability of the information of the product is not guaranteed as the platform does not validate the identity of the seller. In our market study, another common phenomenon is that the knowledge of the materials, such as the cultivation and the processing method, is lessened from the farmers to the customers. For example, the herbalists in TCM pharmacy usually need to ask the supplier or the middlemen about the origin of the product; the suppliers or pharmaceutical companies often do not know the cultivation or processing method of the herb. It indicates that the information flow between the supply chain members is fragmented along the chain.

Blockchain system: strategy of improving supply chain

With the rise of blockchain technology, some researchers raise the potential applying blockchain to food and herbal product industry (Ahmed and Broek, 2017; Galvez,

Mejuto and Simal-Gandara, 2018; Heinrich *et al.*, 2019; Salah *et al.*, 2019). The current practice of herbal supply chain applies centralised controls. This proves vulnerable to both data modification and management; it encounters a data fragmentation problem.

Blockchain comprises of encrypted "blocks" or public ledger of executed activities in the chain of product or service. The "blocks" are verified by consensus of a majority of participants involved, and the verified records are irreversible. As all the participants form a network of information; and synchronise the "blocks" periodically, blockchain system does not require centralised authority. In addition, the algorithm used in this technology increases the difficulty of being hacked, manipulated or compromised. It increases food security, and prevents food falsification or adulteration (Galvez, Mejuto and Simal-Gandara, 2018).

Blockchain enables data transparency to each sector including the origin of the products, batch number, cultivation and processing methods, additives, storage condition, and more. Someone may argue that centralised authority is free to disclose all the information in order to achieve data transparency, but the accountability of blockchain is superior as a blockchain forms with the trust of groups of participants; whereas the trustworthiness of current practice relies on one specific authority.

The transparency of information from the blockchain system will not only enhance the trust of customers, but it will also be applied for resolving the sustainability problem. According to the national protected wild medicinal species in China, forty-two Chinese medicinal plants are endangered or threatened, for example, wild *Panax notoginseng* (Burk.) F. H. Chen has disappeared in China; most of the *Salvia miltiorrhiza* in the market are cultivated (Wang *et al.*, 2015; Chi *et al.*, 2017). The transparency of the blockchain system can be utilised in collective agreements to tackle the environment and sustainability problem in herbal medicine, for example, it can be

easily identified the illegal harvest and unsustainable exploitation of *Paris polyphylla*; it can be also used to identify the stock of *Paris polyphylla* to minimise unnecessary harvest (Cunningham *et al.*, 2018).

6.3 Limitations of the experiments

Limitations – value chain study through interviews and observations

Interviews provide qualitative and quantitative analysis for value chain study; however, it subjects to interviewers' opinions and the interaction of interviewer and interviewee. Hence the information can be influenced by privacy issues, and availability and bias are unavoidable. To alleviate bias, engagement is necessary to increase the trust between interviewer and interviewee in order to obtain more information. In this case, a robust framework of interview helps interviews to remain objective and enable to engage the society efficiently. The draft of the interview has been attached in the section "**Error! Reference source not found.**". Another concern is social desirability. In other words, negative responses are usually underestimated due to social expectations unfit to interviewees' personal opinions; hence, interviewees tend to have a "socially respected" answer. In order to get reliable answers, questions in the interview were drafted in open question form to avoid interviewees' subjective ratings. The interviewer also needed to ask further questions if the response was not specific enough and additional time of interview was usually required (Krosnick, 1999; Schaeffer and Presser, 2003).

The observation, on the one hand, provides additional information to interview in value chain study. On the other hand, it may provide evidence for interviewees' answers. There are also some limitations in observation. One crucial and critical restriction in this study is the problem of time. To obtain better information, observation needs to be long enough to let the researcher experience the daily work of the targets.

However, the cycle of Danshen cultivation requires one year and the availability of the researcher and the targets are limited. Therefore, the observation was arranged during summer, the harvesting time for Danshen in order to experience most of the work in cultivating Danshen.

6.4 The confidence of using a metabolomics approach in plant-derived products

Limitations – ^{1}H-NMR metabolomics

One of the problems in metabolomics is that a significant proportion of metabolites which degrade or oxidise over time during the experiment, are started in the presence of media such as water, more commonly, or other solvents. Although the processing time of extraction and analysis cannot be eliminated in ^{1}H-NMR, it minimises the sample preparation and shortens analysis time compared to chromatography-based analysis. It also provides higher resolution and more information compared to direct MS and FTIR. The degradation of metabolites causes not only the increase the primary metabolites but also a decrease of secondary metabolites which are usually considered as the key pharmacological active compounds in herbal medicine. It is essential in metabolomics to decreases the chance of degradation of metabolites and sample quality difference over the passage of time.

The ^{1}H-NMR analysis also allows “real-time” detection with a high diversity of metabolites, including secondary metabolites such as flavonoids, alkaloids, terpenoids, etc. and primary metabolites such as amino acids, sugars, fatty acids, etc. It means that all chemical signals are approximately equivalent in the spectrum. Even though the sensitivity depends on the structure of the metabolites but not as much as MS caused by the favour of ionisation. It is possible to directly compare these among chemicals in the spectrum due to the chemicals being proportional to molecular concentration.

Admittedly, sensitivity compared to MS is much lower while NMR sensitivity is around 50 µM in 500 MHz with 5 mm probe. However, with the continuous scientific development of NMR hardware, for instance, magnet strength and probe head, sensitivity has been improving significantly in a short period of time. Cold probe manufactured by Varian enables to increase sixteen times signal to noise ratio per scan. Microprobe also allows analysis of 2 µl instead of 500 µl in the probe during traditional NMR protocol. It is not only the massive drop of sample volumes that increases the sample availability of metabolomics, but it is also the sensitivity when compared to conventional 5 mm probe and is increased twenty times more.

Another limitation in NMR is that the spectrum is unlikely to transpose the chemical shifts in the same solvent. In other words, if overlapped chemical shifts occur, it is unlikely to adjust the spectrum. It is also true that chemicals with similar chemical structures commonly have similar chemical shifts which may cause overlapping. In chromatography base MS metabolomics, this can be easily avoided through the optimisation of solvent system flow in the chromatography column. In NMR metabolomics, 2D dimensional NMR can be applied to rectify this problem. It is also common to find a characteristic chemical shift among similarly structured chemicals.

In metabolomics, especially in herbal medicine, while thousands of metabolites can be found in a single-plant material, current analytical technologies only enable to analysis of an “ice-berg” portion. In NMR, it is approximately 50 metabolites, while MS is around 100 metabolites. However, regarding the key metabolites, it seems NMR still enables to show a clear picture of the dynamics among metabolites. Moreover, the global metabolite analysis, the robustness and the rapidity in NMR fits the idea of metabolomics that is a holistic metabolite dynamics study within living organisms.

6.5 The rationale and critical evaluation of bioassays used (including MTT, H_2O_2 induced apoptosis in RAW 264.7, NO production induced by LPS in RAW 264.7

Limitations – LPS induced NO assay and H_2O_2 induced apoptosis in RAW 264.7

LPS induced NO assay and H_2O_2 induced apoptosis are commonly used in drug discovery for anti-inflammatory agents and anti-oxidants. These are fast, easy handled and price reasonable assays but both assays are considered as general, meaning that they cannot fully understand the mechanism of inflammation and oxidisation. Additionally, the mechanism of inflammation is a very complicated subject which involves different kinds of factors. In LPS induced NO assay, LPS as a bacterial metabolite initiates a significant immune response from the host which increases the NO level. However, in vascular and cardiovascular diseases which Danshen has been used for, it does not necessarily involve bacterial infections but other factors such as diabetes, physical damages, etc. (Brownlee, 2005; Shiao *et al.*, 2008; Kreuger and Phillipson, 2016). In H_2O_2 induced apoptosis assay, H_2O_2 plays as free radical against the cells to promote apoptosis of both cardiovascular and vascular cells. Oxidative stress is reported as one of the major reasons for having circulatory illnesses (Valko *et al.*, 2007; Hansson and Hermansson, 2011; Chavez-Sanchez *et al.*, 2014). For example, evidence showed the production of reactive oxygen species increases the formation of angiotensin, which regulates blood pressure and fluid balance which, as a consequence increases the chance of having angiotensin II-induced hypertension (Valko *et al.*, 2007; Jones and Grainger, 2009). However, in the experiment, RAW 264.7 are mutated rat macrophages that do not have a direct relationship with human vascular cells or macrophage. The reason for using RAW 264.7 is not only for pharmacological relevance but also feasibility and time constraints. In order to understand the potential of metabolomics linked with pharmacological evidence, large scale study and replication should be applied. Hence,

since there are not many studies using this kind of approach, a time-consuming approach might not be worthwhile.

6.6 Conclusion and prospects

This study is the first interdisciplinary study regarding the correlation of supply chain, phytochemistry, biological activity of Danshen (*Salvia miltiorrhiza* Bunge.) using fieldwork, market study, trace metals analysis, HPTLC, NMR, cytotoxicity and anti-inflammation bioassays.

This study shows that the quality of Danshen varies among different sales channels and that there are adulteration and contamination problems. The risk of heavy metals and variation of chemical compositions have a direct relationship to the processing of the product. It also implies that the current chemical standards in pharmacopeia do not represent the totality of the anti-inflammatory activity in Danshen.

Although ^{1}H-NMR metabolomics has certain challenges needed to be resolved, including sample selection and sensitivity, it is obvious that the Danshen products in the market have huge variations in chemical composition and biological activities. Further investigations using a metabolomics approach to understand the relationship between phytochemistry and biological activity are needed.

Organic farming may boost the yield of secondary metabolites, but there are still some factors may influence the results such as density of crops and soil nutrients. It does not represent enhancing its pharmacological effect. This study also recommends using a blockchain system to monitor the supply as a solution for tackling quality, environmental and sustainability problem.

VII. References

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VIII. Supplementary documents

1.1 Determination of H2O2 concentration

0.1 M of potassium permanganate ($KMnO_4$) was prepared by dissolving 1.58 g $KMnO_4$ in 100 ml distilled water. 0.1 ml of stock H_2O_2 solution with a few drops of 0.1

M HCl was diluted with 50 ml distilled water, then was titrated with permanganate solution until a faint pink colour persists for 30 seconds. The calculation and the record of preparing 1 M of H_2O_2 were showed in the following:

Determination of the concentration of stock H_2O_2:

$$\left(\frac{0.1\ M\ X\ Titration\ volume\ (ml)}{0.1\ \ ml\ (the\ volume\ of\ H_2O_2}\right)\times\frac{2}{5}$$

Determination of preparing 1 ml of 1 M of H_2O_2 in PBS:

$$\frac{1\ ml}{the\ concentration\ of H_2O_2}$$

Test Date	Titration volume (ml)	Stock H_2O_2 concentration (M)	H_2O_2 Added volume to PBS (ul)	PBS volume (ul)
01/12/2016	31.1	12.44	80.4	919.6
15/01/2017	31.6	12.64	79.1	920.9
15/02/2017	31	12.4	80.6	919.4
30/04/2017	30.8	12.32	81.2	918.8
31/05/2017	30.1	12.04	83.1	916.9
30/06/2017	30.5	12.2	82.0	918.0
31/07/2017	30.5	12.2	82.0	918.0
15/01/2018	31.2	12.48	80.1	919.9
15/02/2018	31.2	12.48	80.1	919.9
15/03/2018	30.9	12.36	80.9	919.1
15/04/2018	30.8	12.32	81.2	918.8
15/06/2018	31	12.4	80.6	919.4
15/07/2018	31.5	12.6	79.4	920.6

15/08/2018	32	12.8	78.1	921.9
15/10/2018	31.6	12.64	79.1	920.9
15/11/2018	31.6	12.64	79.1	920.9
15/12/2018	30.9	12.36	80.9	919.1

Table 21 The results of hydrogen peroxide concentration determination

The H_2O_2 solution was kept in under 4°C refrigerator and was tested nearly every month while the Evaluation of H_2O_2 induced apoptosis was being carried out. It was labelled as 30% H_2O_2 (w/w) in the product description but according to the test it was 12.43 M on average which is around 37% (w/w). Experiment data was used instead of product description. There were two reasons: 1) the experimental data was consistent. The range of determined H_2O_2 concentration was between 12.02 to 12.64 M with 17 different trials in different time. This permanganate titration method to determine the molarity of H_2O_2 has been used for a long while and 2) Given that some literatures reported that, the experiment data matched with literature results that more than 50% cytotoxicity to RAW 264.7 occurs at around 1 mM.

1.2 Overview of interviewees and his/her relevance to the supply chain studied

	Supply chain sector	Interviewee Occupation	Year of Experience	Major duty	Relevance to the supply chain	Main business/ service	Main products
1	TCM pharmaceutical	Project Manager	17 years	ODM or OEM product improvement/ development	Secondary processing Wholesaler	1) Manufacture TCM extracts 2) Provide quality assessment	TCM extracts
	Research institute	Deputy Director					

	Quality assessor	CSO				3) Provide product R&D service	
2	TCM pharmaceutical	1) Production technology manager 2) Xindian manufacturer manager 3) Product development manager	12 years	1) Product production 2) Internal product development	Secondary processing Wholesaler	Manufacture TCM extracts	TCM extracts
3	TCM pharmaceutical	International trade department manager	12 years	International trading of TCM extracts	Secondary processing Wholesaler	Manufacture TCM extracts	TCM extracts
4	Farming and manufacturer	Director and founder	38 years 16 years (Organic TCM materials)	Production and business monitoring	Producer/ Farmer Primary processing Secondary processing Wholesaler	1) Sell organic TCM materials 2) Manufacture and sell it derived products as food supplements	Organic TCM materials and products
5	Governmental department	Vice researcher	22 years	1) Agricultural experiments and research 2) Agricultural demonstration for local farmers	Agricultural productivity research	Improve agricultural productivity	N/A
6	Distributor/ supplier	Director	10 years	Distribute the products from	Distributor	Provide TCM materials to	TCM raw materials

				parent company		professional bodies	
7	TCM pharmacy	TCM doctor/ pharmacist	7 years	1) Provide TCM diagnosis to patients 2) Manage the quality of TCM materials	Distributor Secondary processing	1) Traditional TCM materials 2) Traditional TCM decoction process	TCM raw materials

Table 22 Overview of interviewees

1.3 Semi-structured interview framework

Consent form

I am Ka Yui Kum, a PhD student in UCL studying metabolomics, quality and the composition of *Salvia miltiorrhiza* Bunge – Danshen derived products along with its value chains. I would like to interview you on this topic. No personal data will be stored/recorded. The interview will be recorded, is it fine to you?

Before that, I would like to know more about you.

(Asking following information)

Age:

Highest Degree obtained:

Company:

Position:

Year of experience:

Contact:

Today is _____. Interviewee number is _____. Let's start our interview.

Starting questions

1) How important is Danshen in your company compared to your other business?

2) Could you summarise the supply chain of Danshen products in your company and what do you think about it?

3) Please tell me more about the main commercial interests of your company?

4) Please tell me more about your department?

(According to their speciality, move the questions to different sessions)

Management

1) Please summarise the management structure of your company?

2) Is there any the partnership/collaboration with other companies/associations, which is relevant in the context of danshen production? Which is/are the main partnership(s)? (technology, quality control, product design, quality, or marketing)

3) In the whole supply chain, which part is the most challenging from your company's perspective?

4) In the whole supply chain, which part is the strength of your company?

5) In the whole supply chain, which part is being subcontracted or outsourced?

6) How many Danshen related products do your company have?

Cultivation and processing

1) What is the role of your department in the context of cultivation?

2) Could you summarise the cultivation of Danshen as it relates to your company?

3) How is the structure of your farm? Are there any other species that you are cultivating as well?

4) How did you get first the seed from? How do you identify that is the right seed? Whom/how did you obtain the method from? How do you procure seeds or other starting material?

5) What is/are the difficulties in identifying Salvia miltiorrhiza/Danshen?

6) What are the other crops produced on your danshen production sites? If yes, what is/are they for?

7) How do you cultivate Danshen? Whom/how did you obtain the method?

8) What is/are the difficulties in cultivating Danshen products? (Pests, contaminants)

9) What is/are the pesticides you use? How do you monitor the outcome? Do you do a pesticide analysis of the product(s)?

10) What is/are the fertiliser you use? What is the basis for this? How do you monitor the outcome?

11) How do you harvest Danshen? When and what are the specific conditions you need?

12) What is/are the difficulties in harvesting Danshen products?

13) How do you deal with other parts and bad Danshen roots?

14) How do you process Danshen after harvesting?

15) What is/are the difficulties in processing Danshen?

16) How do you categorise your Danshen?

17) What is/are your customer group(s)? What is the price range of Danshen?

18) How much for the cost of cultivating Danshen? That includes money, time, workers.

19) Why do you keep cultivating Danshen?

Processing – Danshen final products

1) Could you summarise the processing of Danshen into your company's final products?

2) What is/are the customer group(s)?

3) How do you obtain Danshen for your products?

4) How many source(s)/suppliers do you have for Danshen? Why do you pick this/these suppliers?

5) How do you resolve problems of sourcing?

6) How do you assure the quality of your products? (method and frequency, external or self- management) Where did you obtain the method from?

7) How do you monitor the quality of your products? (method and frequency, external or self- management)

8) What are the standards you are using? How do you obtain/decide on the standards?

9) What is/are the difficulties or common problems in processing Danshen products?

10) How do you deal with the unusable parts from your supply materials?

11) How long does each process of processing Danshen products take?

12) In your business, what is the percentage of working on processing Danshen products over your whole work?

Packaging and storage

1) What are your routine conditions for storing danshen?

2) For how long would you normally store it prior to selling it? And what is the maximum period you would store it?

3) Could you summarise the packaging of Danshen products?

4) How do you package the Danshen products? Are they outsource/subcontract or part of your company?

5) What kind of materials are you using in packaging Danshen?

6) Where do you obtain the materials from?

7) Why do you have this packaging?

8) What is/are the difficulties/common problems in packaging Danshen products?

9) How do you solve these problems?

10) How long does packaging Danshen products take?

11) In your business, what is the percentage of working on packaging Danshen products over your whole work?

12)

Transport and distribution

1) Could you summarise the transport system for Danshen products?

2) What is/are the customer group(s)?

3) How do you transport Danshen products?

4) Why do you have this transporting method?

5) What is/are the difficulties/common problems in transporting Danshen products?

6) How do you solve these problems?

7) How long does transporting Danshen products take to the destination?

8) In your business, what is the percentage of working on transporting Danshen products over your whole work?

Marketing

1) Could you summarise the local market of Danshen products?

2) Could you summarise the international market of Danshen products?

3) What is/are the customer group in your company? (Patients, Domestics, Industrial, Institutional?) (Local and international?)

4) Is there any competitor for your company? Which is/are the main competitor(s)?

5) How do you promote Danshen products to your service target?

6) What is/are the difficulties in marketing Danshen products?

7) What is the price range of Danshen products? What is/are the factors/parameters of the price?

8) How strong would you say the market of Danshen products in your company compared to other business?

This is the end of the interview. Thank you for your participation.

^{1}H-NMR validation

the protection from H_2O_2 induced apoptosis in RAW 264.7 validation

the inhibition from NO production induced by LPS in RAW 264.7 validation

PCA analysis validation