OVERCOMING THE CHALLENGES OF HERBAL ADULTERATION IN A GLOBALIZED WORLD

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OVERCOMING THE CHALLENGES OF HERBAL ADULTERATION IN A GLOBALIZED WORLD

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EDITORIAL
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Editorial: Overcoming the Challenges of Herbal Adulteration in a Globalized World

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Editorial on the Research Topic

Overcoming the Challenges of Herbal Adulteration in a Globalized World

The adulteration of plant based products is a global problem and one that cannot be addressed without combined efforts from industry, academia and regulators. This research topic focused on the problems of quality in the broadest sense as well as looking at specific established and new techniques to help improve the detection of sub-standard products. The issues investigated included species authentication, adulteration and contamination, value chains, agricultural practices and sustainability. Authors and reviewers contributed from Asia, the Americas and Europe, with a high percentage of published papers coming from Chinese institutions.

Of the fourteen papers that were accepted for publication, three papers focussed on documenting and highlighting the scale of the problem. Ichim and Booker in their review assessed the scale of the problem globally and found that 37 countries had significant adulteration problems. An opinion piece provided by Palhares et al., the authors described the situation in Brazil and particularly in relation to sustainability and the protection of minority groups and their traditional knowledge. Luo et al. looked specifically at the on-going and increasing problem of heavy metal contamination in Chinese herbal products, something that is often affected by increased industrial pollution of the land and rivers as well as being due to cultivation or collection in areas where they are naturally occurring e.g., in cinnabar containing rock, risking mercury contamination in crops.

There were several papers that explored the value chains of medicinal plants and Kum et al. used Salvia miltiorrhiza as an example of how incorrect species might enter the supply chain and how different processing and storage techniques can affect the end quality of products. Whereas Bi et al. investigated the value chains of Astragalus membranaceus var. mongholicus and assessed the quality in relation to the geographical origin of the material and the effects of using different systems of production and suggested sustainable cultivation as a workable alternative to wild Research Topic. Cultivation may be a viable sustainable option to uncontrolled wild collection, however start up costs and lack of infrastructure can inhibit this process and more governmental and industrial support is needed for these practices to be established long-term.

Other researchers provided new ways to assess the quality of herbal products. Woodley et al. conducted a review of chemical methods and described how mitochondrial activity could be used as a new measure for assessing the biological potency of herbal products and gave detailed methodology on how this could be achieved. Ichim and De Boer described the variation in Ginseng quality and how there were other problems apart from incorrect species, including the wrong part of the plant used and incorrect age of the plant at the time of harvesting (ginseng should be at least 5 years old prior to harvesting in order to obtain the desired amounts of metabolites). There were three papers

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on shotgun metabarcoding and Yu et al. presented this as a powerful tool for the molecular identification of biological ingredients. This was endorsed by Liu et al. who described how adulterating fungi could be detected in complex herbal mixtures using this technique. Xie et al. showed how shotgun metabarcoding could overcome biased PCR amplification and be used to authenticate herbal mixtures with distinct variation of dosages of individual ingredients. Similarly Yu et al. described specific mini- DNA barcode techniques for use with highly processed herbal products.

Some of the new techniques (or known techniques repurposed) focussed on single plants and Yu et al. described how a method was developed for the analysis of Ophiopogon japonicus that incorporated quantitative microscopy. This allowed for the differentiation of quality within the same species and highlighted the concept of da di (superior quality) herbs in the Chinese herbal supply chain. Ichim et al. also looked at how microscopy could be used as a cost-effective and rapid method to identify impurities within mixtures although with the caveat that it was difficult to use with highly processed materials e.g., extracts. Zhao et al. described vector control quantitative analysis as a new method for analysing complex formulations. A PCR strategy was explained that rapidly generated the integrated DNA fragments needed from multiple targeted species. A technique with much potential for future use within the industry.

It is clear that there are global initiatives in place to improve the detection of poor quality and adulterated material in herbal medicines and there has been advances in many analytical techniques in an effort to overcome the difficulties encountered in analysing complex mixtures of medicinal plants. There appears to be growing emphasis on investigating quality problems along the length of the value chain as opposed to focussing on the finished products and with this comes an increased awareness of good collection and cultivation practices and a greater resolve to tackle issues of sustainability. Overall the papers emphasise the need for effective quality management along the length of the supply chain and the continued application of well known and new techniques to improve herbal quality and safety.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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MINI REVIEW
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Microscopic Authentication of Commercial Herbal Products in the Globalized Market: Potential and Limitations

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Herbal products are marketed and used around the globe for their claimed or expected health benefits, but their increasing demand has resulted in a proportionally increase of their accidental contamination or intentional adulteration, as already confirmed with DNA-based methods. Microscopy is a traditional pharmacopoeial method used for plant identification and we systematically searched for peer-reviewed publications to document its potential and limitations to authenticate herbal medicines and food supplements commercially available on the global market. The overall authenticity of 508 microscopically authenticated herbal products, sold in 13 countries, was 59%, while the rest of 41% were found to be adulterated. This problem was extending over all continents. At the national level, there were conspicuous differences, even between neighboring countries. These microscopically authenticated commercial herbal products confirm that different magnifying instruments can be used to authenticate crude or processed herbal products traded in the global marketplace. The reviewed publications report the successful use of different magnifying instruments, single or in combinations with a second one, with or without a chemical or DNA-based technique. Microscopy is therefore a rapid and cost-efficient method, and can cope with mixtures and impurities. However, it has limited applicability for highly processed samples. Microscopic authentication of commercial herbal products will therefore contribute to raise public awareness for the extent of adulteration and the need to safeguard consumer safety against the challenges of globalization.

Keywords: light microscopy, electron microscopy, authentication, plant identification, food supplement, traditional medicine, herbal product, consumer safety

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INTRODUCTION

Herbal products are a heterogeneous category of goods which are produced, marketed, and used around the globe for their claimed or expected health benefits. Their commercial names depend on their final declared use (i.e., medicines or foods), and the prevailing legal frameworks and regulatory requirements (Simmler et al., 2018), such that they are sold under different names, such as herbal

drugs, botanical drugs, botanicals, phytomedicines, traditional medicines, herbal medicines, traditional Chinese medicines (TCMs), traditional herbal medicinal products, natural health products, or plant food supplements (Ichim, 2019).

The use of herbal medicinal products and supplements has increased tremendously over the past decades with a substantial proportion of the world's population relying on them as element of primary healthcare (Ekor, 2014). The global market for herbal products is rapidly expanding and expected to reach 115,000 million US$ in 2020 (Raclariu et al., 2018a) while the trade of medicinal plants will continue to advance with annual growth rates of 15–25% (Booker et al., 2012). This increasing demand for plant-based products has resulted in a proportionally increase of peer-reviewed reports of accidental contamination or intentional, economically motivated adulteration (de Boer et al., 2015; Ichim et al., 2018; Simmler et al., 2018; Grazina et al., 2020; Grosu and Ichim, 2020). A global analysis of nearly 6,000 herbal products sold in 37 countries has revealed that 27% of the products contain undeclared contaminants, substitutes, or filler species (Ichim, 2019). To address this problem, DNA barcoding as powerful strategy has recently attracted considerable attention and, along with chemical methods (de Boer et al., 2015; Pawar et al., 2017; Sgamma et al., 2017), has started to enter the regulatory systems for quality control (Pharmacopoeia Committee of P. R. China, 2015; British Pharmacopoeia Commission, 2018).

However, DNA-based diagnostics of processed food products or of mixtures from different plants can sometimes be challenging (Raclariu et al., 2018b; Grazina et al., 2020), such that this principally powerful approach has to be complemented by alternative methods (Sgamma et al., 2017). Microscopy has long been used to identify herbal products in many countries, as recorded in many pharmacopoeias, because of its advantages of small amount of sample needed, speed, reliability, simplicity, and low costs (Au et al., 2009). Moreover, histochemical techniques have been used to reveal the characteristics of tissue structure and cellular features that can be used as markers for species identification (Au et al., 2009).

Since they are often taxon-specific, in some cases even down to the species level, the morphological, anatomical, and microscopic characteristics of plant species are of great value for the purposes of scientific investigation and botanical quality control (Grazina et al., 2020). These characteristics can be used to verify plant authenticity, and to detect contaminations, adulterations, and substitutions in plant products (Michetti et al., 2019). Microscopic authentication refers to the analysis of structural, cellular and molecular features of herbal products using different forms of microscopy (Tam et al., 2006). While macroscopic and microscopic examinations are easily applied to fresh whole plants or plant parts, dried products, as those typically sold on the market, are generally difficult to identify, as many useful diagnostic characteristics are lost during dehydration (Joharchi and Amiri, 2012). In addition, often macroscopic or microscopic examinations will fail because a preparation consists of multi-component powdered samples that have been processed beyond the extent that would allow morphological characterizations (Pawar et al., 2017).

Rather than to extend the rampant increase of peer-reviewed single-case reports on adulteration of commercial herbal products, this mini-review strives to give a survey on the documented ultimate real-case scenario of all: the global market of herbal products. What are potential and limitations of microscopic food diagnostics in detecting substitution and adulteration in order to safeguard consumer safety in a globalized economy?

METHODS

Search Strategy

We systematically searched four databases for relevant peer-reviewed studies following the PRISMA guidelines (on 17 January 2020) (Moher et al., 2009). Combinations of relevant keywords, Boolean operators and wildcards were used: [("medicinal plant" OR herbal OR botanical OR nutraceutical OR TCM) AND (microscop* OR histolog* OR morpholog*) AND (identification OR authentication OR adulteration)] for Web of Science, PubMed, and Scopus, and [("medicinal plant" OR herbal OR botanical OR nutraceutical) AND (microscopy OR microscopical OR histological) AND (identification OR authentication)] for ScienceDirect, due to limitations imposed by the latter search engine. The option "search alert" was activated for all the databases, to receive weekly updates after the search was performed.

Selection Process and Criteria

Identification: 4,406 records were identified through database searching (WoS = 998, PubMed = 846, Scopus = 2,426, and ScienceDirect = 136), and additional 174 records through other sources.

Screening: 2,326 records were retrieved and their abstracts screened after the duplications had been removed. After screening, 2,062 records were excluded for not reporting data relevant to the microscopic authentication of herbal products.

Eligibility: 264 full-text articles were assessed and screened based on the following eligibility criteria:

1. The reported samples had to be "herbal products" *sensu lato*. A wide range of commercial names was searched for and accepted for inclusion into our analysis, all falling under two main categories: medicines or foods, with claimed or at least expected health benefits.
2. The products had to be clearly allocated to a "country" or "territory" (i.e., Hong Kong).
3. The conclusion "authentic"/"adulterated" (or similar) had to be drawn by the authors of the analyzed studies. Our involvement was restricted to operations such as counting the samples, transforming percentages in absolute numbers, without reinterpreting the experimental results in any way.
4. The samples had to be authenticated with a magnifying instrument. A wide variety of instruments were accepted,

from hand lenses to electronic microscope. The morphological authentication with the naked eye was excluded from our analysis.

5. The products had to have a commercial value. Studies where the analyzed samples were "collected", obtained "cost-free", a "gift" or "donated" by a person, institution or company, were excluded.

The set of retrieved full-text articles was further reduced by 236 that did not meet all five eligibility criteria.

Included: 28 peer-reviewed articles.

RESULTS

Our systematic literature search identified 28 peer-reviewed publications reporting the use of magnifying instruments for the successful authentication of 508 commercial herbal products. In average, the reviewed articles reported the results for 18 herbal products, the number varying from 1 product (Liu et al., 2013; Czigle et al., 2018) to as many as 86 commercial samples (Walker and Applequist, 2012). In 13 articles, the light microscope was reported as the only instrument used for authentication, being successfully used for a total of 226 products, while the remaining studies included a second magnifying instrument, from hand lenses and stereo-microscopes till scanning electron microscopes, for the analysis of the remaining 282 products. Besides, in 20 articles at least one additional technique (DNA- or/and chemistry-based methods) was used to test authenticity. Yet, in eight studies, the exclusive use of microscopy was successfully authenticating a total of 358 commercial herbal products (**Table 1**).

We have reviewed the authenticity results for 508 herbal medicines and food supplements traded in thirteen countries or territories (i.e., Argentina, Brazil, China, Egypt, Hong Kong, Germany, Greece, India, Iran, Peru, Thailand, Turkey, and USA), geographically representing all inhabited continents, except Australia. For two countries (i.e., Argentina and USA), more than 100 commercial herbal products have been analyzed and microscopically authenticated from their respective national marketplace. For additional six countries (i.e., Brazil, China, Germany, Hong Kong, Iran, and Thailand), more than 10 herbal products were analyzed, while for Egypt, Greece, India, Peru, and Turkey, 10 products or less were reported (**Figure 1**).

The overall authenticity of the microscopically authenticated commercial herbal products from the global marketplace was 59% (n = 300 products), while the rest of 41% (n = 208 products) were found to be adulterated (**Table 1**). All or at least most (>70%) products were reported to be authentic in Argentina (n = 135), China (n = 40), Germany (n = 38), Thailand (n = 4), and Egypt (n = 2). In the USA (n = 128) and Peru (n = 2), a substantial part (> 30%) was wrongly declared, and a third group of countries, comprising Iran (n = 70), Brazil (n = 30), India (n = 10), Turkey (n = 10), and Greece (n = 1) showed authenticity score is lower than 40%. It should be kept in mind that the numbers of samples was geographically heterogeneous: At continental level, the highest number of commercial herbal products was reported for South America (n = 167), followed very closely by Asia (n = 164), by North America (n = 128) and, distantly, by Europe (n = 49). Instead, almost half (49%) of the total products (n = 167) microscopically authenticated in Asia were reported to be adulterated, followed by South America (40%), Europe (39%), and more distantly by North America (33%).

DISCUSSION

Our survey on the extent of herbal adulteration as detected by microscopical diagnostics supports a previous study using DNA-based authentication (Ichim, 2019). On the global scale, almost half of herbal products did not contain, what they declared. However, there are substantial differences between the different national markets, sometimes even between neighboring countries, such as Argentina (with a high authenticity score among the 135 tested products) and Brazil (with a low authenticity score among the 30 tested products). In the following, we will discuss to what extent this startling outcome is robust against unavoidable sampling bias, and what the potential and the limitations of microscopic diagnostics are. Using two case studies, we will address possible reasons for this high degree of adulteration, and conclude by deriving some suggestions, how consumer protection can be safeguarded in times of globalization.

Sampling Heterogeneity and Its Reasons

The frequency of adulteration in the current study exceeds that seen for the study based on DNA authentication (Ichim, 2019). This might stem from the fact that the sample size is different (DNA authentication: n = 5,957; microscopic authentication: n = 508) by a factor of ten. There are different reasons for this obvious sampling bias: to publish results from a traditional method in peer-reviewed journals is much more difficult as compared to publishing DNA-based, "new" diagnostic approaches. The countries with a functional consumer safety system might be underrepresented as the authentication results of the commercial samples screened by the respective institutions will be published in internal bulletins or protocols, rather than in peer-reviewed journals. There is a second, methodological reason, though: microscopic diagnostics requires not only patience, exact observation, and long experience, but also deep knowledge of plant anatomy and plant biodiversity. This expertise is currently lost rapidly in most countries, and may be one reason, why some countries are not represented in our sampling. The comparison of Brazil (n = 30, most adulterated) and Argentina (n = 178, most authentic) indicates a further reason for sampling bias: the lack of methodological expertise may also correlate with a lack of consciousness that there is a serious problem.

Prospects and Limitations of Microscopic Diagnostics

Our current study clearly confirms previous results (Gao et al., 2017) that a substantial proportion of commercial herbal

TABLE 1 | Microscopy-based authentication of commercial herbal products.

Country/ Continent	Products/authenticated species	Reference material used & vouchers deposited (Y/N)	Products (no.)			Magnifying instrument	Other techniques used	Ref.
			Total	Authentic	Adulterated			
China	Traditional herbal tea "Ku-Ding-Cha" from markets & manufacturers (intact or fragmented dried leaves or powders)/*Ilex kudingcha* C.J.Tseng, *I. latifolia* Thunb., *Ligustrum robustum* (Roxb.) Blum, *Clerodendrum fortunatum* L., *Ehretia acuminata* R.Br.	plant samples (Y)	19	19	0	light microscope, polarized light microscope	n/a	(Tam et al., 2006)
China	Radix Polygoni Multiflori (Heshouwu)/dried root tuber of *Reynoutria multiflora* (Thunb.) Moldenke	n/a	12	12	0	light microscope	TLC, HPLC	(Zhang et al., 2005)
China	traditional "Xihuangcao" herbal tea bags from retail stores/*Isodon lophanthoides* (Buch.-Ham. ex D.Don) H.Hara, *I. lophanthoides* var. *graciliflorus* (Benth.) H.Hara, *I. serra* (Maxim.) Kudô	collected reference plant baches (Y)	8	0	8	light microscope	UPLC-ESI-QTOF-MS	(Wan et al., 2016)
China	Menispermi Rhizoma from a drug store/dried rhizome of *Menispermum dauricum* DC.	n/a	1	0	1	light microscope	UPLC-DAD-MS	(Liu et al., 2013)
Egypt	slimming herbal tea products/*Cichorium intybus* L., *Urtica dioica* L., *Origanum majorana* L., *Senna alexandrina* Mill. leaves, *Glycyrrhiza glabra* L. roots, *Apium graveolens* L. fruits, *Calendula officinalis* L. flowers, *Foeniculum vulgare* Mill., *Cichorium intybus* L.	standard herbal tea mixtures prepared from collected herbs (N)	2	2	0	light microscope	HPLC, LC-MS-MS, GC-MS	(Abdel Kawy et al., 2012)
Hong Kong	*Cordyceps sinensis* purchased fermented samples and supplements (capsules)/*C. sinensis*, *C. hawkesii*, *C. liangshanensis*, *C. militaris*	collected samples (Y)	15	4	11	light microscope, polarized light microscope	n/a	(Au et al., 2012)
Hong Kong	traditional crude drug "Wuzhimaotao" (Radix Fici Hirtae) (primarily dried roots of *Ficus hirta* Vahl)/*F. hirta* Vahl, *F. simplicissima* Lour., *F. esquiroliana* H.Lév	plants (Y)	5	3	2	light microscope	n/a	(Au et al., 2009)
India	Ayurvedic crude drug "Daruharidra" (roots of *Berberis aristata* DC.) from drug markets/*B. aristata* DC., *B. asiatica* Roxb. ex DC., *B. chitria* Buch.-Ham. ex Lindl., *B. lyceum* Royle	plants (N)	10	0	10	light microscope	HPTLC	(Srivastava and Rawat, 2013)
Iran	crude raw material of herbal drugs/27 cases of herbal drugs	n/a	78	30	48	dissecting microscope	n/a	(Joharchi and Amiri, 2012)
Thailand	white "KwaoKrua" products from local markets/*Pueraria candollei* Benth.	plant leaves (Y)	7	7	0	light microscope	ARMS-PCR, HPLC	(Intharuksa et al., 2020)
Thailand	strains of Thai medicinal fungus *Cordyceps militaris* collected from different commercial farms/*C. militaris*	n/a	7	7	0	light microscope, scanning electron microscope	DNA barcoding	(Nopparat et al., 2018)
Asia			***164***	***84***	***80***			
Germany	"Goji" products (dried fruits)/*Lycium barbarum* L., *L. chinense* Mill., *L. ruthenicum* Murray fruits, *L. europaeum* L., *L. chilense* Bertero, *L. ameghinoi* Speg.	whole plants (Y), fruits, DNA (Y)	19	19	0	stereo microscope, light microscope	DNA barcoding, ARMS-PCR	(Wetters et al., 2018)
Germany	three bamboo teas and five fruit teas containing bamboo leaves/bamboo (*Bambusoideae*), lemongrass (*Cymbopogon*)	plants (Y)	8	4	4	stereo microscope, light microscope	DNA barcoding	(Horn and Häser, 2016)
Germany	commercial tea mixtures containing 'Lemon Myrtle'/*Backhousia citriodora* F.Muell., *Leptospermum petersonii* F.M.Bailey	plants (Y)	4	4	0	stereo microscope, light microscope	RAPD	(Horn et al., 2012)

(Continued)

TABLE 1 | Continued

Country/ Continent	Products/authenticated species	Reference material used & vouchers deposited (Y/N)	Products (no.)			Magnifying instrument	Other techniques used	Ref.
			Total	Authentic	Adulterated			
Germany	Holy Basil 'Tulsi' mixtures (mixture teas, cut leaf fragments)/*Ocimum tenuiflorum* L., *O. basilicum* L., *O. serratum* (Schltr.) A.J.Paton, *O. gratissimum* L.	plants (Y)	4	0	4	stereo microscope, light microscope	DNA barcoding	(Jürges et al., 2009)
Germany	tea mixtures/*Dracocephalum moldavica* L.	plants (Y)	3	3	0	stereo microscope, light microscope	ARMS-PCR, RFLP	(Horn et al., 2014)
Greece	powdered Ginkgo leaf food supplement purchased in local community pharmacy/*G. biloba* L.	n/a	1	0	1	light microscope	HPLC-UV, LC-MS/MS	(Czigle et al., 2018)
Turkey	leaf products from different herbal shops/*Eucalyptus camaldulensis* Dehnh., *E. globulus* Labill., *E. grandis* W.Hill	plants (Y)	10	0	10	light microscope	TLC	(Tombul et al., 2012)
Europe			***49***	***30***	***19***			
USA	unprocessed products containing botanicals purchased from retail outlets/*Arnica Montana* L., *Arnica* sp., *Matricaria chamomilla* L., *Crataegus* sp., *Juniperus communis* L., *Tilia* sp., *Hypericum perforatum* L., *Schisandra* sp., *Scutellaria lateriflora* L., *Illicium verum* Hook.f.	n/a	86	65	21	hand lenses, dissecting microscope	n/a	(Walker and Applequist, 2012)
USA	"buchu" commercial raw materials and finished products/*Agathosma betulina* (P.J.Bergius) Pillans, *A. crenulata* (L.) Pillans, *A. serratifolia* (Curtis) Spreeth	plants (whole/powder) (Y)	27	10	17	light microscope, scanning electron microscope	HPTLC	(Raman et al., 2015)
USA	commercial "yohimbe" raw products/*Pausinystalia johimbe* (K.Schum.) Pierre ex Beille	plant bark (Y)	12	9	3	light microscope, scanning electron microscope	UPLC-UV-MS	(Raman et al., 2013)
USA	*Hoodia gordonii* (Masson) Sweet ex Decne. capsules/*Hoodia* sp., *Opuntia ficus-indica* (L.) Mill., *Ceropegia dichotoma* Haw., *Cynanchum* sp., *Edithcolea grandis* N.E.Br., *Huernia* sp., *Orbea* sp., *Piaranthus globosus* A.C. White & B. Sloane, *Stapelia* sp., *Tridentea choanantha* (Lavranos & Harry Hall) L.C. Leach	herbarium vouchers, collected plants (Y)	3	2	1	light microscope	PCR	(Joshi et al., 2009)
North America			***128***	***86***	***42***			
Argentina	single ingredient herbal products (mostly fragmented)/various species	n/a	64	44	20	light microscope	n/a	(Cuassolo et al., 2010)
Argentina	herbal drugs "canchalagua"/*Schkuhria pinnata* (Lam.) Kuntze ex Thell., *Scoparia montevidensis* (Spreng.) R.E. Fr.	herbarium vouchers, collected plants (Y)	59	40	19	stereo microscope, optic microscope	n/a	(Molinelli et al., 2014)
Argentina	fine cut (tea bags) and thick cut (fragmented herbs) mixtures/21 different species	n/a	11	7	4	stereo microscope, optic microscope	n/a	(Michetti et al., 2019)
Argentina	dietary supplements from local market/*Arthrospira* sp.	n/a	1	0	1	light microscope, polarized light microscope	FT-IR, TLC, 1D-2D NMR, PLC-MS/MS	(Redko et al., 2018)

(Continued)

TABLE 1 | Continued

Country/ Continent	Products/authenticated species	Reference material used & vouchers deposited (Y/N)	Products (no.)			Magnifying instrument	Other techniques used	Ref.
			Total	Authentic	Adulterated			
Brazil	products from drugstores/*Smilax goyazana* A.DC., *S. rufescens* Griseb., *S. brasiliensis* Spreng., *S. campestris* Griseb., *S. cissoids* M.Martens & Galeotti, *S. fluminensis* Steud., *S. oblongifolia* Pohl ex Griseb., *S. polyantha* Griseb.	roots, leaves (Y)	15	0	15	light microscope, scanning electron microscope	TLC, DNA barcoding, SSR markers	(Martins et al., 2014)
Brazil	herbal drugs "carqueja"/*Baccharis trimera* (Less.) DC.	plants (Y)	15	8	7	magnifying glass, microscope	GC-FID	(De Ferrante et al., 2007)
Peru	*Hoodia gordonii* (Masson) Sweet ex Decne. tablets (white)/*Hoodia* sp., *Opuntia ficus-indica* (L.) Mill., *Ceropegia dichotoma* Haw., *Cynanchum* sp., *Edithcolea grandis* N.E.Br., *Huernia* sp., *Orbea* sp., *Piaranthus globosus* A.C. White & B. Sloane, *Stapelia* sp., *Tridentea choanantha* (Lavranos & Harry Hall) L.C. Leach	herbarium vouchers, collected plants (Y)	2	1	1	light microscope	PCR	(Joshi et al., 2009)
South America			***167***	***100***	***67***			
Total			**508**	**300**	**208**			

products are adulterated. Despite the *caveat* on sampling bias, it can also be concluded that microscopic diagnostics can detect adulterations more thoroughly than DNA-based authentication alone. The microscopically authenticated products were purchased from a wide variety of markets and shops, and were processed for quite diverse purposes. Thus, microscopic authentication is robust enough to be used along local, national, and international value chains for herbal products (Booker et al., 2012; Heinrich et al., 2019). Qualitative cellular and anatomical details, such as calcium oxalate crystals, idioblasts, xylem cells, stone cells, or stomatal complexes are well covered by monographies forming the base for pharmacopeias (Tam et al., 2006). In difficult cases, morphological identification can be supported by histochemical analysis to detect diagnostic features of the specimen (Au et al., 2009). In addition, microscopy allows to trace contamination by non-plant materials, such as insects or even inanimate matter, which would go unnoticed in a DNA-based test, such as pieces of wire, nylon, soil, stones, or sand (Cuassolo et al., 2010; Michetti et al., 2019). Despite these undisputable advantages, microscopic diagnostics also has to face several challenges: for the often exotic species, monographies on their diagnostic characteristics are not available and have to be newly established. A precondition for this endeavor is the availability of authentic reference material, either from wild or cultivated resources, Botanical Gardens, germplasm centers, or herbarium vouchers. The validation and verification of such reference materials is an absolute must. Unfortunately, a substantial fraction of these materials are mislabeled as well (Goodwin et al., 2015), because *"Rapidly increasing numbers of specimens in an increasing numbers of herbaria are not being revived because there are too few taxonomists"*. Some microscopical techniques, such as SEM, require expensive equipment, which may not be affordable in a lab. TEM in particular, requires extensive sample processing, such as fixation, embedding, ultrasectioning, and contrasting, which easily can introduce artifacts that are misleading, if the user is not very experienced. A further challenge for microscopic diagnostics are heavily processed products, such as finely cut material or ground powders. Although one can try to process the collected reference plant material in a similar manner to match the commercial products, often the diagnostic features are lost.

Herbal Adulteration as Product of Economic Incentive and Ambiguous Nomenclature

There seem to be mainly two drivers for adulteration: (i) limited supply and high dynamics of demand; (ii) ambiguities of traditional versus scientific nomenclature. In the following, both factors are exemplarily discussed. Optical microscopy can help here to screen out in a robust and cost-effective way, samples that appear suspicious and might deserve closer scrutiny also involving other, more laborious, and more expensive, methods. The connection between limited supply, sky-rocketing demand, and the use of light microscopy can be illustrated using the *Cordyceps* case.

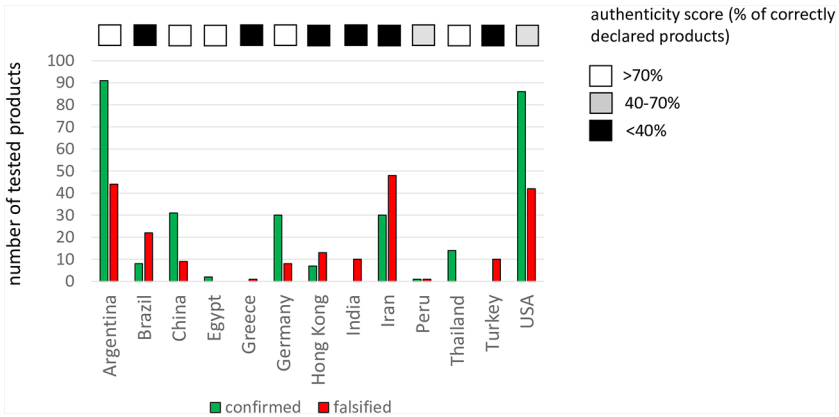

FIGURE 1 | Numbers of commercial herbal products verified by microscopical diagnostics over different countries. Products, where authentication confirmed the declared content are given in green, products, where the declaration was falsified, in red. The fraction of correctly declared products in a given country is indicated by the authenticity score.

Optical Microscopy—A Cheap Technique to Operate Can Detect Adulteration in a Multi Billion Industry

The insect-parasitic fungus *Cordyceps sinensis* is widely used and valued in Asian traditional medicine (Elkhateeb et al., 2019), but endemic to a very restricted area in the Tibetan plateau (Li et al., 2011). In the meantime, it has been recognized to be not related to other members of the genus and has been renamed as *Ophiocordyceps sinensis*. During the last decade, *Cordyceps* had been discovered as "superfood" and is now amply used as supplement for food supplements. The combination of restricted habitat and increasing demand have led to over-harvesting and a dramatic rise in its price (Liu et al., 2011). The market volume of traded products declared as *Cordyceps* exceeds the annual harvest of *O. sinensis* by a factor of 20 and has reached 470 million USD in 2018. Around 70% seems to be in fact the only distantly related parasitic species *C. militaris* (GlobeNewswire, 2019). The economic gain of this adulteration is tremendous: the crude drug was already at 13,000 USD/kg in 2008, but has skyrocketed to USD $20,000 to 40,000 per kg within 5 years (Lo et al., 2013). In this context, light microscopy was successful to authenticate these products, no matter, whether marketed, as raw fruiting body (Nopparat et al., 2018), or in fermented or processed form as capsules (Au et al., 2012).

Traditional Nomenclature Can Cause Non-Intentional Adulteration: The Case of Bamboo Tea

Traditional nomenclature is not based on phylogenetic relationship, but on common use (Berlin et al., 1966). Globalization shifts plants out from their traditional functional context, and this represents a major source for non-intentionally adulteration, as exemplarily addressed for the product Bamboo tea (Horn and Häser, 2016), which appeared on the European market from the mid of the last decade. Bamboo belongs to the Poaceae with characteristic dumbbell-shaped guard cells. When leaf fragments of this product were investigated by bright-field microscopy, instead, kidney-shaped guard cells with two subsidiary cells, arranged in parallel rows, were seen. This diacytic stoma type was definitely not consistent with the declared Poacean ingredients, but rather pointed to a specimen of the genus *Dianthus* (carnation). This could be confirmed using the plastidic markers matK and rbcL. Different accessions from this genus were then purchased from different Botanical Gardens and commercial sources and taxonomically verified to serve as references. When the histology of completely developed leaves from these plants was investigated by microscopy, the samples could be assigned to the species *Dianthus chinensis* L. The background of this curious case of adulteration (bamboo and carnation are evolutionarily far apart) is to be sought in the problematic use of vernacular names in commercial products. The young leaves of the bamboo species *Sasa palmate* (Burb.) E.G.Camus, *Sasa kuriliensis* (Rupr.) Makino & Shibata, and *Lopaterum gracile* have a long history in TCM and are supposed to support the breakdown of body sediments including fat. They are traded under the name *Dan Zhu Ye*. Also Chinese carnation (*Dianthus chinensis* L.) is used in TCM, but for a different purpose (for instance, it acts diuretically). Since the leaves of this plant resemble young bamboo leaves, and since it is a traditional component of Chinese Stone Gardens, it is often called *Shi Zhu* ("Stone Bamboo"). In the context of TCM, it is fairly clear, what plant is handled. However, the rapid increase in consumer demand had caused bottlenecks in the producing countries. When supply is under constraint, the internet search for Bamboo tea can easily lead to the purchase of products that are branded as "Bamboo Carnation Tea". This had happened in the current case, and again underlines, how important it is to install a systematic authentication of commercial products, preferably already in the harbors, where the material enters the industrial supply chain.

Concluding Remarks

The use of microscopy and molecular markers as diagnostic tools for the authentication of plant food ingredients is rarely

used in combination. However, this would be important, because each of the two approaches has individual advantages and disadvantages. Microscopy is a rapid and cost-efficient method, and can cope with mixtures and impurities. However, it has limited applicability for highly processed samples. It is important to keep this art alive as essential part of university curricula. It is also important to raise public awareness for the extent of adulteration and the need to safeguard consumer safety against the challenges of globalization.

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MI performed the literature systematic search. MI, AH, and PN wrote and approved the submitted manuscript.

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ORIGINAL RESEARCH
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Species Quantification in Complex Herbal Formulas—Vector Control Quantitative Analysis as a New Method

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Product mislabeling and/or species fraud in Traditional Chinese Medicine (TCM) not only decrease TCM quality, but also pose a potential health issue to the end user. Up to now, methods to control TCM quality have been developed to detect specific metabolites or identify the original species. However, species quantification in complex herbal formulas is rarely concerned. Here, we reported a simple Vector Control Quantitative Analysis (VCQA) method for flexible and accurate multiplex species quantification in traditional Chinese herbal formulas. We developed PCR-based strategy to quickly generate the integrated DNA fragments from multiple targeted species, which can be assembled into the quantitative vector in one round of cloning by Golden Gate ligation and Gateway recombination technique. With this method, we recruited the nuclear ribosomal DNA Internal Transcribed Spacer (ITS) region for the quantification of *Ligusticum sinense* "Chuanxiong," *Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav., *Notopterygium incisum* K. C. Ting ex H. T. Chang, *Asarum sieboldii* Miq., *Saposhnikovia divaricata* (Turcz.) Schischk., *Nepeta cataria* L., *Mentha canadensis* L., and *Glycyrrhiza uralensis* Fisch. ex DC. in ChuanXiong ChaTiao Wan, a classic Chinese herbal formula with very long historical background. We found that, firstly, VCQA method could eliminate the factors affecting such as the variations in DNA extracts when in combination with the use of universal and species-specific primers. Secondly, this method detected the limit of quantification of *A. sieboldii* Miq. in formula products down to 1%. Thirdly, the stability of quality of ChuanXiong ChaTiao Wan formula varies significantly among different manufacturers. In conclusion, VCQA method has the potential power and can be used as an alternative method for species quantification of complex TCM formulas.

Keywords: complex herbal formulas, vector control quantitative analysis, internal transcribed spacer region, species quantification, limit of quantification, traditional Chinese medicine quality control

INTRODUCTION

Traditional Chinese Medicine (TCM) is the key element of traditional Chinese medical system and its culture and civilization have evolved over thousands of years. Despite the advanced medical technologies in recent years, TCM is still playing an important role in primary healthcare for Chinese people and even worldwide. Indeed, many herbal remedies of TCM have made it into modern medicines through drug development and their use continues to increase. China possesses a diverse and rich flora to form the basis of traditional medicine, and more than 3,200 herbs are used in different formulas (Daxue Consulting, 2015). Just in the Pharmacopoeia of the People's Republic of China, more than 500 herbal plants and their extracts and almost 300 complex herbal formulations are recorded (State Pharmacopoeia Committee, 2015), not to mention the other ethnic pharmacopoeias in China. Herbal formula usually contains multi-ingredients from two or more plant species and has the competence to systematically heal illness. Different to those herbal medicines that contain only one herb, the complex formula uses one or two main ingredients as pioneers to target and treat the core symptom, while the other ingredients are used to treat other symptoms caused by the disease, helping the main herbs to enhance their positive effects and finally eliminate the signs and symptoms of disease exactly (Wang et al., 2008; Daxue Consulting, 2015). Owing to this excellent characteristic, it is not surprising that herbal formulas have become more and more popular and attract consumers' preferences around the world. A statistics from hexa research reported that the global herbal medicine market value reached USD 71.19 billion in 2016 and is expected to exhibit profitable growth over the forecast period (Hexa Research, 2017). In addition, the rapid industrialization and modernization also pose profound effects on herbal medicine supply (e.g., expanding the commercialization of herbal medicinal products throughout various markets, including the Internet) (Li et al., 2016a; Xiong et al., 2018). In this context, quality control of herbal medicine products is a crucial part that cannot be overlooked. Although the Pharmacopoeia has strict species dosage listed for herbal formulas and recommend the manufacturer to produce accordingly, no stringent stipulations of quality control have been established. This situation leaves loopholes for some unscrupulous manufacturers who tend to use fake herb species or incorrectly label species percentage in the products (Chen et al., 2014; Osathanunkul et al., 2015a; Li et al., 2016b). Any case of herbal medicine fraud not only decreases product quality but also causes potential health issue to the end user.

To date, methods to control herbal medicine quality have been developed, involving morphological and microscopic identification and chemical constituents' analysis. These classical methods could identify plant species in herbal medicine product based on morphological characteristics or determine specific chemical components based on related reference substance (Franz et al., 2007; Liu et al., 2017). Remarkably, several new reviews highlighted the efficiency and increasing application of DNA-based methods in herbal medicine identification (Sucher and Carles, 2008; Chen et al., 2014; Li et al., 2016a). From their reviews, we notice that DNA barcoding methods which are originally developed to identify organisms have been widely adopted for the identification of single species in herb product (Techen et al., 2014; Mishra et al., 2016). However, due to the shortcoming of Sanger sequencing, current DNA barcoding methods are not sufficient to simultaneously identify multispecies in complex medicinal preparations (i.e., herbal formulas). In the Chinese Pharmacopoeia, herbal formulas are formulated with two or more species in prescribed proportions and are processed into different dosage forms such as tablets, capsules, powders, extracts, pastes, gels, and oils (**Figure 1**). It is almost impossible to identify certain biological ingredients in herbal formulas through Sanger sequencing. In addition, the present DNA barcoding methods are emphasizing on species authentication of herbal medicine, yet little concern has been focused on the species quantification in herbal formulas, which we believe to be an important yet underappreciated factor in quality control. As we know, some herbal formulas contain toxic species (e.g., ChuanXiong ChaTiao Wan contains *Asarum sieboldii* Miq.; ShuFeng DingTong Wan contains *Strychnos nux-vomica* L.; YuZhen San contains *Arisaema heterophyllum* Blume; LiuWei MuXiang San contains *Rhododendron molle* (Blume) G.Don). Excess toxic ingredients in formula may lead to chronic injury on the human body (Wu et al., 2015). In principle, DNA quantification methods target either low copy genes in nuclear genome or single copy genes in plastid DNA. However, contrary to nuclear DNA, the quantity of ptDNA exhibits high variation among different tissue; hence, quantification cannot be based on ptDNA; instead a low copy nuclear DNA target is appropriate for herbal formulas. The Internal Transcribed Spacer (ITS) sequence has been used as a core barcode for plant identification and classification because this locus has high mutation rate (Chen et al., 2010; Li et al., 2011). According to these studies, we hypothesis that ITS region may fulfill the requirements for the species quantification of complex herbal formulas.

In the past, most of the studies preferred to use relative quantitative real-time PCR to quantify meat species in food; however, this method is affected by many factors (Laube et al., 2007). One such factor is the unavailability of an exogenous standard robust enough for use in a complex product like sample mixed with different type tissue. In absolute quantification, although some studies used PCR or restriction digestion to perform quantitative analysis of parasite or virus in blood, none of them can simultaneously quantify more than three targets (Workenhe et al., 2008; Kamau et al., 2013; Gotia et al., 2016). In this work, we developed a Vector Control Quantitative Analysis (VCQA) method that enables efficient quantification of multiple species in herbal formula products (**Figure 1**). VCQA is amenable to high throughput but with reduced costs, it has several advantages over previous quantitative analyses: 1) VCQA uses PCR-based procedures to quickly produce multiple species-specific DNA fragments with designated *Bsa*I enzyme digestion sites at their two ends, which could assemble into a single quantitative vector in one round of cloning by Golden Gate ligation and Gateway

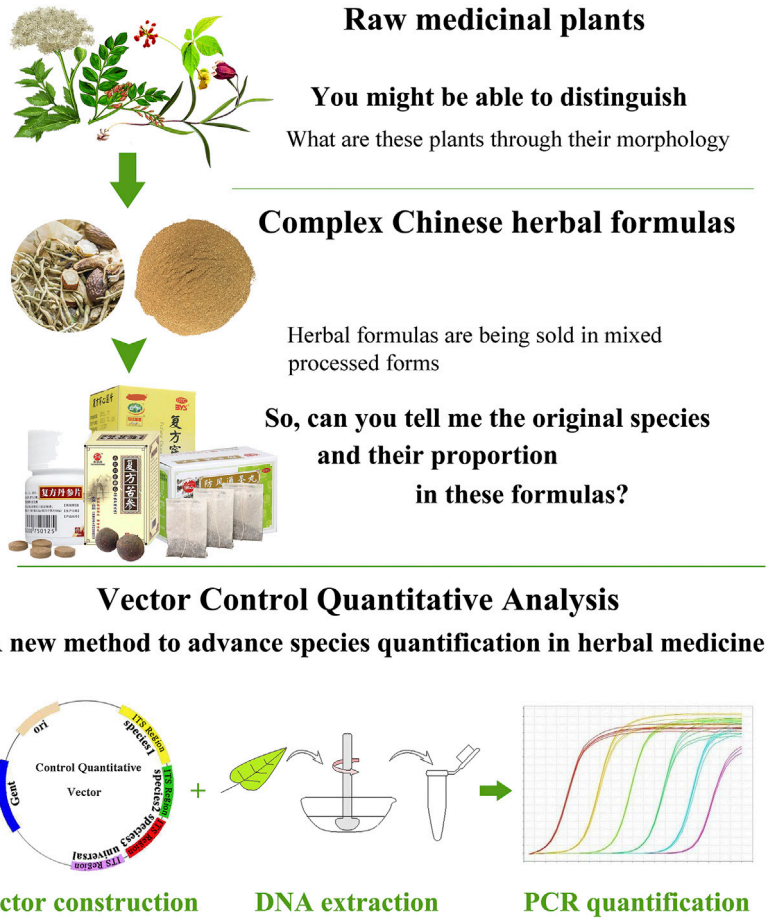

FIGURE 1 | VCQA method shows a potential power in species quantifying for herbal medicine formulas.

recombination. 2) VCQA could eliminate the factors affecting such as the variations in DNA extracts when in combination with the use of universal and species-specific primers. 3) The data produced by VCQA can change into mg/mg for species quantification, which is consistent with the norms described in the Chinese Pharmacopoeia. To elucidate the workflow of quantification of this approach, we described a systematic pipeline for the quantitative determination of ChuanXiong ChaTiao Wan formula in this study.

MATERIALS AND METHODS

Reference Sample and Commercial Products Collection

To test the accuracy and repeatability of VCQA method, a lab-made reference ChuanXiong ChaTiao Wan sample was formulated with authenticated herbal materials according to the prescription documented in the Chinese Pharmacopoeia. Eight herbal materials were collected from Tong Ren Tang drug store, including *Ligusticum sinense* 'Chuanxiong', *Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav., *Notopterygium incisum* K.C.Ting ex H.T.Chang, *Asarum sieboldii* Miq., *Saposhnikovia divaricata* (Turcz.) Schischk., *Nepeta cataria* L., *Mentha canadensis* L., and *Glycyrrhiza uralensis* Fisch. ex DC. (**Table 1**). All samples were authenticated using DNA barcoding method according to the protocol described in the Chinese Pharmacopoeia (Chen et al., 2014; State Pharmacopoeia Committee, 2015). All of the corresponding voucher materials were deposited in Guangxi Institute of Botany. The reference ChuanXiong ChaTiao Wan sample was processed as follows: the eight herbal materials were respectively ground into powder, and then the powder was sieved and evenly mixed according to the official proportion (**Table 1**; **Figure 2**). To test the sensitivity of VCQA method, an increasing quantity of *Asarum sieboldii* Miq. powder (0.1, 1, 2, 5, and 10%) was added to a standard mixture of ChuanXiong ChaTiao Wan formula which does not contain *Asarum sieboldii* Miq. Three repetitions performed in one run were used for the ChuanXiong ChaTiao Wan mixtures. The limit of quantification is defined as the lowest percentage of *Asarum sieboldii* Miq. that could be stably detected in all qPCRs. To evaluate the practical application capacity of VCQA method, ten commercial products of ChuanXiong ChaTiao formula produced by different

TABLE 1 | Sample list of the botanical species in ChuanXiong ChaTiao Wan.

Species name	Weight (g)	Part used	Voucher number
Ligusticum sinense	120	Rhizome	RF01LC01~05
Angelica dahurica	60	Root	RF02AD01~05
Notopterygium incisum	60	Rhizome and root	RF03NI01~05
Asarum sieboldii	30	Root and rhizome	RF04AS01~05
Saposhnikovia divaricata	45	Root	RF05SD01~05
Nepeta cataria	120	Stem and leaf	RF06NC01~05
Mentha canadensis	240	Stem and leaf	RF07MC01~05
Glycyrrhiza uralensis	60	Root and rhizome	RF08GU01~05

FIGURE 2 | Reference samples of ChuanXiong ChaTiao Wan used for VCQA.

manufacturers were purchased from different drug stores for this study.

DNA Extraction and Primer Design

Total DNA was extracted according to the protocol of CTAB as modified by Sun (Sun et al., 2016). DNA concentration was quantified using a Qubit 3.0 spectrophotometer (Invitrogen, USA) and adjusted to 50 ng/μl working concentration and stored at −20°C until needed. The ITS region was amplified using universal primers ITS4 and ITS5 (**Supplementary Table S1**). PCR was performed in a volume of 20 μl containing 10 μl of 2 × Taq PCR mix (Sangon Biotech Co., China), 0.5 μl of DNA template, 1 μl of each primer (final concentration of 0. 25 μM), and 7.5 μl of ultrapure water. The following PCR program was used: 95°C for 3 min; 32 cycles at 95°C for 20 s, 55°C for 20 s, and 72°C for 30 s; and a final extension cycle at 72°C for 5 min. PCR product was sequenced in two directions by Sanger sequencing. Based on the sequencing results, we designed species-specific primers and universal primers for *Ligusticum sinense* "Chuanxiong," *Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav., *Notopterygium incisum* K.C.Ting ex H.T.Chang, *Asarum sieboldii* Miq., *Saposhnikovia divaricata* (Turcz.) Schischk., *Nepeta cataria* L., *Mentha canadensis* L., and *Glycyrrhiza uralensis* Fisch. ex DC., respectively (**Supplementary Table S1**). The sequence alignment was carried out by MUSCLE in software MEGA 5.0. Regions of species divergence were selected for designing primer to amplify specific product for a particular species, while the universal primer sites were chosen at conserved sites to give a PCR product size of 120 bp.

Cloning of Multiple Target Sequences in Quantitative Vector

A restriction–ligation–recombination reaction (15 μl) was prepared to contain 1x *Bsa*I reaction buffer (NEB) plus 1.5 μl of 10x NEB ligation buffer (which contains 10 mM ATP), 5 U of *Bsa*I, 20 U of T4 DNA ligase (NEB), and the purified PCR products (10 ng for each species) amplified with the Pps and Pgs primers. The reaction was incubated in a thermocycler for 15 cycles (37°C 2 min, 10°C 3 min, and 20°C 5 min) and then terminated at 65°C for 5 min; when the reaction cooled down to room temperature, 20 ng of pDONR207 plasmid and 3 μl of BP Clonase™ buffer (Invitrogen, USA) were added to the reaction and incubated at 25°C for more than 2 h. However, in cases with five or more species-specific fragments and with the reactions not optimally set, the ligation efficiency may be relatively poor. Therefore, as an optional step the ligated products could be used as templates to amplify the linked fragments using two flanking primers (i.e., Pps-LcITS_F and Pgs-GuITS_R, **Supplementary Table S1**). A PCR (25 μl) with 3.0 μl of the ligated product and 12.5 μl 2x PrimeSTAR® Max DNA Polymerase was set up and thermal cycling was performed with 15 cycles (98°C, 10 s, 58°C, 15 s, and 72°C 3 min). Specific PCR product was purified from agarose gel using a gel purification kit (Tiangen, China), and 20 ng of the PCR product and 20 ng of the pDONR207 plasmid were subjected to Gateway BP recombination as described above.

The reaction products with multiple specific fragments (up to eight for ChuanXiong ChaTiao Wan formula) were directly used to transform *E. coli* competent cells. Positive colonies on an LB-agar medium plate containing 50 mg/L of Gentamycin were selected for further analysis by PCR and sequencing using flanking primer pDONR_F and pDONR_R, and species-specific primers were used as internal primers.

Preparation of Plasmid DNA Standards

The positive colony was grown up overnight in 10 ml LB liquid cultures, with shaking at 180 rpm at 37°C, and plasmid was subsequently extracted using a plasmid extraction kit (Tiangen, China). In order to generate standard curves, the plasmid was linearized by digesting with EcoR V enzyme (NEB) in a 50 μl reaction volume following the manufacturer's protocol. The linearized DNA was then purified using the PCR product purification kit (Tiangen, China) and was quantified

using a Qubit 3.0 spectrophotometer. With the molecular weight of the plasmid known, it is able to calculate the copy number by the following formula (Kamau et al., 2013): Number of copies/μl = 6.022×10^{23} (molecules/mole) × DNA concentrations (g/μl)/ number of bases pairs × 660 daltons.

Based on the concentration of plasmid DNA and its copy number, the accurate amount of molecules added to subsequent real-time PCR runs can be computed to establish a standard curve for the quantification of specific DNA (Cao and Luo, 2016).

Quantification of Specific Species in Herbal Formula Using Vector Control Quantitative Analysis Combined With qPCR

RT-qPCR was performed on LightCycler 480 (Roche) real-time PCR machine. Briefly, the 20 μl reaction containing 10 μl 2x SYBR Premix Ex Taq II Mix, 1 μl diluted DNA template, and 1 μl of each primer (final concentration of 0. 25 μM) was added to each well. Samples were amplified for 40 cycles of 95°C for 15 s, 58°C for 10 s, and 72°C for 20 s. After the last reaction cycle, melting curve analysis was carried out immediately from 55 to 95°C in 0.15°C/s increments to determine the specificity of the RT-PCR products. For construction of the standard curves, tenfold dilution series of linearized plasmid DNA starting from 10^{10} to 10^5 specific copies/ μl were used as DNA template to construct the standard curve. In order to use standard curves to quantify the specific species in ChuanXiong ChaTiao Wan formula, plasmid DNA was run alongside the DNA from single species, reference formula, and commercial samples. A standard curve was drawn by plotting the threshold cycle (Ct) against the natural log of concentration (copies/μl). Ct value was calculated using default settings in the LightCycler series software. The quality of standard curves was judged by the slope of the standard curve and the coefficient of determination (R^2). According to the slope of each standard curve, the efficiency (E) of PCR amplification can be calculated based on the equation $E = 10^{(-1/\text{slope})} - 1$. Besides, in the reaction of SYBR® Green I RT-qPCR, melting curve analysis was used to check the specificity of the RT-PCR product.

RESULTS

Primer Design and Evaluation

In order to establish a robust method for the quantitative detection of plant species in herbal formula, species-specific and universal primers (used as endogenous control) were combined into a single method in this study. The ITS region is a well-established target for plant species discrimination (Fritsch and Cruz, 2012; Yan et al., 2015; Li et al., 2016c). ITS region has a high mutation rate, which provides the degree of sequence variation required for plant species identification. Therefore, sequence divergence in ITS can be selected to design species-specific primers. In addition, the use of universal primers can provide an endogenous control of qPCR-quantitative DNA presented in the mixed sample. The use of such primers to simultaneously detect a universal fragment is very important for quantitative analysis because it allows accounting for possible amplification differences among the sample extracts due to variations in DNA recovery and quality of the extracts as a result of matrix effects and industrial processing (Iwobi et al., 2015). By a comparison of the species-specific versus endogenous control signal obtained from the samples, the inaccuracies caused by the use of different DNA from different batches can be reduced. To evaluate the specificity of the primer pairs for VCQA, both species-specific and universal primers were tested for their selectivity and cross-reactions by the analysis of DNA obtained from the eight individual species of ChuanXiong ChaTiao Wan. Firstly, the PCR amplified products were detected using electrophoresis in 1.5% agarose gel. Only those primer pairs produced the expected amplicon in corresponding species and no nonspecific bands in any other species were selected for further analysis (data not shown). Secondly, in real-time PCR, the specificity of the primers can be evaluated by the melting temperature (Tm) of the amplification products immediately after the last reaction cycle. The Tm refers to the temperature at which 50% of the DNA amplicon is in the single-stranded configuration. It can be determined when an additional procedure of slow heating from 55 to 95°C in 20 min is recruited in the qPCR reaction. During this period, fluorescence intensity decreased rapidly due to the denaturation of amplicons, because SYBR green could not bind to single-stranded DNA. A good primer pair results in a single amplicon, distinguished by generating a single melting curve peak but not in nonspecific products such as nonspecific band and primer-dimers (**Supplementary Figure S1**).

Finally, as a target site for species-specific quantification of ChuanXiong ChaTiao Wan, 180, 407, 342, 365, 410, 195, 387, and 362 bp fragment were amplified for *Ligusticum sinense* "Chuanxiong," *Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav., *Notopterygium incisum* K. C. Ting ex H. T. Chang, *Asarum sieboldii* Miq., *Saposhnikovia divaricata* (Turcz.) Schischk., *Nepeta cataria* L., *Mentha canadensis* L., and *Glycyrrhiza uralensis* Fisch. ex DC., respectively, while the target site for the endogenous control (which can be amplified using the new designed universal primers for these eight species) consisted of a ~120 bp DNA fragment.

Preparation of Quantitative Vector Constructs for Species Quantifying

To generate quantitative vector containing the target sequences, firstly we amplified the target fragment using site-specific primers (Pps, Pgs), which include different *Bsa*I-cutting sites at their 5′-end for Golden Gate ligation, and the flanking primers with *att*B sites for Gateway recombination (**Supplementary Table S1**). Golden Gate ligation uses the special cleavage feature of type IIs restriction endonucleases, such as *Bsa* I, to design and generate distinct, nonpalindromic sticky ends of sequences, which can avoid self-ligation and noncompatible end ligation (Ma et al., 2015). Therefore, this approach is efficient to link multiple DNA fragments in a designed order in a single reaction (**Figure 3**). Gateway Cloning Technique allows transfer of DNA fragments into a plasmid with two flanking recombination sequences called

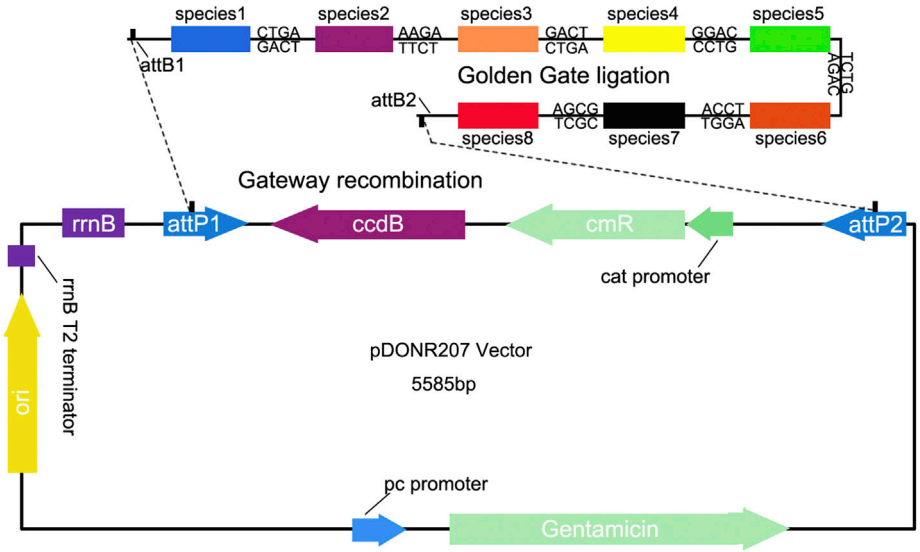

FIGURE 3 | Cloning of eight quantitative fragments into the pDONR207 vectors.

"attL 1" and "attL 2," to develop a "Gateway recombination clone." Using these cloning strategies, we can construct a quantitative vector containing one or multiple species-specific sequences. To test whether this VCQA method can effectively quantify species in herbal formula, we prepared a pDONR207-based construct carrying eight species-specific sequences from the eight target species in ChuanXiong ChaTiao Wan (**Figure 3**).

Real-Time PCR System Set Up for Vector Control Quantitative Analysis

To evaluate the applicability of VCQA method for the quantification of plant species in ChuanXiong ChaTiao Wan, both linearized plasmid and standard reference sample were used to construct standard calibration curves based on the parallel amplification of species-specific and/or universal sequences for each target species. Because there are no formal requirements for species quantification of herbal medicine products at present, we recruited some performance criteria of real-time PCR assays for VCQA method. Accordingly, VCQA should be specific to respond exclusively to the target species, which has been comprehensively demonstrated in above section, the standard test including the following criteria according to the document of method performance requirements released by the European Network of GMO Laboratories (ENGL, 2015) with some modification. The coefficient of determination (R^2) should be above 0.98, and the average value of the slope for the standard curves should be in the range of −3.1 and −3.6, corresponding to amplification efficiencies of 110 to 90%. The limit of quantification refers to the minimum amount of a target species in the product that can be stably quantified within an acceptable level of confidence of 95%, ensuring the false negative results are less than 5%.

The reliability of standard samples for absolute qPCR is a determining factor. Researchers have paid a lot of effort to develop appropriate standards (e.g., PCR amplified target sequences, plasmids insert with the target sequence) for quantitative analysis (Dhanasekaran et al., 2010). It is accepted that plasmid DNA, especially linearized plasmid, is more reliable for absolute qPCR. Therefore, it is very important to linearize plasmid DNA produced in this study to improve its reliability. After digestion, the absolute qPCR analysis performance of each target sequence could be determined by linearized plasmid. To evaluate the efficiency, we diluted the linearized plasmid DNA 6 times from 10^{10} to 10^5 specific copies/μl with tenfold serial gradient and analyzed it in 3 replicates (**Figure 4**). The R^2 and the slope values of each curve were used to determine the efficiency of each performance, while the standard deviation of each standard calibration curve was employed to determine the assay precision. Consequently, most of the absolute qPCR analysis of the target fragments was performed with efficiency of greater than 90%, R^2 values were 0.99 or higher, and the standard deviation of each standard calibration curve was lower than 0.7.

In absolute quantification, copy number is used to represent the sample amount. However, for herbal formula, species proportion is commonly expressed as mg/mg according to species dosage as measured by weighing instrument. When performing absolute qPCR for herbal formula product, it is important that species proportion should be expressed as herbal medicine industry norms. Here, a purpose was laid out to determine the amount of copy numbers that is equivalent to mg/mg. The Ct values generated from absolute and relative qPCR assays were correlated to calculate the amount of copy numbers that is equivalent to mg/mg. To determine the species proportion in ChuanXiong ChaTiao Wan, reference samples with known species weight were made. All the eight plant species were detected by real-time PCR analyses using serially diluted plasmid DNA (for absolute qPCR) and genomic DNA (for relative qPCR). The related calibration curves were generated by plotting the calculated Ct value against the logarithm of sample

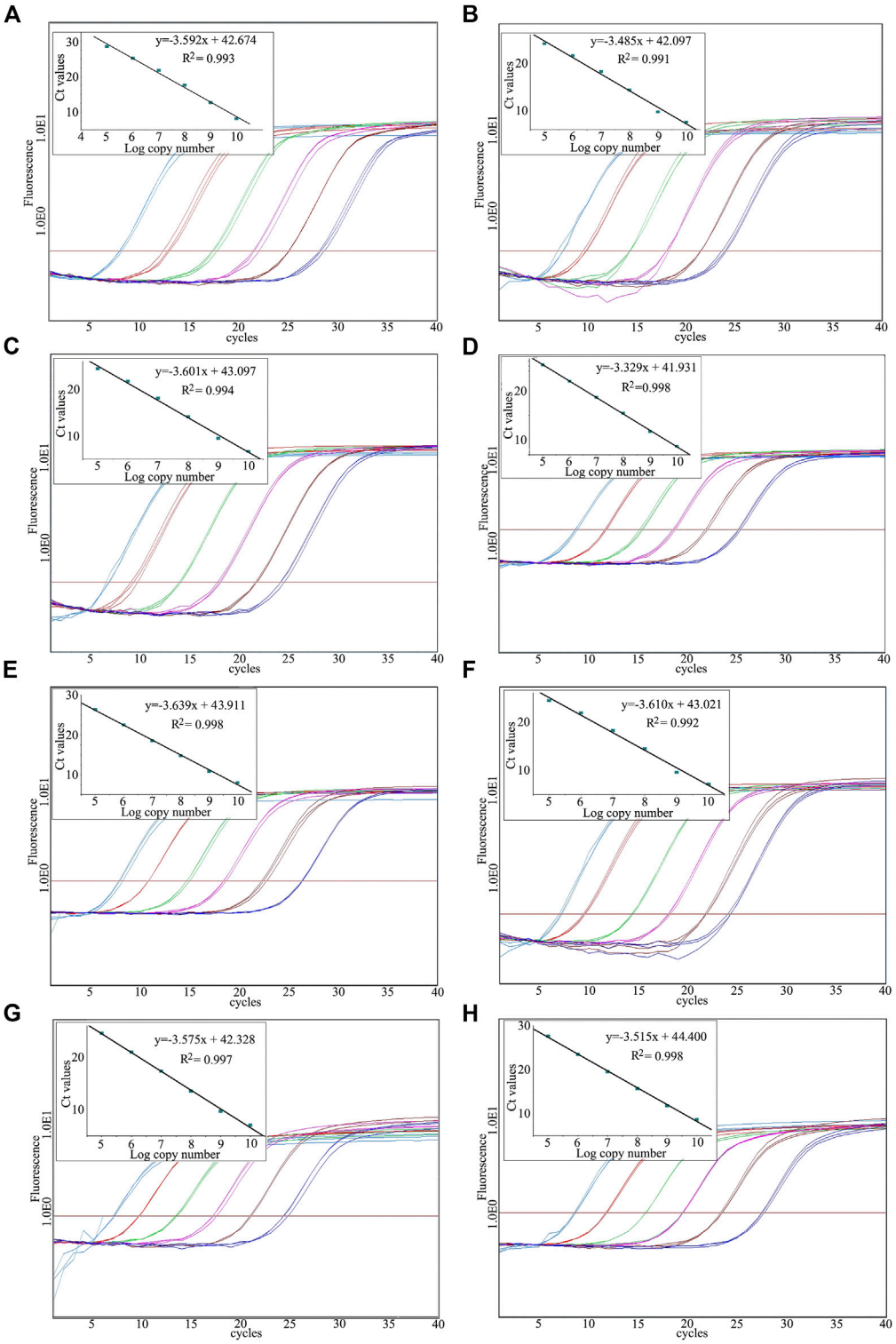

FIGURE 4 | Amplification graph and standard curve constructed using the dilute linearized plasmid. **(A)** *Ligusticum sinense*, **(B)** *Angelica dahurica*, **(C)** *Notopterygium incisum*, **(D)** *Asarum sieboldii*, **(E)** *Saposhnikovia divaricata*, **(F)** *Nepeta cataria*, **(G)** *Mentha canadensis*, **(H)** *Glycyrrhiza uralensis*.

weight. When the two obtained curves exhibited the same linear ranges, we can say that the Ct levels for both curves were equivalent. Therefore, to calculate the amount of copy numbers that is equivalent to mg/mg from the relative qPCR assay, the Ct values obtained from relative qPCR assays were interpolated as unknown copy numbers from the linear

regression standard curve of the absolute qPCR assays to obtain equivalent mg/mg. The amount of copy numbers that is equivalent to species weight in mg was calculated according to the averages obtained from series dilutions for qPCR analyses. For the *Ligusticum sinense* "Chuanxiong" absolute qPCR assay, 1208.5 copy numbers corresponds to 1 mg; for the *Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav. absolute qPCR assay, 1355.7 copy numbers correlates to 1 mg; for the *Notopterygium incisum* K. C. Ting ex H. T. Chang absolute qPCR assay, 3275.5 copy numbers corresponds to 1 mg; for the *Asarum sieboldii* Miq. absolute qPCR assay, 1664.3 copy numbers correlates to 1 mg; for the *Saposhnikovia divaricata* (Turcz.) Schischk. absolute qPCR assay, 2389.1 copy numbers correlates to 1 mg; for the *Nepeta cataria* L. absolute qPCR assay, 1214.9 copy numbers correlates to 1 mg; for the *Mentha canadensis* L. absolute qPCR assay, 4661.6 copy numbers correlates to 1 mg; for the *Glycyrrhiza uralensis* Fisch. ex DC. absolute qPCR assay, 1338.7 copy numbers correlates to 1 mg.

The estimated weight of each species in the reference formula sample was listed in **Table 2**. The coefficients of variation corresponding to the repeatability of results obtained under experiment conditions varied from 2.0 to 18.6%, indicating the accuracy of VCQA over the tested dynamic range (<25%). The measure of trueness (i.e., bias) is within ± 25% of the accepted reference value over the whole dynamic range, which demonstrates a close proximity between the estimated and real values (ENGL, 2015). The closeness of agreement between estimated and real values suggests that the developed VCQA method can competently be applied to calculate the species proportion in complex herbal formula products.

The absolute quantification limit of VCQA method was determined using an increasing quantity of *Asarum sieboldii* Miq. (0.1, 1, 2, 5, and 10%) in standard mixture of ChuanXiong ChaTiao Wan formula. The experimental data showed that *Asarum sieboldii* Miq. could be reliably quantified to 1% level. The results exhibited high performance in terms of linearity (R^2 = 0.998) and PCR efficiency (98.5%). In addition, we noticed that linearity was vulnerable under the lowest percentage (0.1%) of *Asarum sieboldii* Miq. in formula mixtures (**Figure 5**). When the level of 0.1% (w/w) was included, PCR efficiency did not comply with the performance criteria for VCQA method any longer (ENGL, 2015). Based on these results, we speculated that the VCQA method allows the quantification and detection of level down to 1% and 0.1% of *Asarum sieboldii* Miq., which correspond to 1664.3 and 166.4 copy numbers, respectively.

Application of Vector Control Quantitative Analysis Method to Commercial Herbal Formula Products

In order to demonstrate the practicability of VCQA method, 10 commercially available ChuanXiong ChaTiao Wan products randomly selected and purchased from different manufacturers were analyzed. The results indicated the quality of ChuanXiong ChaTiao Wan formula varies significantly among different manufacturers (**Table 3**). Generally, the proportion of quantified *Nepeta cataria* L. and *Mentha canadensis* L. was higher in relation to the other quantifiable species in most of the products. This case was expected as these two species, being the cheapest biological ingredients of all, would be added preferentially with larger amounts in the production of ChuanXiong ChaTiao Wan. Surprisingly, in one sample (COM_09), the percentage of *Asarum sieboldii* Miq. was unexpectedly almost 7% higher than the prescript of the Chinese Pharmacopoeia. In order to rule out that this result was not caused by artificial error, we reanalyzed this sample but found no significant deviation. A possible explanation for this perceived discrepancy may be a self-patent preparation of the product. Although no stringent stipulations require the manufacturer to list the species dosage in herbal medicinal product, a higher concentration of toxic component may pose a serious health issue to the end users. In addition, the quantitative results also reveal that another two products

TABLE 2 | Results of reference formulas for the validation of VCQA method.

Species name	Species weight (mg/100 mg)		SD	CV (%)	Bias
	Actual	VCQA predicted			
Ligusticum sinense	16.33	14.19	2.83	18.6	−13.1
Angelica dahurica	8.16	7.71	1.26	16.3	−5.5
Notopterygium incisum	8.16	7.04	0.71	8.8	−12.5
Asarum sieboldii	4.08	4.00	0.09	2.3	−1.9
Saposhnikovia divaricata	6.12	6.68	0.13	2.0	9.1
Nepeta cataria	16.33	14.97	1.75	11.7	−8.3
Mentha canadensis	32.65	31.75	2.28	7.2	−2.7
Glycyrrhiza uralensis	8.16	8.53	0.91	10.9	4.5

*Note: SD, standard deviation. CV, coefficient of variation = (SD/mean) * 100%. Bias = ((mean value-true value)/true value * 100).*

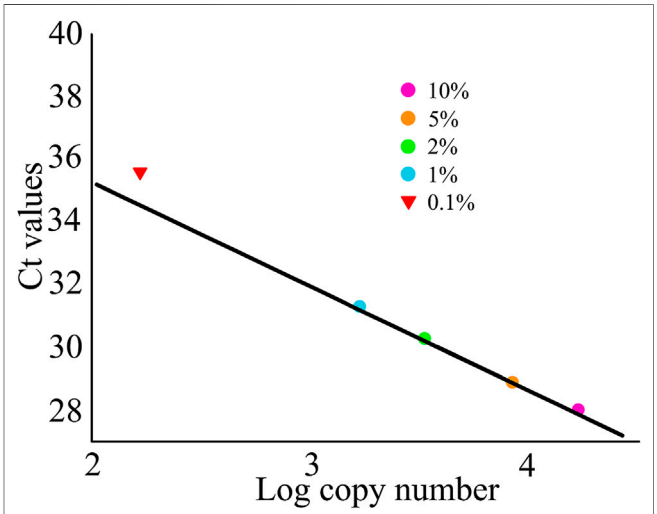

FIGURE 5 | Linear regression plots for the limit quantification of *Asarum sieboldii* in the reference model mixtures of ChuanXiong ChaTiao Wan.

TABLE 3 | Application of VCQA method for the quantitative determination of various commercial ChuanXiong ChaTiao Wan products.

Samples	Species content (mg/mg)							
	Ligusticum sinense	*Angelica dahurica*	*Notopterygium incisum*	*Asarum sieboldii*	*Saposhnikovia divaricata*	*Nepeta cataria*	*Mentha canadensis*	*Glycyrrhiza uralensis*
COM_1	0.1038 ± 0.0092	0.0517 ± 0.0047	0.0545 ± 0.0026	0.0258 ± 0.0016	0.0394 ± 0.0019	0.1259 ± 0.006	0.2420 ± 0.0113	0.0529 ± 0.0019
COM_2	0.1271 ± 0.0066	0.0629 ± 0.0041	0.0626 ± 0.0041	0.0297 ± 0.0008	0.0466 ± 0.0022	0.1418 ± 0.0101	0.2985 ± 0.0055	0.0679 ± 0.0022
COM_3	0.0956 ± 0.0048	0.0475 ± 0.006	—	0.0234 ± 0.0032	0.0363 ± 0.0014	0.1200 ± 0.0095	0.2411 ± 0.0063	0.0474 ± 0.0006
COM_4	0.1182 ± 0.0103	0.0591 ± 0.0074	—	0.0311 ± 0.005	0.0445 ± 0.0027	0.1435 ± 0.0092	0.3427 ± 0.0218	0.0590 ± 0.0018
COM_5	0.1388 ± 0.0035	0.0703 ± 0.0083	0.0485 ± 0.006	0.0318 ± 0.0028	0.0588 ± 0.005	0.1574 ± 0.0133	0.3166 ± 0.01	0.0711 ± 0.0076
COM_6	0.1376 ± 0.0061	0.0669 ± 0.0029	0.0601 ± 0.0018	0.0351 ± 0.0016	0.0509 ± 0.0006	0.1710 ± 0.002	0.2915 ± 0.0085	0.0614 ± 0.0008
COM_7	0.1020 ± 0.0073	0.0524 ± 0.0064	0.0450 ± 0.0033	0.0246 ± 0.0007	0.0369 ± 0.0013	0.1150 ± 0.0082	0.2306 ± 0.0093	0.0548 ± 0.0044
COM_8	0.1407 ± 0.0114	0.0700 ± 0.0099	0.0617 ± 0.0072	0.0362 ± 0.0055	0.0536 ± 0.0029	0.1594 ± 0.0125	0.3517 ± 0.0127	0.0774 ± 0.0082
COM_9	0.1156 ± 0.0088	0.0546 ± 0.0071	0.0517 ± 0.0047	0.0308 ± 0.0029	0.0463 ± 0.0018	0.1271 ± 0.008	0.2478 ± 0.0203	0.0577 ± 0.0045
COM_10	0.1447 ± 0.0109	0.0722 ± 0.0068	0.0718 ± 0.0051	0.0364 ± 0.0024	0.0532 ± 0.0006	0.1676 ± 0.0165	0.2883 ± 0.0074	0.0804 ± 0.0026

Note: values are the means of three replicate analyses.

(COM_3 and COM_4) were much likely not containing any *Notopterygium incisum* K. C. Ting ex H. T. Chang since the presented Ct values were all lower than the limit of detection. After careful verification, we found that the price of these two products was almost a third lower than other products, which indicated that while *Notopterygium incisum* K. C. Ting ex H. T. Chang is the most expensive of all the tested species, the possibility exists for manufacturers to fraudulently increase the competitiveness of their products.

A Practical Research Flow Chart for Species Quantification in Herbal Formula Using Vector Control Quantitative Analysis

As the results described above, VCQA could be used to quantify the species composition in reference herbal formula and commercial products. Based on the protocol used in this study, we develop a practical research flow chart for species quantification in herbal formula using VCQA (**Figure 6**). First, genomic DNA from the original plant of each species is extracted respectively. The ITS sequence is then amplified by PCR using the extracted DNA. Second, ITS sequences from all the target species are aligned to find conserved sites for designing universal primers, as well as to identify species divergence sites for designing species-specific primers. Third, the species-specific primers are crosschecked among all the target species to ensure their specificity and amplification efficiency. Fourth, multiple target sequences are assembled into the pDONR207 vector to construct a quantitative plasmid by using a restriction–ligation–recombination reaction strategy. Fifth, a preliminary test is conducted to evaluate the quantified capacity of VCQA in reference experimental herbal formula and then apply it to quantify the species composition in commercial herbal formula products. Finally, if the results of tested commercial products deviate from the theoretical characterization of the official statement, metabolite profiling methods can be alternatively used to assist in metabolomic characterizing to verify and guarantee the VCQA results.

DISCUSSION

As highlighted by the recent authenticity survey of herbal medicines sold in China, Thailand, and North American, herbal medicine products are particularly vulnerable to commercial frauds (Newmaster et al., 2013; Osathanunkul et al., 2015b; Han et al., 2016). Although progress has been made in species-level authentication of most single herbs via DNA barcoding, species identification and quantification in complex herbal formulas are still rarely concerned. Although there are two reports that used Next Generation Sequencing (NGS) approach to identify and quantify species in herbal formulas (Cheng et al., 2014; Xin et al., 2018), it is difficult to widely apply it in herbal medicine detection mainly due to the sequence data and its analysis need highly skilled individuals and specialized bioinformatics programs. Moreover, although

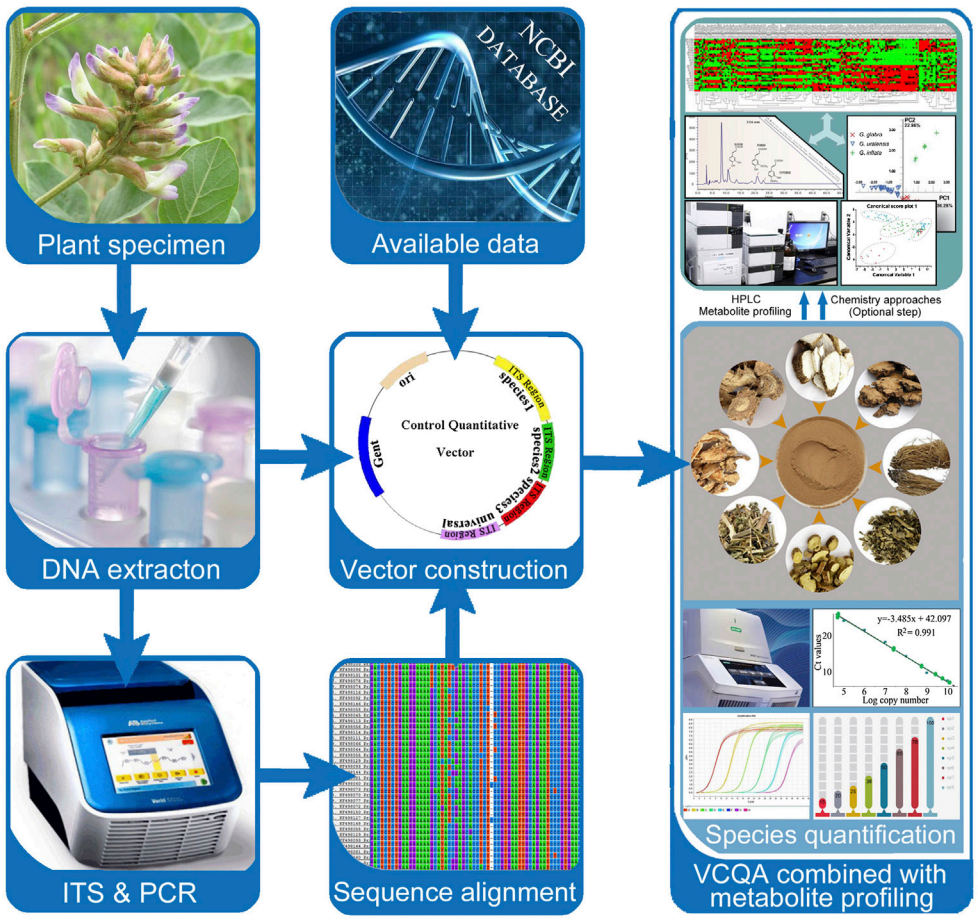

FIGURE 6 | The research flow chart for species quantification in herbal formula using VCQA.

the cost per read for NGS approach is actually much lower than for Sanger sequencing, the price of equipment and consumables is not easily accessible for those starting out in molecular methods. Actually, most of these kinds of studies are conducted by external companies, which will lead to more time consumption. Thus, the development of more convenient and economical methods for the detection of species ingredient in complex herbal products is of great significance for herbal industry.

To meet such needs, a novel VCQA method is developed for quantifying multiple plant species in complex herbal product in this study. The backbone of our quantitative vector is based on the commonly used pDONR207 vector, which is suitable for inserting relatively long fragments (up to approximate 5 kb) by using Gateway recombination method. We used PCR-based strategy to quickly generate multiple species-specific fragments with designated adapters. Although many herbal formulas contain several plant species, the Golden Gate ligation combined with Gateway recombination techniques could simultaneously deliver multiple species-specific fragments (up to eight in this study) into the pDONR207 vector in a single cloning reaction (**Figure 3**). Notably, if the ligated multiple fragments were further processed by an additional PCR and then Gateway recombination was performed with the quantitative vector (see Methods), the efficiency of cloning can be significantly enhanced. Based on the comprehensive evaluation of eight target fragments in qPCR analysis, we found that VCQA method has an accurate quantifying capacity for ChuanXiong ChaTiao Wan formula. All the quantitative results present in this study showed high efficiencies of more than 90%, high R^2 values ($R^2 \geq 0.99$), and very low standard deviation in each target fragment.

As we know, a big challenge in the quantitative evaluation of processed herbal product using DNA-based method is requiring a good quality of the initial DNA product against the backdrop of the diverse properties of medicinal parts and different production style of herbal medicine formulas. DNA isolated from different formula products was highly variable in quality and concentration. In order to address this issue, the universal primers and species-specific primers were introduced for the quantification. A major advantage of VCQA is that this method considers the factors affecting such as DNA degradation and inhibition when performing quantitative assessment. Without using universal primers, it

would be difficult to determine whether variations in species-specific primer response were caused by the differences in species content or other factors such as DNA degradation or inhibition or differences in the amount of DNA added to the qPCR. By allowing both comparative measurements (Ct from species-specific against Ct from universal signal), the normalized calibration curves can be obtained. This process will reduce the inaccuracies caused by the uncertainty factors as described above.

Although the VCQA method has a potential power for biological ingredients quantification of herbal medicine formulas, there are still some limitations, such as the fact that some closely related species may not be quantified due to the limit sequence divergence in ITS region among these species, while current research advancement has not yet exploited optimal barcode markers that exhibit enough divergence for most closely related species. In addition, the accuracy of the VCQA method could be affected by DNA degradation and the presence of different plant tissue which potentially yielded different DNA amounts. More critical testing on VCQA using blind samples and different control samples is necessary to assess the accuracy of this technique. It is worth noting that the VCQA method is not suitable for quantifying the herbal medicine formulas which just contain plant extracts. We recommend combining the DNA molecular approaches for species quantification with analytical chemistry approaches for compounds determination to ensure the quality of herbal medicine formulas in a more thorough manner.

CONCLUSION

VCQA method has proven to be a highly efficient, easy-to-use technique to quantify eight plant species in ChuanXiong ChaTiao Wan formula in this study. The fragment quantified using the ITS region had enough sequence divergence for designing species-specific primer, and it also had conserved sites for designing universal primer to amplify a short size fragment. By constructing a quantitative plasmid, absolute quantification of ChuanXiong ChaTiao Wan formula is presented. Most importantly, the species content is described in mg/mg, consistent with the norms described in the Chinese Pharmacopoeia. The VCQA method with its reliance on the ITS universal fragment and multiple quantification nature (i.e., multiple species are quantified in parallel relative to the universal fragment content) provides a unique alternative for the quantification of various herbal medicine formulas, especially the complex herbal formulas comprising multiple plant species.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

BZ, XD, and WS conceived and designed the experiments. CX and JL performed the experiments. BZ and CX analyzed the data. DZ and YS contributed reagents/materials/equipment. BZ and CX wrote the paper. XD and WS revised and approved the final version of the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.488193/full#supplementary-material

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ORIGINAL RESEARCH
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Quality Control of Radix Astragali (The Root of *Astragalus membranaceus* var. *mongholicus*) Along Its Value Chains

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Radix Astragali (RA), the root of *Astragalus membranaceus* var. *mongholicus* (Bunge) P.K. Hsiao, known as "Huangqi" in Chinese, has been used as a traditional herbal medicine or food in China for more than 2,000 years and is now consumed globally. Unfortunately, the increasing demand for RA has led to the overexploitation of its wild stock, as well as quality problems, including adulteration and contamination. Therefore, the sustainable cultivation of RA is urgently needed. In the present research, semi-structured interviews and key informant interviews were conducted, over a 2-year period, to collect data from stakeholders in the main production areas; moreover, a targeted chemical analysis-based quality assessment strategy was applied to understand the quality of RA. Accordingly, 10 different types of value chains (VCs) were identified in RA production; meanwhile, the contents of the main active ingredients (astragaloside and calycosin-7-O-β-D-glucoside) were analyzed by HPLC-ELSD-UV and the yield of medicinal material was demined and further analyzed using *k*-means clustering analysis. The results show that the tight relationship between quality of the RA and stakeholders' revenues among the VCs, which reflects a more general trend in the production system. Over the past few decades, vertical coordination has emerged increasingly in VCs of RA, which leads to a more coherent traceability system and rigorous regulations in the supply chains. *Daodi* herbs can be considered to be a standard that is distinctive with good quality characteristics that emphasize the origins of the medicinal plants. We find that the suitability of geographical areas and vertical integration can improve the VCs of RA, which further contributes to its quality control, as well as its sustainable production.

Keywords: radix astragali, daodi herb, value chain, quality, grade

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INTRODUCTION

Radix Astragali (RA), the root of *Astragalus membranaceus* var. *mongholicus* (Bunge) P.K. Hsiao or *A. membranaceus* (Fisch.) Bge. is often used in traditional Chinese medicine. It was classified as top grade in *Shennong Bencao Jing* (*Shennong's Materia Medica Classic*), which means adverse side effects from the medicine material are uncommon and can be taken for a long time. It has been widely used in foods, teas, drinks, wines, cosmetics, and so on (Guo et al., 2018). In fact, according to

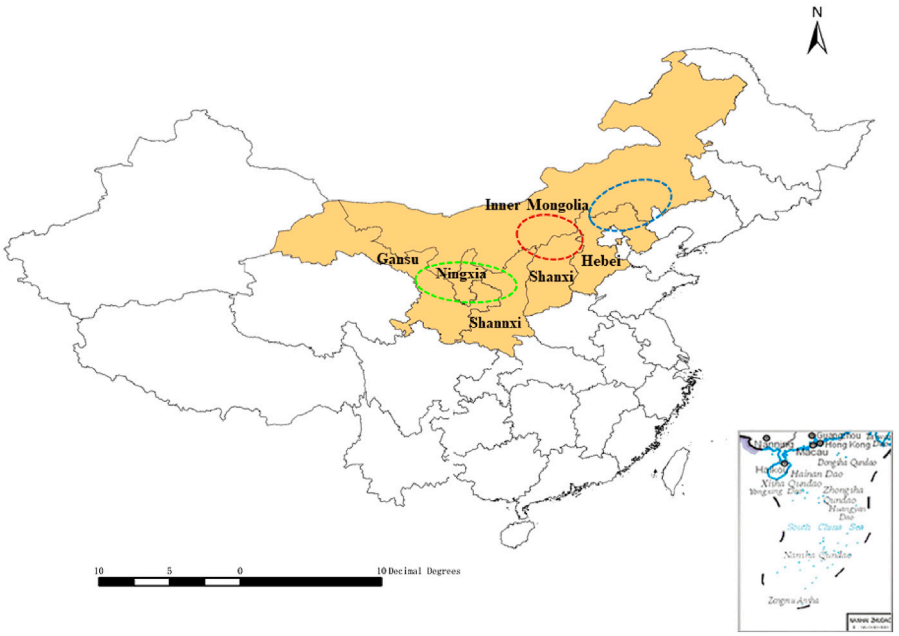

FIGURE 1 | Radix Astragali production areas in China (mapped using ArcGIS v10.4.1). Red circle: center of the *daodi* area of Radix Astragali (Midwest Inner Mongolia and north Shanxi), blue ellipse: main production areas of Radix Astragali in the east (east Inner Mongolia and parts of Hebei), green ellipse: main production areas of Radix Astragali in the west (Midwest Gansu and parts of Ningxia and Shaanxi).

the statistics, RA is used in more than 192 Chinese medicine prescriptions and over 1,045 kinds of health products (Cheng et al., 2019). In recent years, RA is also increasingly consumed by the global market beyond China. The global consumption of RA is vast, for example, 4,477.17 tons of RA have been exported from China in 2015 (Wang et al., 2018).

As the demand for medicinal plants in the domestic and international pharmaceutical markets has increased, artificial cultivation has had a positive effect on the protection of wild resources and has allowed market demands to be met (Kling, 2016; Cunningham et al., 2018). Since 1950, cultivated RA products have gradually replaced wild materials. The cultivated *A. membranaceus* var. *mongholicus* is a major source of RA, which accounts for an 80% share of the market (Sinclair, 1998; Qin et al., 2013). Based on their particular natural conditions, ecological environments, cultivation technology, and postharvest processing methods involved (Huang et al., 2011), the Midwest area of Inner Mongolia and north Shanxi Province have been considered as "*daodi*" areas for RA (an area producing top quality herbal medicine). While the demand for RA is increasing, its cultivation has expanded from *daodi* areas to many other production areas, such as Gansu, Shaanxi, Ningxia, and Hebei Provinces (Zhang et al., 2019). The cultivation area and *daodi* region of RA are illustrated in **Figure 1**.

The cultivation of medicinal plants is helpful for the conservation of their wild resources – it can also be of benefit to farmers economically, and provides social benefits (Booker et al., 2016; Kling, 2016); however, certain quality problems arise when unsuitable planting habitats and intensive input of herbicides, fertilizers, and pesticides are used, and such changes have subsequently resulted in the quality and yield of the medicinal material becoming uneven (Booker et al., 2014; Heinrich, 2015; Yao et al., 2018b). While the supply system of medicinal plants is much affected by its VCs (Booker et al., 2012; Yao et al., 2018b), hence, in order to meet the steadily increasing demand for RA, reconsider the quality measurement system and VCs among suppliers currently used needs to be reconsidered.

VCs describe the different activities involved in different production approaches, i.e., starting from a farmer preparing their raw materials, to the sale of the finished product and the customer (Chopra and Meindl, 2004). They also emphasize the relationships between primary producers and other stakeholders in different production systems and their impacts on the social economy. They have been referred to as a 'centerpiece of agricultural policy' in the *World Development* Report 2008 (World Bank, 2007) and are considered to be an important measure for achieving poverty reduction in developing countries.

By understanding the relationship between production and supply, we can identify the product flows, financial flows, and information flows involved in different models. It is then possible to get a better understanding of why the quality and price of herbal medicines vary widely in different markets. In VCs, companies can employ both vertical and horizontal integration as they undertake their production collaborations, export, and e-commerce activities. Hence, VCs can have a series of impacts on the quality of the products, resulting in economic benefits, and effects on patients (Booker et al., 2012; Yao et al., 2018a). Presently, the focus is not just on the economic benefits of a VC, but also on achieving a balance with its effect on the ecological environment and product quality.

Published research shows that chemical components were identified in the RA, include saponins, flavonoids, nitrogen containing chemicals, and aminophenols (Zhang et al., 2019). Among the many compounds present in RA, astragaloside and calycosin-7-O-β-D-glucoside are considered to be the main active ingredients. They have potent pharmacological activity and are often used as target compounds to assess the quality of the RA (Zhan et al., 2017). They are also used as "marker components" in various standards in the Chinese Pharmacopoeia (CPC, 2015), and astragaloside is recorded in the European Pharmacopoeia and British Pharmacopoeia (HMSO, 1963; European Pharmacopoeia, 1993).

From the perspective of VCs, the study aims to achieve the quality control strategy of RA. The study was mainly carried out in Inner Mongolia, which is the main production and *daodi* area for RA, in terms of economic, medicinal value and traded volume of plants. This work was based on information arising from an investigation conducted in the main areas of RA production, including Gansu, Shanxi, Shaanxi, Hebei, etc. Differences of the stakeholders, financial flows, and information about RA from production to consumer among its VCs were elucidated. The quality of RA was measured based on its main active ingredients (Cao et al., 2019b). We further described the effects of geographical indication on the quality and yield of the medicinal material and the competitive advantage of vertical coordination. Hopefully, the study will be helpful for the sustainable supplement of high-quality RA.

MATERIALS AND METHODS

Reagents and Chemicals

The acetonitrile used in the high-performance liquid chromatography (HPLC) system was purchased from Thermo Fisher (Thermo Fisher Scientific, United States). Purified water was obtained using Water Purification Systems (Shenyang, China). Formic acid was purchased from the first chemical company of Nanjing (Jiangsu, China); all other reagents were of analytical grade. The reference compounds of astragaloside and calycosin-7-O-β-D-glucoside were purchased from the Pufei De Biotech Company (Chengdu, China). All solvents and samples were filtered through a 0.45-μm filter before injection into the HPLC.

The reagents used for analysis of heavy metal pollutants were HNO_3 and $HClO_4$ both at suprapure-grade. Other reagents were of analytical reagent grade unless otherwise stated. The element standard solutions used for assay of the contents of Pb, Cd, As, Hg, and Cu were supplied by National Institute of Metrology (Beijing, China).

All the analytical standards of the studied pesticides were of high purity and certified upon purchase from the Agro-Environmental Protection Institute, Ministry of Agriculture and Rural Affairs (Beijing, China), as being at a purity of greater than 99%. The solvents, acetonitrile (ACN), acetone, methanol, and n-hexane, were of HPLC-grade (Thermo Fisher Scientific, United States). Magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) obtained from Aladdin (China) were used, with a purity exceeding 99%. Other reagents were of analytical reagent grade unless otherwise stated.

Plant Material

Thirty-one batches of RA samples were collected from 16 habitats across Inner Mongolia. A few samples from Shanxi and Gansu Provinces were used as a control. Firstly, samples purchased in medicinal markets were separated, as their information was thought to be confusing and their origins were uncertain. Secondly, the collected samples were numbered using digital information related to the time and serial number of the collection location. Meanwhile, voucher specimens of the plants were collected and deposited at Baotou Medical College, University of Inner Mongolia. Retaining their plant morphological characteristics allowed them to be examined by personnel with extensive expertise in plant knowledge so as to yield the best possible botanical identification of the samples. Finally, we analyzed 31 samples with complete information after identification.

Fieldwork

The RA harvest stretches from September to November. The fieldwork presented in this work was carried out during the cultivation and harvesting periods in 2018–2019, and involved a variety of locations in China: Guyang County, Wuchuan County, Helin County, Duolun County, Shangdu County, Naiman County, Siziwang County, etc. in the autonomous region of Inner Mongolia, and Min County and Longxi County in Gansu Province. Based on the characteristics of RA in the supply chain, we modified and designed three different structural questionnaires (Yao et al., 2018a). Semi-structured interviews were also taken from different stakeholders in Gansu, Shanxi, Inner Mongolia, etc. (**Figure 1**). These have been the main areas of RA production, where cultivation of RA needs 2–3 years. Semi-structured interviews and key informant interviews were conducted with experienced participants in the RA industry. In total, 34 farmers, 16 members of agricultural cooperatives or planting companies, three middlemen, 10 managers of processing companies, and 21 retailers provided information on the production, processing, and retail aspects of the RA industry.

The investigation into the average yield and price of RA in local outlets was supported by the Information and Technology Service Center for Modern Chinese Materia Medica Resources Dynamic Monitoring (founded in 2012) and China Agriculture Research System CARS-21 (founded in 2017). Before the investigation, staff using these two platforms conducted extensive investigations on the cultivation and marketing of RA, respectively, providing us with the basic information we required on the stakeholders. In addition, a large number of RA samples were obtained from the *daodi* and other production areas.

Quantitative Analyses of Astragaloside in Radix Astragali

A total of 31 batches of RA were analyzed by a HPLC system equipped with an evaporative light scattering detector and diode-

array detector. Analysis was performed using a Thermo Fisher Ultimate 3000 HPLC system, and an Agilent C18 column (250 mm × 4.6 mm, 5 μm) with a flow rate of 1.0 ml/min (Du et al., 2014; Chen et al., 2015). The mobile phase consisted of acetonitrile and deionized water (32%:68%). The injection volume was 10 μl, and the column component was set to a temperature of 30°C.

Quantitative Analyses of Calycosin-7-O-β-D-Glucoside in Radix Astragali

Analysis was performed using a Waters C18 column (250 mm × 4.6 mm, 5 μm) at a flow rate of 1 ml/min. The mobile phase consisted of acetonitrile (solvent A) and water (containing 0.2% methanoic acid, solvent B). Gradient elution was applied as follows: 0–20 min, 80–60% B; 20–30 min, 60% B. The injection volume was 10 μl, and the temperature of the column was maintained at 30°C. Detection was performed at a wavelength of 260 nm. Each sample was assayed in triplicate.

Detection of Heavy Metal Contaminants

Due to the possibility of heavy metal contamination, it is necessary to determine the contents of harmful substances, thus to ensure the safety of the products. Amount of Pb, Cd, As, Hg, and Cu in RA were analyzed by atomic absorption spectrometry (AAS) (CPC, 2015). A Thermo ICE 3000 atomic absorption spectrometer with deuterium background corrector was used in this study. Pb and Cd contents in plant samples were determined by HGA graphite furnace using argon as the inert gas. Cu was assayed out in an air-acetylene flame. Other measurements were based on cyanide complex processing (Tüzen, 2003; CPC, 2015).

Detection of Pesticide Residues

The organochlorine residues including total BHC (α-BHC, β-BHC, γ-BHC, δ-BHC), DDT (pp'-DDE, pp'-DDD, op'-DDT, pp'-DDT), and pentachloronitrobenzene (PCNB) were determined in accordance with the following gas chromatography tandem mass spectrometry method (GC-MS) (CPC, 2015). A Thermo TRACE 1300 gas chromatograph was used. Nitrogen with a purity of 99.99% was used as the carrier gas. A DB-5MS capillary column (0.25 mm × 30 m × 0.25 μm) was used. A full auto-tune of the mass spectrometer was performed before the analysis of each set of samples. The transfer-line temperature was 300°C, the manifold temperature was 50°C, and the ion-trap temperature was 250°C. The flow rate was 1.0 ml/min and the sample injection volume was 1 μl.

Value Chain Analysis

The analysis of the different VCs was undertaken in four stages: 1) identify the main VCs and add the stakeholders to the corresponding procedures; 2) calculate the market price of each trading link based on the interview data and information from the RA industry and then convert this into CNY/kg; 3) analyze the strengths and weaknesses of the VCs in relation to safety, quality, and geographical indication; 4) draw up a framework for the VCs to relate the main production activities to the stakeholders. Additionally, the production behavior, quality, and financial performance of the RA in VCs were analyzed according to Yao et al. (2018b).

Clustering Analysis

The geographical indication of a medicinal plant is related to the geographical location where the plant is grown. *Daodi* herbal medicines have adapted to their environments over long periods of time and have self-adapting characteristics. They are thus thought to feature superior qualities, including superior active ingredients and yields.

The *k*-means algorithm is a clustering method based on partitioning which is capable of effectively dealing with large-scale data but is easy to understand (Diao, 2012). Contents of the two measured constructs and yields of the fresh roots were combined into a data frame. The R-tree indexing algorithm was then used to analyze the spatial index in the *k*-means clustering method.

RESULTS AND DISCUSSION

Industrial Structure and Value Chains

RA cultivation began in the 1950s and was intended to mitigate the sharp decline in wild populations while meeting the demands of commercial enriches. Later on, RA was artificially cultivated in Gansu, other parts of Inner Mongolia, and Shanxi on a large scale. *A. membranaceus* var. *mongholicus* is the main source of RA, and is cultivated much more than *A. membranaceus*, providing 80% of the RA medicinal market. Therefore, the present study launched a series of investigations into the production of *A. membranaceus* var. *mongholicus*.

Inner Mongolia has long been considered as a *daodi* area of RA, with high production rates and superior quality. As such, RA was popularly used in traditional Chinese medicinal practice. With its long production and supply history, the practice of RA production is found to consist of 10 mature VCs, which can be distinguished by their various composite patterns of stakeholders. Typically, RA goes through six production stages until it reaches the herbal wholesale and retail markets shown in **Figure 2**.

Although RA is available in a diverse range of forms, dried roots and traditional Chinese decoction pieces are still the main consumables. The price varies with quality, and is consistent with the size and active ingredients. The Chinese Pharmacopoeia (CPC, 2015) gives the standards for the quality control of RA. In addition to public standards, there are also a number of different private market standards and these can be broadly divided into four grades. Commonly, based on size, different prices are often found for RA that is "small" (0.35–0.8 cm), "medium" (0.8–1.2 cm), "large" (>1.2 cm), and "huge" (>1.5 cm), as illustrated in **Figure 3**.

Figure 4 demonstrates the 10 primary VCs identified for RA and the stakeholders involved. Different stakeholders may have various roles, and the division of labor is also slightly different. Among the 10 VCs, middlemen or agricultural cooperatives play intermediary roles in VCs 1–4, while in VCs 5–10 production is made to order which weakens the role of middlemen.

FIGURE 2 | Radix Astragali goes through six stages before it reaches the consumer.

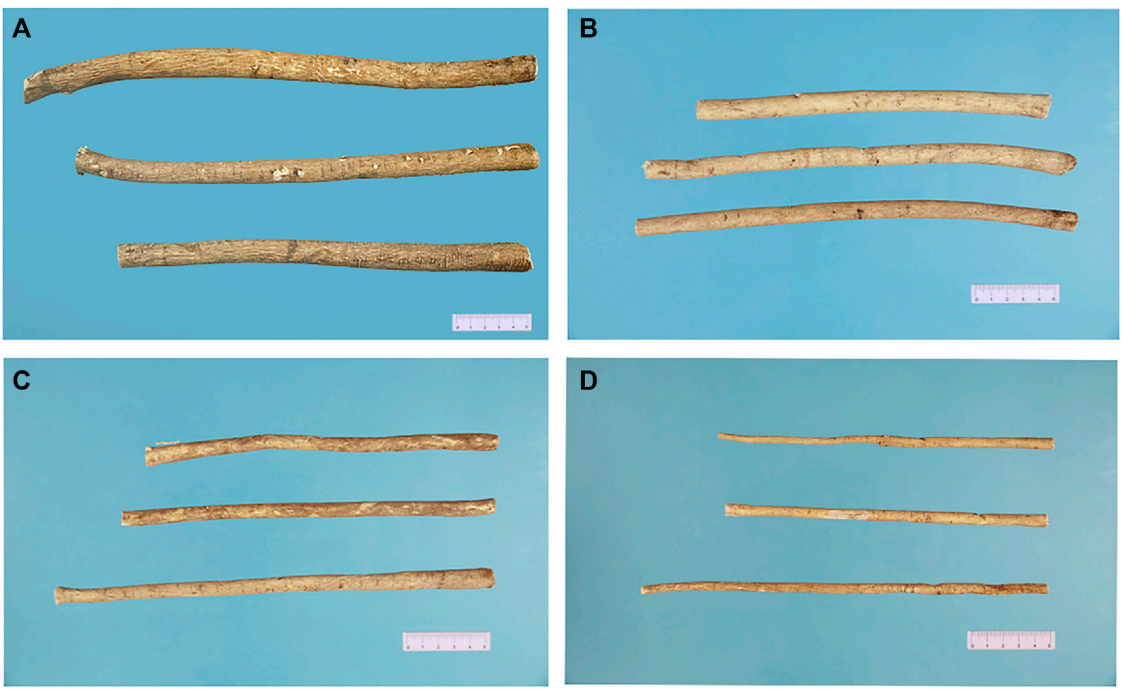

FIGURE 3 | Commercial grades of Radix Astragali found at a local market in Inner Mongolia: **(A)** huge (d > 1.5 cm), **(B)** large (*d* > 1.2 cm), **(C)** medium (1.2 cm ≥ *d* > 0.8 cm), and **(D)** small (0.8 cm ≥ *d* ≥ 0.35 cm).

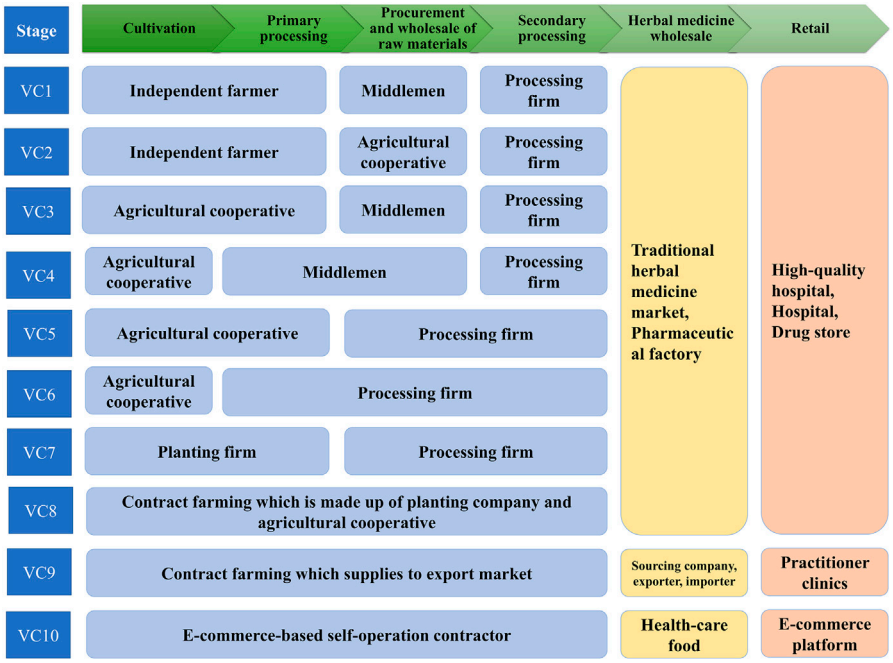

FIGURE 4 | Primary value chains and stakeholders involved in Radix Astragali produce.

VCs 1 and 2 begin with independent farmers with relatively small RA fields (<2 ha). These tend to be of traditional, small-scale, peasant economy form, and have been an important part of RA production for decades. Most of the time, the RA is delicately processed and graded in order to gain more profit, while the exception is when cash is scarce and they are sold as fresh roots. In these VCs, farmers often sell RA through large suppliers, such as middlemen, cooperatives, etc., and these procedures lead to high transaction costs and switching cost. Although the yields of RA products are sometimes higher than in other stages, procurement difficulties limit farmers' incomes. Farmers near traditional herbal medicine markets or processing companies can sell their products directly to increase profits earned from cultivation of this medicinal plant. The model is especially prevalent in the parts of Gansu and eastern Inner Mongolia where RA is cultivated.

VCs 3–6 begin with agricultural cooperatives that have relatively large fields (~30 ha). These are gatherings of several farmers who took over large fields and invested in more machinery than independent farmers. Cooperatives purchase equipment and facilities to cultivate and process for the common good. Farmers involved in this stage would produce RA material individually or cooperatively and make it wholesale. The farmers can easily benefit from financial and technical supports from local government, and gain financial resources in the form of bank credits. In addition, after a few years, many cooperatives that participate in RA planting become inclined to sell their fresh roots to middlemen or processing companies, thereby achieving rapid capital gains for the following year's planting.

VC 7 begins with planting companies. These are large plantations (>60 ha) with high degrees of mechanization, comprehensive sprinkler–irrigation systems, and storage warehouses. The planting companies buy seeds from large breeders or specialized seed companies and hire farm workers to do the farm work. This model is especially prevalent in Midwest Inner Mongolia, as much land is available for RA cultivation therein.

VC 8 is based on contract farming which involves vertical coordination within the chain. The planting company is involved in all the nodes of the VC, from production, through processing, to wholesale. This involves the formation of verbal or written agreements by the planting company, which bridges the gaps between farmers and trading companies or other production enterprises. Owing to fewer intermediate links existing between suppliers with retailers, the costs have been reduced and the economic efficiency is improved. All stages are all traceable, including the production chains and planting techniques, the application of fertilizers and pesticides, and quality inspection. In this model, the company can provide farmers with production targets and standards before the annual production process begins, as well as fixing the current purchase price. However, there are signs that farmers are starting to form alliances with local planting or processing companies (thus to change the traditional route of selling products locally) for the purpose of getting better quality products and entering more profitable markets. Studies have also shown that some procurement agencies (planting or processing companies) prefer to work with large-scale farmers rather than smallholder farmers because of the higher transaction costs associated with dealing with the latter. Commercial markets are highly competitive, with

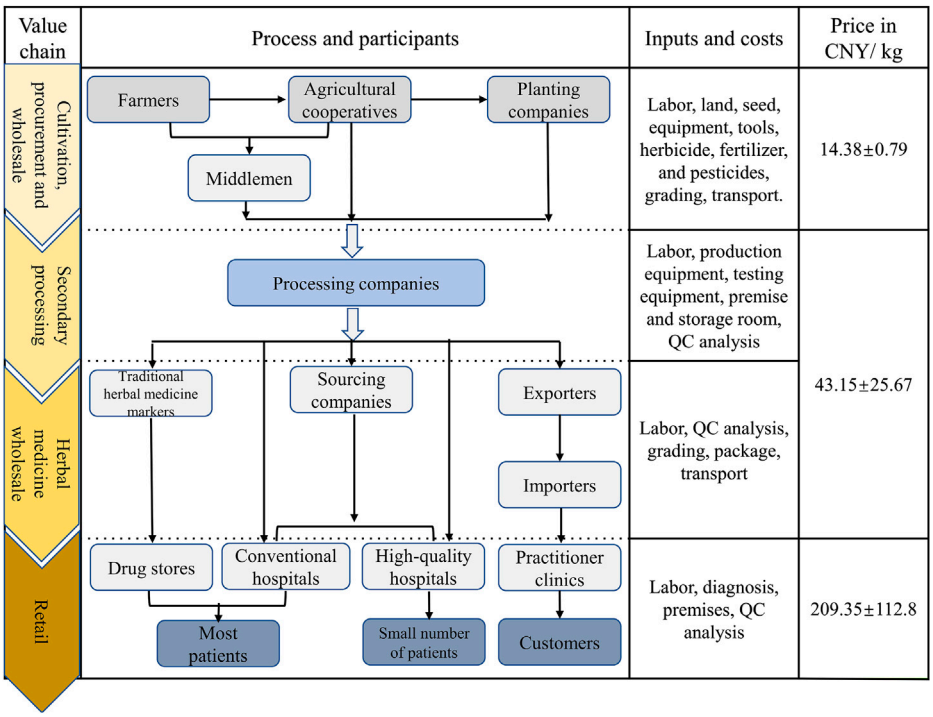

FIGURE 5 | Herbal practitioner value chains for crude botanical materials, beginning with a grower in Inner Mongolia and finishing with the consumers. The data were derived from informants questioned after the Radix Astragali harvest in 2019. Value are expressed as Mean ± Srandard deviation (SD).

high quality standards and requirements of consistent, timely deliveries, which smallholders struggle to achieve (Reardon et al., 2009; Wainaina et al., 2012; Da Silva and Rankin, 2013; Namulindwa, 2018).

VC 9 involves another form of integrated vertical coordination in which products are directly supplied to export markets. In VC 9, the botanical raw materials begin with medicinal material growers in China, which are then exported to consumers in foreign countries via the export markets. In this VC, some of the RA is used for medicine for treatment or health care under the guidance of traditional practitioners, and some is finely processed by the drug manufacturer. Furthermore, "added value" occurs due to the processing which is carried out by the farmers, processers, export and import middlemen, suppliers, and foreign herbal practitioners. The planting company becomes linked with the sourcing companies (i.e., joint ventures or big pharma) in order to enter the export market. In the process, the stakeholders need to pay careful attention to the quality of the products in order to improve the reliability of the RA production system and achieve reputational and marketing goals. RA agriculture also encourages producers to implement the Hazard Analysis and Critical Control Point System (HACCP). However, the RA produced in this VC is exported through a complicated series of quality inspection and intermediate sales procedures, without second processing, and is finally sold in the export market as prescription drugs or health food.

VC 10 is a relatively new model based on e-commerce. Although the sales information accompanying the goods often states that the RA was "collected in the wild," in fact this is not necessarily true. In the process of VC, the contractor (in the form of a middleman or small-scale medical processing plant) is the main participant. They procure RA with good appearance, process it into pieces or powder, and package it more attractively before selling it on the network platform. In theory, e-commerce should provide higher profit than conventional markets as operating costs are highly reduced; however, the quality control of such products sold via the network platform needs to be greatly improved.

Financial Performance of Stakeholders

The monetary value of herbal medicine is created by the stakeholders' production activities and is generated during the trading process. Labor and non-labor inputs promote the value of the products to different extents in different VCs. Stakeholders also play different roles at the same stage, so they can take different risks and accrue various benefits in the process (Yao et al., 2018b). **Figure 5** illustrates the value variability of RA products along its way from farmer to consumer.

At the cultivation stage, stakeholders are attracted to RA agriculture due to the greater monetary value attached to medicinal plants (12,000–22,500 CNY/ha) compared to crop production (7,500–12,000 CNY/ha). However, prices in the medicinal-materials market are subject to intense fluctuations. The difference has a strong influence on the acreage devoted to such raw materials (both positive and negative). All of the costs are relatively high during the cultivation stage. Non-labor costs at

this stage consist of land, seed, equipment, tools, irrigation pipes, herbicides, fertilizers, and pesticides, etc. There is an enormous demand for labor as the seeding, weeding, and harvesting machinery is semi-automatic, especially for the green production practices. Labor is required to care for the field management, transplant, harvest, dry, process, and transport the materials, and required for manual grading, etc. Collectively, local farmers need to pay 130–200 CNY to each person per day for such labor services.

In addition, some stakeholders (farmers, agricultural cooperatives, and planting companies) process the plants themselves, except when there is large economic pressure whereupon they will sell the fresh roots at low prices. Among the various models, the independent farmers in VCs 1 and 2 will carry out breeding by themselves and use their own farmland for cultivation purposes. This will help them to reduce non-labor costs, so as to save input while ensuring higher returns. Additionally, during the harvesting process, the drying and primary processing processes can all be performed by family members in order to save labor costs. In contrast, the large-scale agricultural operations involved in VCs 3–9, which are becoming more popular, including agricultural cooperatives and planting companies, pay more attention to their economic benefits and production efficiency.

Procurement and wholesale of raw materials are key processes in the VCs. Some VCs use middlemen in these processes but sometimes the functions of such middlemen are omitted or replaced by agricultural cooperatives. At this point, suppliers that are closer to the processing companies and get more information about transactions are more likely to make direct contact with the contractor, without the need for involving middlemen. Procurement costs include procuring and reselling labor, traveling costs, and transportation costs. Middlemen need to invest a part of their original capital in the procurement process, so financial business credit is very important to them. Also, as the product quality of the RA from different sources varies, and considering the ease with which information can be communicated, the pricing structure of the RA is affected by the locality of the cultivation area.

The second processing and wholesale stages are also labor-intensive and include washing, cutting into pieces, re-drying, hand selecting, size-based grading, and transportation. Only VC 9 does not require the RA to be cut into. Before they carry out the second processing stage, processing companies have to spend a lot of money on production and testing equipment, production workshops, and storage rooms. In addition, their factories need to perform quality testing (according to the Chinese Pharmacopoeia) for each batch of traditional Chinese medicine processed. This is also where a large part of the input is made in the management of the factory.

The middle stage of the transformation process involves the wholesale of the herbal medicine. The stakeholders are engaged by the traditional herbal medicine market, pharmaceutical factory, sourcing company, and contractor. After processing, the herbal medicine is sold wholesale to different markets according to the sizes and contents of the active ingredients. The aim is to supply high-quality RA to their customers at a favorable price which fluctuates between 20 and 100 CNY/kg.

Retail is the last stage in the RA value chain shown in **Figure 5**. Here, the resources flow to high-quality hospitals, traditional herbal medicine markets, export markets, and online markets. The price of the RA now ranges from 50 to 330 CNY/kg. Differentiated commodity values are reflected in the different markets, so that the selling price to the export market and high-quality hospitals is more than three times that to other retailers. Studies of the trends in prices show that they are related to the dynamic balance between demand and supply. It is thus thought that as the demand for RA products grows, progressively more and more of the RA will be harvested. Then, the prices (and farmers' financial gains) will decline.

Geographical Indication

Daodi herbs are famous in traditional Chinese medicine. They have a high and consistent quality which, in addition to their place of origin and its ecological conditions, should be defined by their traditional methods of culture and production, and even their specific germplasm (Booker et al., 2012).

During our study, samples were analyzed to see if there are any differences between the RA from *daodi* and other areas. Quality of RA from different origins and quality standards are commonly adjudged by the index composition of astragaloside or calycosin-7-glucoside (Cao et al., 2019a; Cao et al., 2019b; Xue et al., 2019; Tian et al., 2020). Liu et al. (2019) shows that compared with flavonoids and other isoflavones, calycosin-7- glucoside makes a significant contribution to distinguishing RA from different origins. Therefore, we analyzed 31 batches of RA and classified them using a classification scheme based on three variables including the yield of medicinal material, and the contents of astragaloside and calycosin-7-glucoside (Supplementary Material show the chromatography).

Table 1 displays the results of the content analyses. Clustering analysis (using the *k*-means algorithm and R v3.6.1 software package) was then adopted to compare the samples. The results are illustrated in **Figure 6** in the form of a dendrogram. In **Figure 6**, the samples collected from Inner Mongolia, Shanxi and Gansu Province are divided into different clusters (labeled Ⅰ and Ⅱ). Cluster Ⅱ is further divided into three subgroups (labeled a, b and c) based on geographic variation. The contents of active ingredients in the RA from Gansu Province are found to be lower than that in the products from Inner Mongolia; however, the yield of medicinal material is higher. The RA *daodi* herbal medicines from specific areas of Guyang, Helin, Wuchuan, and Wulateqian counties are of better quality than that from other habitats due to their natural geographical environments and human factors, etc. The results of our geographical indication evaluation were universally accepted by the stakeholders involved in the production and supply chains. The RA also includes other saponins, flavonoids, polysaccharides, amino acids, etc. Researchers have tried to establish comprehensive quality evaluation protocols for RA by using fingerprinting methods (Zhang et al., 2016). Future research will we need to explore the comprehensive influence of multi-index components on quality control indices and evaluate resources for RA.

TABLE 1 | The results of the content analyses performed on the 31 batches of Radix Astragali samples.

Sample no	Cluster	Longitude	Latitude	Calycosin-7-O-β-D-glucoside (%)	Astragaloside (%)	Yield (kg/mu)
2019001GS	I	E104°14′54.27″	N35°0′15.28″	0.034	0.032	750
2019002GS	I	E104°02′3.19″	N34°26′50.11″	0.041	0.076	800
2019003GS	I	E104°35′35.64″	N34°59′55.64″	0.034	0.042	775
2019004GS	I	E103°58′9.44″	N34°21′13.7″	0.032	0.063	750
2019005IM	II c	E113°11′29.69″	N41°23′49.50″	0.049	0.159	600
2019006IM	II b	E111°14′12.04″	N41°33′22.08″	0.043	0.087	590
2019007IM	II b	E116°34′08.86″	N42°20′57.02″	0.024	0.053	540
2019008IM	II a	E110°15′01.69″	N41°21′10.24″	0.064	0.035	640
2019009IM	II a	E110°35′12.10″	N40°50′09.45″	0.064	0.025	625
2019010IM	II a	E109°56′03.12″	N40°37′40.66″	0.058	0.026	640
2019011IM	II a	E111°47′41.95″	N40°20′14.27″	0.061	0.050	665
2019012IM	II a	E111°51′17.16″	N40°24′14.43″	0.058	0.047	665
2019013IM	II a	E111°39′51.05″	N40°16′21.62″	0.060	0.050	655
2019014IM	II a	E111°45′22.29″	N40°06′22.07″	0.070	0.040	655
2019015IM	II a	E112°0′31.96″	N40°06′17.19″	0.065	0.065	655
2019016IM	II a	E112°10′24.61″	N40°27′9.07″	0.057	0.047	655
2019017IM	II b	E120°48′10.48″	N42°21′06.52″	0.046	0.067	525
2019018IM	II b	E113°51′55.26″	N41°34′03.00″	0.023	0.055	525
2019019IM	II a	E111°38′12.17″	N41°37′50.87″	0.069	0.041	550
2019020IM	II a	E110°39′24.78″	N40°24′43.84″	0.077	0.057	625
2019021IM	II c	E110°44′07.09″	N40°42′14.23″	0.057	0.133	630
2019022IM	II c	E111°11′14.06″	N40°16′22.04″	0.058	0.094	650
2019023IM	II a	E109°36′12.46″	N40°56′42.16″	0.081	0.084	625
2019024IM	II a	E109°49′51.91″	N40°53′02.79″	0.056	0.031	610
2019025IM	II a	E109°00′05.35″	N40°51′21.68″	0.042	0.061	620
2019026IM	II a	E111°20′16.89″	N41°12′4.38″	0.055	0.06	625
2019027IM	II a	E111°35′58.07″	N41°04′46.71″	0.078	0.046	610
2019028IM	II a	E110°54′35.45″	N40°59′49.19″	0.079	0.044	610
2019029IM	II b	E115°47′40.67″	N42°52′49.60″	0.023	0.064	525
2019030IM	II b	E115°48′42.67″	N42°52′09.90″	0.022	0.066	525
2019031SX	II c	E113°40′0.88″	N39°42′54.81″	0.032	0.102	725

IM, Inner Mongolia; GS, Gansu; SX, Shanxi.

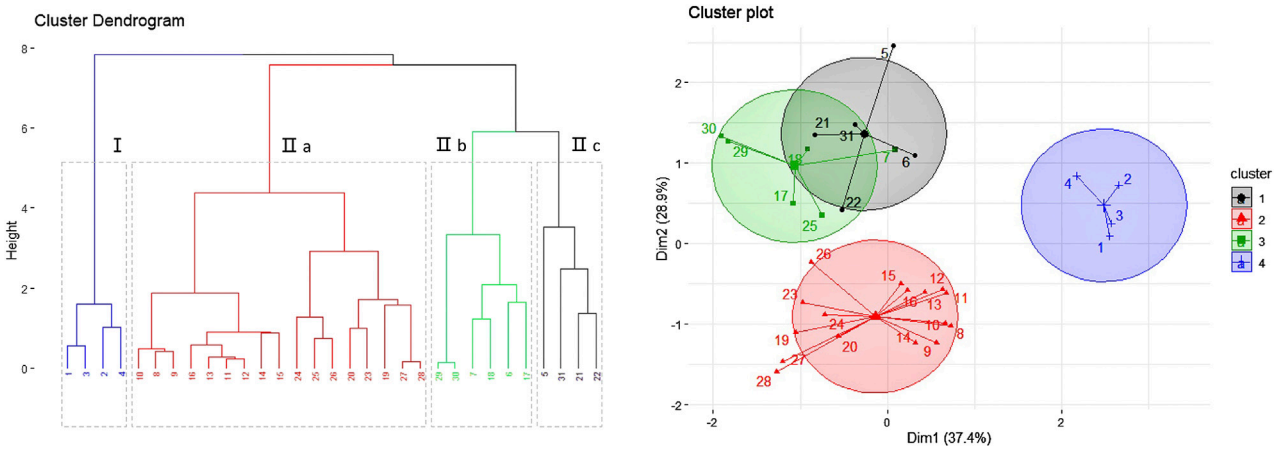

FIGURE 6 | Results of the geographical indication analysis (based on 31 batches of Radix Astragali from 16 habitats).

Radix Astragali Quality in the Different VCs

Various voluntary and mandatory standards, regulations, and requirements are used in the production and retailing of herbal medicines to control the quality and safety of the final products. Measures that can be used to evaluate the quality of RA have been established in pharmacopoeia. Characterization tends to be made according to the appearance, transection, nature, and flavor of RA, and on certain tests, including chemical identification, quantification analysis, and residual amounts of pesticides and heavy metals (CPC, 2015).

TABLE 2 | The contents of the heavy metal and pesticide residues on the 31 batches of Radix Astragali samples.

Sample no	Heavy metal (mg/kg)					Pesticide residues (mg/kg)		
	Pb	Cd	As	Hg	Cu	BHC	DDT	PCNB
2019001GS	0.6571	—	0.0416	0.1018	6.3993	—	—	—
2019002GS	0.1265	—	0.0335	0.0078	7.6253	0.0418	—	—
2019003GS	0.1466	0.0113	0.0640	0.0012	6.9752	0.0409	0.0023	—
2019004GS	0.5724	0.0001	0.0501	0.0312	7.1044	0.0238	0.0023	—
2019005IM	0.0811	0.0052	0.0956	—	7.8815	0.0095	—	—
2019006IM	0.1914	0.0095	0.0255	0.0572	9.2858	0.0488	0.0012	0.0010
2019007IM	0.0739	—	0.0112	—	6.3453	—	—	—
2019008IM	0.0609	—	0.1447	0.0156	8.6778	0.0091	—	—
2019009IM	2.4061	0.0369	0.1631	0.0638	8.7489	—	—	0.0012
2019010IM	0.3471	0	0.0960	0.1125	8.5240	0.0057	—	—
2019011IM	—	—	0.2023	0.0124	4.7928	–	–	—
2019012IM	1.1124	0.0065	0.2213	0.0061	6.5630	0.0061	0.0010	—
2019013IM	0.7809	—	0.0561	—	8.5231	0.0067	—	—
2019014IM	0.5661	0.0091	0.0984	0.0095	5.3323	0.0417	—	—
2019015IM	0.4678	0.0006	0.0675	0.0022	6.3645	0.0074	0.0012	0.0010
2019016IM	0.0923	0.0012	0.0649	—	4.8126	—	—	—
2019017IM	0.9518	0.0057	0.1654	0.0057	5.5663	0.0069	—	—
2019018IM	0.6841	0.0035	0.0129	0.0067	6.0265	0.0218	0.0015	—
2019019IM	0.8594	—	0.0613	0.0912	6.3138	0.0069	—	—
2019020IM	1.6125	0.0073	0.1170	0.0138	8.9820	0.0073	—	—
2019021IM	0.3682	0.0005	0.1280	0.0061	8.0072	—	—	—
2019022IM	0.1215	0.0015	0.0352	0.0483	7.1362	0.0135	0.0023	—
2019023IM	0.0793	0.0047	0.0951	0.0764	7.6247	0.0094	—	—
2019024IM	0.5226	0.0068	0.0642	0.0576	4.8623	0.0063	0.0023	0.0010
2019025IM	0.3601	0.0026	0.1690	0.0915	4.9561	0.0077	—	—
2019026IM	0.9120	0.0034	0.0662	—	8.8135	—	—	—
2019027IM	—	0.0018	0.0268	0.0017	7.8915	—	—	—
2019028IM	0.8941	0.0009	0.0544	0.0152	7.1783	0.0161	0.0017	0.0015
2019029IM	0.6354	0.0003	0.0934	0.0369	6.7128	0.0065	—	—
2019030IM	1.5079	—	0.0142	0.0658	7.6173	0.0092	—	—
2019031SX	0.6892	0.0048	0.0636	0.0052	6.654	0.0069	0.0012	—

In high-quality RA, the roots have cuticles that are smooth and pliable. Their cross-sections are dense and their textures are solid, and the center of the xylem is light yellowcolor. The product quality and safety of the plants can be accurately determined using a combination of traditional and modern identification and analytical quantification techniques. As a result, the supply of these globalized commodities is improved considerably.

The contents of total Pd, Cd, As, Hg, and Cu vary between 0 and 2.4061 mg/kg, 0 and 0.0369 mg/kg, 0.0112 and 0.2213 mg/kg, 0 and 0.1125 mg/kg, 4.7928 and 9.2858 mg/kg, with an average concentration of 0.6165, 0.0054, 0.0839, 0.0362, and 7.0418 mg/kg, respectively. The contents of heavy metals in RA comply with the limit standard of Chinese Pharmacopoeia, as well as the standards of the natural traditional Chinese Medicine materials, and standards of China Hong Kong, Japan, China Taiwan, United Kingdom and the United States (BS, 2015). From the measured pesticide residues, it was observed that the residues of BHC, DDT, and PCNB were found in samples at levels below 0.0488, 0.0023, and 0.0015 mg/kg, which were below permissible limits. The concentration of organochlorine pesticide residue was found to be within safe limits. Heavy metal and pesticide residues were found to be up to the standard as found in the study (**Table 2**).

It has been shown that there is a strong relationship between good manufacturing practice (GMP) and the traceability of the quality of the RA (Qi et al., 2015). Although the risk-assessment standards used in GMP certification were canceled in 2019, they remain one of the most important tools ever used to guarantee the quality of the manufacturing process. Instead of having to get GMP certification every year, processing companies now have to ready themselves for standard inspections. Quality control measures have been raised during these inspections, and the finished products are guaranteed to be of acceptable quality before they are released for sale (Govindaraghavan, 2008; He et al., 2015).

In more recent times, voluntary standards have also been further developed. These include standards relating to: excellent germplasm [Chinese medicinal plant seeds and seedlings—Seeds of Menggu Radix Astragali (*Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao, 2017]; the distribution of *daodi* areas (*Daodi* herbs—Beiqi, 2018); breeding and cultivation standards [The Seedling transplanting technical specification of *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) in Midwest Inner Mongolia, 2018]; the classification of medicinal materials (Commercial grades for Chinese Materia Medica – ASTRAGALI RADIX, 2018), etc.

In addition to published standards, some planting companies and pharmaceutical factories also impose their own regulations to ensure the quality of their herbal medicines. There are many standards that can be used for quality control, but independent farmers are not proactive enough technologically to access these. In contrast, farmers involved in vertically-integrated VCs can get technological advice and support from various companies in the VCs.

In order to meet consumer demand for RA, core stakeholders have attempted to establish traceability frameworks that cover the whole of the supply chain (including planting companies, processing companies, and various markets) based on their domestic and international experience with traceability systems constructed for agricultural and food products. However, a major impediment to advancing the safety of raw RA is the failure of producers to consider their products as agricultural commodities rather than traditional Chinese medicines.

As VCs 1–6 involve multiple sources and intermediary nodes, the information that needs to be collected is more widely dispersed which makes it much more difficult to check the integrity of the traceability chain. VCs 7–8 incorporate traceable management information platforms that record a certain amount of electronic data (covering breeding, cultivation, production processing, and circulation) for the whole supply chain according to specified requirements. Unfortunately, almost all the current traceability technologies in place only provide production or sales information. That is, it excludes information about habitat (precautions taken before planting and details of the natural and geographical environments) and input of agricultural materials (e.g., fertilizers, pesticides, and herbicides). Only the products in VC 9 that link directly to export markets are developed under complete product-to-market control.

TABLE 3 | Quality of the Radix Astragali for the different value chains and likelihood of risks being made to its quality during its production.

VC	Traceability	Certify	Control	Heavy metal	Pesticide residue	Likelihood of hazard occurring		
						Cultivation	Processing	Procurement
1	No	No	Weak	Seldom	Seldom	Probable	Improbable	Very probable
2	No	No	Weak	Seldom	Seldom	Probable	Improbable	Very probable
3	No	No	Medium	Seldom	Seldom	Probable	Improbable	Very probable
4	No	No	Medium	Seldom	Seldom	Probable	Improbable	Very probable
5	No	No	Medium	Seldom	Seldom	Probable	Improbable	Probable
6	No	No	Medium	Seldom	Seldom	Probable	Improbable	Probable
7	Maybe	Maybe	Strong	Rare	Rare	Improbable	Improbable	Probable
8	Maybe	Maybe	Strong	Rare	Rare	Improbable	Improbable	Probable
9	Yes	Maybe	Strong	Rare	Rare	Improbable	Improbable	Improbable
10	Maybe	Maybe	Strong	Rare	Rare	Improbable	Improbable	Probable

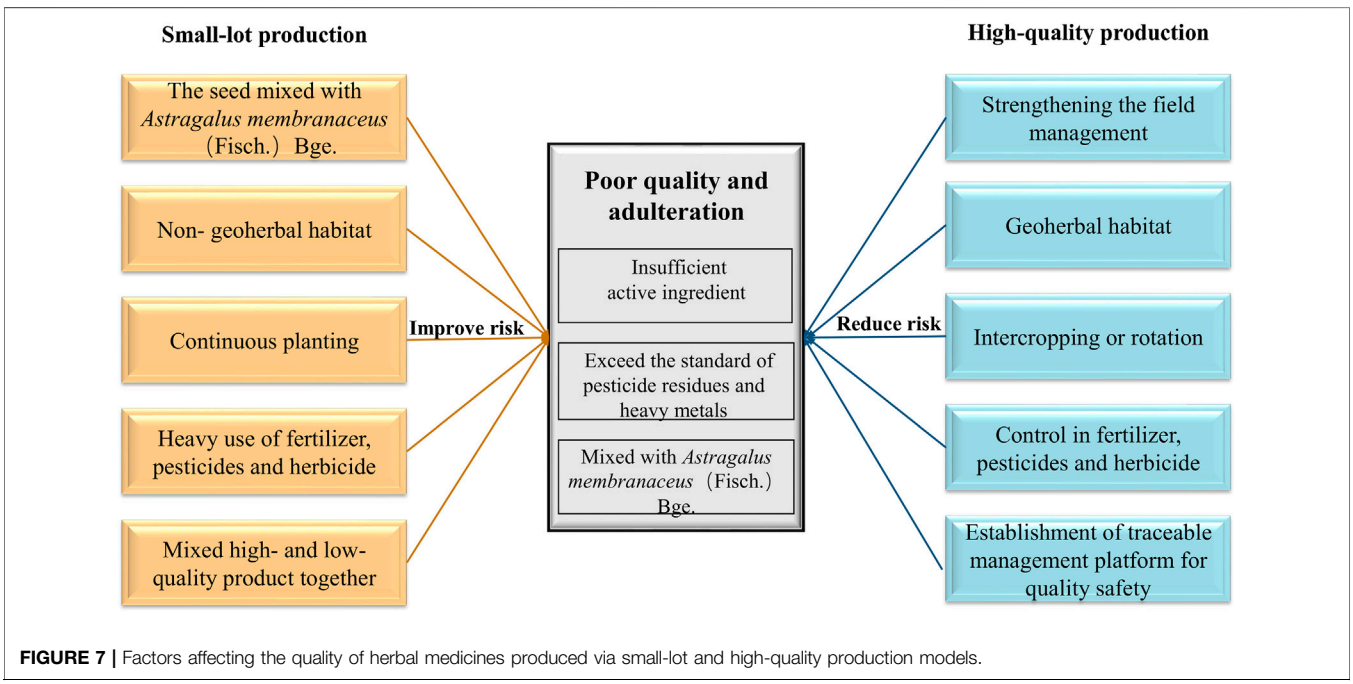

FIGURE 7 | Factors affecting the quality of herbal medicines produced via small-lot and high-quality production models.

In VCs that are not fully vertically integrated, consumers and markets cannot be told the true year and area of production of the RA as this information is unknown due to the incomplete nature of their traceability systems. In addition, the assessment of RA quality will be subject to different hazard risks in different VCs (**Table 3**). The use of vertically-integrated VCs in medicine production therefore shows some differences compared to the general markets.

Relationship Between Behavior, Revenue, and Quality

RA is a traditional herbal medicine and is included in a broad range of products including foods, beverages, cigarettes, toiletries, etc. As demand for RA has grown, the standards used to specify its purity and identification have not always kept pace with the expansion process. As a result, there has been a decline in its quality in the supply chains. Production processes driven by economic value can result in the products having poor quality and suffering from adulteration. In the current context, this means the RA products contain an insufficient amount of the active ingredients, an excessive amount of pesticide residues and/or heavy metals or be mixed with *A. membranaceus* in the seed stage. Herbal medicines with high concentrations of active ingredients are more likely to be purchased by pharmaceutical companies. They are also more likely to be used in adulteration schemes. The problem is more likely to occur in the traditional herbal medicine market, but the form of adulteration only works in the short term. In the longer term, it will seriously affect the credibility of such unscrupulous sellers and the prices they are able to charge.

At present, due to the introduction of technology, the development trends in RA production are gradually forming into three main markets: traditional markets, high-quality markets, and export markets. The remaining output is generally employed to produce extracts. The different models

show large differences in their VCs and behaviors, as illustrated in **Figure 7**.

Stakeholders in all the VCs will increase their profits in different ways. In traditional markets, growers are more likely to cause quality issues as they take steps to reduce their costs and increase their profits. VCs 1 and 2 are typical examples of traditional markets. In these VCs, in order to achieve a high yield without the help of professional guidance, excessive amounts of chemical drugs may be used, and some unreasonable measures degrade the quality of the RA. Therefore, quality problems are common in these VCs and the RA produced can readily enter the traditional markets but provide relatively low income.

VCs 3–6 lie in between the traditional and high-quality market models. They have a lot of variability available to them and will determine the quality of the medicine according to the market environment.

In high-quality markets, the companies involved participate more completely in some form of self-regulation. As a result, their brand names and reputations improve which raises their overall value. VCs 7 and 8 produce high-quality products that enter the high-quality markets, although the companies involved may not have quality certification. Their production behavior is mainly self-regulated but the reliability and traceability of their products allow them to establish good credibility.

In export markets, the companies involved spend a great deal of time and energy on tracing the quality of the RA from the very beginning (germplasm selection and testing the soil of the planting area) to ensure that their products meet the inspection standards required by the foreign markets.

VC 9 produces high-quality RA as effective quality control measures are implemented during cultivation and production. This brings high financial returns to the stakeholders. Therefore, the producers in high-quality and export markets tend to receive higher incomes from their consumers while traditional market stakeholders receive less.

Selling medicinal materials via the e-commerce platform in VC 10 can greatly reduce store costs. However, there are still many gaps in the supervision process, so there will be some irregular and uncontrolled components. However, high-quality medicinal materials from the same source may also be divided into different levels and circulate into the markets of different channels. Therefore, the behavior and benefits of the stakeholders, as well as the quality of the products and their target markets are all closely related.

According to our survey, the price of RA in traditional markets (50–150 CNY/kg) is lower than that in high-quality or export markets (about 300 CNY/kg). The price of the RA sold ranges from 50 to 550 CNY/kg and it may consist of a mixture of cultivated and wild materials. Moreover, with the improvements made with regard to professional membership of organizations pertinent to traditional Chinese medicine, better guidance is becoming available in different markets. Thus, higher requirements are being placed on the quality of medicinal materials, and the input costs will also be higher. As a result, the price of RA in city hospitals is much greater than that in the Chinese counties. In addition, the price of the RA sold in hospitals is generally greater than that in drug stores, as the products sold in hospitals tend to have a complete chain of quality-inspection links.

CONCLUSION

While the increasing demand of RA in the global market has led to the expansion of its production, its supply system is becoming more diverse. One of the purposes of this study is to demonstrate the 10 VCs currently used to produce the RA medicines that are commonly encountered in supplying. Among them, we found that the behavior of stakeholders, the suitability of geographical areas used for cultivation, and the target market all exert critical influences on the quality of RA.

Large-scale stakeholders, such as planting companies are more able to control the development of RA. They are provided with the benefits of large-scale production and quality control to enable quality and stability: they are supported by technology and management structures. On the contrary, small and medium-sized stakeholders are under pressure to invest, and are often too dispersed for good management, and are limited to small-scale production.

The flow of goods and services from producers to consumers matches supply and demand into different markets. Based on morphological characteristics, and active ingredients, the development trends in RA production are gradually forming into three main markets: traditional markets, high-quality markets, and export markets. Besides these, the safe use of herbicides, fertilizers, and pesticides will also affect the choice of target market.

Daodi herbs are typical products of geographical origin indication according to good quality and better therapeutic effect in clinical application. The Midwest area of Inner Mongolia and north Shanxi are considered to be suitable for *daodi* RA production. Through the utilization of chemical analysis and a clustering method, *daodi* RA is found to be distinguishable from other products.

During the course of our interviews and quality analyses, some key issues were found in the RA agriculture, processing procedures, and wholesale and retail activities that have largely been overlooked and can reduce the value of the products. Furthermore, we identified the relationship between quality of RA and stakeholder revenues among VCs: these reflected a general trend in the production system. There also seems to be situations in which poor quality and adulteration (intentionally or accidentally) occur due to poor quality control measures in the supply chain.

VCs with vertical integration are found to have more coherent traceability systems. Furthermore, imposing rigorous regulations in the supply chains is beneficial to the implementation of effective quality assurance measures in the manufacturing process. This can efficiently improve the quality and economic value of the final products delivered to consumers. Therefore, there is a strong need to improve the market linkages between producers and customers to create vertically-integrated VCs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

YB and CZ collected data and wrote the manuscript; ML and RY Initiated the concept and supervised the study; HB, RY, and ML analyzed data and revised the manuscript.

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Development of a Specific Mini-Barcode From Plastome and its Application for Qualitative and Quantitative Identification of Processed Herbal Products Using DNA Metabarcoding Technique: A Case Study on *Senna*

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Herbal products play an important role globally in the pharmaceutical and healthcare industries. However, some specific groups of herbal products are easily adulterated by confused materials on the market, which seriously reduces the products' quality. Universal conventional DNA barcodes would function poorly since the processed herbal products generally suffer from varying degrees of DNA degradation and DNA mixing during processing or manufacturing. For quality control purposes, an accurate and effective method should be provided for species identification of these herbal products. Here, we provided a strategy of developing the specific mini-barcode using *Senna* as an example, and by coupling with the metabarcoding technique, it realized the qualitative and quantitative identification of processed herbal products. The plastomes of Senna obtusifolia (L.) H.S.Irwin & Barneby and Senna occidentalis (L.) Link were newly assembled, and the hypervariable coding-regions were identified by comparing their genomes. Then, the specific mini-barcodes were developed based on the identified hypervariable regions. Finally, we applied the DNA metabarcoding technique to the developed mini-barcodes. Results showed that the lengths of plastomes of *S. obtusifolia* and *S. occidentalis* were 162,426 and 159,993 bp, respectively. Four hypervariable coding-regions *ycf1*, *rpl23*, *petL*, and *matK* were identified. Two specific mini-barcodes were successfully developed from *matK*, and the mini-barcode of primer 647F-847R was proved to be able to qualitatively and quantitatively identify these two processed *Senna* seeds. Overall, our study established a valuable way to develop the specific mini-barcode, which may provide a new idea for the quality control of processed herbal products.

Keywords: mini-barcode, plastome, metabarcoding, species identification, Herbal products, *Senna*

INTRODUCTION

Herbal products use medicinal plants as raw materials for herbal medicines, herbal extracts and dietary supplements. In recent years, there was a tremendous increase in the global demand of herbal products, making herbal products adulteration and counterfeiting a global problem (Mackey and Liang, 2013; Gromek et al., 2015; Gaudiano et al., 2016). Hence, the authentication of herbal products has become an important topic within and beyond the pharmaceutical and healthcare industries. DNA barcoding is a technique for authenticating species using a standard DNA region, aiming to provide rapid, automatable, and cost-effective methods for accurate identification at the species-level. Studies on DNA barcoding have made remarkable progress in species identification (Naeem et al., 2014; Zhang et al., 2016; Viglietti et al., 2019). Initially used as an identification tool, DNA barcoding is now applied in the industrial quality assurance context to identify herbal products (Mosa et al., 2018; Amritha et al., 2020). However, DNA barcoding faces practical limitations that exclusively restrict it to identifying single ingredient herbal products. Once the plants undergo a series of extraction and processing steps resulting in DNA mixing, DNA barcoding would not be a suitable choice. Unfortunately, herbal products generally suffer from varying degrees of DNA mixing during processing or manufacturing. For a suitable solution to this case, DNA metabarcoding appeared to be an alternative choice.

DNA metabarcoding, combining the next-generation sequencing and DNA barcoding, enables simultaneous multi-taxa identification by using the total DNA extracted from complex samples containing DNA from different origins (Taberlet et al., 2012; Staats et al., 2016). It has generally been assumed that the proportion of reads obtained for a given species is proportional to the contribution of species biomass (Amend et al., 2010; Egge et al., 2013). Hence, species reads obtained from DNA metabarcoding can reflect the richness of species in the community to a certain extent, which makes DNA metabarcoding a widely used tool for biodiversity studies (Egge et al., 2013; Klindworth et al., 2013; Duke and Burton, 2020). Nowadays, DNA metabarcoding has also proved to be applicable for authentication of species diversity in herbal products, and has been used to investigate the level of discrepancy between the expected and detected plant species in the herbal product market (Cheng et al., 2014; Coghlan et al., 2015; Ivanova et al., 2016). However, although DNA metabarcoding is superior to DNA barcoding in the assessment of complex herbal mixtures, it still faces some limitations similar to DNA barcoding. For instance, the selection of barcodes has always been a challenge. Barcodes that are too short may not provide sufficient resolution for identification of multiple-taxa, e.g., P6 loop of *trnL* intron (Taberlet et al., 2007). In contrast, conventional barcodes like *matK*, *rbcL*, *trnH-psbA*, and *ITS2* are generally longer than 500 bp, which is very unfavorable for PCR amplification when facing DNA degraded samples including the processed herbal products (Gao et al., 2019). In this case, a short but informative DNA barcode could be a solution, and that is called a mini-barcode.

The DNA mini-barcode is a short DNA fragment, 100–250 bp in length, with sufficient variable sites for species identification (Little, 2014b). Due to the significantly reduced length of barcode regions, PCR amplification success could be much improved, but the barcode resolution would thus be limited. So, it is necessary to design a specific mini-barcode for accurate species identification of close-related species. For angiosperms, it is now realistic to find a specific DNA mini-barcode by searching the whole plastome owing to the ease of next-generation sequencing (Dong et al., 2013). The plastomes of most land plants exhibit a typical quadripartite structure with stable gene content and gene order (Li et al., 2017). Owing to their characteristics of maternally inherited, multi-copy, and moderate evolutionary rate, the plastome sequences are widely used for molecular marker development (Park et al., 2018; Yu et al., 2019; Li et al., 2020). Moreover, the abundant interspecific sequence diversity makes the plastome sequence a particularly useful tool for providing high-resolution barcodes for close-related species (Jiao et al., 2019; Xia et al., 2019). The utility of mini-barcodes has been successfully demonstrated in a number of specific taxa, such as *Panax* (Dong et al., 2014), *Hypericum* (Costa et al., 2016), and *Phyllanthus* (Srirama et al., 2014).

The seed of Senna obtusifolia, called Juemingzi in China, is regarded as a dual-use material for food and medicine by China Food and Drug Administration. Studies on Juemingzi showed that it has various pharmaceutical properties such as hypertension regulation (Li and Guo, 2002), hepatoprotective effect (Kim et al., 2009) and eyesight improvement (Yang et al., 2012), which have made it a very popular herbal product in China and some other Asian countries. Since the wide applications in pharmaceutical and healthcare industries, the demand for Juemingzi increased rapidly, and the proportion of its adulterant increased at the same time. Among these adulterants, the most common and indistinguishable one is the seed of *S. occidentalis*. However, just like the conditions of other herbal products generally suffering from varying degrees of DNA degradation during processing, the conventional DNA barcodes for Juemingzi was difficult to amplify as well. Moreover, mixing seeds could make DNA barcoding function poorly. Therefore, it is necessary to design the specific mini-barcode and combine with the metabarcoding technique for quality control of Juemingzi.

The aims of our study included: 1) to provide a strategy of developing the specific mini-barcode using *Senna* as an example; 2) to test the feasibility of combining mini-barcodes and DNA metabarcoding techniques for qualitative and quantitative identification of processed herbal products. In this study, the plastomes of *S. obtusifolia* and *S. occidentalis* were first assembled, then the hypervariable coding-regions were further sought out by comparing their genomes. Subsequently, the length and position of suitable mini-barcodes were determined, and finally, we applied DNA metabarcoding techniques to the developed mini-barcodes.

MATERIALS AND METHODS

Plant Material

The fresh plant of *S. obtusifolia* was collected from the Medicinal Botanical Garden of Tianjin University of Traditional Chinese Medicine, Tianjin City (117.06°E,

38.96°N), China. The ungerminated seed of *S. occidentalis* was collected from Baoding City (115.33°E, 38.42°N), China. They were identified by Prof. Tianxiang Li from School of Chinese Materia Medica, Tianjin University of Traditional Chinese Medicine. The voucher species were deposited in Tianjin State Key Laboratory of Modern Chinese Medicine, Tianjin University of Traditional Chinese Medicine, and the voucher numbers were JMZ201806 (*S. obtusifolia*) and WJN201808 (*S. occidentalis*). The Extract Plant DNA kit (Sangon Biotech Co., Ltd., Shanghai, China) was used to extract the total genomic DNA from the fresh leaves of *S. obtusifolia* and the whole seed of *S. occidentalis*. In addition, four batches of processed seeds of *S. obtusifolia* and *S. occidentalis* were purchased from Anguo medicine market to construct experimental mixtures. In particular, the seeds were identified by Prof. Tianxiang Li to avoid the possibility of adulteration. DNA purity was checked using NanoPhotometer®spectrophotometer (IMPLEN, CA, United States). Concentrated DNA was measured using Qubit® DNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, United States). Sequencing library was generated using Truseq Nano DNA HT Sample preparation Kit (Illumina United States) following the manufacturer's recommendations. The library was sequenced by Illumina HiSeq X Ten platform (Novogene, Nanjing, China) and 150 bp paired-end reads were generated.

Plastome *de Novo* Assembly and Annotation

Raw data of fastq format of DNA was processed by removing adapter sequences, removing reads with the ratio of N (N indicates that base information cannot be determined) greater than 10%, and removing low-quality reads in which >50% of the bases had a quality value Qphred<=5. Subsequent analyses were based on the filtered high-quality sequences. The plastomes of *S. obtusifolia* and *S. occidentalis* were assembled via combination of *de novo* and reference-guided assembly approaches following the procedure described by Niu *et al* (Niu et al., 2017). The plastome of *S. tora* (NCBI accession number NC_030193) was used as a reference. Then, the finished plastomes of *S. obtusifolia* and *S. occidentalis* were annotated using GeSeq (Tillich et al., 2017), coupled with manual corrections for start and stop codons. Finally, the plastomes of *S. obtusifolia* and *S. occidentalis* were visualized using OGDRAW (Lohse et al., 2013).

Identification of Hypervariable Coding-Regions and Design of Primers

To find the hypervariable coding-regions, the protein-coding genes of two plastomes were respectively extracted and aligned using PhyloSuite_v1.1.15 (Zhang et al., 2020). Then, the nucleotide variability (Pi) values of protein-coding genes were calculated using DnaSP version 6.11.01 software (Rozas et al., 2017). The regions with high Pi values were selected as the candidate regions for mini-barcode development. The Primer Premier V6.0 was used to design primers for the mini-barcode, and the parameters were as follows: product size between 150 and 300 bp, primer size between 18 and 30 bp, melting temperature (Tm) between 40° and 70°C, GC content between 30 and 70%. Then, the physicochemical properties of the designed primers such as hairpin structure, primer dimer, and annealing temperature were evaluated using Oligo seven software, and primers that were likely to have hairpin structures, primer dimers, or excessive annealing temperature were abandoned.

Experimental Mixtures Construction, Next-Generation Sequencing, and Analysis of Amplicon Sequence Variants (ASVs)

To test the qualitative and quantitative capacity of developed mini-barcodes in the processed mixture of *S. obtusifolia* seeds and *S. occidentalis* seeds, we prepared four experimental mixtures (for the biomass of each species in the four experimental mixtures, please see **Supplementary Table S1**). Each experimental mixture contained processed seeds (seeds were fully crushed by a powder mill to facilitate sample mixing) of *S. obtusifolia* and *S. occidentalis*. Then, the genomic DNA was extracted from each experimental mixture using the Extract Plant DNA kit (Sangon Biotech Co., Ltd., Shanghai, China), respectively. The target regions were amplified using two pairs of fusion primers with matching tag sequences (for detailed tag sequences, please see **Supplementary Table S2**) to ensure that tag jumps would not result in false assignments of sequences to samples (Schnell et al., 2015). Then, PCR reaction was conducted in a 25 μL reaction with 12.5 μL of 2 × Gflex PCR Buffer (containing 1 mM Mg^{2+} and 200 μM dNTP), 0.5 μL of each primer, 0.5 μL Tks Gflex™ (Takara Biomedical Technology Co., Ltd., Beijing, China) DNA Polymerase (1.25 units/μl), 2 μL template DNA, and approximately 9 μL ddH_2O. The PCR protocol was as follows: preheating at 94°C for 1 min, 30 cycles at 98°C for 10 s, annealing at 55°C for 15 s and elongation at 68°C for 30 s, and final extension at 68°C for 5 min. The negative controls were included in every run. Then, the PCR products were detected on 2% agarose gels. Subsequently, equimolar concentrations of PCR products were pooled to construct the library. Finally, the library was sequenced with 2 × 150 bp reads on the Illumina Hiseq X Ten platform.

The fastq-multx (Aronesty, 2013) was used to split the generated data according to the tag sequences. Then, the primer sequences were trimmed using Cutadapt (Kechin et al., 2017). To construct ASVs (sequences with 100% similarity will be assigned into each ASV), denoize and quality control were performed using DADA2 (Callahan et al., 2016). Then, BlastN was used to search the sequences of ASVs from the chloroplast genomes of *S. obtusifolia* and *S. occidentalis*. Finally, the relationship between species reads and species biomass was analyzed using R package ampvis2 (Andersen et al., 2018).

RESULTS

Plastome Features

The plastomes of *S. obtusifolia* and *S. occidentalis* were 162,426 and 159,993 bp in length, respectively (**Figure 1**). Either of the two plastomes displayed a typical quadripartite structure consisting of a pair of IR regions (26,791 bp in *S. obtusifolia*, 26,101 bp in *S.*

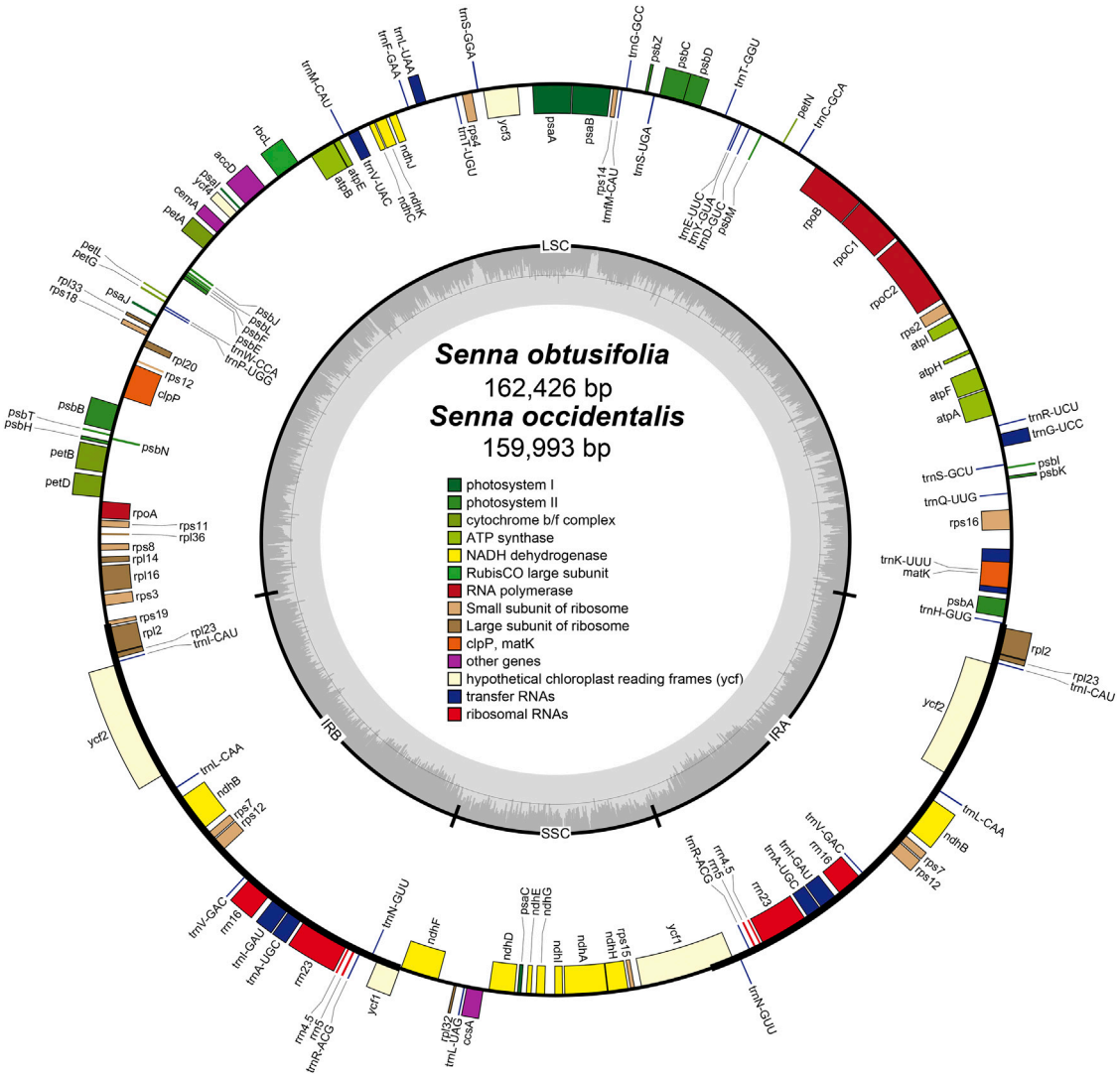

FIGURE 1 | Plastome map of *S. obtusifolia* and *S. occidentalis*. Genes inside the circle are transcribed clockwise, whereas those on the outside are transcribed counter-clockwize. Genes belonging to different functional groups are color-coded. The darker gray in the inner circle represents the GC content, while the lighter gray represents the AT content.

occidentalis) separated by the LSC (90,843 bp in *S. obtusifolia*, 89,322 bp in *S. occidentalis*) and SSC (26,791 bp in *S. obtusifolia*, 26,101 bp in *S. occidentalis*) regions. Both plastomes exhibited a low GC content (36.0% and 36.2% in *S. obtusifolia* and *S. occidentalis*, respectively). The two *Senna* plastomes encoded an identical set of 129 predicted functional genes, 111 of which were unique, and 18 were duplicated in the IR regions. Overall, the genome structure, gene type, gene content, and GC content of the two *Senna* plastomes were highly similar.

Selection of Hypervariable Coding-Regions and Design of Primers

To find the hypervariable coding-regions for mini-barcode development, the Pi values of the shared 77 protein-genes of two plastomes were calculated using DnaSP. As shown in **Figure 2A**, *ycf1* had the highest Pi values (0.04363), followed by *rpl23* (0.04301), *petL* (0.03226), and *matK* (0.03206). These observed hypervariable genes were regarded as the candidate regions for mini-barcode development. Then, the primers for the mini-barcode were designed based on these selected hypervariable regions. As depicted in **Table 1**, two primer pairs were successfully designed from *matK*. However, limited by the length and variability of the barcode regions, as well as the physicochemical characteristics and conservation of the primers, no suitable primers were designed from *rpl23*, *petL* and *ycf1*. The amplicon sizes for primer 647F-847R and 478F-629R were 200 and 151 bp, respectively (**Figures 2B,C**). Both mini-barcode of primer 647F-847R and 478F-629R had seven variable sites, suggesting a great capacity for identifying the two different *Senna* species.

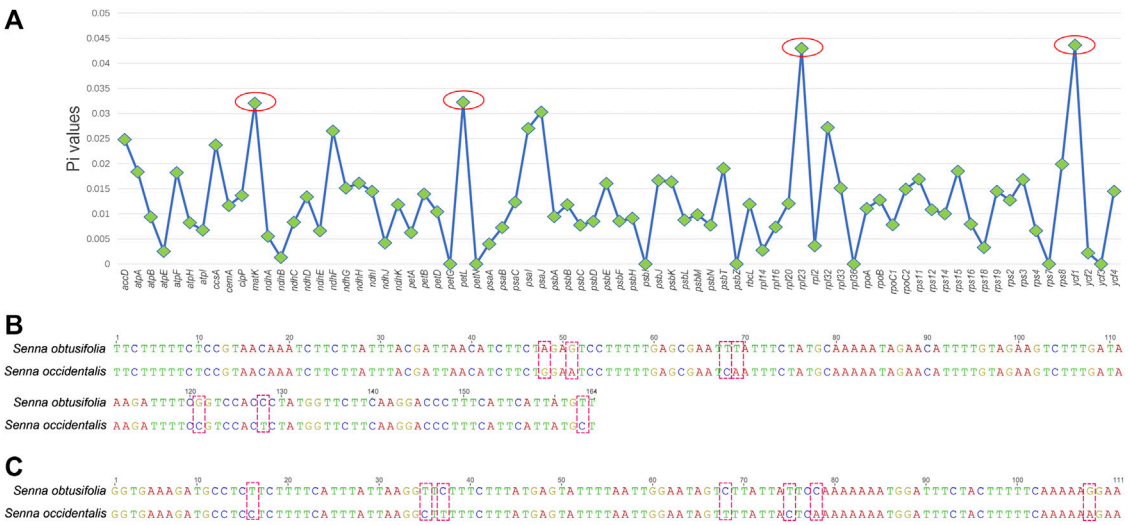

FIGURE 2 | Selection of hypervariable coding-regions and examination of variable sites in two mini-barcodes **(A)** The Pi values of the shared 77 protein-genes of two plastomes **(B)** The mini-barcode of primer 647F-847R, red dotted frames present variable sites **(C)** The mini-barcode of primer 478F-629R, red dotted frames present variable sites.

TABLE 1 | Primers developed from *matK*.

Primer name	647F-847R	478F-629R
Forward primer sequence 5′ to 3	GTGAATACGAATCTATCT	GTTCAAACCCTTCGATACTG
Reverse primer sequence 5′ to 3	GGATTTTCCTTGATATCT	GGAACAGGAAAAATCTTGGA
Amplicon size (bp)	200	151
Variable sites of mini-barcode	7	7
Sequence excluding primers (bp)	164	111
GC% (For/Rev)	37.5/31.2	33.3/33.3
Tm (For/Rev)	45.2/42.4	45.7/45.5

TABLE 2 | BLAST results and sequencing reads of ASV.

Primer name	Sequencing reads of ASV in four experimental mixtures					Blast result	Identity (%)
	ASV	JM1	JM2	JM3	JM4		
647F-847R	ASV1	56,065	564,176	11,901	400,029	*C. occidentalis*	100
	ASV2	57,978	31,078	321,102	348,064	*C. obtusifolia*	100
478F-629R	ASV3	108,018	288,609	6,666	65,827	*C. occidentalis*	100
	ASV4	27,160	2,937	79,349	3,718	*C. obtusifolia*	100

Qualitative and Quantitative Identification of Two *Senna* Species in Experimental Mixtures by DNA Metabarcoding Technique

Two designed primer pairs were used to amplify the barcode regions in four experimental mixtures. Illumina sequencing results of the amplified products showed that primer 647F-847R generated 1,790,393 reads, which were subsequently clustered into two ASVs (**Table 2**). These two ASVs were identified as *S. obtusifolia* (ASV1) and *S. occidentalis* (ASV2), with 100% similarity against the corresponding barcode regions. Similarly, for primer 478F-629R, a total of 582,284 reads were generated and clustered into two ASVs, which were identified as two *Senna* species (100% similarity) (**Table 2**). These two primer pairs successfully amplified the target barcode regions in four experimental mixtures, indicating a qualitative capacity of two mini-barcodes in identifying these two *Senna* species.

To validate the quantitative capacity of the mini-barcodes, the relationship between species reads proportion and species biomass proportion was evaluated in four experimental mixtures (**Figure 3A**). For primer 478F-629R, there was a significant difference between species reads proportion and species biomass proportion in JM1 and JM4, suggesting a poor quantitative capacity of this mini-barcode in identifying two *Senna* species. For primer 647F-847R, the species reads proportion were almost identical to species biomass proportion in four experimental mixtures. Further correlation analysis (**Figure 3B**) showed a significant correlation of species reads proportion and species biomass proportion in four experimental mixtures ($R^2 = 0.9975$ in two *Senna* species), which indicated that the mini-barcode of primer 647F-847R demonstrated a relatively accurate quantitative ability in identifying two *Senna* species.

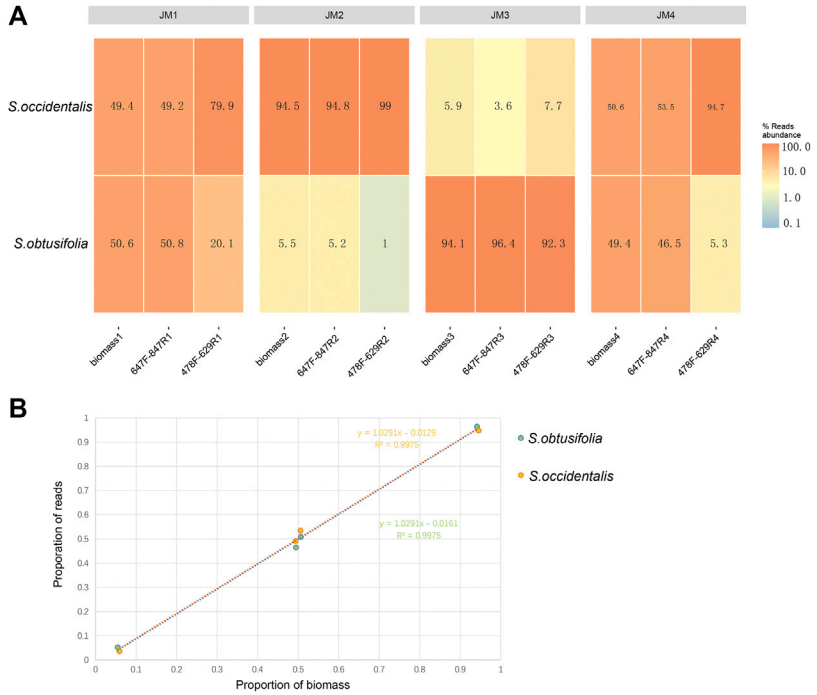

FIGURE 3 | Analysis of species reads abundance and species biomass abundance **(A)** Heat map of reads abundance of two primer pairs in four experimental mixtures **(B)** For primer 657F-858R, the correlation analysis of species reads proportion and species biomass proportion in four experimental mixtures. *X*-axis, proportion of biomass; *Y*-axis, proportion of reads.

DISCUSSION

Practically, the length of a barcode has always been an issue of concern. A barcode can be generally classified into four types according to its length, for example, micro-barcode within 100 bp (Taberlet et al., 2007), mini-barcode of 100–250 bp (Little, 2014b), standard barcode of 400–800 bp (Hebert et al., 2003), and the whole genome as a super-barcode (Chen et al., 2018). However, micro-barcodes can provide limited resolution for species identification. Although the standard barcode and super-barcode can provide strong resolution, they are difficult to be obtained from the degraded samples due to length limitation. Compared with the three types mentioned above, mini-barcode is the best choice for identifying processed and confused herbal products due to its sufficient variable sites and short in length. However, mini-barcode of high resolution is not easily found, and that is why the whole genome is indispensable for the development of mini-barcodes.

An ideal mini-barcode should meet two requirements: one is a barcode region with sufficient variable sites and short in length, and the other is a primer pair with conservation and meeting physicochemical properties. It is necessary to incorporate the physicochemical properties of the primer into consideration because the hairpin structure, primer dimer, and annealing temperature are all important factors that affect the success of primer amplification (Xie et al., 2019). In addition, insertion and deletion of nucleotides, as well as excessive length variations, are frequently observed in the intergenic region of the chloroplast genome (Bortiri et al., 2008; Liu et al., 2019), which is not conducive to primer conservation. Moreover, the intra-specific variation in the intergenic region is much larger than that in the coding-region (Jiang et al., 2017). Any intra-specific variation may affect the quantitative capacity of the mini-barcode when there are multiple individuals of target species in the mixture. Therefore, the intergenic region was excluded when screening potential DNA barcode regions based on the chloroplast genome.

*mat*K is a recommended gene as the candidate DNA barcode because of its high evolution rate (Lahaye et al., 2008). At the family and genus levels, *matK* provides the high support value of phylogenetic tree and the high-resolution of species discrimination in a given taxon (Ji et al., 2007; Sun et al., 2012). In some groups like *Juglans* (Stanford et al., 2000) and *Fagopyrum* (Ohsako and Ohnishi, 2000), *matK* has also played a useful role in studying intra- and interspecific phylogeny. However, a standard barcode length of *matK* is approximately 800 bp, which is not suitable for identifying severely degraded samples. So, there have been studies focused on the development of mini-barcodes from *matK* and have achieved good results in some specific taxa. For example, Costa et al. (2016) suggested that *matK* was an adequate mini-barcode region for the differentiation of two *Hypericum* species in herbal infusions. Little (2014a) developed a 166 bp mini-barcode from *matK* to authenticate *Ginkgo biloba* herbal dietary supplements. Our study developed two specific mini-barcodes and successfully applied them to identify two *Senna* species, which once again verified the feasibility of developing a specific mini-barcode from *matK*.

We recommend that *matK* can be regarded as a preferred option for specific mini-barcode development in subsequent studies.

Primer bias is a well-known factor that substantially influences results in metabarcoding studies. For instance, primer bias has been shown to skew the relative abundance of amplified DNA from experimental mixtures such as stonefly species and some terrestrial arthropods (Elbrecht and Leese, 2015; Piñol et al., 2015). Although our results showed that both primer 647F-847R and 478F-629R provided a positive relationship between species reads abundance and species biomass abundance in the experimental mixtures, which was consistent with other metabarcoding studies (Thomsen et al., 2012; Evans et al., 2016). However, the primer 478F-629R exhibited the large difference between observed reads proportion and given biomass proportion in JM1 and JM4 (i.e., biomass1 vs. 478F-629R1 and biomass4 vs. 478F-629R4). This large variability is most likely resulted from primer bias, and primer binding efficiency and inherent species-specific differences could lead to primer bias in metabarcoding studies (Duke and Burton, 2020). Although the quantitative ability of metabarcoding has always been tested due to primer bias (Elbrecht and Leese, 2015; Bista et al., 2018), our results showed that metabarcoding could provide a relatively accurate quantitative relationship between species read abundance and species abundance when there was no primer bias inference (e.g., primer 647F-847R).

The currently available quality control assessment methods for herbal products are mainly chemical methods, such as thin-layer chromatography, high-performance liquid chromatography, and mass spectrometry (Turova et al., 2018; Mukherjee, 2019). However, the chemical properties of herbal products can be affected by many factors, such as processing methods, storage conditions, and geographic location (Parveen et al., 2016), posing difficulties for proper chemical analyses and objective judgment (de Boer et al., 2015). Moreover, chemical methods may not be able to distinguish closely related species since they generally share chemical compounds (Raclariu et al., 2017), making it challenging to find representative chemical markers for authentication. Compared with chemical methods, DNA mini-barcoding can provide stable quality control assessment for herbal products due to the stability of DNA, and its short barcode region can overcome the difficulties caused by DNA degradation during processing and manufacturing of herbal products. Besides, our study has proved that specific mini-barcodes combined with the metabarcoding technique can realize the qualitative and quantitative identification of closely related species (i.e., *S. obtusifolia* and *S. occidentalis*). From an economic perspective, in our experiment, genomic DNA extraction takes about 1 h, PCR amplification takes about 2 h, and sequencing relies on the Illumina HiSeq X Ten platform, which takes about three days. The cost of each sample is about $100, of which the main cost is library construction and data generation (about $80). However, in the past 20 years, the cost per megabase of DNA sequencing has dropped from about $10,000,000 to about $0.01 (National Human Genome Research Institute, 2020), and it will continue to decline in the future. Moreover, with the introduction of portable sequencers, such as MinION and DNBSEQ E series, the sequencing time has been greatly shortened, and even real-time sequencing can be achieved. Thus, it is conceivable that this molecular technique will become an efficient and economical method for the quality control of herbal products driven by technological development in the near future.

It has always been a concern of the herbal products market whether there is adulteration and how much adulteration since it is not uncommon that the adulterants of expensive or shortage materials are not only similar but also cheaper or easily available. A specific DNA mini-barcode combined with the metabarcoding technique can realize the qualitative and quantitative identification of samples with DNA degradation, which is suitable for processed herbal products like *S. obtusifolia* seeds since they are frequently adulterated with *S. occidentalis* seeds and generally suffer from varying degrees of DNA degradation during harvesting, storage and processing. Nevertheless, not all herbal products require the mini barcodes for quality control assessment. Whether a mini barcode or a regular barcode is needed depends largely on the degree of DNA degradation. Besides, whether specific or universal barcodes are required is also a question to be considered since the quantitative ability of barcodes is greatly interfered by primer bias. Therefore, we recommend that in follow-up studies, different types of herbal products need to be evaluated for the degree of DNA degradation and the suitability of primers to find an optimal quality control strategy.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MK817504, https://www.ncbi.nlm.nih.gov/genbank/, MK817505.

AUTHOR CONTRIBUTIONS

XT and LM designed the study; XY and WT assembled, annotated and analyzed the plastomes; HG and XY performed the experiment; XY drafted the manuscript; XT and LM revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.585687/full#supplementary-material.

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MINI REVIEW
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A Review of Authenticity and Authentication of Commercial Ginseng Herbal Medicines and Food Supplements

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Ginseng traditional medicines and food supplements are the globally top selling herbal products. *Panax ginseng*, *Panax quinquefolius* and *Panax notoginseng* are the main commercial ginseng species in herbal medicine. Prices of ginseng products vary widely based on the species, quality, and purity of the used ginseng, and this provides a strong driver for intentional adulteration. Our systematic literature search has reviewed the authenticity results of 507 ginseng-containing commercial herbal products sold in 12 countries scattered across six continents. The analysis of the botanical and chemical identity of all these products shows that 76% are authentic while 24% were reported as adulterated. The number of commercial products as well as the percentage of adulteration varies significantly between continents, being highest in South America (100%) and Australia (75%), and lower in Europe (35%), North America (23%), Asia (21%) and Africa (0%). At a national level, from the five countries for which more than 10 products have been successfully authenticated, the highest percentage of adulterated ginseng products were purchased from Taiwan (49%), followed by Italy (37%), China (21%), and USA (12%), while all products bought in South Korea were reported to be authentic. In most cases, labeled *Panax* species were substituted with other *Panax* species, but substitution of ginseng root, the medicinally recommended plant part, with leaves, stems or flowers was also reported. Efficient and practical authentication using biomarkers to distinguish the main ginseng varieties and secondary metabolite spectra for age determination are essential to combat adulteration in the global marketplace.

Keywords: ginseng, Panax, identification, adulteration, contamination, herbal product, herbal medicine, food supplement

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INTRODUCTION

Ginseng is used collectively to refer to several plant species, mainly in the Araliaceae genera *Panax* L. and *Eleutherococcus* Maxim. In China, the ginseng preparations have been used for thousands of years in traditional medicine (Robbins, 1998). Demand for ginseng roots in the 18th century intensified the wild harvest of the main species *Panax ginseng* C. A. Mey. (*Araliaceae*) (Korean ginseng), and nearly extirpated it from the wild (Millspaugh, 1974), but also fuelled a rapid expansion in wild-harvesting of *Panax quinquefolius* L. (*Araliaceae*) (American ginseng) that in turn destroyed

wild populations in North America (Kimmens, 1975). There are currently 13 recognized ginseng species, but new taxa at species, subspecies and variety level are continuously published (Manzanilla et al., 2018). The most commonly used species in the genus are *P. ginseng*, *P. quinquefolius*, *Panax notoginseng* (Burkill) F. H. Chen (*Araliaceae*) (Chinese ginseng), *Panax japonicus* (T. Nees) C. A. Mey. (*Araliaceae*) (Japanese ginseng), *Panax pseudoginseng* Wall. (*Araliaceae*) (Himalayan ginseng) and *Panax vietnamensis* Ha and Grushv. (*Araliaceae*) (Vietnamese ginseng) (Yang and Wu, 2016). The majority of commercialized ginseng material is from cultivation and controlled sustainable wild harvest, whereas material from uncontrolled depletive wild harvesting plays a minor and decreasing role. *P. ginseng, P. quinquefolius* and *P. notoginseng* are the three most widely used species in herbal medicine, and are traded as either red ginseng (Ginseng Radix et Rhizoma Rubra), white ginseng (Ginseng Radix et Rhizoma), American ginseng (Panacis Quinquefolii Radix), or notoginseng (Notoginseng Radix et Rhizoma). These ginseng varieties are sold in different stages of processing from raw materials through highly processed products that have lost their botanical morphological characteristics to extracts differentiated mainly by ginsenoside content (Yang and Wu, 2016). In the global market, many different formulations of these herbs are available, including gelcaps, capsules, teas, tinctures, slices to eat in salads, powders, as well as entire roots. There are a wide variety of products that contain ginseng such as toothpaste, cigarettes, soaps, cosmetics, beverages (including beer), coffee, baby food, candies, and gum (Morgan and Cupp, 2010).

In the global medicinal plant trade, the economic value of ginseng is estimated to be more than US$ 2.1 billion (Manzanilla et al., 2018). Prices vary widely based on the quality and the quantity of the ginseng root. The ginseng cultivar, locality, growth condition (cultivated or harvested from the wild), and plant age are some of the most important factors which influence the quality and properties of *P. ginseng* (Kim et al., 2012). It was shown previously that *P. ginseng* possesses different active ingredients and differentiated curative effects depending on the age of the plants, that is why the older ginseng plants are sold at a much higher price than the younger ones (Kim et al., 2011; Yang et al., 2012; Pan et al., 2013). Tenfold and more price differences among the main *Panax* processed varieties is the main driver for intentional adulteration (Choi et al., 2007). Adulteration using low-price varieties, low grade material from above-ground parts or processing waste, alternative species, or nothing at all, could bring huge profits through deceptive or illegal trade (Huang et al., 2017). The authentic herbal medicines and food supplements should be non-adulterated (Simmler et al., 2018) while the inadvertent or intentional adulteration (Simmler et al., 2018) includes contamination, product substitution and the use of fillers (Shanmughanandhan et al., 2016).

Efficient discrimination methods for ginseng varieties is necessary to combat fraud. Product adulteration and substitution are severe and widespread problems in the ginseng market, given the significant difference in medicinal value and economic benefits of different ginseng preparations (Zhao et al., 2020). The adulteration interferes with the proper and correct use of ginseng products and compromises the credibility of the whole supply chain. The development of efficient and practical authentication using biomarkers to distinguish the main ginseng varieties and secondary metabolite spectra for age determination are essential to combat adulteration. This review aims to provide an overview of the state of the art in ginseng authentication, while also highlighting current strengths and limitations.

METHODS

Search Strategy

Four databases (Web of Science, PubMed, Scopus, and ScienceDirect) were systematically searched for peer reviewed articles using keywords combined with Boolean operators: [(ginseng OR Panax) AND (identification OR authentication OR authenticity OR authentic OR adulteration OR contamination OR substitution)] following the PRISMA guidelines (Moher et al., 2009). After the search was performed (on 17 February 2020), weekly updates were received and taken into consideration as the option "search alert" has been activated for all four literature databases.

Selection Process and Criteria

A total of 3,683 records were identified, with WoS = 1,277, PubMed = 692, Scopus = 1,480, ScienceDirect = 234 respectively, as well as another 49 records from other sources. Out of these a total 1,023 records were retained after removing duplicates. After screening, 869 records were excluded. The highly diverse reporting formats of the authenticity results made it necessary to define unifying criteria for selection and retain only the relevant articles in our review. The remaining 154 full-text articles were assessed for eligibility using the assessment criteria: 1) The reported samples had to be "herbal products" in the broad sense. The widest possible range of commercial names was used for the searches and accepted for inclusion in the review; 2) The analyzed products had to be "commercial." The "cost-free," "gifts" or the "donated" products were excluded from our analysis; 3) The products had to be allocated specifically to a "country" or "territory" (i.e., Hong Kong, European Union); 4) The "authentic" or "adulterated" conclusion was drawn by the authors of the reviewed articles; 5) All authentication methods were accepted. After applying these selection criteria, 120 articles were excluded because they did not report authentication results of commercial herbal products. This objective literature search identified 34 peer-reviewed publications reporting authentication of a total of 507 ginseng-containing commercial herbal products (**Table 1**). Authentication of botanical identity was reported for 468 products in 29 studies, and 13 of them used only DNA-based methods, mostly DNA barcoding. In another 13 studies only chemical methods were used, while the remainder used a combination of genetic and chemical authentication methods. Fifty-seven percent of studies (17 out of 30) reported authentication results for ginseng products purchased from China. The integrity of the chemical composition of 39 ginseng products was reported in five additional peer-reviewed studies.

TABLE 1 | The authenticity of ginseng commercial herbal products sold on the global market.

Country/ territory	Labeled and authenticated Panax sp./type of herbal product	Identified adulterant	Product composition	Products			Authentication methods	Ref
				Total	Authentic	Adulterated		
				no	no	no		
		Botanical identity						
Brazil	*P. ginseng*	*Pfaffia* spp.	Root products (0.2% ginsenoside Rg1 and 0.1% ginsenoside Rb1, HPLC)	5	0	5	DNA barcoding	(Palhares et al., 2015)
China	*P. ginseng, P. quinquefolius, P. notoginseng*	n/a	Batches of TCM compound preparations (e.g. pill, bag, injection, capsule, tablet, powder, dripping pill)	40	38	2	LC–MS	(Yang et al., 2016)
China	*P. ginseng*	*P. quinquefolius*	Batches of CPMs containing ginseng products	24	19	5	DNA barcoding	(Liu et al., 2016)
China	*P. ginseng*	*P. quinquefolius*	Ginseng Radix et Rhizoma samples	15	12	3	DNA barcoding	(Han et al., 2016)
China	*P. notoginseng*	n/a	Baches of CPM Shuxiong tablets prepared from Notoginseng Radix et Rhizoma), Carthami Flos, and Chuanxiong Rhizoma	12	12	0	UPLC/QTOF-Fast DDA	(Yao et al., 2016)
China	*P. ginseng, P. quinquefolius*	n/a	CPMs containing ginseng or American ginseng	11	8	3	MAS-PCR	(Cheng et al., 2015)
China	*P. ginseng, P. quinquefolius, P. notoginseng*	n/a	TCHMs multi-ingredient ginseng preparations, Sheng Mai Yin (SMY) capsule and granules	11	6	5	HPTLC	(Xie et al., 2006)
China	*P. notoginseng*	flower of *P. notoginseng*	TCM preparations with P. notoginseng powder (dry roots and rootstock)	10	9	1	UPLC/Qtof MS	(Liu et al., 2015)
China	*P. ginseng*	n/a	Ginseng containing samples	10	8	2	PCR	(Zhou et al., 2016)
China	*P. ginseng*	*P. quinquefolius, Platycodon grandiflorum* (Jacq.) A.DC. (*Campanulaceae*), *Physochlaina infundibularis*Kuang (*Solanaceae*), *Phytolacca acinosa* Roxb. (*Phytolaccaceae*)	Root samples	8	5	3	NIR barcode	(Dong et al., 2020)
China	*P. ginseng*	n/a	Ginseng Radix et Rhizoma (dried roots and rhizomes of *P. ginseng*)	3	3	0	DNA barcoding	(Zhang et al., 2019)
China	*P. ginseng, P. notoginseng*	n/a	steamed roots of *P. ginseng*; powdered roots of *P. notoginseng*	2	2	0	DNA barcoding	(Dong et al., 2014)
China	*P. ginseng*	n/a	Shihu Yeguang Pills containing Ginseng Radix Et Rhizoma	1	1	0	DNA barcoding	(Jiang et al., 2019)

(Continued)

TABLE 1 | (*Continued*) The authenticity of ginseng commercial herbal products sold on the global market.

Country/ territory	Labeled and authenticated Panax sp./type of herbal product	Identified adulterant	Product composition	Products			Authentication methods	Ref
				Total	Authentic	Adulterated		
				no	no	no		
China	*P. ginseng*	n/a	Batches of Asian ginseng	11	11	0	UPLC/Q-TOF-MS	(Li et al., 2010)
China	*P. quinquefolius*	n/a	Batches of American ginseng	7	7	0		
Canada	*P. quinquefolius*	n/a	Batches of American ginseng	5	5	0		
United States	*P. quinquefolius*	n/a	Batches of American ginseng	4	4	0		
China	*P. quinquefolius*	*P. ginseng*	Batches of American ginseng preparations	13	0	13		
China	*P. ginseng*	leaf/stem	Root extract	1	0	1	HPTLC, HPLC	(Govindaraghavan, 2017)
European Union	*P. ginseng*	*P. ginseng* leaf or other plant parts, *P. quinquefolius* roots	Root extracts, stem/ leaf extract, berry extract	12	6	6		
Australia	*P. ginseng*	*P. ginseng* leaf or other plant parts	Capsules (extract or herb), tablet	4	1	3		
South Korea	*P. ginseng*	n/a	*P. ginseng* extract, tea, capsule	3	3	0	Multiplex PCR	(Tian et al., 2020)
China	*P. ginseng, P. quinquefolius*	n/a	*P. ginseng* extract, tea, capsule; *P. quinquefolius* extract, tea, capsule	6	6	0		
United States	*P. ginseng, P. quinquefolius*	n/a	*P. quinquefolius* extract, tea, capsule	3	3	0		
Hong Kong	*P. ginseng*	n/a	Ready-to-serve ginseng soup	1	1	0	Multiplex PCR, DNA sequencing	(Lo et al., 2015)
Hong Kong	*P. ginseng, P. quinquefolius*	n/a	Dried roots, powders, tea granules	7	7	0	PCR, HAD	(Jiang et al., 2014)
Italy	*P. ginseng, P. quinquefolius*	n/a	Raw materials (as body root, root tails and root prongs), capsules and tablets (containing dried extract)	19	12	7	RFLP, HPLC	(Del Serrone et al., 2006)
South Korea	*P. ginseng*	n/a	decoctions, beverages, capsules, tablets	61	61	0	HPLC, UPLC–DAD–ESI-IT-TOF-MS	(Choi et al., 2018)
South Korea	*P. ginseng*	n/a	Bak-Ho-Ga-Insam-Tang resources containing *P. ginseng*	14	14	0	Gradient PCR	(Shim et al., 2005)
Taiwan	*P. ginseng*	not composed of 6 years old ginseng radix only	White ginseng radix sliced material, powder, capsules	7	6	1	1H-NMR	(Lin et al., 2010)
China	*P. ginseng*	n/a	white ginseng radix sliced material	1	1	0		
Taiwan	*P. ginseng*	Panacis quinquefolii Radix	Chinese medical preparations containing Ginseng Radix	58	27	31	nested PCR, RFLP, DNA sequencing	(Lu et al., 2010)
United Kingdom	*P. ginseng, P. quinquefolius, P. notoginseng*	n/a	American ginseng, white Asian ginseng, sanchi ginseng	8	8	0	LC-MS	(Kite et al., 2003)
United States	*P. ginseng*	*Astragalus propinquus* Schischkin (*Leguminosae*)	Single ingredient HMP	1	0	1	DNA barcoding	(Molina et al., 2018)

(Continued)

TABLE 1 | (*Continued*) The authenticity of ginseng commercial herbal products sold on the global market.

Country/ territory	Labeled and authenticated Panax sp./type of herbal product	Identified adulterant	Product composition	Products			Authentication methods	Ref
				Total	Authentic	Adulterated		
				no	no	no		
United States	*P. ginseng, P. quinquefolius*	n/a	Ground *P. quinquefolius* root (capsule), red *P. ginseng* root extract (liquid vial)	2	2	0	UPLC/QTOF-MS	(Yuk et al., 2016)
United States	*P. quinquefolius*	*P. ginseng*	American ginseng products	6	4	2	HPLC	(Yu et al., 2014)
United States	*P. ginseng, P. quinquefolius*	soybean	American and Korean ginseng fresh or dried roots, powders, capsules, tablets	24	22	2	PCR, RAPD, HPLC	(Mihalov et al., 2000)
China	*P. ginseng, P. quinquefolius*	n/a	American and Korean ginseng dried root	2	2	0		
United States and Canada	*P. ginseng, P. quinquefolius*	*P. quinquefolius*	NHPs containing Red Korean, Korean, American, sand ginseng (capsules, tablets, roots, carved roots, extracts, teas, and dried and shredded products	36	22	14	DNA barcoding	(Wallace et al., 2012)
Total				***468***	***358***	***110***		
		Chemical composition						
China	Functional food for relieving physical fatigue	Testosterone, adalafil, sildenafil	Fur seal ginseng pills with complex herbal composition, including *P. ginseng*	16	13	3	HPLC	(Wang et al., 2019)
China	Antidiabetic functional food	Tolbutamide, glimepiride, metformin	Bitter melon and ginseng soft gels containing also containing American ginseng root	16	13	3	UPLC-Q-Orbitrap-MS/MS	(Xie et al., 2019)
China	Herbal medicines for male sexual health	Sildenafil, hongdenafil, vardenafil, homosildenafil	Complex herbal composition, including Radix et rhizoma ginseng or Radix et rhizoma ginseng rubra (tablet, capsule, pills, soft gel capsule)	4	0	4	TLC, HPLC-MS	(Cai et al., 2010)
Saudi Arabia	Dietary supplements for weight loss, slimming and as a stimulant or stamina enhancer	Theobromine, theophylline, pseudoephedrine, caffeine, hydrochlorothiazide, yohimbine	Ginseng extract (capsule), Korean Ginseng (capsule)	2	2	0	UHPLC–DAD	(Ahmad et al., 2020)
Sweden	Dietary supplement	Ephedrine	Ginseng preparation	1	0	1	n/a	(Cui et al., 1994)
Total				***39***	***28***	***11***		
TOTAL				**507**	**386**	**121**		

RESULTS

The analysis of the botanical and chemical identity of 507 ginseng-containing commercial herbal products shows that 76% ($n = 386$) are authentic while 24% ($n = 121$) were reported as adulterated. These ginseng commercial herbal products were purchased from 12 countries scattered across six continents: Asia ($n = 375$), North America ($n = 81$), Europe ($n = 40$), South America ($n = 5$), Australia ($n = 4$), and Africa ($n = 2$). Among continents, the number of commercial products as well as the percentage of adulteration varies significantly, being the highest in South America (100%, $n = 5$) and Australia (75%, $n = 3$), and lower in Europe (35%, $n = 14$), North America (23%, $n = 19$), Asia (21%, $n = 80$) and Africa (0%) (**Table 1**).

TABLE 2 | National distribution of the ginseng commercial ginseng herbal products and their authenticity.

Country	Products (total)	Authentic/adulterated			
	no	no	%*	no	%*
China	224	176	79%	48	21%
South Korea	78	78	100%	0	0%
Taiwan	65	33	51%	32	49%
United States	40	35	88%	5	12%
Italy	19	12	63%	7	37%
Hong Kong	8	8	100%	0	0%
United Kingdom	8	8	100%	0	0%
Brazil	5	0	0%	5	100%
Canada	5	5	100%	0	0%
Australia	4	1	25%	3	75%
Saudi Arabia	2	2	100%	0	0%
Sweden	1	0	0%	1	100%

**The percentage values were rounded to the nearest whole number.*

Analysis of authentication results per country ($n = 459$ commercial ginseng products were clearly allocated to a single country) show that the number of samples purchased and tested for each country varies. In China, 224 ginseng products were successfully authenticated, representing almost half (49%) of all reported samples worldwide, while South Korea (n = 78), Taiwan (n = 65) and United States (n = 40) follow distantly. All of these countries, except Taiwan, are the three major ginseng cultivating countries in the world. This data also suggests the importance of these herbal products in Asian traditional medicine systems is mirrored by the interest of the scientific community to develop and test new methods on market products. Much smaller numbers of tested commercial ginseng products were reported for Italy (n = 19), Hong Kong (n = 8), United Kingdom (n = 8), Brazil (n = 5), Canada (n = 5), Australia (n = 4), Saudi Arabia (n = 2), and Sweden (n = 1), but they support and reconfirm the widespread interest for ginseng-containing products on the global market.

The ginseng authenticity reported for the 12 countries represented in our review range widely from country to country. The five countries for which more than 10 products have been successfully authenticated account together for 84% of all ginseng products analyzed worldwide. Out of these countries, the highest percentage of adulterated ginseng products were purchased from Taiwan (49%), followed by Italy (37%), China (21%), United States (12%), while all the products bought from the South Korean market were reported to be authentic. Notably, six out of the seven remaining countries, each with less than 10 authenticated commercial samples, have all their products reported either as authentic (i.e., Canada, Hong Kong, Saudi Arabia, United Kingdom), or adulterated (i.e., Brazil, Sweden) while the ginseng products purchased from Australia were reported to be 25% authentic and 75% adulterated (**Table 2**).

DISCUSSION

Authenticity of Commercial Ginseng Herbal Products Sold on the Global Market

Overall, the peer-reviewed publications show that one in each four ginseng containing herbal products sold on the market is adulterated with respect to the labeled species or declared chemical composition. Adulterated ginseng products are present across many regions and countries, and this confirms a recent global analysis of DNA-based authenticity testing, both in terms of adulteration percentage and geographic spread (Ichim, 2019). In addition, our analysis further confirms the widespread presence of adulterated commercial herbal products in traditional medicine systems, including Traditional Chinese Medicine (TCM) (Han et al., 2016) and Ayurvedic medicine (Seethapathy et al., 2019).

In most cases, labeled *Panax* species are substituted with other *Panax* species. *P. ginseng* is replaced by *P. quinquefolius* in China (Han et al., 2016; Liu et al., 2016; Dong et al., 2020) and Taiwan (Lu et al., 2010), and vice-versa in United States (Li et al., 2010; Yu et al., 2014), so that the species most highly valued in that country is substituted by other ginseng species. The adulteration of traditional ginseng medicinal products included the substitution of ginseng root, the medicinally recommended plant part, with leaves, stems (Govindaraghavan, 2017) or even flowers (Liu et al., 2015), all replacing the high value herbal material with less costly ones. Liu et al. (2017) showed that ginsenoside ratios could be used to determine the age of cultivated *Panax ginseng* with 30-fold differences in some ginsenosides with age. Premium ginseng commercial products sold on the Taiwanese market have been reported to be adulterated with inferior plant material (Lin et al., 2010). Economically motivated adulteration of commercial ginseng products includes the use of soybean as the only plant ingredient identified in commercial products sold in the United States (Mihalov et al., 2000) thus confirming the reported widespread adulteration of commercial herbal products on the North American market (Newmaster et al., 2013).

The widespread use of the ginseng vernacular name across many countries and continents, including for species not from the same genus or even family (Osathanunkul and Madesis, 2019), is a catalyst conducive to adulteration. In Brazil for example, ginseng products have been found to be adulterated with a completely unrelated species from another family, *Pfaffia* spp., albeit locally known as Brazilian ginseng (Palhares et al., 2015). The inadvertent contamination through plant misidentification during harvesting or cross-contamination during processing but also the intentional and fraudulent use of filler species or cheaper substitutes, present on the herbal product market (Jordan et al., 2010; Sgamma et al., 2017; Ichim et al., 2020), also affects the highly valued ginseng-containing food supplements and traditional medicines across the globalized market. The presence of substitutes or filler species in some cases reflects intentional, economically motivated and fraudulent practices by producers or vendors although the European Pharmacopoeia (Ph. Eur.) and the United States Pharmacopeia (USP) as well as some monographs for herbal raw materials, allow a certain amount (e.g., 2% in USP) of foreign organic matter (Parveen et al., 2016; Sgamma et al., 2017) as acceptable accidental contamination. Counterfeiting by adding synthetic prescription drugs as chemical adulterants is another form of falsification, and Calahan et al. (2016) found that 28% of authenticated ginseng

products in their study were adulterated. Apart from the many cases of adulteration and general low quality of commercial herbal products, *P. ginseng* was also reported, along with some other plant species, as adulterant of a herbal product sold in Australia, supposedly to contain only *Eucalyptus radiata* A. Cunn. ex DC. (*Myrtaceae*) and *Melaleuca alternifolia* (Maiden and Betche) Cheel (*Myrtaceae*). This latter case suggests either accidental contamination through poor manufacturing process or intentional adulteration for achieving an expected physiological or pharmacological effect (Hoban et al., 2020).

Ginseng-Drug Interactions, Abuse and Negative Effects of Long-Term Use on Human Health

The presence of unlabeled species, plant extracts or synthetic chemical compounds might negatively interact with other medicinal plants, food supplements or prescription drugs and will pose significant risks for human health (Jordan et al., 2010). Adverse drug reactions (ADRs) due to herb–drug interactions (HDI) can appear in patients taking herbs and prescribed medications concomitantly (Awortwe et al., 2018). Ginseng can interfere with various drugs, such as digoxin, insulin, anticoagulants, and monoamine oxidase inhibitors (Sellami et al., 2018). Pharmacovigilance relies heavily on ADR reporting, and despite initiatives to stimulate reporting of suspected ADRs associated with herbal medicines, numbers of herbal ADR reports are relatively low. ADRs Under-reporting is likely to be specific for herbal medicines, since their users usually do not look for medical advice about their use of such products, or report if they experience any adverse effects (Barnes, 2003).

The specific active constituents in Panax herbs, the ginsenosides, have been shown to improve immune function, reduce mental stress, and stabilize blood pressure while ginseng products are used as an endurance performance enhancer (Sellami et al., 2018). Nevertheless, authentic ginseng products does not represent a doping concern for athletes, as there were no positive tests for any International Olympic Committee (IOC) banned or restricted substances in any of the subjects after the ingestion of commercially available, proprietary ginseng root extract product (Goel et al., 2004).

Adverse drug reactions to ginseng are associated with high doses and long-term usage (Kiefer and Pantuso, 2003; Sellami et al., 2018). The ginseng abuse syndrome includes edema, decreased appetite, depression, and hypotension (~10%), hypertension (17%), sleeplessness (20%), nervousness (25%), skin eruption (25%), and morning diarrhea (35%) (Paik and Lee, 2015). Long term use may cause blood clotting (Mohammed Abdul et al., 2018). Ginseng reduces the blood levels of warfarin and alcohol as well as induced mania if taken concomitantly with phenelzine, a non-selective and irreversible monoamine oxidase inhibitor used as an antidepressant and anxiolytic agent (Chen et al., 2011). Because the ginsenosides have a chemical structure similar to that of testosterone, estrogen, and glucocorticoids, the ginseng may also produce effects similar to those of estrogen (Anadón et al., 2016). Moreover, women may experience additional side effects, such as vaginal bleeding and breast tenderness. Most of these side effects are serious enough to warrant stopping taking ginseng in breast cancer patients (Sellami et al., 2018). A rare adverse drug reaction to herbal and dietary supplementation, the drug-induced liver injury (DILI), was reported as result from ingestion of ginseng for premenopausal symptoms (Lin et al., 2018). Recently, a website with a critically reviewed database presenting reported cases of ginseng-drug interactions was publicly launched (Wu et al., 2019).

The herbal medicines differ considerably from conventional medicines, and they pose a variety of challenges to their pharmacovigilance. For herbal pharmacovigilance, four main challenges: 1) Substitution and adulteration; 2) Nomenclature of herbals and ingredients of plant origin; 3) Lack of monitoring; and 4) Standardization, have been identified (de Boer et al., 2015). This review highlights that all four apply to ginseng commercial products, and support the challenge of detecting significant adverse drug reactions in a timely manner to protect consumers.

CONCLUSION

Despite being the most valuable herbal product in terms of market value share, ginseng products are poorly regulated. Several authentication studies have shown that adulteration is not uncommon and not limited to any specific country of origin. The high market value of ginseng provides an incentive for fraudulent actors to generate profits at the expense of gullible consumers and honest producers, wholesalers and retailers. Although authentication using traditional, pharmacopoeial, analytical methods such as TLC, HPLC and NMR can be used for advanced quality control, the standard authentication protocols are insufficient for efficiently detecting species adulteration, adulteration with synthetic pharmaceuticals and spiking of low quality products with marker compounds. Cutting-edge approaches enable distinction of age-specific metabolite spectra, quantification of active ingredients and accurate identification of ginseng species, but these are not yet in widespread use.

AUTHOR CONTRIBUTIONS

MI performed the literature systematic search and analyzed the results. MI and HB wrote the manuscript together.

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Heavy Metal Contaminations in Herbal Medicines: Determination, Comprehensive Risk Assessments, and Solutions

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Heavy metal contamination in herbal medicines is a global threat to human beings especially at levels above known threshold concentrations. The concentrations of five heavy metals cadmium (Cd), lead (Pb), arsenic (As), mercury (Hg) and copper (Cu) were investigated using Inductively Coupled Plasma Optical Mass Spectrometry (ICP-MS) with 1773 samples around the world. According to Chinese Pharmacopoeia, 30.51% (541) samples were detected with at least one over-limit metal. The over-limit ratio for Pb was 5.75% (102), Cd at 4.96% (88), As at 4.17% (74), Hg at 3.78% (67), and of Cu, 1.75% (31). For exposure assessment, Pb, Cd, As, and Hg have resulted in higher than acceptable risks in 25 kinds of herbs. The maximal Estimated Daily Intake of Pb in seven herbs, of Cd in five, of Hg in four, and As in three exceeded their corresponding Provisional Tolerable Daily Intakes. In total 25 kinds of herbs present an unacceptable risk as assessed with the Hazard Quotient or Hazard Index. Additionally, the carcinogenic risks were all under acceptable limits. Notably, As posed the highest risk in all indicators including Estimated Daily Intake, Hazard Index, and carcinogenic risks. Therefore further study on enrichment effect of different states of As and special attention to monitoring shall be placed on As related contamination.

Keywords: heavy metal, risk assessment, safety and quality, herbal mecidine, extrinsic contamination

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INTRODUCTION

Having been utilized as traditional folk remedies for thousands of years (Maiga et al., 2005), medicinal plants gained an increasingly important role in the pharmaceutical, health food, and natural cosmetic industries (Kim et al., 2016). It was reported that a total of 60,107 COVID-19 cases (85.20% of the total cases) in China were treated by Chinese herbal medicines with positive results in all infection stages, including significant symptom management, lower rates of deterioration and mortality, faster recovery as well as disease prevention on February 17, 2020 (Ministry of Science and

Technology, 2020). However, concerns grew regarding the safety of herbal medicines after studies indicated that high levels of heavy metals were present in some herbal medicines. This was a problem more frequently encountered in traditional medical therapy, considered to be a cause of several health disorders (Maiga et al., 2005; Brookes et al., 2019). Samples taken from both developed and developing countries have shown high levels of potentially toxic heavy metals in products available to the public (Awodele et al., 2013). It is known that numerous traditional medicines can give rise to severe adverse renal pathology, the mechanism of which is yet not fully certain but has been associated with heavy metal toxicity (Awodele et al., 2013). Uptake of heavy metals by plants and subsequent accumulation along the food chain is a potential threat to animal and human health (Jiwan and Kalamdhad, 2011). Particularly as heavy metals are known to have low renal excretion rates, potentially resulting adverse effects in humans even at very low concentrations (Jamroz et al., 2015). They are not easily metabolized by body and are found to accumulate in the soft tissues (Jiwan and Kalamdhad, 2011). They produce toxic effects due to their interference in many known normal biochemical and metabolic processes (Kurban et al., 2015). Several health problems were linked to excessive uptake of dietary heavy metals, including decreased immunity, cardiac dysfunction, fetal malformation, impaired psychosocial and neurological behavior (Harris et al., 2011). Pb and Cd are not essential elements that are required neither in the human body nor in plants, and which cause various bimolecular adverse functional effects at low level doses (Maiga et al., 2005). Though an essential component of many enzymes, excessive intake of Cu can cause dermatitis, irritation of the upper respiratory tract, abdominal pain, nausea, diarrhea, vomiting, and liver damage (Harris et al., 2011). While As and Hg can damage pulmonary, nervous, renal and respiratory systems, as well as causing skin pathology (Jarup, 2003; Mahurpawar, 2015). It may also induce disorders in the central nervous system, liver, lungs, heart, kidney and brain. Leading to hypertension, abdominal pain, skin eruptions, intestinal ulcer and is associated with various types of cancers (Paulsen et al., 2012). It is therefore necessary and a matter of urgency to conduct a comprehensive risk assessment of heavy metal contamination in herbal medicines.

To explore and guarantee the safety of herbal medicines, multiple studies regarding heavy metal contamination in herbal medicines have been carried out in China (Ye et al., 2013; Li et al., 2018), India (Singh et al., 2016), Iran (Behnam et al., 2017), Egypt (Abou and Abou Donia, 2000), South Africa (Mulaudzi et al., 2017), United States (Harris et al., 2011), Brazil (Leal et al., 2013), and Australia (Kim et al., 2016), etc. Though a number of studies have been conducted regarding heavy metal contamination in herbal plants, most were with limited sample numbers and categories. Given the severe consequence it may bring to health and environment, exposure risk assessment (Kirkham et al., 2010; Okatch et al., 2012), hazard quotient (Li et al., 2018), and ecological risk assessment (Awodele et al., 2013), have been conducted by researchers, showing that heavy metal contamination in herbal medicines is an area requiring immediate attention, with potential risk to human health having now being demonstrated (Sandilyan and Kathiresan, 2014). Though studies now show that exposure of heavy metals through general dietary consumption contribute negatively to human health (Wei and Cen, 2020), very few have conducted comprehensive health risk assessments, with large sample numbers focusing on herbal medicines. Furthermore, specific identification of metals is required for accurate diagnosis due to considerable overlap between various clinical syndromes associated with heavy metal poisoning (Kurban et al., 2015). Therefore a study of accurately quantified heavy metal contents in herbal medicines appears necessary to further assess and justify of the dosage of herbal formulas. This study assesses contamination levels and the health risk to humans posed by heavy metals more specifically in herbal medicines, providing an evidence base on which to further build prevention measures, establish relative standards, and control external contamination. Through investigation and suggested recommendations in able to significantly reduce or eliminate the levels of heavy metals in herbal medicines.

MATERIALS AND METHODS

Sample Collection and Detection

A total of 1773 crude plant extract samples from 2014 to 2019, representing 86 different kinds of commonly used herbal medicines were collected for examination of heavy metals. As a part of a heavy metal detection project for Chinese Pharmacopoeia (2020 edition) (National Pharmacopoeia Commission, 2020) and based on the principle that only herbal medicines from large-scale production areas would be considered, at least three samples for each herbal medicine were collected from one to 13 different sampling locations, which were purchased in four major Chinese herbal markets (Anhui, Henan, Chengdu, Hebei). Sampling locations were chosen according to areas of traditional production of the herbal medicines, without regard to possible pollution sources. Each bulk sample was harvested, cleaned, and processed according to the method required by Chinese Pharmacopoeia (National Pharmacopoeia Commission, 2020). Precisely 0.5 g sample (Electronic balance, Mettler Toledo) was first ground into powder, then soaked in polytetra fluoroethylene (PTFE) tank with 6 mL nitric acid (HNO_3) added overnight. It was then predigested for 1 h on a temperature controlled electronic hotplate with 1 mL hydrogen peroxide solution (H_2O_2) added. After cooling down, more HNO_3 was added to up to volume of 7 mL. The amount of each metal was added before microwave digestion. The PTFE tank was then placed in Multiwave PRO Microwave Digestion Apparatus (Anton Paar) for further digestion before being placed on a 130°C electronic hotplate, until reduced to 1 mL. Microwave digestion was set on a digestion program. For the first stage, 1100 W, maintained for 10 min then further, for the second stage, 1,400 W for 15 min and held for 20 min (Gonzalez-Martin et al., 2018). The detailed methodology is listed in the appendix (**Supplementary Table S1**). The tank was then removed for cooling and 50 mL of the digested liquid transferred to a

graduated flask. The tank was flushed with low volume amounts of water multiple times and the complete washings added to the graduated flask, 200 μL of single-element standard solution (1 mg L^{-1}) was added. Then diluted up to the mark of the graduated flask with water, shaken and set aside. The blank solution was prepared in the same way except that no standard solution of single element and sample powder were added (Pohl, 2016).

The resulting solution was analyzed using a Scientific X Series Inductively-Coupled Plasma Mass Spectrometer (ICP-MS) (Thermo Fisher Scientific, Waltham, MA). Heavy metal results from the ICP-MS were quantified against standard curves generated from 1 blank and at least 4 standard reference solutions (High-Purity Standards, Charleston, SC) run separately. Quality control was assessed by running a laboratory reagent blank after every 10 samples. The detection limit was based on consideration of the blank runs, concentration of the low standard in the calibration curve and the sample preparation procedure. Based on this method, the limit of detection was considered equivalent to the limit of quantification (Wang and Hansen, 2004; Pereira et al., 2010). The blank solution was run for 14 times and the first three were discarded considering they were not stable. The limit of detection (LOD) was automatically calculated with standard deviation of the remaining 11 runs, divided by the slope of the standard curve. The LOD achieved for each metal was 0.1 mg kg^{-1} for Cu, 0.01 mg kg^{-1} for As, 0.005 mg kg^{-1} for Cd, 0.001 mg kg^{-1} for Hg, and 0.01 mg kg^{-1} for Pb. Detailed parameters could be found in supplementary materials (**Supplementary Tables S1.1–S1.23**).

In our experiment, standard solutions of Cu (GSB04-1725, 1,000 mg L^{-1}), As (GSB04-1714, 1,000 mg L^{-1}), Cd (GSB04-1721, 1,000 mg L^{-1}), Hg (GSB04-1729, 1,000 mg·L), Pb (GSB04-1742, 1,000 mg L^{-1}) were purchased from the National Nonferrous Metals and Electronic Materials Analysis and Testing Center, while Analytical Reagents (AR) and nitric acid (HNO_3) were purchased from Merck Co., Ltd., and guaranteed reagents (GR) hydrogen peroxide and hydrochloric acid from Sinopharm Chemical Reagent Co., Ltd. Each single-element standard solution was measured precisely and then diluted with 5% HNO_3 to make a mixed solution containing 1 μg mL^{-1}. For a reference stock solution, single-element standard solutions of the five metals were separately taken and diluted with 5% nitric acid to make solutions containing 5 μg of Pb and As, 50 μg of Cu, 2.5 μg of Cd, and 0.5 μg of Hg. For preparation of a reference standard curve, precise measurements were taken of the above stock solution diluted with 5% nitric acid to make standard mixtures (ng·mL^{-1}): 0, 1, 5, 10, 20, and 50 ng mL^{-1} As or Pb; 0, 10, 50, 100, 200, and 500 ng mL^{-1} Cu; 0, 0.5, 2.5, 5, 10, and 25 ng mL^{-1} Cd; 0, 0.1, 0.5, 1, 2, and 5 ng mL^{-1} Hg (Harris et al., 2011).

The conditions for Inductively coupled plasma mass spectrometry (ICP-MS) conditions were: Radio Frequency (RF) power: 1400 W; sampling depth: 15 mm; auxiliary gas (argon) flow rate: 0.8 L min^{-1}; cooling gas flow rate: 13.0 L min^{-1}; peristaltic pump speed: 30.0 L min^{-1}; channel three; repeat for three times; scan for 100 times, automatic detection. Before samples were measured, the instrument was optimized to perform under the optimal conditions.

Quantification of Heavy Metal Contamination

Mean concentrations, general detection rate, and detection rates of each metal were calculated. Figures were plotted using *R* language [no IDE (integrated development environment), *R* from the linux terminal *R* version 3.5.1. (2018-007-02)--"Feather Spray" Copyright (C) 2018, the *R* Function for Statistic Computing Platform: x86_64-pc-linux-gnu (64-bit)] (Wang et al., 2009; The R core team, 2020).

Over-limit Ratio of Five Heavy Metals

In total, 27 currently available permissible limits containing five heavy metals of herbal medicines were obtained from 20 countries or regions and seven international organizations (**Supplementary Table S2**). The detailed calculation of both general over-limit ratio and that of each metal in different producing areas were shown in appendix (**Supplementary Table S3**). Numbers of over-limit samples and metals were calculated from within across five medicinal herbal properties, including: flos (flower), folium and cortex (leaf and bark), fructus and semen (fruit and seed), herba and others (the whole plant), and radix and rhizoma (root and rootstock).

$$\text{Over-Limit ratio} = (C_{\text{detected}} - \text{Limit}_{\text{metal}}) \times 100\% \quad (1)$$

$$\text{Times Over Permissible Limits} = \frac{C_{\text{detected}} - \text{Limit}_{\text{metal}}}{\text{Limit}_{\text{metal}}} \times 100\% \quad (2)$$

The Health Risk Assessments of Heavy Metal Contamination in Herbal Medicines

Exposure assessment (EDI), non-carcinogenic risk assessment (HQ, HI), and carcinogenic risk assessment (CR) were employed to explore the potential health impacts from heavy metal contamination in herbal medicines. The U.S. Environmental Protection Agency (EPA) has replaced the Tolerable daily intake (TDI) with RfD, which is defined as "an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure for the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime." Use of the term RfD is intended to avoid any implication that exposure to the chemical is completely "safe" or "acceptable" (Peter et al., 2015; Gunnar et al., 2019). We adopted provisional tolerable daily intake (PTDI) in exposure assessment, to compare the estimated daily intake (EDI) with PTDI. While for non-carcinogenic risk assessment, we adopted the Oral reference dose (RfD), to evaluate it by comparing an exposure level over a specified time period (eg, lifetime) with a reference dose derived for a similar exposure period (Sawut et al., 2018; USEPA, 1989; USEPA 2009; USEPA 2012). Additionally, the minimal, mean and maximal concentrations of each metal in each herbal medicines were applied for calculations with the equations below:

Exposure Assessment

$$EDI = \frac{C \times IRD}{BW} \quad (3)$$

The estimated daily intake (EDI, mg kg^{-1} day^{-1}·bw) of each metal in each sample was calculated, before comparison with its corresponding provisional tolerable daily intake (PTDI). The PTDIs (mg·kg^{-1}·d^{-1}) of As, Cu, Hg, Pb, and Cd are 0.00214, 0.5, 0.00057, 0.00357, and 0.00083, respectively (Sawut et al., 2018). *C* (mg·kg^{-1}) in the equation here refers to the concentrations detected of each metal in herbal medicines (maximal, mean and minimal concentrations were all considered). IRD (kg·day^{-1}) refers to daily ingestion rate, which signifies the daily dosage of herbal medicines. Here the maximal dosage specified in CP (2020 edition) was applied. BW (kg) is body weight, and average human weight of 60 kg was applied in the equation (Zhao et al., 2010).

Non-carcinogenic Risk Assessment

The non-cancer risk was evaluated by comparing an exposure level over a specified time period (eg, lifetime) with a reference dose derived for a similar exposure period. The non-cancer risk can be characterized as a hazard quotient (HQ) (USEPA, 1989; USEPA 2009; USEPA 2012; Sawut et al., 2018).

$$HQ = \frac{C \times IR \times Ef \times Ed \times t}{AT \times BW \times RfD} \quad (4)$$

IR (kg·day^{-1}) is the daily dosage of herbal medicine, and according to a questionnaire on herbal medicine consumption of 20917 people, the 95th percentile of daily dosage of general herbal medicine consumption is 0.5 kg; Ef (day) is exposure frequency, here the 95th percentile of annual consumption on herbal medicine was adopted which was set 90 days per year; Ed (year) is the exposed days over a lifetime which was set as 20 years; AT (day) is the average lifetime = 365 days × 70 years, while t is the transfer rate of heavy metal to herbal detection, which is 14% for Cd, Cu, and Pb, 35% for As, and 24% for Hg; The transfer rates were referred from the investigation conducted by Zuo et al. (2019), which were estimated based on several fine quantification researches on transfer rates of heavy metals in herbal medicines conducted in China (Wang et al., 2014; Gan et al., 2016; Jin et al., 2017; Pan et al., 2017; Zuo et al., 2017). RfD refers to Oral reference dose (mg^{-1}·kg^{-1}·day), which is 0.0035 for Pb, 0.0005 for Cd, 0.0003 for As, 0.0003 for Hg and 0.04 for Cu (Li et al., 2019).

HQs of five heavy metals in each herbal medicine were summed up to obtain non-carcinogenic Hazard Index (HI). If HQ or HI is less than 1, there will not be obvious risk for exposed population from metal exposure in herbal medicine. If HQ or HI is equal to or above 1, the risk will be considered unacceptable. As the HQ or HI increases, the risk also does. The contributions of HQs of each metal (HQ_m) to the total HI were calculated to explore which metal contributed the most serious risks (Farmer et al., 2019).

$$HI = \sum HQ \quad (5)$$

$$\text{Contribution of } HQ_m \text{ to HI} = (HQ_m - HI) \times 100\% \quad (6)$$

Carcinogenic Risk Assessment

$$CR = \frac{C \times IR \times Ef \times Ed \times t \times SFo}{AT \times BW} \quad (7)$$

SFo (mg·kg^{-1}·day^{-1}·bw) is the Oral Slope Factor signifying cancer severity, only three heavy metals were proven with certain SFs: 6.1 for Cd, 1.5 for As and 0.0085 for Pb, while 10^{-6} is the conversion factor (USEPA, 1989; USEPA 2009; USEPA 2012; Liu et al., 2013; Farmer et al., 2019). CR of these three metals in the same herbal medicine was also summed up to give total CR of single herbal medicine. If CR is higher than 10^{-6}, which means one case of cancer over one million exposed people, it is considered unacceptable (Fakhari et al., 2017; Onyele and Anyanwu, 2018).

RESULTS

The Content of Five Heavy Metals in 1773 Herbal Medicines

Heavy metals were detected in all 1773 samples. The order of detection rates of heavy metal concentrations in herbal medicines is Cu (1771, 99.89%) > Pb (1755, 98.98%) > Cd (173, 97.74%) >As (1,679, 94.70%) > Hg (1,497, 84.43%). The contents (mg·kg^{-1}) of each medicinal property were also calculated, the highest content of Cu, Cd and Hg was 11.12 ± 2.73, 0.439 ± 0.686 and 0.081 ± 0.667, respectively, in Flos (n = 340); the highest content of As and Pb was 1.06 ± 1.56 and 3.23 ± 3.83, in Herba and others (n = 380) (**Table 1**). The orders of mean concentrations detected of five heavy metals in five plant properties are: for Cu, flos > herba and others > folium and cortex > radix and rhizoma > fructus and semen; for As, herba and others > folium and cortex > flos > radix and rhizoma > fructus and semen; for Cd, flos > herba and others > radix and rhizoma > folium and cortex > fructus and semen; for Hg, flos > fructus and semen > herba and others > folium and cortex > radix and rhizoma; while for Pb, herba and others > folium and cortex > flos > radix and rhizoma > fructus and semen (**Figure 1**). The highest concentration detected for Cu was in herbal medicine *Schisandra chinensis* (Turcz.) Baill. (34.01 mg kg^{-1}), the highest concentration of As was in *Plantago asiatica* L. (14.53 mg kg^{-1}), of Cd was in *Curcuma longa* L. (6.20 mg kg^{-1}), of Hg was in *Chrysanthemum indicum* L. (8.69 mg kg^{-1}), of Pb was in *Tetradium ruticarpum* (A.Juss.) T.G.Hartley (50.11 mg kg^{-1}) (**Table 1**). In conclusion, all five heavy metals were widely detected in herbal medicines, particularly, Cu and Pb, most notably in flos and herba parts of medicinal plants.

Over-Limit Ratio of Five Heavy Metals in 1773 Herbal Medicines

In a total of 541 samples (30.51%) were detected at levels over the CP (2020 edition) standard, and 433 samples detected with one

TABLE 1 | Health risk assessment scores of top risk-inducing herbal medicines.

Herbal medicine	Medicinal plant property	Max EDI ($mg \cdot kg^{-1} \cdot day^{-1} \cdot bw$)				Max HQ				HI	Max CR			
		As	Cd	Hg	Pb	As	Cd	Hg	Pb		As	Cd	Pb	Total CR
Mentha canadensis L.	Herba and others					1.54				2.28				
Plantago asiatica L.	Herba and others	0.007			0.024	9.95			1.11	11.47	1.34E–06			
Andrographis paniculata (Burm.f.) Nees	Herba and others		0.001			5.51				7.02				2.18E–06
Isatis tinctoria L.	Folium and cortex					1.64				2.21				
Pueraria montana (Lour.) Merr.	Radix and rhizoma					1.03				1.35				
Grona styracifolia (Osbeck) H.Ohashi and K.Ohashi	Herba and others		0.001	0.001	0.008	1.79				3.47				
Carthamus tinctorius L.	Flos					2.02				2.41				
Chrysanthemum indicum L.	Flos			0.001		1.90		4.08		6.31				
Tussilago farfara L.	Flos					2.24		1.89		2.65				
Forsythia suspensa (Thunb.) Vahl	Fructus and semen			0.001		1.29				3.51				
Ligustrum lucidum W.T.Aiton	Fructus and semen					1.74				2.91				
Taraxacum officinale (L.) Weber ex F.H.Wigg.	Herba and others	0.002			0.004	6.82		`		8.62				
Cornus officinalis Siebold and Zucc.	Fructus and semen	0.002				8.05				8.66				
Ziziphus jujuba Mill.	Fructus and semen					1.58				1.83				
Tetradium ruticarpum (A.Juss.) T.G.Hartley	Fructus and semen			0.001	0.004	1.69		6.31	1.18	9.53			1.23E–07	
Schisandra chinensis (Turcz.) Baill.	Fructus and semen					1.07				1.49				
Houttuynia cordata Thunb.	Herba and others		0.002		0.004	1.97				3.42				
Gardenia jasminoides J.Ellis	Fructus and semen					2.93		1.14		4.57				
Citrus × *aurantium* L.	Fructus and semen					1.08				1.28				
Perilla frutescens (L.) Britton	Folium and cortex					2.64		1.02		4.02				
Curcuma longa L.	Radix and rhizoma		0.001				1.02			1.43		1.55E–06		
Coptis chinensis Franch.	Radix and rhizoma													
Lonicera japonica Thunb.	Flos				0.005					2.12				
Chaenomeles lagenaria (Loisel.) Koidz.	Fructus and semen									1.48				
Lonicera confusa DC.	Flos		0.001		0.004					1.81				

The estimated daily intakes (EDI, $mg \cdot kg^{-1} \cdot day^{-1} \cdot bw$) above their corresponding provisional tolerable daily intakes (PTDI) were shown (The PTDIs ($mg \cdot kg^{-1} \cdot day^{-1}$) of As, Hg, Pb, and Cd are 0.00214, 0.00057, 0.00357, and 0.00083, respectively). The non-carcinogenic hazard quotient (HQ) and non-carcinogenic Hazard Index (HI) above one were shown. The carcinogenic risks (CR) higher than 10^{-6}, which means one case of cancer over one million exposed people, is considered unacceptable, thus shown here. All scores in this table were calculated with maximal concentrations of each herbal medicine.

over-limit metal, 75 samples with two over-limit metals, three samples with 24 over-limit metals and nine samples with four over-limit metals (**Figure 2**). The order of over-limit ratio of five heavy metals based on CP (2020 edition) standard is Pb (102, 5.75%) > Cd (88, 4.96%) > As (74, 4.17%)> Hg (67, 3.78%) > Cu (31, 1.75%). The times of highest concentration detected over the standards of CP (2020 edition) were 1.70 for Cu (*Schisandra chinensis* (Turcz.) Baill.), 6.27 for As (*Plantago asiatica* L.), 5.20 for Cd (*Curcuma longa* L.), 66.17 for Hg (*Chrysanthemum indicum* L.), and 9.02 for Pb (*Tetradium ruticarpum* (A.Juss.) T.G.Hartley). The highest concentration detected over the limit of European Union (EU) and United Kingdom (U.K.) is Hg (133.35 times). As per over-limit ratios of each metal in five medicinal properties, 40.00% samples were detected with over-limit concentrations in flos, 34.39% in folium and cortex, 7.69% in fructus and semen, 58.16% in herba and others, 37.20% in radix and rhizome. For Cu ($n = 31$), there are 12.90% samples detected over-limit in flos, 9.68% in folium and cortex, 16.13% in fructus

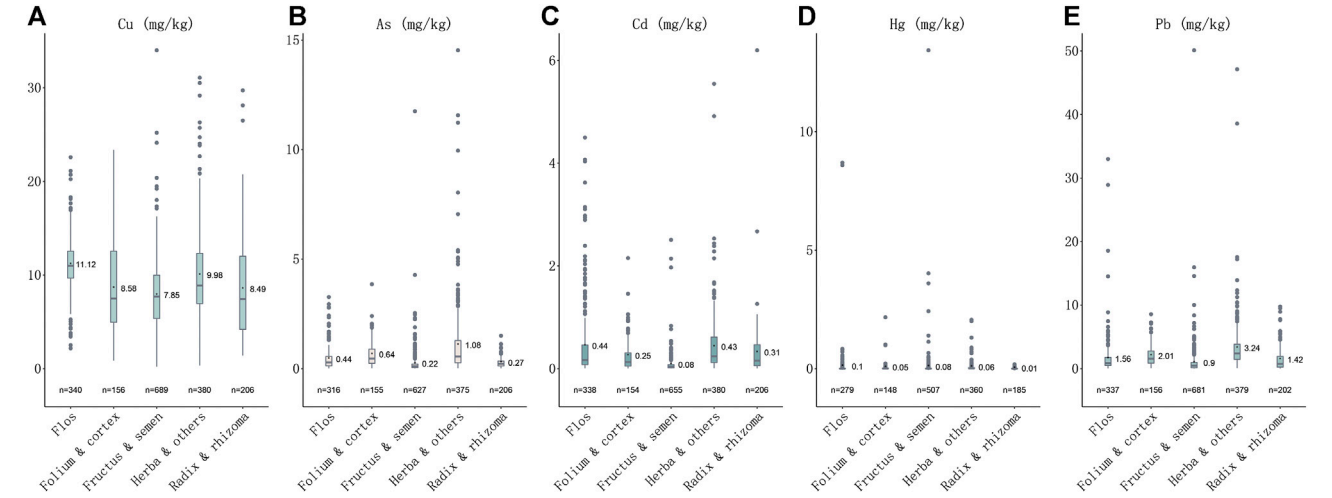

FIGURE 1 | Plotbox showing concentrations of five heavy metals in five medicinal properties. **(A)** Concentrations of Cu in five medicinal properties. **(B)** Concentrations of As in five medicinal properties. **(C)** Concentrations of Cd in five medicinal properties. **(D)** Concentrations of Hg in five medicinal properties. **(E)** Concentrations of Pb in five medicinal properties.

and semen, 41.94% in herba and others, and 19.35% in radix and rhizome; For As (n = 74), 9.46% samples were detected with concentrations above the threshold in flos, 6.76% in folium and cortex, 8.11% in fructus and semen, 75.68% in herba and others, and none in radix and rhizome; For Cd (n = 416), 27.16% samples detected over-limit in flos, 10.10% in folium and cortex, 5.29% in fructus and semen, 39.90% in herba and others, and 17.55% in radix and rhizome; For Hg (n = 67), there are 25.37% samples detected over-limit in flos, 5.97% in folium and cortex, 34.33% in fructus and semen, 34.33% in herba and others, and none in radix and rhizome; For Pb (n = 102), there are 11.76% samples detected over-limit in flos, 9.80% in folium and cortex, 9.80% in fructus and semen, 59.80% in herba and others, and 8.82% in radix and rhizome. Of note, we found that heavy metals prefer to accumulate in fructus and semen, while herba and others were detected with the highest over-limit ratio. Notably, heavy metal Pb was presented with the highest over-limit ratio, followed by Cd and As.

Risk Assessments of Five Heavy Metals in Herbal Medicines

As per exposure assessment, though the majority of herbal medicines were considered within the acceptable limit, the concentrations detected in all five heavy metals in few herbal medicines have surpassed their corresponding Provisional Tolerable Daily Intakes (PTDI), demonstrating unacceptable risk to health. The EDIs of three heavy metals (Cd, Hg, and Pb) in *Desmodii styracifolii* herba have surpassed their corresponding PTDIs (**Figure 3**; **Supplementary Figure S1**; **Table 2**). Notably, a total of 12 herbal medicines out of 86 have presented with EDIs above their corresponding PTDIs. The maximal EDI of As in three herbal plants *Plantago asiatica* L. (0.007), *Taraxacum officinale* (L.) Weber ex F.H.Wigg. (0.002), and *Cornus officinalis* Siebold and Zucc. (0.002); of Cd in five herbal plants *Desmodium styracifolium* (Osbeck) Merr. (0.001), *Andrographis paniculata* (Burm.f.) Nees (0.0008), *Curcuma longa* L. (0.001), *Lonicera japonica* Thunb. (0.001), and *Houttuynia cordata* Thunb. (0.002), of Hg in four herbal plants *Desmodium styracifolium* (Osbeck) Merr. (0.001), *Chrysanthemum indicum* L. (0.001), *Forsythia suspensa* (Thunb.) Vahl (0.001), and *Tetradium ruticarpum* (A.Juss.) T.G.Hartley (0.001); and of Pb in seven herbal plants *Plantago asiatica* L. (0.024), *Taraxacum officinale* (L.) Weber ex F.H.Wigg. (0.004), *Desmodium styracifolium* (Osbeck) Merr. (0.008) *Lonicera japonica* Thunb. (0.004), *Houttuynia cordata* Thunb. (0.004), *Tetradium ruticarpum* (A.Juss.) T.G.Hartley (0.004), *Lonicera japonica* Thunb. (0.005) exceeded their corresponding PTDIs (**Figure 3**).

For non-carcinogenic risk, the majority of the herbal medicines were calculated with risks within the acceptable limit (<1). The HIs of a total of 86 herbal medicines ranged from 11.47 *Plantago asiatica* L. and 0.02 *Chaenomeles lagenaria* (Loisel.) Koidz. and HIs in a total of 25 out of 86 kinds of herbal medicines (29.07%) showed values over 1, thus considered unacceptable risk. HQs of As in 20 herbal medicines, of Hg in five herbal medicines, of Pb in two herbal medicines, and of Cd in one herbal medicine exceeded 1, considering as unacceptable risks. It was also shown that heavy metal As contributed the most in HQ > 1 herbal medicines. The highest was 92.94% in *Cornus officinalis* Siebold and Zucc. Heavy metal As has shown the highest non-carcinogenic (HQ = 9.95), presenting more severe risks than other four heavy metals (**Figure 3**; **Table 2**; **Supplementary Figure S2**).

For carcinogenic risks, all CRs were found to be within the acceptable limit (10^{-4}) (Alidadi et al., 2019). The highest risk of As was found in *Plantago asiatica* L (1.34E−06), the lowest was in *Ziziphus jujuba* Mill. (9.36E−10); the highest of Cd was in *Curcuma longa* L. (1.55E−06), the lowest in *Citri Grandis Exocarpium* (1.25E−09); and the highest of Pb was in

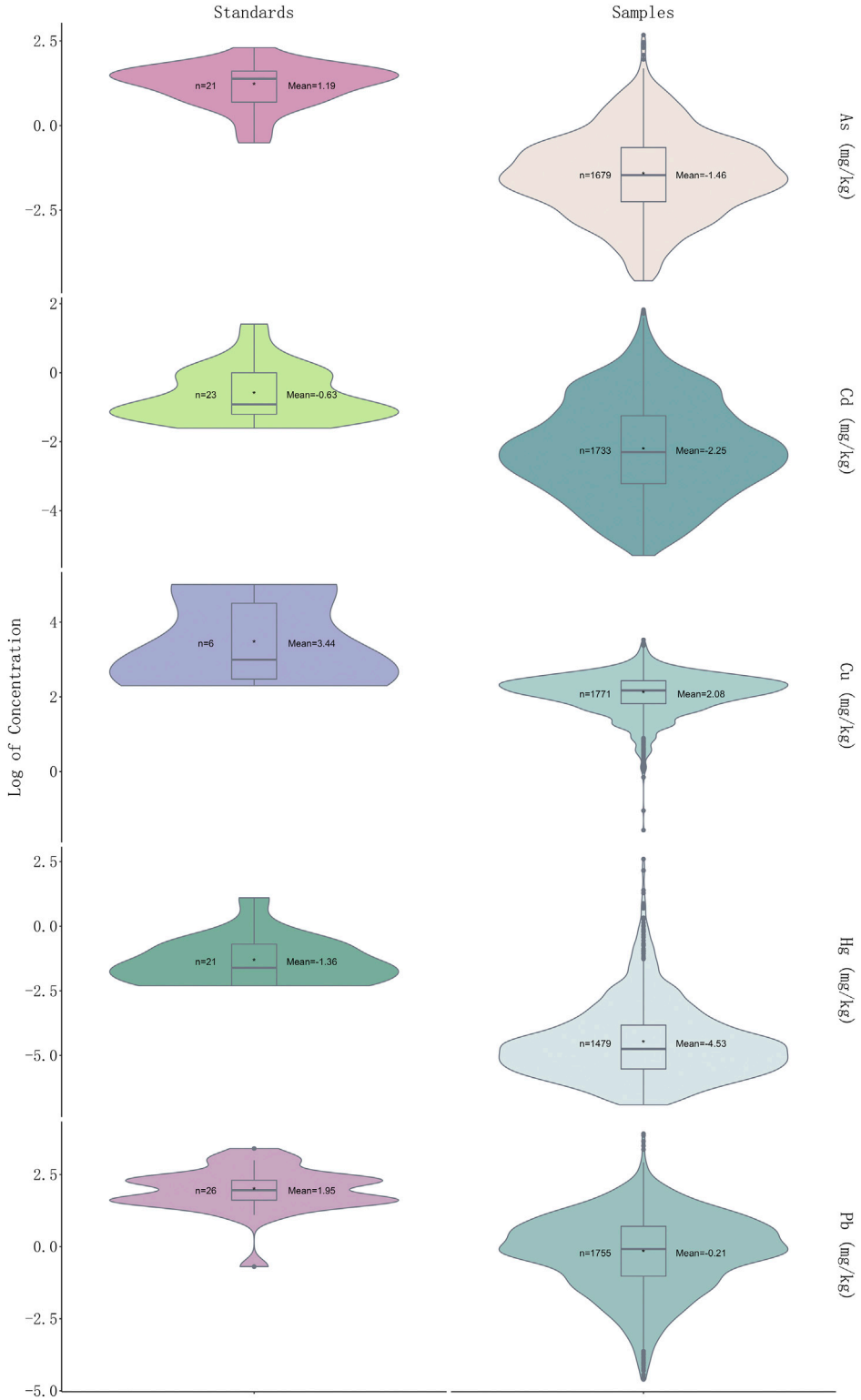

FIGURE 2 | Violin plot showing $\log_2$ (concentration) values of five heavy metals in 1773 samples and permissible limits in different countries of each heavy metal (see **Supplementary Table S3** for detailed standards of each heavy metal).

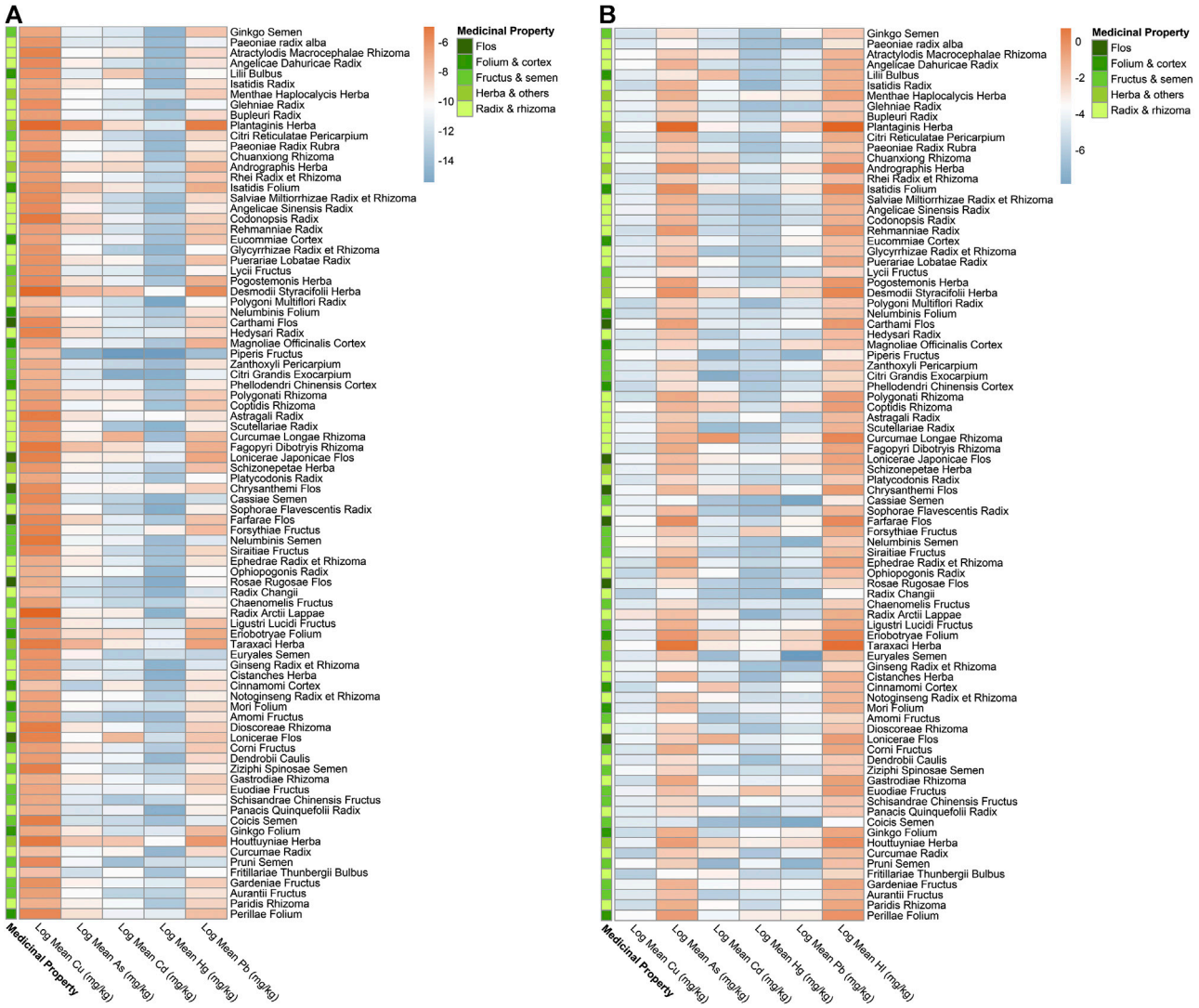

FIGURE 3 | Heatmap of EDI and HI by mean concentrations detected for five heavy metals in 86 kinds of herbal medicines. **(A)** Heatmap showing $\log_2$ (Estimated Daily Intake, EDI) values by mean concentrations detected of five heavy metals in 86 herbal medicines. **(B)** Heatmap showing $\log_2$ (Hazard Index, HI) values by mean concentrations detected of five heavy metals in 86 herbal medicines.

Tetradium ruticarpum (A.Juss.) T.G.Hartley (1.23E−07), the lowest were in *Pueraria montana* (Lour.) Merr. and *Ziziphus jujuba* Mill. (2.45E−11). *Andrographis paniculata* (Burm.f.) Nees presented with the highest total carcinogenic risk (2.18E−06), while *Ziziphus jujuba* Mill. the lowest (2.31E−09). Among these top risk-inducing herbs, nine belong to fructus and semen (**Figure 4**). Heavy metal As and Cd have shown more serious carcinogenic risks (CR = 1.34E−06, 1.55E−06).

In total here, 25 (29.07%) different kinds of herbal medicines (n = 86), presented with unacceptable risks based on exposure assessment, among which, nine belonged to fructus and semen, six belonged to herba and others, five belonged to the flos category, three belonged to radix and rhizoma, and two belonged to the folium and cortex. *Plantago asiatica* L. presented with the highest non-carcinogenic risk (HI = 11.47), while *Andrographis paniculata* (Burm.f.) Nees with the highest carcinogenic risk (CR = 2.18E−06). Heavy metal As has shown the highest non-carcinogenic (HQ = 9.95) while Cd the carcinogenic risk (1.55E−06) in herbal medicines. As these particular herbal medicines and heavy metals have the potential to cause health problems, they are in need of special monitoring to reduce potential risk (**Table 2**).

DISCUSSION

Based on this study with a large-spatio-temporal-scale herbal medicine samples, 30.51% (541) of samples were detected with at least one over-limit heavy metal. Five heavy metals (As, Pb, Cu, Hg and Cd) were widely detected in cultivated herbal medicines

TABLE 2 | Health risk assessment scores of top risk-inducing herbal medicines.

Herbal medicine	Medicinal plant property	Max EDI ($mg \cdot kg^{-1} \cdot day^{-1} \cdot bw$)				Max HQ				HI	Max CR			
		As	Cd	Hg	Pb	As	Cd	Hg	Pb		As	Cd	Pb	Total CR
Mentha canadensis L.	Herba and others					1.54				2.28				
Plantago asiatica L.	Herba and others	0.007			0.024	9.95			1.11	11.47	1.34E–06			
Andrographis paniculata (Burm.f.) Nees	Herba and others		0.001			5.51				7.02				2.18E–06
Isatis tinctoria L.	Folium and cortex					1.64				2.21				
Pueraria montana (Lour.) Merr.	Radix and rhizoma					1.03				1.35				
Grona styracifolia (Osbeck) H.Ohashi and K.Ohashi	Herba and others		0.001	0.001	0.008	1.79				3.47				
Carthamus tinctorius L.	Flos					2.02				2.41				
Chrysanthemum indicum L.	Flos			0.001		1.90		4.08		6.31				
Tussilago farfara L.	Flos					2.24		1.89		2.65				
Forsythia suspensa (Thunb.) Vahl	Fructus and semen			0.001		1.29				3.51				
Ligustrum lucidum W.T.Aiton	Fructus and semen					1.74				2.91				
Taraxacum officinale (L.) Weber ex F.H.Wigg.	Herba and others	0.002			0.004	6.82		`		8.62				
Cornus officinalis Siebold and Zucc.	Fructus and semen	0.002				8.05				8.66				
Ziziphus jujuba Mill.	Fructus and semen					1.58				1.83				
Tetradium ruticarpum (A.Juss.) T.G.Hartley	Fructus and semen			0.001	0.004	1.69		6.31	1.18	9.53			1.23E–07	
Schisandra chinensis (Turcz.) Baill.	Fructus and semen					1.07				1.49				
Houttuynia cordata Thunb.	Herba and others		0.002		0.004	1.97				3.42				
Gardenia jasminoides J.Ellis	Fructus and semen					2.93		1.14		4.57				
Citrus × *aurantium* L.	Fructus and semen					1.08				1.28				
Perilla frutescens (L.) Britton	Folium and cortex					2.64		1.02		4.02				
Curcuma longa L.	Radix and rhizoma		0.001				1.02			1.43		1.55E–06		
Coptis chinensis Franch.	Radix and rhizoma													
Lonicera japonica Thunb.	Flos				0.005					2.12				
Chaenomeles lagenaria (Loisel.) Koidz.	Fructus and semen									1.48				
Lonicera confusa DC.	Flos		0.001		0.004					1.81				

The estimated daily intakes (EDI, $mg \cdot kg^{-1} \cdot day^{-1} \cdot bw$) above their corresponding provisional tolerable daily intakes (PTDI) were shown (The PTDIs ($mg \cdot kg^{-1} \cdot day^{-1}$) of As, Hg, Pb, and Cd are 0.00214, 0.00057, 0.00357, and 0.00083, respectively). The non-carcinogenic hazard quotient (HQ) and non-carcinogenic Hazard Index (HI) above one were shown. The carcinogenic risks (CR) higher than 10^{-6}, which means one case of cancer over one million exposed people, is considered unacceptable, thus shown here. All scores in this table were calculated with maximal concentrations of each herbal medicine.

according to our experiment, which is in accordance with the other published results (Ernst, 2002; Kim et al., 2013). In Nigerian herbal remedies, 100% of the samples also contained elevated amounts of heavy metals (Obi et al., 2006), which revealed that the Nigerian herbs contained high levels of Fe, Ni, Cd, Cu, Pb, Se, and Zn sufficient to cause adverse health effect when regularly taken as recommended. In our study, 27 (31.40%) different kinds of herbal medicines, mostly with fructus and semen part with medicinal applications posed unacceptable health risk due to heavy metal accumulation though herba and others were detected with the highest over-limit ratio. Toxic element As posed the most serious health risk according to exposure, carcinogenic and non-carcinogenic risk assessments, as health risk assessment employed by Ren, indicated that As and Pb generated from industrial sites and traffic sites has a potential to pose serious health risks (Saifullah et al., 2015). It was also found that As was the major metal found for water pollution (Alidadi et al., 2019), exceeding its permitted daily exposure dosages and suggested a potential health risk for *Panax notoginseng* (Burkill) F.H.Chen consumers (Eisenberg et al., 2018). Therefore, we can conclude that As might be the one of five heavy metals necessitating special attention, possibly due to various states of As accumulation and absorption from soil and water. Furthermore, Bolan employed a study and asserted that the concentrations of Cd, Hg, and Pb in Ayurvedic medicines exceeded their daily intake amounts (Bolan et al., 2017). According to the study by Lee SD, levels of Cd

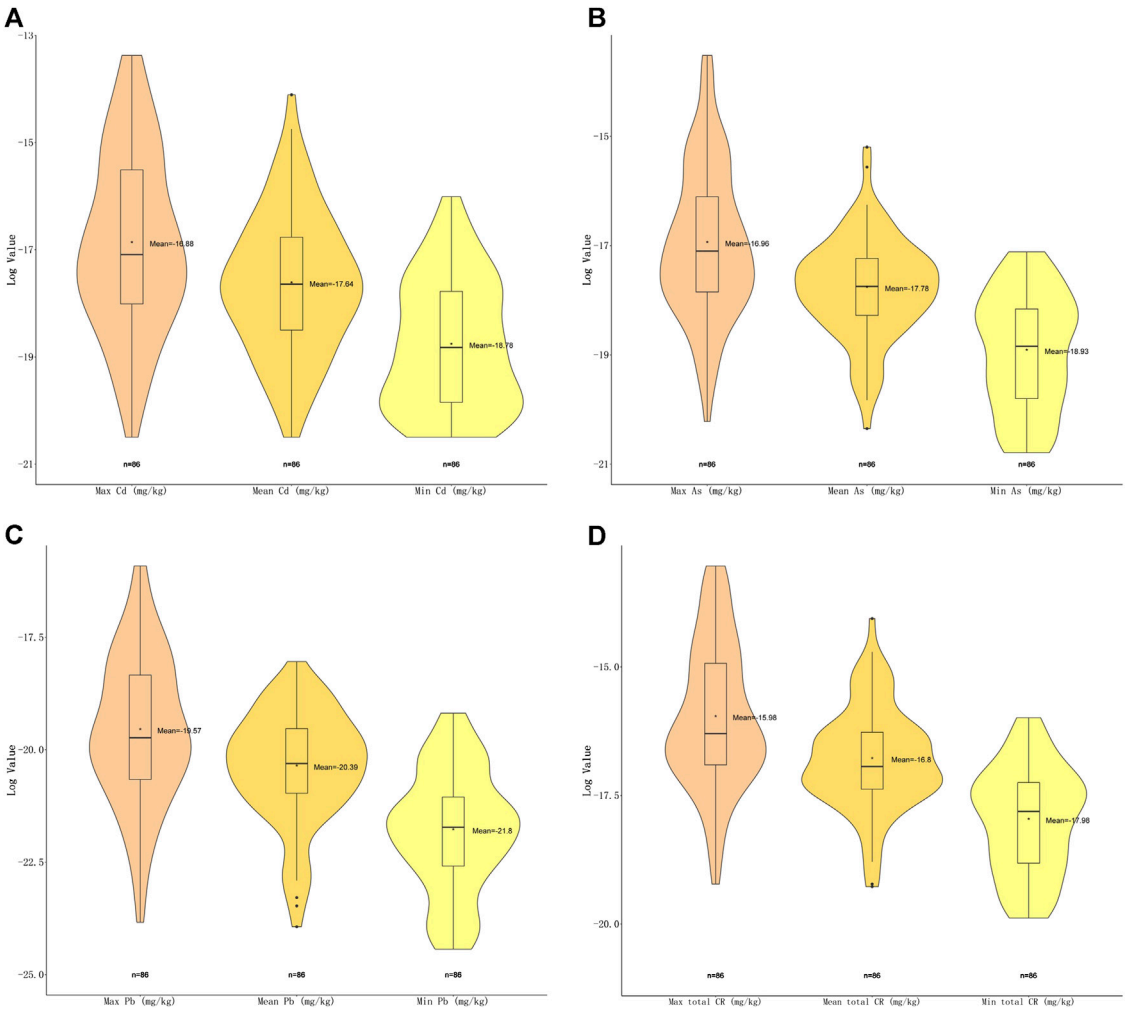

FIGURE 4 | Violin plot of carcinogenic risk (CR) of three carcinogenic metals with maximal, minimal and mean concentrations detected. **(A)** Violin plot showing $\log_2$ (CR) values of Cd with maximal, minimal and mean concentrations. **(B)** Violin plot showing $\log_2$ (CR) values of As with maximal, minimal and mean concentrations. **(C)** Violin plot showing $\log_2$ (CR) values of Pb with maximal, minimal and mean concentrations **(D)** Violin plot showing total $\log_2$ (CR) values with maximal, minimal and mean concentrations.

exceeding WHO reference values were observed in 10 samples and the weekly intakes of Pb, Cd, Cr, Cu, Hg from herbs (Lee et al., 2012). While in Iran, maximum bioaccumulate of Pb and Hg was noted in *artemisia dracunculus* L. and *Spinacia oleracea* L., respectively (Fu et al., 2018). The non-carcinogenic risks target hazard quotients (THQs) of Al and Cr from individual herbs were over 1, which might impart risk for human consumption (Kohzadi et al., 2018). It could be concluded that heavy metals As, Pb, Cd, and Hg all impose significant risk to health due to herbal consumption. The highest HI was presented in herbal medicine *Plantago asiatica* L. (HI = 11.47) and highest CR in *Andrographis paniculata* (Burm.f.) Nees (CR = 2.18E−06), which are in need of special dosage control and monitoring. Furthermore, the highest over-limit ratios of five heavy metals based on different producing areas are Cu (7.69%) in Chongqing, As (20.21%) in Gansu province, Cd (0.77%) in Chongqing, Hg (9.89%) in Hunan province, and Pb (25.00%) in Fujian province (**Supplementary Table S3**). However, Principle Components Analysis (PCA) didn't showcase that there was significant statistic difference regarding the five heavy mental accumulations in five medicinal plant properties. While according to Pearson Correlation Analysis, Pb and As were correlated to flos, folium and cortex, fructus and semen, and herba and others, while Pb and Cd in radix and rhizoma (**Supplementary Figures S1, S2**). Additionally, based on the Analysis of Similarities (ANOSIM), it was indicated that the difference within groups of five medicinal plant properties is less significant than the one throughout the five groups ($R = 0.165$, $p = 0.001$) (**Supplementary Figures S3, S5**).

Heavy metals may induce a variety of pathologies damaging our health. They may also react directly with DNA, inducing a variety of DNA lesions including both DNA strand damage and DNA protein cross-linkage (Harris et al., 2011). Generally, five

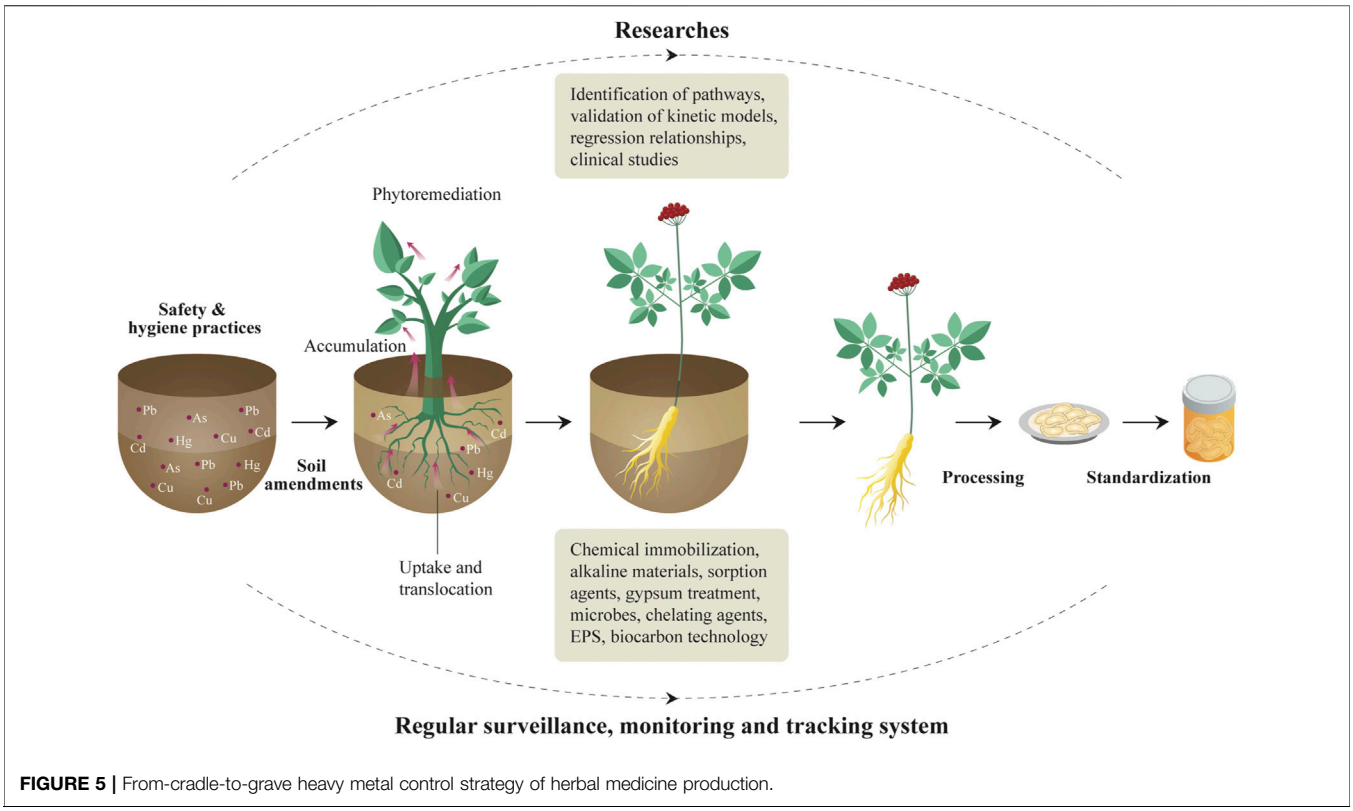

FIGURE 5 | From-cradle-to-grave heavy metal control strategy of herbal medicine production.

reasons explain the levels of heavy metal contents in herbal medicines. The first is the variable exposure to environmental pollution including industrial encroachment, contaminated soil or atmosphere (Filipiak-Szok et al., 2015). The physicochemical properties of soil including pH, temperature, redox potential, translocation exchange capacity and organic matter may influence the availability of metal to plants. Secondly, the phytological characteristics of medical plants themselves such as reduced biomass, root length and shoot length are common indicators of heavy metal toxicity. Furthermore, the interactions of soil-plant roots-microbes play vital roles in regulating heavy metal movement from the soil to edible plant parts. Certain plants are "hyper-accumulators" which grow on metalliferous soils and accumulate extraordinarily high levels of heavy metals without displaying phytotoxic effects. Thirdly, herbal plants could be contaminated during manufacturing and agronomic processes (Oliveira et al., 2018) including growing, harvesting, transportation, processing and storage, due to pesticide formulations, chemical fertilizers and irrigation with poor-quality water. (Harris et al., 2011; Bolan et al., 2017; Filipiak-Szok et al., 2015). For example, Cd and Pb may enter the soil due to fertilizer impurities (Rai, 2012), non-ferrous smelters, lead and zinc mines, sewage slug application and combustion of fossil fuels (Khan et al., 2008). Additionally, fumigants containing heavy metals may also be applied for preventing rats and mildew (Fujita et al., 2016). Fourthly, plant uptake is one of the major routes of dietary exposure to heavy metals in the soil, and the wide variations in metal concentrations in the analyzed herbs could be attributed to differences in the plant metal uptake and translocation capabilities. Studies have shown wide variations in concentration factor for different metals among different plant species and sampling sites. Certain species have higher tendency to accumulate Cd (Harris et al., 2011). Lastly, the bioavailability of heavy metals could have an impact on their concentrations, such as soil pH, the metal levels already resident in the soil, the oxidation-reduction potential of the soil, and other chemical and physical factors (**Supplementary Table S4**).

Here we propose a solution for heavy metal control in herbal medicines (**Figure 5**). We consider, given the results found here and those of others previously there is an urgent need to implement a regular monitoring and surveillance program, controlling extrinsic contamination of herbal medicines along the supply chain from field to consumer (Bolan et al., 2017). Secondly, research, such as identifying ways in which heavy metals reach herbal products; development and validation of kinetic models linking processing techniques with metal speciation and bioavailability; bioavailability tests of heavy metals in herbal medicines; experiments on regression relationships between speciation and bioavailability of heavy metals, clinical studies examining the toxicity of heavy metals, etc. (Harris et al., 2011). Soil amendments, including mitigation and preservation management for the growth performance of biomass and metal accumulation in contaminated soils, is necessary (Kim et al., 2016). Thirdly, international and universal standards related to risk assessments and further permissible limits are in urgent need. For example, the transfer rate of heavy metals varies greatly among medicinal materials, but there is no international guideline that gives a

general rule. While in our research, we calculated all the risks with minimal, mean, and maximal concentrations of five heavy metals detected in each kind of herbal medicines to cover the most-likely scenarios to the worst-case scenarios. Therefore, a universal transfer rate is necessarily needed and will be convenient for the development of international standards. Lastly, tolerant medicinal plants with high phytoremediation potential (Khattak et al., 2015) and capability for phytostabilization and phytoextraction (Kim et al., 2016) can be cultivated as an approach for the management and targeted bio-extraction of heavy metals from moderately polluted lands (Fakhari et al., 2017), together with a combination of different agents such as pH change-inducing chemical immobilization, alkaline materials including lime based materials, fly ash, and biochar, calcite, dolomite, oyster and egg shell (Kim et al., 2016). Sorption agents such as phosphate materials, compost, zeolite and iron compounds, activated carbon, and bentonite, or materials that decrease dissolved organic carbon such as gypsum treatment, Solanum nigrum, microbes, chelating agents, Extracellular polysaccharides or Exopolysaccharides (Rehman et al., 2017), and eco-friendly biocarbon technology (Augustina and Adriana, 2014). These materials increase soil pH, favor deprotonation and the formation of oxides, metal-carbonate precipitates, complexes and secondary minerals that all decrease the phytoavailable heavy metal concentrations (Maiga et al., 2005; Kranthi et al., 2018). Phytoremediation has been perceived to be a more low-cost, low-impact, low-tech alternative, visually benign and environmentally sound comparing to more active and intrusive remedial methods (Augustina and Adriana, 2014).

In conclusion, heavy metal contamination in herbal medicines was borderline or higher than the safety level. There are 30.51% samples detected with at least one over-limit heavy metal according to Chinese Pharmacopoeia (CP, 2020 edition) standards (National Pharmacopoeia Commission, 2020). The risk assessments have demonstrated that the majority (70.93%) of the herbal plants were within acceptable risks. Notably, As posed the highest risk in all indicators including EDI, HI, and CR, inducing the most serious risks in all five metals. Herbal medicines *Tetradium ruticarpum* (A.Juss.) T.G.Hartley, *Plantago asiatica* L., and *Desmodium styracifolium* (Osbeck) Merr. were considered the most risk-inducing herbal medicines. Contamination in herbal medicines is well demonstrated and clearly poses a serious potential risk to health. Furthermore, trace metals play a significant role in reactions which lead to formation of the active chemical plant constituents and are, therefore, responsible in-part for their curative as well as toxic properties. The analysis of toxic metals can be useful to evaluate the dosage of the herbal drugs prepared from these plants (Parveen et al., 2013). Therefore, it is of great advantage to establish universal standards and quality requirements for hazardous elements in herbal medicines so that this natural resource can continue and expand further, to benefit health globally (Luo et al., 2019).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SC and LD conceived the study. LD and LL collected and sorted the samples. BW conducted the heavy metal analysis. LL, JJ, MF, QH, and ZY analyzed the data. LL, JJ, QH, HL, and JZ plotted the figures. LL, JW, CY, and HZ created the tables and supporting materials. SC, LD, and LL contributed to drafting the first version of the manuscript, and all authors proved the final text.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.595335/full#supplementary-material.

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OPINION
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Medicinal Plants and Herbal Products From Brazil: How Can We Improve Quality?

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INTRODUCTION

Brazil has widely diverse flora, rich in medicinal plants, which are an important part of the Amerindian traditional knowledge (Levis et al., 2017). Some Brazilian plants were included decades ago in different Pharmacopoeias because they provide important substances used in medical practice worldwide. Examples are *Carapichea ipecacuanha* (Brot.) L. Andersson (ipecac), source of the emetic and amoebicide alkaloid emetine, and *Pilocarpus microphyllus* Stapf ex Wardlew, source of the antiglaucoma pilocarpine (Nogueira et al., 2010). More recently, Açaí (*Euterpe oleracea* Mart.), native from the Amazon rainforest, became notorious in the international market as a nutraceutical (Carey et al., 2017). Despite its potential, the native vegetation of Brazil has been undergoing intense destruction: all the ecosystems, including the Amazon rainforest, have been quickly replaced by monocultures of sugarcane, soybeans, eucalyptus, and livestock, leading to an intense process of genetic and cultural erosion. On the other side, more recently, the development of bioproducts from Brazilian plants has been stimulated, aiming at a market based in the bioeconomy, which not only brings health benefits but also is important for conservation of biodiversity and consequent mitigation of climate changes (Dinerstein et al., 2020). However, due to the current precarious situation of the herbal products market in Brazil, many steps need to be taken until such a goal is achieved.

QUALITY OF HERBAL PRODUCTS FROM BRAZIL

The denomination herbal products (HP), in Brazil, includes dried plants, sold as tea, and also the finished products used as medicine, nutraceutical, or cosmetics. In this internal market, it is possible to find HP from plant species from three different origins: i) native species that are collected in the local ecosystems, ii) cultivated exotic species, and iii) imported species (mainly dry plants and extracts). HP from i) are mainly commercialized in local markets while HP from ii) and iii) can be found in pharmacies and natural products shops. Since the 1960s, there are regulatory rules for the correct identification of medicinal plants for the commerce in Brazil (Brasil 1967), and more recently, new legislation was launched for regulating the commerce of plants used in traditional knowledge (Carvalho et al., 2018). Despite such efforts, studies done using classical analytical procedures, provided by monographs from Brazilian Pharmacopoeia and Pharmacopoeia from other countries, have shown the existence of serious problems.

Studies with species from the group i) uncovered different problems. Contamination with potentially pathogenic bacteria (enterobacteria and other Gram-negative bacteria) and fungi

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(*Aspergillus* spp. and *Penicillium* spp.) was found in samples of Brazilian ginseng (*Pfaffia glomerata* (Spreng.) Pedersen and *P. paniculata* Mart.) (Zaroni et al., 2004) and "mate" (*Ilex paraguariensis* A.St.-Hil.) (Borges et al., 2002). Adulteration in leaves of "guaco" (*Mikania glomerata* Spreng. and *M. laevigata* Sch. Bip. ex Bak) was detected in different studies that have shown species substitution and presence of other parts of the plant, insects, and sand (Alvarenga et al., 2009; Melo and Sawaya 2015; Palhares et al., 2015). Samples of "guaraná" (*Paullinia cupana* Kunth) presented low levels of methylxanthines and presence of inorganic materials (sand and earth) and nonessential metals (Araújo et al., 2006; Bara et al., 2006; Sousa et al., 2011). Parts of insects and nonessential minerals were also found in samples of "carqueja" (*Baccharis crispa* Spreng) (Ferrante et al., 2007), "pata-de-vaca" (*Bauhinia forficata* Link) (Engel et al., 2008), and "espinheira-santa" (*Maytenus ilicifolia* Mart. ex Reissek, syn. *Monteverdia ilicifolia* (Mart. ex Reissek) Biral) (Leal et al., 2013). Our group also showed that, from a total of 252 samples of native plants with historical use in Brazilian traditional medicine, purchased from popular markets in all the regions of Brazil, only 50.2% corresponded to the original species (Brandão et al., 2013). It is important to note that *P. glomerata*, *P. paniculata*, *I. paraguariensis*, *M. glomerata*, *M. laevigata*, *P. cupana*, *B. crispa*, *B. forficata*, and *M. ilicifolia* are also commercialized in other countries, suggesting that the problems found in the Brazilian market may be present in the international market as well.

More recently, we added DNA barcoding techniques to the classical analytical studies. One of our studies showed that samples of barks of the Brazilian quina (*Remijia ferruginea* (A.St.-Hil.) DC. and *Strychnos pseudoquina* A. St.-Hil.) were substituted by other species without any correlation to traditional medicine (Palhares et al., 2014). In a similar study, we have analyzed 257 commercial samples of the native species i) *M. ilicifolia* and *Mikania* spp. as well as exotic species ii) *Matricaria recutita* L. and *Passiflora incarnata* L. and imported species iii) *Hamamelis virginiana* L., *Panax ginseng* C. A. Mey, *Peumus boldus* Molina, and *Valeriana officinalis* L. This study showed that substitutions may be as high as 71% (Palhares et al., 2015). In a recent review, Ichim (2019) also showed the results of studies using DNA-based methods for species identification in herbal products commercialized in 37 countries. In Brazil, besides our results (Palhares et al., 2015), the author showed that a study using DNA barcode and a wider range of markers (ITS, trnL, trnL-trnF, psbA-trnH, matK, and rbcL) evidenced substitutions in "quebra-pedra" (*Phyllanthus* spp.) (Inglis et al., 2018). In another study done also with *M. ilicifolia* using PCR-RFLP technique, substitution was also an issue (Nakamura et al., 2013). On the other hand, when analyzing the complete ITS/5.8S region in commercial samples of "Brazilian arnica" (*Egletes viscosa* (L.) Less), no substitutions were found (Batista et al., 2012). In his review, Ichim concludes that the highest percentage of adulterated commercial HPs among all countries was reported for Brazil.

In another review, Ichim et al. (2020) show results of studies on authenticity using microscopic analysis on 508 herbal medicines and food supplements traded in thirteen countries or territories. All or at least most (>70%) herbal products were reported to be authentic in Argentina, China, Germany, Thailand, and Egypt. In the United States and Peru, a substantial part (>30%) was wrongly declared, and a third group of countries, comprising Iran, Brazil, India, Turkey, and Greece, showed authenticity score lower than 40%. Overall, almost half (49%) of the total products (n = 167) microscopically authenticated in Asia were reported to be adulterated, followed by South America (40%) and Europe (39%) and more distantly by North America (33%). Other studies also show that very known and used medicinal plants marketed in Europe, Asia, and the United States such as *Hypericum perforatum* L. (Raclariu et al., 2017), *Echinacea* spp. (Raclariu et al., 2018), and *M. recutita* (Guzelmeric et al., 2017) are often adulterated or show low quality. Other problems were described for Ayurvedic herbal products sold in Norway, Romania, and Sweden (Seethapathy et al., 2019), HP sold in Canada and United States (Newmaster et al., 2013), and Traditional Chinese Medicines (TCM) entering the Australian market (Coghlan et al., 2012), among other countries.

DISCUSSION

Considering the current situation of the HP sold in Brazil, some points must be considered to reach the full potential and benefits from it:

(1) Develop Pharmacopoeia Monographs for Brazilian native plants including DNA barcode. In the last years, DNA barcoding from different organisms has gained rapid acceptance in the scientific community from various fields, including studies of plant identification (CBOL Plant Working Group, 2009). Despite DNA barcoding being relatively new, pharmacopoeias around the world, such as Ayurveda (Indian), British, Chinese, and Korean, have already introduced protocols for DNA barcoding authentication. In the last decade, significant advances regarding DNA sequencing were made, making this technique more viable to be used for the purpose of species identification in complex samples. Herbal products may contain several species in their composition, and even when the product is declared to contain only one species, we may encounter adulteration by the addition of other species. The methodological advances in high-throughput sequencing (HTS) were crucial to the analysis of those samples. Different from Sanger sequencing, which has the limitation of only analyzing one sample per reaction, HTS made the parallel sequencing of thousands of samples possible simultaneously in a cost-effective manner, besides being more sensitive and faster (Coghlan et al., 2012; Ivanova et al., 2016). Combining the use of DNA barcoding and HTS, the metabarcoding technique was developed. Metabarcoding is the use of universal PCR primers to mass-amplify barcodes from DNA extracted from complex samples (de Boer et al., 2015). This technique has been used more and more, allowing the study of thousands of samples of HPs simultaneously to evaluate their authenticity and safety (Ivanova et al., 2016; Seethapathy et al., 2019; Urumarudappa et al., 2020). Despite its benefits, DNA

barcode is not able to identify which part of the plant was used for the preparation of HPs or even if the material was well preserved. Therefore, DNA barcoding should be used as a complementary method to improve the quality and safety of HPs (de Boer et al., 2015; Palhares et al., 2015).

(2) List the resilient useful Brazilian species and create a database with different information about them. Adulteration and substitutions may be occurring for several reasons, for example, cross-contamination and fraudulent practices for financial gain. The problem might also be amplified by a lack of inspection by health agencies. Another cause for the substitution of a native species, or even the plant parts used for the HP preparation, is the lack of knowledge about the plants, caused by the genetic and cultural erosion occurring in some regions in Brazil. In fact, studies performed by us in recently deforested regions of Minas Gerais have shown that the population no longer knows or uses native species from traditional medicine (Brandão and Montemór 2008; Prates et al., 2020). In 2010, we created the Dataplamt database (www.dataplamt.org.br) with information about traditional uses of Brazilian plants, recovered from references published until 1950. After this date, the use of native plants has declined, as a consequence of the installation of foreign pharmaceutical industries in Brazil (Manhã et al., 2008). To date, Dataplamt has information on 3,400 Brazilian plant species with more than 150 different uses. In another recent work, we have developed a strategy to identify resilient useful species, that is, plants used for the same purpose along the five centuries of the written history of Brazil (Ricardo et al., 2018). We argue that a database with traditional, pharmacopoeia, chemical, pharmacological, toxicological, and DNA barcode (Liu et al., 2017; Wong et al., 2018) data for these resilient species should be created, in order to support actions on pharmacovigilance. An international cooperative network for researches studying these plants, specially those that are commercialized in other countries, could contribute to filling the lacks.

(3) It is imperative to respect the regulations to protect Amerindian and other traditional communities. Brazil has specific legislation that protects traditional knowledge but there have been few institutional advances to achieve these objectives (Hanazaki et al., 2018). In August 2020, Brazil ratified the Nagoya Protocol, a multilateral agreement accessory to the Convention on Biological Diversity, created during the United Nations Conference on Environment and Development held in Rio de Janeiro in 1992 (Eco-92) (Agência Brasil, 2020). This international legislation regulates access and benefit-sharing and aims at the intellectual property protection of genetic resources and traditional knowledge. To defined measures to comply with the rules and especially to protect Amerindian knowledge are urgent and necessary.

(4) It is necessary to teach the society to value and protect the Brazilian biodiversity. Ichim (2019) showed that adulteration in HP marketed in Asia and Africa is less common than in other countries. This occurs because in these continents traditional medicines are strongly recognized by population. Since 2014, our research group is also doing a set of works with school teachers and students living in small cities, showing them the importance of plant biodiversity and traditional knowledge associated with them (Prates et al., 2020). It is strongly necessary to promote such activities in all regions of Brazil; nobody protects or values what they do not know!

CONCLUSION

The adulteration found in Brazilian HP reflects a trend that can be seen throughout the world. The points listed can contribute to improving their quality, especially those from native Brazilian species.

AUTHOR CONTRIBUTIONS

All authors contributed to the bibliographic survey and preparation of the manuscript, each writing about their area of knowledge. MGLB was also responsible for coordinating the group and funding acquisition.

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A Novel Method to Identify Three Quality Grades of Herbal Medicine Ophiopogonis Radix by Microscopic Quantification

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Maidong, the root tuber of *Ophiopogon japonicus* (Thunb.) Ker Gawl., is a commonly used herbal medicine in China. There are three quality grades of Maidong according to traditional opinion and modern research studies: superior quality (Zhe-Maidong), medium quality (Chuan-Maidong), and poorest quality (Chuan-Maidong with paclobutrazol, which is a kind of plant growth regulator). However, no efficient way to distinguish the three quality grades of Maidong exists; thus, the herbal markets and botanical pharmacies are flooded with Chuan-Maidong with paclobutrazol. To ensure the safety and quality of Maidong, a comparative microscopic study was performed on three quality grades of Maidong. The result was to establish a microscopic quantification method based on the area ratio between xylem and pith to distinguish the three quality grades of Maidong. Subsequently, Maidong from regional markets was evaluated by this method. In this study, we developed a novel quantification method to identify the three quality grades of Maidong, which could in turn make efforts on the quality improvement of Maidong. Our study is the first to demonstrate that microscopic technology could be used to distinguish different quality grades of a specific herbal medicine.

Keywords: light microscope microscopy, herbal medicine, botanical identification, quality identification, microscopic quantification, ophiopogonis radix

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INTRODUCTION

Maidong (Ophiopogonis Radix), which is the dried root tuber of *Ophiopogon japonicus* (Thunb.) Ker Gawl (family: Liliaceae) (the State Pharmacopoeia Committee of China, 2020), is one of the most important herbal medicines and has been used for over 2000 years in China. Maidong has been approved as a functional food ingredient (Lin et al., 2011) and as the main ingredient in patented drugs, such as ShenMai granule and XuanMai Gan Jie capsule/granule (the State Pharmacopoeia Committee of China, 2020). Recent studies have shown that Maidong mainly contains polysaccharides, steroidal saponins, and homoisoflavonoids (Nguyen et al., 2003; Chen et al., 2011; Zhang et al., 2012; Chen et al., 2016). Pharmacological research has revealed the anti-inflammatory, antioxidant, immunoregulatory, cardiovascular protective, and venous thrombosis inhibition effects of Maidong (Kou et al., 2005a; Kou et al., 2005b; Xiong et al., 2011; Zhang et al., 2016). Historically, the dried root tubers of *Liriope spicata* Lour. and L. *muscari* (Decne.) L. H. Bailey were used as Maidong, which resulted in nomenclatural confusion in herbal markets. In 2010, the

microscopic characteristics have been described and helped distinguish authentic Maidong from the adulterant species (Mo, 2010). Therefore, distinguishing genuine or counterfeit Maidong is no longer an important issue.

The quality of Maidong is noteworthy and is influenced by at least two factors. The first influencing factor is the cultivation region. There are two main cultivation regions of Maidong in China: Zhejiang province and Sichuan province. Maidong cultivated in Zhejiang province is called Zhe-Maidong; that cultivated in Sichuan province is called Chuan-Maidong. Zhe-Maidong has been widely recognized as "daodi medicinal material," a quality standard indicating that it has superior quality compared with Chuan-Maidong (Zhao et al., 2012; Li et al., 2016b). Modern research found that ophiopogonin B and ophiopogonin D contents in the tubers of Zhe-Maidong were higher than those in the tubers of Chuan-Maidong (Li et al., 2016a). Moreover, Zhe-Maidong showed higher promoting rates in macrophage phagocytosis and gastrointestinal motility than Chuan-Maidong, suggesting that the former has stronger immunomodulatory activities (Lu et al., 2017). The second influencing factor is the cultivation mode. According to our field survey, Zhe-Maidong needs 3-4 years for growth in the field, only a few of Chuan-Maidong is cultivated for 2 years, and most of Chuan-Maidong is cultivated for 1 year only and is overdosed with the plant growth regulator paclobutrazol to increase the yield two to three times. Based on a previous report, a flavonoid and four steroidal saponins were significantly decreased in Maidong after spraying paclobutrazol. In addition, different levels of paclobutrazol residue were detected in Maidong, soil and water samples, and the detection rate of paclobutrazol in Maidong was 100% (Zhang et al., 2019). The indiscriminate use of paclobutrazol leads to a growing concern about its safety.

When analyzing genetic differences between species, molecular biology techniques mainly rely on gene sequence comparison and calculation of conserved regions on chloroplasts and mitochondria. When analyzing genetic differences within species, biologists generally use gene expression profiles, but the long storage of herbal medicine makes it difficult to obtain plant gene expression profiles, which greatly limits the possibility of using genetic analysis to classify the quality grades of herbal medicine. Moreover, techniques based on chemical component analysis to identify the three quality grades have not been established, and thus no method to distinguish the different quality grades of Maidong exists. Based on this situation, Zhe-Maidong and Chuan-Maidong could barely be found in markets and Chuan-Maidong with paclobutrazol is rampant in the market, thereby reducing the medicinal quality of Maidong. In addition, the long planting cycle of Zhe-Maidong minimized the farmers' economic interests; superior germplasm resource of Zhe-Maidong is reducing and vanishing gradually (Li et al., 2016b). Therefore, establishing a method to distinguish the three quality grades of Maidong is vital.

Microscopic technology has the advantages of speed, simplicity, reliability, requires small samples amounts, and is low cost. This method is applied in many pharmacopoeias (Zhao et al., 2006) and has been successfully used to identify herbal medicines and authenticate Chinese prescriptions (Liu et al., 2011; Kang et al., 2012; Xu et al., 2015). Moreover, histological techniques based on microscopic examination have been used to reveal the characteristics of tissue structure and arrangement of cells that could be used as markers for identifying original sources of plant-derived drugs, such as *Aloe vera* var. *chinensis* (Shen et al., 2001) and *Dendrobium officinale* Kimura et Migo (Yu et al., 2017). While microscopic and histological studies in quantitative analysis are limited, they are effective. For example, Wuzhimaotao (Radix Fici Hirtae) was identified by laticifer quantification (Au et al., 2009). However, distinguishing the same herbal medicine from different cultivation locations and with different cultivation modes based on microscopic techniques is not been developed yet, and the histological differences due to plant growth regulators have not been reported completely.

In this study, we established an analytical method to quantify microscopic characteristics; the quantification method was subsequently applied to examine Maidong from different cultivation regions and with different cultivation modes. This study aimed to provide scientific and objective data using microscopic quantification to identify the quality grades of Maidong, which could in turn promote the use of the best herbs and thus ensure the effectiveness and safety of Maidong.

MATERIALS AND METHODS

Plant Material

Eighteen batches of Maidong, including four batches of Zhe-Maidong, four batches of Chuan-Maidong, and ten batches of Chuan-Maidong with paclobutrazol, from the field were used in this study. The details of each sample are presented in **Table 1**. To validate the method established by this study and evaluate the quality of Maidong in market, twelve batches of Maidong were obtained from herbal markets and botanical pharmacies in different provinces in China (**Table 2**). All samples were authenticated by Dr Shuai Kang (Institute for Control of Chinese Traditional Medicine and Ethnic Medicine, National Institutes for Food and Drug Control), and the voucher specimen (no. CH-056-100) was deposited in the National Institute for the Control of Pharmaceutical and Biological Products, National Institutes for Food and Drug Control, Beijing, China.

Apparatus

All transverse sections of the materials were prepared using Leica Jung Biocut 2035 (Leica Instruments, Germany). A light microscope (Olympus BX51, Japan) equipped with an Olympus DP71 digital camera (Olympus, Tokyo, Japan) was used for image acquisition. Images were processed with a Zeiss AX10 equipped with a Zeiss AxioCam ICc five camera and analyzed by ZEN 2.3 lite (Zeiss, Germany). Area was measured in μm^2.

Liquid chromatography/mass spectrometry (LC/MS) analysis was conducted using Agilent 1200 and Agilent 6410 Triple Quad LC/MS systems (Agilent Technologies, Santa Clara, CA,

TABLE 1 | Data of the three quality grades of Maidong.

Maidong	Source	Batch no.	Collection area	GPS coordinate	Collection date
Zhe-maidong	Field	Z1	Hangzhou, Zhejiang	E 118°753′, N 29°124′	March 2013
		Z2	Cixi, Zhejiang	E 121°279′, N 30°175′	September 2010
		Z3	Cixi, Zhejiang	E 122°563′, N 31°564′	May 2006
		Z4	Cixi, Zhejiang	E 121°751′, N 30°945′	August 2009
Chuan-maidong	Field	C1	Nanchong, Sichuan	E 105°367′, N 30°575′	April 2018
		C2	Nanchong, Sichuan	E 105°624′, N 30°863′	April 2018
		C3	Nanchong, Sichuan	E 106°157′, N 31°116′	April 2018
		C4	Nanchong, Sichuan	E 105°421′, N 31°254′	May 2018
Chuan-maidong with paclobutrazol	Field	CP1	Mianyang, Sichuan	E 104°561′, N 30°577′	November 2015
		CP2	Mianyang, Sichuan	E 105°241′, N 31°784′	August 2018
		CP3	Mianyang, Sichuan	E 103°875′, N 32°218′	August 2018
		CP4	Mianyang, Sichuan	E 105°116′, N 32°511′	August 2018
		CP5	Mianyang, Sichuan	E 104°335′, N 31°782′	August 2018
		CP6	Mianyang, Sichuan	E 104°951′, N 30°756′	April 2018
		CP7	Mianyang, Sichuan	E 105°223′, N 32°155′	April 2018
		CP8	Mianyang, Sichuan	E 104°596′, N 31°321′	April 2018
		CP9	Mianyang, Sichuan	E 105°332′, N 32°544′	April 2018
		CP10	Mianyang, Sichuan	E 103°651′, N 30°965′	May 2015

TABLE 2 | Data of Maidong from the herbal markets and botanical pharmacies.

Batch no.	Collection area	Collection date
MS1	Chengdu, Sichuan; herbal market	May 2017
MS2	Hefei, Anhui; botanical pharmacies	May 2017
MS3	Shanghai; botanical pharmacies	May 2017
MS4	Mianyang, Sichuan; herbal market	May 2017
MS5	Wuhan, Hubei; botanical pharmacies	June 2017
MS6	Anguo, Hebei; herbal market	June 2017
MS7	Shijiazhuang, Hebei; botanical pharmacies	Nov 2020
MS8	Beijing; botanical pharmacies	Nov 2020
MS9	Bozhou, Anhui; herbal market	Nov 2020
MS10	Bozhou, Anhui; herbal market	Nov 2020
MS11	Guangzhou, Guangdong; herbal market	Nov 2020
MS12	Foshan, Guangdong; botanical pharmacies	Nov 2020

United States). A high-performance liquid chromatography (HPLC) analytical column (4.6 × 250 mm, 5.0 μm, ZORBAX SB-C18, Agilent, United States) was used at 40°C.

Reagents

Chloral hydrate test solution was prepared using 50 g of chloral hydrate powder (Sinopharm Chemical Reagent, China), 15 ml of distilled water, and 10 ml of glycerinum (Sinopharm Chemical Reagent, China). Phloroglucinol test solution, which is a classic dye used to stain lignified cell walls red, was prepared using 0.5 g of phloroglucinol powder (Sinopharm Chemical Reagent, China) and 25 ml of 95% ethanol (Sinopharm Chemical Reagent, China). Diluted glycerin was prepared using 33 ml of glycerinum and 67 ml of distilled water. After the preparation, the aforementioned three solutions were filtered into dark-colored bottles and kept at room temperature. A paclobutrazol standard was purchased from Aladdin Industrial Corporation (P109932-250 mg, Shanghai, China); HPLC-grade acetonitrile were purchased from Sinopharm Chemical Reagent. Pure water was prepared using a Milli-Q water purification system (Millipore, Burlington, MA, United States).

Preparation of Standard and Market Sample Solution for LC/MS Analysis

The reference compound of paclobutrazol was weighed accurately and dissolved in acetonitrile to produce standard solutions. The samples from herbal markets and botanical pharmacies were powdered, and each powdered sample of 2 g (accurately weighed) was ultrasonicated with 20 ml of acetonitrile containing 0.1% formic acid for 30 min. Each solution was centrifuged at 3,000 rpm for 10 min. The supernatant was saved and filtered through a 0.45 μm filter for qualitative analysis.

METHOD

For each batch of root tuber of *O. japonicus*, four samples were investigated. To investigate the microscopic characteristics of each sample in different section locations, tissues from the middle, upper, and lower sampling sites were sectioned and compared (**Figure 1**).

Samples were sectioned using Leica Jung Biocut 2035 to 30 μm thickness. Slides were prepared by adding one to two drops of chloral hydrate solution, rapidly heating to boiling repeatedly, and cleaning away excess chloral hydrate solution. One drop of phloroglucinol solution and hydrochloric acid stain, which was allowed to sit for 3 min, was used to stain lignified cells, and excess solution on the slide was washed away with diluted glycerin. Thereafter, the slides were sealed with diluted glycerin and observed under a microscope. The xylem and pith area of each sample was measured with ZEN 2.3 lite, and then data were analyzed using Statistical Package for the Social Sciences (SPSS).

The conditions for chromatographic separations were as follows: mobile phase consisted of water containing 0.1% formic acid and acetonitrile, and the isocratic elution program was 20% acetonitrile (20 min). The flow rate was

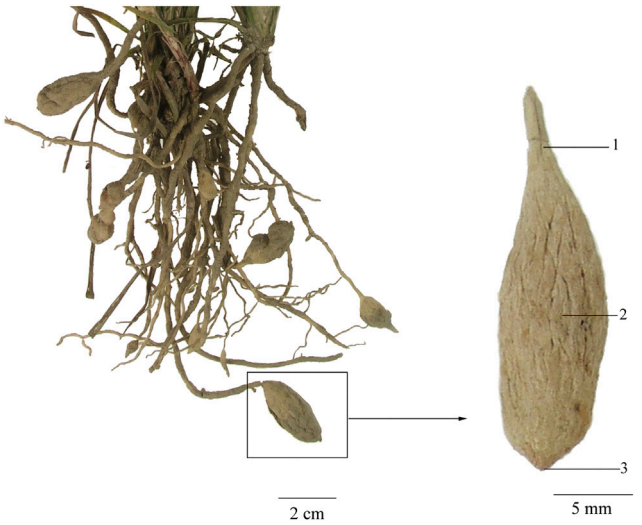

FIGURE 1 | Sampling sites of Maidong: 1) upper site, 2) middle site, and 3) lower site.

0.3 ml/min, and the injection volume was 5 µl. Mass spectra were detected in the positive mode. Moreover, the source parameters were as follows: dry gas (N_2) temperature 350°C; flow rate 8 L/min; sheath gas flow 8 L/min with heater at 350°C; nebulizer pressure 45 psi; and capillary voltage 3500 V. The dwell time for each ion pair was 20 ms, and each sample was analyzed in triplicate.

RESULTS

Testing and Identifying Field Samples

Transverse section: Velamen consisting of 3–5 layers of lignified cells and cortex broad, showing scattered mucilage cells containing raphides of calcium oxalate, which seldom thickened to 5–10 µm in diameter; endodermal cells with evenly thickened and lignified walls, with subrounded cell cavity; and a layer of stone cells lying at the outside of endodermis, the inner and lateral walls thickened, and finely and densely pitted. Stele is relatively small, and 16–22 phloem bundles were noted. Protoxylem stellate and metaxylem linking up in a ring were observed. Pith cells were small, and parenchymatous cells were subrounded. According to Mo (Mo, 2010), thickened raphides of calcium oxalate, subrounded cell cavity of endodermal cells, and number of phloem bundles are the three main characteristics that distinguish genuine from adulterated Maidong (**Figure 2**).

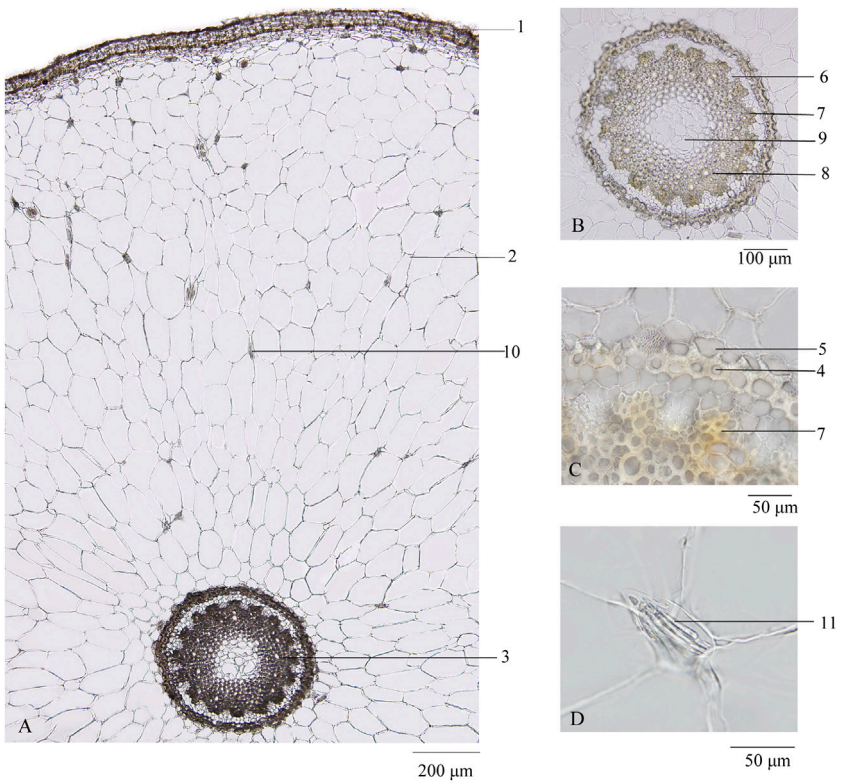

FIGURE 2 | Transverse section of Maidong: **(A)** root transverse section; **(B)** stele; **(C)** endodermis; **(D)** thickened raphides of calcium oxalate. 1) Velamen, 2) cortex, 3) stele, 4) endodermis, 5) stone cells, 6) phloem, 7) protoxylem, 8) metaxylem, 9) pith, 10) raphides of calcium oxalate, and 11) thickened raphides of calcium oxalate.

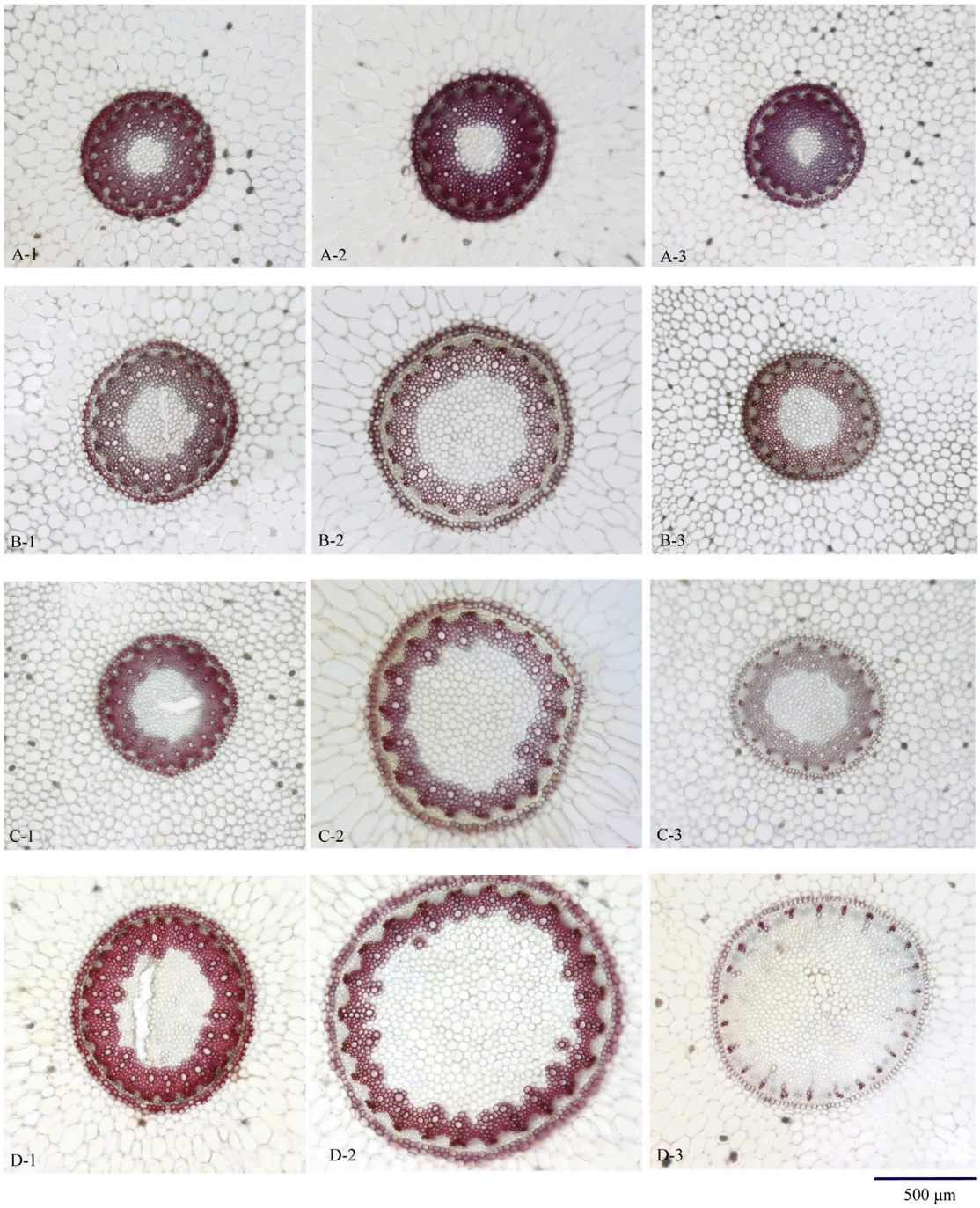

FIGURE 3 | Transverse section of Maidong from different sampling sites: **(A)** Zhe-Maidong, **(B)** Chuan-Maidong, and **(C,D)** Chuan-Maidong with paelobutrazol. 1) Upper site, 2) middle site, and 3) lower site.

The transverse sections of all the samples were investigated to ensure accuracy of the species.

Investigation of Sampling Sites

To explore the influence of microscopic characteristics by sampling sites, the upper, middle, and lower sites were investigated separately (**Figure 1**).

Across all samples, the velamen and cortex were stable and showed no difference among the three quality grades of Maidong. Thus, this study focused on the microscopic characteristics of the endodermis and stele.

Zhe-Maidong

The diameter of stele and pith in the upper and lower sampling sites was smaller than that in the middle sampling sites.

Microscopic elements including the thickness and lignification of endodermis and stone cells, the shape of protoxylem, and lignification of xylem showed no difference among the upper, lower, and middle sampling sites (**Figure 3A**, A-1, 2, 3).

Chuan-Maidong

The diameter of stele and pith in the upper and lower sampling sites was smaller than that in the middle sampling sites. The shape of the protoxylem in the upper and lower sampling sites was clearly distinct; the protoxylem in the upper sampling site was obtusely rounded and that in the lower sampling site showed acute angles. The thickness and lignification of endodermis and stone cells and the lignification of xylem showed no difference among the upper, lower, and middle sampling sites. In addition, the pith area was larger in Chuan-Maidong than in Zhe-Maidong, and the thickness and lignification of endodermis and stone cells were lower in Chuan-Maidong than in Zhe-Maidong (**Figure 3B**, B-1, 2, 3).

Chuan-Maidong With Paclobutrazol

The diameter of stele and pith in the upper and lower sampling sites was smaller than that in the middle sampling site. The shape of the protoxylem in the upper and lower sampling sites was clearly distinct. The protoxylem in the upper sampling site was obtusely rounded and that in the lower sampling site showed acute angles (**Figure 3C**, C-1, 2, 3). Furthermore, the metaxylem was undeveloped and could not link up to a ring in 48% (19 of 40) of the Chuan-Maidong with paclobutrazol (**Figure 3D**, D-3). The thickness and lignification of endodermis and stone cells and the lignification of xylem in the lower sampling site were much less than those in the upper and middle sampling sites. In addition, only a few stone cells with thickened and lignified cell walls were located outside the endodermis in middle and lower sampling sites in some of the Chuan-Maidong with paclobutrazol samples. The pith area was larger in Chuan-Maidong with paclobutrazol than in Zhe-Maidong, and the thickness and lignification of endodermis and stone cells in Chuan-Maidong with paclobutrazol were less than those of Zhe-Maidong (**Figure 3**).

Microscopic Quantification

The microscopic characteristics of Maidong were influenced by the sampling sites in our study (see Investigation of Sampling Sites section). Thus, in the comparative study among the three quality grades of Maidong, the microscopic characteristics in the middle sampling site were investigated.

As shown in **Figures 3A–D** (A-2, B-2, C-2, D-2), the pith and xylem area are clearly different among the three quality grades of Maidong. After staining with phloroglucinol test solution, only the xylem was stained red; thus, examining the boundary between xylem and pith/phloem was easy. The actual area of the selected scope was measured using the analysis function of ZEN software (**Figure 4**).

The pith and xylem areas in the middle sampling site of all samples were measured and calculated (**Table 3**). The pith area ranged from 24,675 to 188,958 μm^2 for Zhe-Maidong, 140,699 to 383,775 μm^2 for Chuan-Maidong, and 264,706 to 1,114,243 μm^2 for Chuan-Maidong with paclobutrazol. The xylem area ranged from 152,195 to 446,337 μm^2 for Zhe-Maidong, from 232,651 to 446,472 μm^2 for Chuan-Maidong, and from 214,702 to

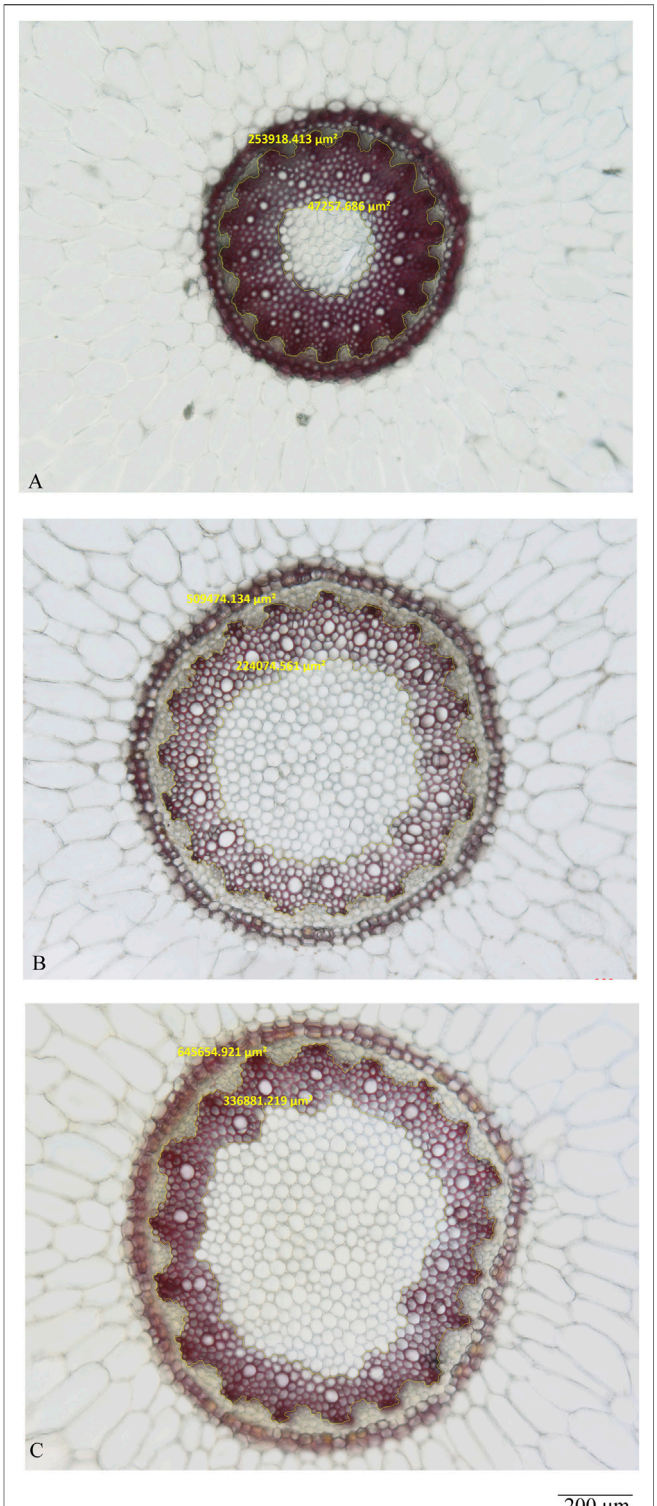

FIGURE 4 | Measurement analysis by ZEN of the middle site: **(A)** Zhe-Maidong, **(B)** Chuan-Maidong, and **(C)** Chuan-Maidong with paclobutrazol.

TABLE 3 | Measurement data of the three quality grades of Maidong in the middle sampling site.

Sample no.	Ia	IIb	IIIc	III/Id	Sample no.	I	II	III	III/I
Z1-1	81,090	358,251	277,161	3.42	CP2-1	469,260	814,575	345,315	0.74
Z1-2	47,258	253,918	206,660	4.37	CP2-2	338,453	642,992	304,539	0.90
Z1-3	36,115	250,914	214,799	5.95	CP2-3	388,940	662,765	273,825	0.70
Z1-4	188,958	635,295	446,337	2.36	CP2-4	518,334	858,267	339,933	0.66
Z2-1	170,782	512,002	341,220	2.00	CP3-1	604,054	961,707	357,653	0.59
Z2-2	114,201	454,009	339,808	2.98	CP3-2	673,572	1,160,622	487,050	0.72
Z2-3	116,845	380,068	263,223	2.25	CP3-3	626,398	993,179	366,781	0.59
Z2-4	24,675	215,227	190,552	7.72	CP3-4	917,850	1,364,305	446,455	0.49
Z3-1	157,485	470,416	312,931	2.00	CP4-1	870,824	1,253,498	382,674	0.44
Z3-2	29,963	182,158	152,195	5.08	CP4-2	636,175	958,408	322,233	0.51
Z3-3	96,574	376,058	279,484	2.89	CP4-3	540,641	865,545	324,904	0.60
Z3-4	107,117	392,831	285,714	2.67	CP4-4	293,372	559,209	265,837	0.91
Z4-1	145,391	476,613	331,222	2.28	CP5-1	515,175	958,198	443,023	0.86
Z4-2	126,981	382,109	255,128	2.00	CP5-2	341,307	592,817	251,510	0.74
Z4-3	165,293	496,785	331,492	2.00	CP5-3	494,032	857,986	363,954	0.74
Z4-4	115,890	453,956	338,066	2.92	CP5-4	264,706	499,548	234,842	0.89
C1-1	224,075	509,474	285,399	1.27	CP6-1	443,379	799,688	356,309	0.80
C1-2	255,424	534,656	279,232	1.10	CP6-2	408,180	743,671	335,491	0.82
C1-3	227,877	526,606	298,729	1.31	CP6-3	280,642	550,861	270,219	0.96
C1-4	140,699	373,350	232,651	1.65	CP6-4	679,345	979,165	299,820	0.44
C2-1	285,660	634,466	348,806	1.22	CP7-1	471,043	829,813	358,770	0.76
C2-2	374,893	799,515	424,622	1.13	CP7-2	437,266	785,005	347,739	0.80
C2-3	383,775	830,247	446,472	1.16	CP7-3	592,120	970,300	378,180	0.64
C2-4	334,601	660,323	325,722	0.97	CP7-4	336,881	645,655	308,774	0.92
C3-1	230,592	571,813	341,221	1.48	CP8-1	298,457	586,455	287,998	0.96
C3-2	210,726	522,545	311,819	1.48	CP8-2	363,258	654,104	290,846	0.80
C3-3	368,176	802,156	433,980	1.18	CP8-3	785,879	1,155,160	369,281	0.47
C3-4	299,166	659,963	360,797	1.21	CP8-4	721,453	1,012,287	290,834	0.40
C4-1	197,351	460,312	262,961	1.33	CP9-1	769,307	1,174,854	405,547	0.53
C4-2	210,724	490,761	280,037	1.33	CP9-2	746,678	1,166,125	419,447	0.56
C4-3	160,184	421,191	261,007	1.63	CP9-3	978,576	1,374,474	395,898	0.40
C4-4	258,882	532,082	273,200	1.06	CP9-4	1,114,243	1,606,081	491,838	0.44
CP1-1	583,132	1,005,278	422,146	0.72	CP10-1	465,625	802,405	336,780	0.72
CP1-2	951,800	1,665,864	714,064	0.75	CP10-2	525,828	933,723	407,895	0.78
CP1-3	329,114	543,816	214,702	0.65	CP10-3	387,022	674,291	287,269	0.74
CP1-4	450,713	750,615	299,902	0.66	CP10-4	320,777	584,877	264,100	0.82

[a]*The area of pith.*
[b]*The area of xylem plus pith.*
[c]*The area of xylem (*II *minus* I*).*
[d]*The area ratio between xylem and pith.*

714,064 μm^2 for Chuan-Maidong with paclobutrazol. The range of the area ratio between xylem and pith was as follows: 2.00–7.72 for Zhe-Maidong, 0.97–1.65 for Chuan-Maidong, and 0.40–0.96 for Chuan-Maidong with paclobutrazol.

After factoring in the area ratio of each sample, the mean and standard deviation (SD) of the three grades of Maidong were calculated (**Table 4**). A chart was generated to show the relationship of the area ratio between xylem with the pith and three quality grades of Maidong. According to these numeric values, the chart indicates a method by which the three grades of Maidong could be quickly and efficiently distinguished by the area ratio between xylem and pith (**Figure 5**).

Variance analysis of the area ratio between xylem and pith of the three quality grades of Maidong was performed with SPSS ($F = 62.842$, $p < 0.001$). Results show a highly significant difference between Zhe-Maidong and Chuan-Maidong and Zhe-Maidong and Chuan-Maidong with paclobutrazol (both $p < 0.001$) and a significant difference between Chuan-Maidong and Chuan-Maidong with paclobutrazol ($p = 0.041$).

TABLE 4 | Mean and SD of the area ratio between xylem and pith of each grades.

Maidong	Area ratio between xylem and pith
Zhe-Maidong	3.306 ± 1.663 (mean ± SD, n = 16)
Chuan-Maidong	1.282 ± 0.196 (mean ± SD, n = 16)
Chuan-Maidong with paclobutrazol	0.691 ± 0.161 (mean ± SD, n = 40)

Evaluation Maidong in Herbal Markets and Botanical Pharmacies

Twelve batches of Maidong obtained from Chinese herbal markets and botanical pharmacies were analyzed. All samples

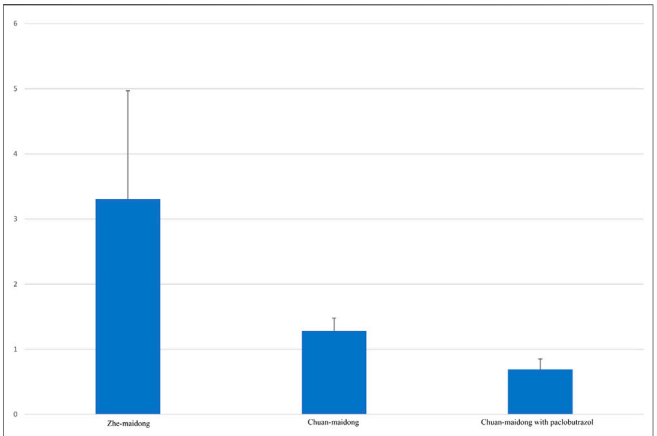

FIGURE 5 | Area ratio between xylem and pith in different quality grades of Maidong. The values are mean ± SD, n = 16 in Zhe-Maidong and Chuan-Maidong and n = 40 in Chuan-Maidong with paclobutrazol.

were crude drugs without cultivation information. Results showed that the area ratio between xylem and pith of all samples ranged 0.41–1.06 (**Table 5**).

Moreover, the area ratio between xylem and pith was pairwise compared between Maidong in the market and three grades of Maidong with SPSS: Maidong in the market vs. Zhe-Maidong ($p < 0.001$), Maidong in the market vs. Chuan-Maidong ($p < 0.001$), and Maidong in the market vs. Chuan-Maidong with paclobutrazol ($p = 0.915$). Data of the market samples were not significantly different from those of Chuan-Maidong with paclobutrazol. Therefore, it was deduced that all the samples from the market were Chuan-Maidong with paclobutrazol.

Method Validation by LC/MS

To validate the accuracy of the method to distinguish the three quality grades of Maidong based on microscopic quantification, the market samples were tested by LC/MS. Qualitative analysis of paclobutrazol was conducted using the multiple reaction monitoring mode, in which monitoring of precursor ion to product ion transitions of m/z 294 → m/z 70 and m/z 125 for paclobutrazol was performed. The MS spectra of the analytes are shown in **Figure 6**. Results revealed that the twelve batches of Maidong from the herbal markets and botanical pharmacies had the same ion chromatograms as those of paclobutrazol standard solutions, thereby confirming that Maidong samples from the market were Chuan-Maidong with paclobutrazol. The LC/MS findings proved the accuracy of microscopic quantification in identifying the quality grades of Maidong.

Method Extension

This study established an analytical method to identify and evaluate three quality grades of Maidong using the ZEISS microscope and ZEN software. The critical quantification information is the area ratio between xylem and pith. The ratio is a relative rather than an absolute value.

TABLE 5 | Measurement data of Maidong from the herbal markets and botanical pharmacies.

Sample no.	I[a]	IIb	IIIc	III/Id	Sample no.	I	II	III	III/I
MS1-1	761,648	1,160,868	399,220	0.52	MS7-1	367,018	584,475	217,457	0.59
MS1-2	349,188	568,929	219,741	0.63	MS7-2	493,800	766,032	272,232	0.55
MS1-3	420,024	751,610	331,586	0.79	MS7-3	406,414	679,591	273,177	0.67
MS1-4	1,172,162	1,650,899	478,737	0.41	MS7-4	698,200	1,117,552	214,176	0.61
MS2-1	501,344	786,097	284,753	0.57	MS8-1	419,892	653,733	233,841	0.56
MS2-2	335,461	595,213	259,752	0.77	MS8-2	431,640	764,408	332,768	0.77
MS2-3	497,161	826,628	329,467	0.66	MS8-3	714,762	1,349,919	635,157	0.89
MS2-4	408,744	695,316	286,572	0.70	MS8-4	424,400	741,024	316,624	0.75
MS3-1	852,114	1,300,519	448,405	0.53	MS9-1	660,896	1,052,320	391,424	0.59
MS3-2	364,316	623,910	259,594	0.71	MS9-2	510,832	774,384	263,552	0.52
MS3-3	341,386	556,751	215,365	0.63	MS9-3	482,832	712,928	230,096	0.48
MS3-4	335,953	644,354	308,401	0.92	MS9-4	342,560	634,768	292,208	0.85
MS4-1	272,036	493,928	221,892	0.82	MS10-1	834,652	1,360,482	525,830	0.63
MS4-2	568,621	948,040	379,419	0.67	MS10-2	675,437	1,154,997	479,560	0.71
MS4-3	488,984	728,193	239,209	0.49	MS10-3	413,458	748,358	334,900	0.81
MS4-4	333,198	630,627	297,429	0.89	MS10-4	365,905	684,242	318,337	0.87
MS5-1	456,148	743,759	287,611	0.63	MS11-1	254,019	497,877	243,858	0.96
MS5-2	435,293	679,153	243,860	0.56	MS11-2	453,317	788,771	335,454	0.74
MS5-3	313,637	555,578	241,941	0.77	MS11-3	543,809	919,037	375,228	0.69
MS5-4	147,068	304,076	157,008	1.07	MS11-4	379,567	713,586	334,018	0.88
MS6-1	258,416	490,179	231,763	0.90	MS12-1	756,490	1,202,819	446,329	0.59
MS6-2	365,085	592,346	227,261	0.62	MS12-2	694,456	1,159,741	465,285	0.67
MS6-3	430,287	687,642	257,355	0.60	MS12-3	574,239	970,463	396,224	0.69
MS6-4	244,198	479,189	234,991	0.96	MS12-4	338,910	647,318	308,408	0.91

[a]*The area of pith.*
[b]*The area of xylem plus pith.*
[c]*The area of xylem (*II *minus* I*).*
[d]*The area ratio between xylem and pith.*

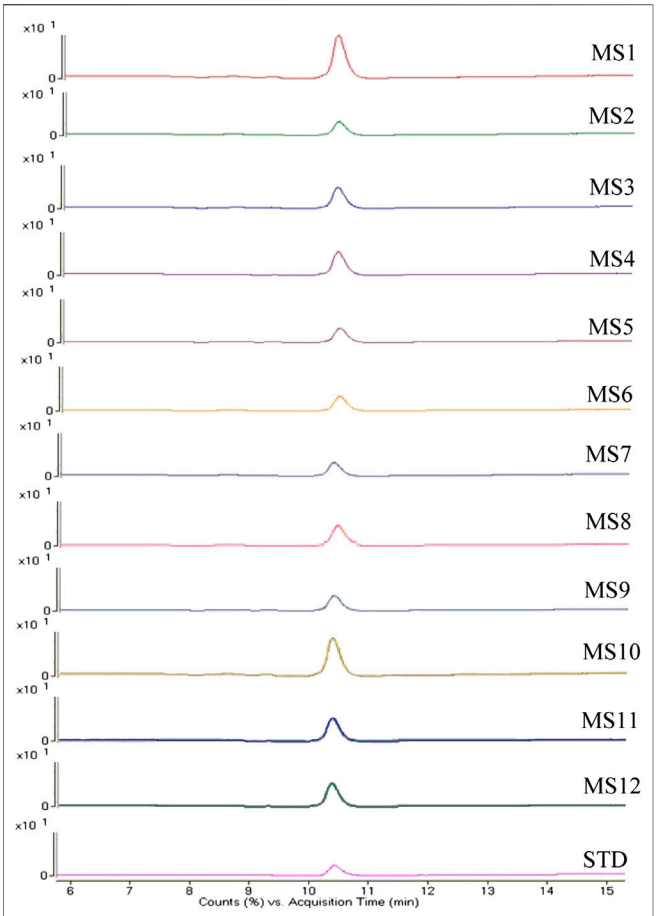

FIGURE 6 | Mass spectrometry (MS) spectra of paclobutrazol in herbal markets and botanical pharmacies samples and standard substance.

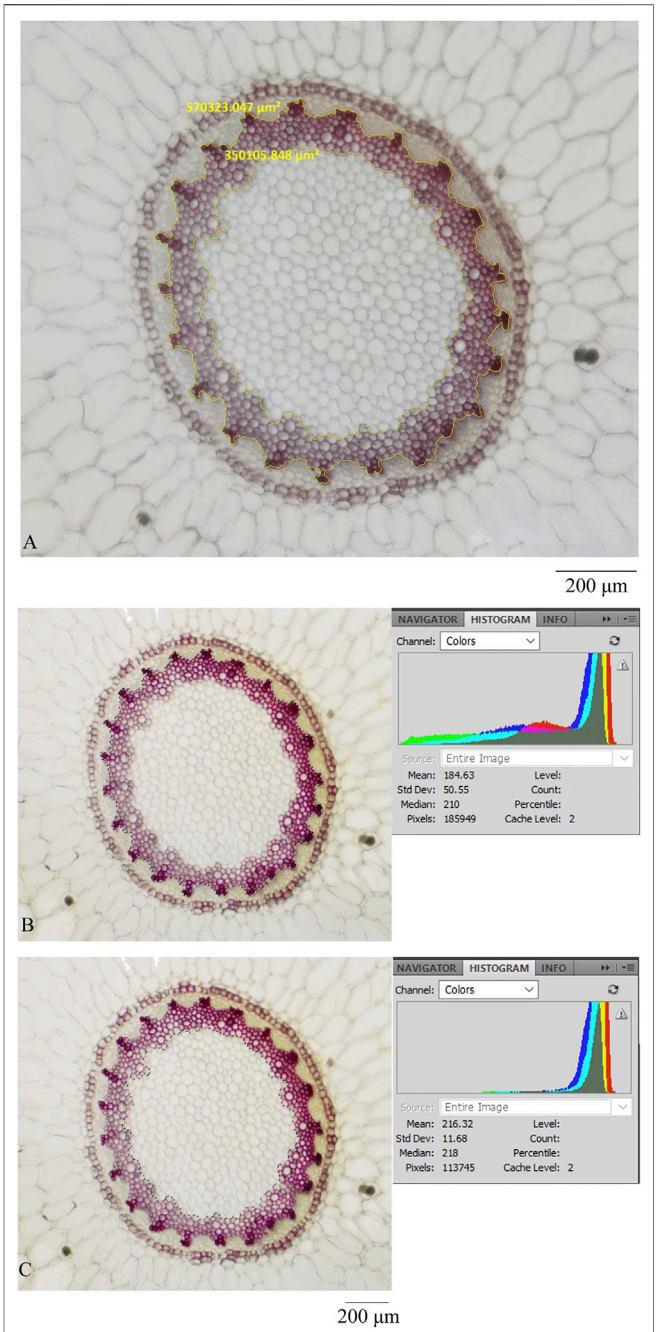

FIGURE 7 | Transverse section was processed by different microscopes and software: **(A)** ZEISS and ZEN, **(B)** OLYMPUS and photoshop (showing the pixel of xylem and pith), and **(C)** OLYMPUS and photoshop (showing the pixel of pith).

In theory, if image software could indicate information such as pixels of a selected area, then the pixel ratio should be equal to the area ratio and thus could be used to identify three quality grades of Maidong.

To verify this assumption, this study selected a section of Maidong randomly from market samples and acquired an image of the stele using an OLYMPUS microscope; thereafter, the image was analyzed this picture with the commonly used image processing software Photoshop CS2 (Adobe, CA). The pith and xylem areas were selected separately using a polygonal lasso tool. The pixel of the selected area was shown in a histogram, and the pixel ratio between xylem and pith was calculated. Results showed that the pixel ratio between xylem and pith was equal to the area ratio between xylem and pith (**Figure 7**, **Table 6**).

The quantification data are based on a relative value; thus, the method could be used to distinguish three grades of Maidong without the constraints associated with the type of themicroscope and processing software.

DISCUSSION

In this study, we evaluated the microscopic characteristics of Maidong from three quality grades; the result showed the pith and xylem area in the middle sampling site, the thickness and lignification of endodermis and stone cells, and the shape of the protoxylem and lignified degree in the lower sampling site different among the three quality grades of Maidong. Our findings reveal that cultivation age, area, and especially plant growth regulator could influence the anatomical characteristics of Maidong. The area ratio between xylem and pith, which is an

TABLE 6 | Measurement data by different microscopes and software.

Microscope+software	Pith	Pith+xylem	Xylem	Xylem/pith
ZEISS+ZEN	350,106 μm^2	570,323 μm^2	220,218 μm^2	0.63
OLYMPUS+photoshop	113,745 pixel	185,949 pixel	73,133 pixel	0.63

objective and practical microscopic characteristic, was first developed to distinguish the different quality grades of Maidong; the area ratio varied according to the quality grades as follows: Zhe-Maidong, 3.306 ± 1.663 (mean ± SD, n = 16); Chuan-Maidong, 1.282 ± 0.196 (mean ± SD, n = 16); and Chuan-Maidong with paclobutrazol, 0.691 ± 0.161 (mean ± SD, n = 40). Consequently, a novel microscopic quantification method to distinguish the three quality grades of Maidong was established.

The identification of three quality grades of Ophiopogonis Radix will make efforts on three aspects: the recovery of superior germplasm resource of Zhe-Maidong, provide evidence to customers and herbalists who want better quality of Maidong, and the improvement quality of Maidong in market and botanical pharmacies. Our study is the first to apply microscopic techniques to test and distinguish Ophiopogonis Radix from different regions and with different cultivation modes simultaneously. It is also the first to use that microscopic quantitative method to classify the quality grades of herbal medicine. This research largely expanded the application range of microscopic technology and provided another way to evaluate the quality grades of herbal medicine.

Microscopic quantification avoids subjective and relative factors in microscope research. Generally, quantification of microscopic characteristics is influenced by sampling site; thus, in most cases, the diameter of the sampling site should be restricted in microscopic quantification analysis, which limits the practical application of this method. For example, the identification of Wuzhimaotao (Radix Fici Hirtae) by quantification used samples around 1 cm in diameter (Au et al., 2009). In our research, the sampling site was considered and investigated. Results showed that the microscopic quantification of the middle sampling site of three quality grades of Maidong is stable, consistent and subject to quantification, and reveals important distinctions. Maidong is a fusiform tuber, and the middle location is easily determined; hence, there is no need to restrict the diameter of sampling site. The practicability of this method could be improved.

Currently, not all microscopes equipped with software can measure actual areas; thus, measurement of actual areas is limited to several types of microscopes and software. In this study, the identification characteristic is based on a ratio, which is a relative value. In theory and in practice, microscopic images from any type of the microscope could be processed using normal image processing software to obtain the pixel value of selected areas. The pixel ratio is equal to the area ratio, which means that our novel method is not limited by the type of the microscope and software, thereby further expanding the scope of application.

For Chuan-Maidong with paclobutrazol, the microscopic characteristics of lower sampling site were not stable, and almost half of samples have undeveloped metaxylem. We assumed that was resulted of short cultivation age and the influence of plant growth regulator. We will confirm this hypothesis in the future studies.

In conclusion, we established an efficient, convenient, and practical method evaluating the quality grades of Maidong based on microscopic quantification, which could in turn improve the quality and safety of Maidong in China, specifically. Furthermore, our study also expands the application of quantitative microscopic techniques and provides another way to identify the quality grades of herbal medicine.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KY conceived and designed the study, did literature research, and prepared the manuscript; WL collected LC-MS data; NZ did the statistical analysis; XC did the data acquisition; SZ collected the plant material; TZ edited the manuscript; SK identified the plant material; FW and SM guaranteed integrity of the entire study and reviewed the manuscript.

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The Species Identification in Traditional Herbal Patent Medicine, Wuhu San, Based on Shotgun Metabarcoding

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Traditional herbal patent medicine typically consists of multiple ingredients, making it challenging to supervise contamination by impurities and the improper use of raw materials. This study employed shotgun metabarcoding for the species identification of biological ingredients in traditional herbal patent medicine, Wuhu San. The five prescribed herbal materials found in Wuhu San were collected, and their reference sequences were obtained by traditional DNA barcoding using Sanger sequencing. Two lab-made and three commercial Wuhu San samples were collected, and a total of 37.14 Gb of shotgun sequencing data was obtained for these five samples using the Illumina sequencing platform. A total of 1,421,013 paired-end reads were enriched for the Internal Transcribed Spacer 2 (ITS2), *psbA* and *trnH* intergenic spacer region (*psbA-trnH*), maturase k (*matK*), and ribulose-1, 5-bisphosphate carboxylase (*rbcL*) regions. Furthermore, 80, 11, 9, and 8 operational taxonomic units were obtained for the ITS2, *psbA-trnH*, *matK*, and *rbcL* regions, respectively, after metagenomic assembly, annotation, and chimeric detection. In the two lab-made mock samples, all labeled ingredients in the Wuhu San prescription were successfully detected, and the positive control, *Panax quinquefolius* L., was detected in the HSZY172 mock sample. Three species, namely *Angelica sinensis* (Oliv.) Diels, *Saposhnikovia divaricata* (Turcz. ex Ledeb.) Schischk., and *Carthamus tinctorius* L., belonging to three labeled ingredients, Angelicae Sinensis Radix (Danggui), Saposhnikoviae Radix (Fangfeng), and Carthami Flos (Honghua), were detected in the three commercial samples. *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav., the original Angelicae Dahuricae Radix (Baizhi) species, was only detected in WHS003. *Arisaema erubescens* (Wall.) Schott, *Arisaema heterophyllum* Blume, or *Arisaema amurense* Maxim., the original Arisaematis Rhizoma (Tiannanxing) species, were not detected in any of the commercial samples, which could be attributed to the fact that this medicinal material underwent extensive processing. In addition, the *Saposhnikovia divaricata* adulterant was detected in all the commercial samples, while 24 fungal genera, including *Aspergillus*, were identified in both the lab-made and commercial samples. This study showed that shotgun metabarcoding provided alternative strategy and technical

means for identifying prescribed ingredients in traditional herbal patent medicine and displayed the potential to effectively complement traditional methods.

Keywords: Wuhu San, shotgun metabarcoding, DNA barcoding, traditional herbal patent medicine, species identification

INTRODUCTION

In recent years, traditional herbal medicine has been widely used to prevent and treat clinical diseases. Many countries have been using traditional herbs to prevent disease or improve health to varying degrees (Barnes et al., 2016; Job et al., 2016; Sammons et al., 2016; Teng et al., 2016). It is difficult to identify the specific content of traditional herbal patent medicines since they mostly consist of multiple mixed ingredients. Microscopic and physicochemical identification are currently the primary methods used for quality control and the determination of traditional herbal patent medicine content (Committee, 2020). However, the microscopic characteristics of the medicinal materials within multiple original plants may be inconsistent (Chen et al., 1998). Furthermore, insufficient professional talent has also restricted the development of microscopic identification. Physical and chemical identification is based on chemical properties. However, the chemical composition of traditional herbal patent medicine is complex, and the correspondence between the chemical composition and different prescription ingredients may not be clear. In addition, many factors, such as the original plant, environment, harvesting, and processing, may affect the content of active ingredients. It is also possible that some manufacturers illegally add chemical substances, complicating the quality control of traditional herbal patent medicine via chemical composition detection (Xu et al., 2014; Li et al., 2015b). With the development of high-throughput sequencing (HTS) technology, shotgun metagenomics based on the genetic information of species has been successfully applied to identify ingredients in mixed samples. This technique involves the untargeted sequencing of all biological ingredient genomes present in a sample (Quince et al., 2017) to break the metagenomic DNA into small fragments, after which bioinformatics methods are used for assembly without the need for PCR amplification. Therefore, potential biases caused during PCR amplification can be eliminated, and multiple DNA barcodes can be obtained simultaneously for further study to produce more comprehensive data. If the information is used to analyze the traditional DNA barcode region, it is known as shotgun metabarcoding. This was applied here for the species identification of the biological ingredients in traditional herbal patent medicine. Furthermore, shotgun metabarcoding can be a powerful supplement to the conventional identification method used for traditional herbal patent medicine.

Currently, shotgun sequencing technology is primarily used in microbiology to study the composition and functions of microbial communities in different environment samples (Tringe et al., 2005; Warden et al., 2016). A comprehensive study of the microbiome during different processing steps in the beef production chain revealed that the relative abundance of common pathogenic and non-pathogenic bacteria decreased significantly in the final stage, while the relative abundance of some bacteria or pathogens increased. The study proved that shotgun sequencing technology could be used to evaluate the microbial community composition during beef production, as well as pathogen population shifts (Yang et al., 2016). Several studies have shown that shotgun sequencing technology is also applicable to the study of microbial communities in food or beverages that require fermentation (Ferrocino et al., 2018; Arikan et al., 2020), as well as human microbes found in the skin (Oh et al., 2014), saliva (Hasan et al., 2014), and gastrointestinal tract (Vangay et al., 2018; Zhao et al., 2018). In addition to research in the field of microbiology, shotgun sequencing technology has also been successfully used in animal diet analysis (Srivathsan et al., 2015), animal diversity (Zhou et al., 2013), and ingredient identification in food (Haiminen et al., 2019). The development of high-throughput sequencing technology has allowed the application of research strategies based on DNA barcodes to identify traditional herbal patent medicine. A previous study used high-throughput sequencing to identify the biological components in Yimu Wan, a traditional patent medicine. The results showed that all the prescription ingredients could be detected based on the ITS2 sequences, indicating that this technique can be used effectively to detect the legality and safety of Yimu Wan (Jia et al., 2017). Another study used single-molecule, real-time sequencing to identify multiple ingredients in Jiuwei Qianghuo Wan, and the result showed that seven prescription ingredients and positive controls were successfully detected in the two reference samples. Adulterants and potential contaminant species were also found in the commercial samples, indicating that this method can effectively detect the biological components of Chinese patent medicines (Xin et al., 2018b). Furthermore, a study based on high-throughput sequencing and ITS2 regions detected some prescription ingredient adulterants (Cangzhu and Tiannanxing) in traditional Ruyi Jinhuang San medicine (Shi et al., 2018). However, minimal studies are available involving the species identification in traditional herbal patent medicine based on shotgun sequencing. Xin et al. reported the first systematic study involving species detection in traditional herbal patent medicine based on shotgun sequencing (Xin et al., 2018a). The results showed that the ITS2 region could detect all the prescription ingredients, as well as the positive control in the mock samples of Longdan Xiegan Wan. This confirms that shotgun metagenomic sequencing can be used to identify the biological ingredients in traditional herbal patent medicine.

Wuhu San was first recorded in the book, *Si He Ting Ji Fang*, written by Ling Huan during the Qing Dynasty (Deng et al., 2000). It is a type of powder patent medicine prepared by mixing five Chinese medicinal materials, namely Angelicae Sinensis

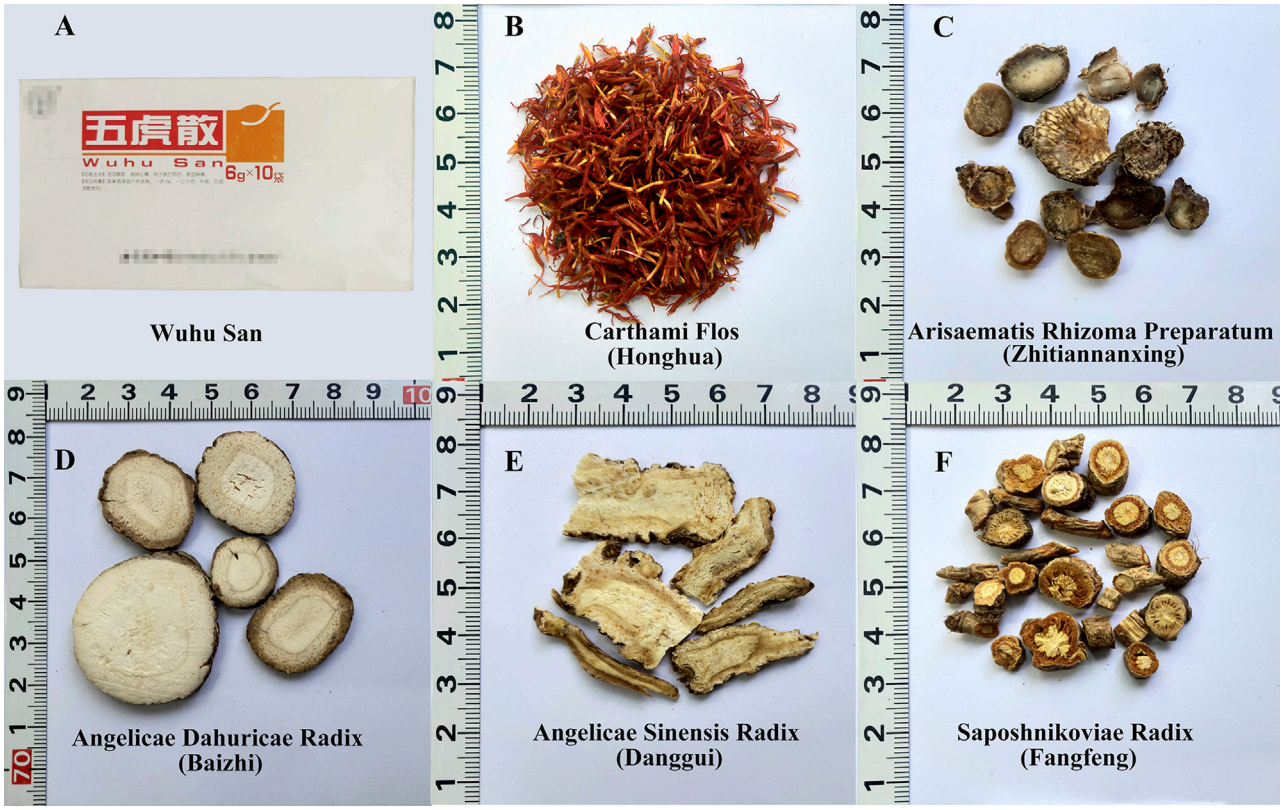

FIGURE 1 | The morphological characteristics of five herbal materials in the prescription of Wuhu San **(A)** Wuhu San **(B)** Carthami Flos (Honghua) **(C)** Arisaematis Rhizoma Preparatum (Zhitiannanxing) **(D)** Angelicae Dahuricae Radix (Baizhi) **(E)** Angelicae Sinensis Radix (Danggui), and **(F)** Saposhnikoviae Radix (Fangfeng).

Radix (Danggui), Saposhnikoviae Radix (Fangfeng), Angelicae Dahuricae Radix (Baizhi), Carthami Flos (Honghua), and Arisaematis Rhizoma Preparatum (Zhitiannanxing). The corresponding Latin names of the original species are shown in **Supplementary Table S1**. It promotes blood circulation, relieves pain, reduces swelling, and disperses blood stasis. Not only can it be used externally with white wine, but it can also be administered with warm yellow rice wine or warm, boiled water. Pharmacological studies have shown that Wuhu San has a relatively apparent anticoagulant effect (Jia et al., 1997), while its alcohol extract displays excellent anti-inflammatory and analgesic properties (Yang et al., 1990). Although both the active substances in Angelicae Sinensis Radix (Danggui) and Carthami Flos (Honghua) have an excellent inhibitory effect on platelet aggregation and can improve blood flow (Li et al., 2009; Zhang et al., 2009), combining these two medicinal materials is more effective in promoting blood circulation (Yue et al., 2017). Moreover, prescription ingredients, such as Saposhnikoviae Radix (Fangfeng), Angelicae Dahuricae Radix (Baizhi), and Arisaematis Rhizoma (Tiannanxing) display certain anti-inflammatory and analgesic properties (Okuyama et al., 2001; Kang et al., 2008; Chunna et al., 2015).

This study uses Wuhu San as an example to evaluate the feasibility and efficacy of using shotgun metabarcoding to identify the biological ingredients in traditional herbal patent medicine. Two mock samples were prepared according to the official species composition listed in the 2015 edition of the Chinese Pharmacopeia and used to verify the feasibility of the shotgun metabarcoding method. This technique was then employed to determine the biological species composition in the commercial Wuhu San samples, aiming to provide different strategies and technical means for the prescription ingredient identification and quality control of traditional herbal patent medicine, such as Wuhu San.

MATERIALS AND METHODS

Herbal Material, Lab-Made Mock Wuhu San, and Commercial Wuhu San Samples

Four kinds of Wuhu San herbal materials, namely Angelicae Sinensis Radix (Danggui), Saposhnikoviae Radix (Fangfeng), Angelicae Dahuricae Radix (Baizhi), Carthami Flos (Honghua), were collected from the Beijing TRT pharmaceutical company. Arisaematis Rhizoma (Tiannanxing) was obtained from Chengde, Hebei Province (**Supplementary Table S2** and **Figure 1**). The herbal materials were authenticated using the morphological and traditional DNA barcoding methods. The lab-made mock samples were prepared according to the prescription ingredients and manufacturing

TABLE 1 | The proportion of the five ingredients listed in the prescription of Wuhu San.

Pinyin name	Latin name	Proportion in the preparation (%)
Danggui	Angelicae Sinensis Radix	21.3
Honghua	Carthami Flos	21.3
Fangfeng	Saposhnikoviae Radix	21.3
Zhitiannanxing	Arisaematis Rhizoma Preparatum	21.3
Baizhi	Angelicae Dahuricae Radix	14.6

method of Wuhu San listed in the 2015 edition of the Chinese Pharmacopoeia (**Table 1**). Of these, Arisaematis Rhizoma (Tiannanxing) was processed in advance following the method described in the Chinese Pharmacopoeia for Arisaematis Rhizoma Preparatum (Zhitiannanxing). The two lab-made mock samples were prepared and labeled as HSZY160 and HSZY172. The Panacis Quinquefolii Radix (Xiyangshen) powder was added to HSZY172 as a positive control at an amount equal to Angelicae Dahuricae Radix (Baizhi), representing the lowest herbal ingredient in the Wuhu San prescription. In addition, the three commercial Wuhu San samples were acquired from pharmacies and labeled WHS001, WHS002, and WHS003.

DNA Extraction, PCR Amplification, Sanger Sequencing, and HTS

The DNA extraction of the herbal material samples was performed according to previous research (Liu et al., 2017) and the DNA barcoding principles for traditional Chinese herbal medicine (Chen et al., 2014) using a plant genomic DNA extraction kit (Tiangen Biochemical Technology (Beijing) Co., Ltd., China). The meta-genomic DNA of Wuhu San was extracted according to the previously published protocols of the CTAB-based method (Cheng et al., 2015) with some changes. A pre-wash buffer was used for pretreatment (Xin et al., 2018a), after which lysis buffer was added. The samples were placed in a 56°C water bath overnight for lysis. Extraction was performed using chloroform/isoamyl alcohol (volume ratio 24:1), and phenol/chloroform/isoamyl alcohol (volume ratio 25: 24:1). The DNA was purified by adding 50 μL of sodium acetate and 1,250 μL of 100% methanol. The extracted DNA quality was estimated using a NanoDrop one ultra-micro spectrophotometer (Thermo Fisher Scientific Inc., USA). The traditional DNA barcoding regions of ITS2, *psbA-trnH*, *matK*, and *rbcL* were amplified with DNA barcoding primer sets and conditions proposed by the barcodes of the traditional Chinese herbal medicine data system (TCM-BOL) (Chen et al., 2014), the CBOL plant working group (Group et al., 2009), and the barcode of life data system (BOLD) (Ratnasingham and Hebert, 2007) using 2 × Taq master mix (AidLab Biotechnologies Co., Ltd., China). The PCR products were bi-directionally sequenced on an ABI 3730xL DNA Analyzer (Thermofisher Co., Ltd., United States). After constructing a PCR-free library, the Wuhu San DNA was sheared into fragments and sequenced using the Illumina NovaSeq platform.

Data Analysis

The Sanger sequencing results were obtained according to the *Standard DNA barcodes of Chinese Materia Medica in Chinese Pharmacopoeia* edited by Chen Shilin (Chen, 2015). The sequence chromatograms were assembled, and the primers were removed using Codoncode aligner v 9.0.1 (CodonCode Corp., Dedham, MA, United States). For the Illumina sequencing data, the sequencing adapter and low-quality reads were filtered using Trimmomatic v0.38 (Bolger et al., 2014). The paired-end reads were enriched using local python scripts (Shi et al., 2019). The enriched reads belonging to ITS2, *psbA-trnH*, *matK*, and *rbcL* were assembled using MEGAHIT v1.2.9 and MetaSPAdes v3.13.2 (Li et al., 2015a; Nurk et al., 2017). Contigs obtained via the two types of software were merged, and duplicates were removed with cd-hit at 100% identity (Li and Godzik, 2006). The ITS2 regions were obtained using the hidden Markov model (HMM)-based annotation method (Keller et al., 2009). The traditional DNA barcoding regions of *psbA-trnH*, *matK*, and *rbcL* were acquired by removing primer sequences based on Cutadapt v2.10 (Kechin et al., 2017). The chimera detection of the annotated contigs was performed using UCHIME v4.2 (Edgar et al., 2011). Sequences belonging to each marker were clustered into OTUs at 100% identity using Usearch v11 (https://www.drive5.com/usearch/), and the representative sequence for each OTU was selected for further analysis. The shotgun paired-end reads were mapped to the OTU representative sequences using bowtie2 v2.4.1 (Langmead and Salzberg, 2012), while the sequencing depth and coverage values were calculated using Samtools v1.10 (Etherington et al., 2015). Poor-quality OTUs were removed when its representative sequences displayed a sequencing depth ≤3 or coverage ≤95%. The remaining high-quality OTUs were used for species assignment by searching the TCM-BOL (Chen et al., 2014), BOLD (Ratnasingham and Hebert, 2007), and GenBank (Benson et al., 2018) databases using the basic local alignment search tool, BLAST (Camacho et al., 2009). Finally, the statistics and taxonomic visualization of the species composition of the traditional herbal patent medicine were performed using MEGAN v6.18.9 (Huson et al., 2016).

After the species in the traditional herbal patent medicine were identified via DNA barcodes, some terms related to the identified species were defined as follows:

Authentic

The species in the medicinal materials are authentic if it is identified as one of the labeled ingredients in the prescription of the traditional herbal patent medicine.

Substitution

Substitutions refer to the species in the medicinal materials with similar characteristics such as efficacy, chemical composition, pharmacological effect, and clinical effect, which are selected instead of authentic medicinal materials according to the clinical medication plan when there is a shortage of these materials (Tang and Huang, 1994; Suo and Chen, 2006).

Adulterant

Adulterants refer to the species in the medicinal materials that are used as authentic although they are similar in appearance or have the same name as the authentic material, but are different regarding the

TABLE 2 | The GenBank accession numbers of the five herbal materials in Wuhu San and the positive control, Panacis Quinquefolii Radix (Xiyangshen).

Herb medicinal material	ITS2	*psbA-trnH*	*matK*	*rbcL*
Angelicae Sinensis Radix (Danggui)	MN727081	MT994327	MN729559	MN746764
Carthami Flos (Honghua)	MN727076	MT994328	MN729561	MN746766
Saposhnikoviae Radix (Fangfeng)	MT821449	MT994331	MW000338	MW000334
Arisaematis Rhizoma (Tiannanxing)	MT821451	—	MW000340	MW000335
Angelicae Dahuricae Radix (Baizhi)	MT821450	MT994330	MW000339	—
Panacis Quinquefolii Radix (Xiyangshen)	MT102865	MT994329	MW000341	MW000333

Note: "—" indicates that the sequence was not successfully obtained.

original plant source, chemical composition, pharmacological effect, and clinical effect (Tang and Huang, 1994).

Contaminant

Contaminants include fungal contamination and impurities.

RESULTS

The Authentication of the Five Herbal Materials in Wuhu San and Their ITS2, *psbA-trnH*, *matK*, and *rbcL* DNA Barcodes

The five herbal materials labeled on Wuhu San prescription were collected from Chengde (Hebei province). They were first identified using the morphological method and then authenticated using DNA barcoding to ensure the accuracy of the mock samples. High-quality DNA was extracted from these materials, after which the ITS2, *psbA-trnH*, *matK*, and *rbcL* DNA barcodes were amplified using their corresponding universal primers. Except for the *psbA-trnH* sequence of Arisaematis Rhizoma (Tiannanxing) and the *rbcL* sequence of Angelicae Dahuricae Radix (Baizhi), all the ITS2, *psbA-trnH*, *matK*, and *rbcL* DNA barcodes of the five herbal materials were successfully amplified and then bi-directionally sequenced using Sanger sequencing technology. The GenBank accession numbers of these sequences are shown in **Table 2**. The ITS2 and *psbA-trnH* sequences obtained via Sanger sequencing were assigned to species by blasting to the TCM–BOL system, while the *matK* and *rbcL* DNA barcodes obtained using the same method were assigned to a species or genus using the BOLD system and GenBank NT database. By combining the identification results of the four DNA barcodes, all five herbal materials were authenticated, and their original Angelicae Sinensis Radix (Danggui), Saposhnikoviae Radix (Fangfeng), Angelicae Dahuricae Radix (Baizhi), Carthami Flos (Honghua), and Arisaematis Rhizoma Preparatum (Zhitiannanxing) species were assigned to *Angelica sinensis* (Oliv.) Diels, *Saposhnikovia divaricata* (Turcz. ex Ledeb.) Schischk., *Carthamus tinctorius* L., *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav., and *Arisaema amurense* Maxim., respectively.

HTS and Shotgun Metabarcoding Data Assembly

The average DNA concentration of the lab-made mock samples and commercial samples was 144.06 ng/μL, while the A_{260}/A_{280} ranged between 1.8 and 2.0 (**Supplementary Table S3**). This indicated that the concentration and purity of the DNA extracted from the traditional herbal patent medicine samples were high. A total of 37.14 G of raw data was obtained via HTS, while 8.54 G and 9 G of raw data were acquired from the HSZY160 and HSZY172 lab-made mock samples, respectively. Additionally, 6.81 G, 6.78 G, and 6.01 G of raw sequencing data were acquired from the WHS001, WHS002, and WHS003 commercial samples. A total of 123,799,141 paired-end sequencing reads were obtained. After removing low-quality sequences, a total of 1,421 013 paired-end sequencing reads were enriched for the ITS2, *psbA-trnH*, *matK*, and *rbcL* regions. The detailed sequencing results are shown in **Supplementary Table S4**. A total of 6,884 unique contigs were generated by assembling and then removing duplications using MEGAHIT v1.2.9 and MetaSPAdes v3.13.2. The DNA barcoding regions of ITS2, *psbA-trnH*, *matK*, and *rbcL* yielded 136, 26, 16, and 21 unique contigs, respectively, after annotating and removing the primers. The cluster analysis of the ITS2 region yielded a total of 80 OTUs, with an average length of 207.5 bp and an average GC content of 54.4%. The number of OTUs obtained via nuclear ITS2 was more than seven times that of chloroplast *psbA-trnH*, *matK*, and *rbcL*. Moreover, the GC content of the ITS2 sequences was higher than that of the *psbA-trnH*, *matK*, and *rbcL* sequences. The specific data results of the four markers are shown in **Table 3**.

TABLE 3 | The results of the data analysis of four marker types.

	ITS2	*psbA-trnH*	*matK*	*rbcL*
Number of unique contig	378	6,416	44	46
Number of DNA barcodes after annotation chimera	136	26	16	21
Number of OTU	80	11	9	8
Average length (bp)	207.5	321.1	836.3	703
GC content (%)	54.4	32.2	34.7	44.1

The Accuracy Verification of the DNA Barcoding Sequences Assembled Using the Shotgun Sequencing Data of the Lab-Made Samples

The DNA barcode assembly results of the labeled ingredients in the prescription of the lab-made mock samples are shown in **Table 4**. To determine the assembly accuracy of the DNA barcode

TABLE 4 | The DNA barcode sequences of five ingredients in the prescription of the lab-made Wuhu San samples, and the positive control, *Panax quinquefolius,* obtained via shotgun metabarcoding.

Species	HSZY160				HSZY172			
	ITS2	*psbA-trnH*	*matK*	*rbcL*	ITS2	*psbA-trnH*	*matK*	*rbcL*
Angelica sinensis	√	√	√	√	√	√	√	√
Carthamus tinctorius	√	√	√	√	√	√	√	√
Saposhnikovia divaricata	√	√	√	√	√	√	√	√
Arisaema amurense	√	—	√	√	√	—	√	√
Angelica dahurica	√	√	—	—	√	√	—	—
Panax quinquefolius					√	√	√	√

Note: "√" shows that the corresponding assembly sequence of this species was obtained; "—" indicates that the corresponding assembly sequence of this species cannot be obtained.

regions assembled via shotgun sequencing, the sequences obtained using shotgun metabarcoding and the reference sequences of ITS2, *psbA-trnH*, *matK*, *rbcL* obtained via Sanger sequencing were analyzed for consistency. The *psbA-trnH* sequence of *Arisaema amurense* and the *rbcL* sequence of *Angelica dahurica* were not obtained with Sanger sequencing.

Regarding the ITS2 sequences, the assembly sequences of all the ingredients in the prescriptions of the two mock samples were obtained. The sequence bases of *Angelica sinensis* and *Angelica dahurica* were identical to their reference sequences. A one base difference was evident between the sequences of *Arisaema amurense* obtained via two sequencing methods. Two *Saposhnikovia divaricata* assembly sequences were obtained, one of which was identical to the reference sequence bases, while the other differed by three bases. Two *Carthamus tinctorius* assembly sequences were obtained, which differed from the reference sequence by 0 and one base, respectively.

For the *psbA-trnH* sequences, the assembly sequences of *Angelica sinensis*, *Saposhnikovia divaricata*, *Carthamus tinctorius*, and *Angelica dahurica* were successfully obtained, but shotgun metabarcoding failed to acquire the *Arisaema amurense* sequence. The assembled sequences of *Angelica sinensis*, *Angelica dahurica*, and *Carthamus tinctorius* were identical to the reference sequences obtained via Sanger sequencing. Two *psbA-trnH* sequences of *Saposhnikovia divaricata* were acquired via shotgun sequencing. Compared with the sequences obtained via traditional DNA barcoding, one is identical, while the other displays two base differences.

Regarding the *matK* sequences, the assembly sequences were acquired for all the species in the two mock samples except for *Angelica dahurica*. A comparison between the *matK* assembly and reference sequences of the four species showed that the sequence bases of *Arisaema amurense* were the same. The two assembled sequences of *Carthamus tinctorius* and *Saposhnikovia divaricata* were obtained via shotgun sequencing and displayed a 0–3 base difference from the reference sequences. A total of two assembled *matK* sequences of *Angelica sinensis* were obtained, which differed from the reference sequences by five and seven bases, respectively.

The assembly sequences of *Carthamus tinctorius* and *Arisaema amurense* were successfully acquired for the *rbcL* region. There were only five mutation sites among the *rbcL* sequences of *Angelica sinensis*, *Saposhnikovia divaricata,* and *Angelica dahurica*. Therefore, these sequences of the three species could not be assembled separately. Only the *rbcL* sequences of *Carthamus tinctorius* and *Arisaema amurense* were obtained using both shotgun and Sanger sequencing. The assembled sequences of the two species were completely consistent with the reference sequences.

In addition, for the positive control, *Panax quinquefolius,* all the ITS2, *psbA-trnH*, *matK*, and *rbcL* sequences that were acquired using the shotgun metabarcoding method were identical to those obtained via the traditional DNA barcode method.

The Plant Species Composition of Commercial Wuhu San Samples Identified Through Shotgun Metabarcoding

Regarding the labeled ingredients in the prescription, combined with the ITS2, *psbA-trnH*, *matK*, and *rbcL* regions, the three commercial samples contained prescription medicinal materials *Angelica sinensis*, *Carthamus tinctorius*, and *Saposhnikovia divaricata*. None of the samples contained *Arisaema erubescens*, *Arisaema heterophyllum*, or *Arisaema amurense*, representing the original Arisaematis Rhizoma (Tiannanxing) species, while only *Angelica dahurica* was detected in WHS003 (**Figures 2**, **3**, **Supplementary Figures S1, S2**). For the ITS2 and *psbA-trnH* sequences, the results showed that *Angelica sinensis* and *Saposhnikovia divaricata* were detected in all three

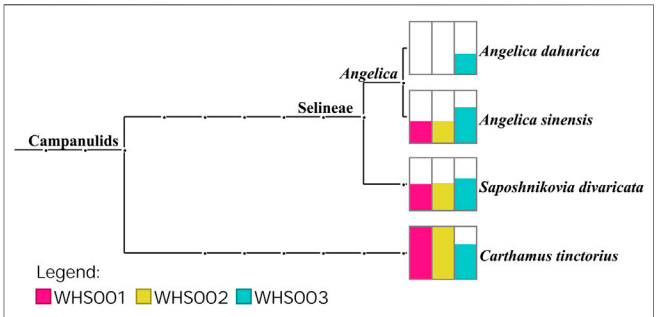

FIGURE 2 | Taxonomic analysis of three samples detected via reads belonging to the ITS2 region. Each taxonomic node is drawn as a bar chart indicating the number of reads assigned to the taxon for each sample.

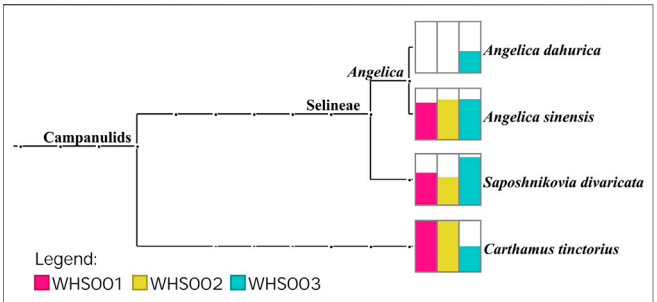

FIGURE 3 | The taxonomic analysis of three samples detected by reads belonging to the *psbA-trnH* region. Each taxonomic node is drawn as a bar chart indicating the number of reads assigned to the taxon for each sample.

commercial samples, while *Angelica dahurica* was only detected in WHS003 (**Figures 2**, **3**). A total of six OTUs were obtained from the *matK* region. Of these, two OTUs were identified as *Carthamus tinctorius*, while the remaining four were only identified as belonging to the Apiaceae family, but the species could not be authenticated (**Supplementary Figure S1**). Six OTUs were obtained from the *rbcL* sequences in the three commercial samples, of which one was identified to species level, namely *Carthamus tinctorius*. The remaining five OTUs could only be identified as belonging to the Apiaceae family, but no species could be determined (**Supplementary Figure S2**). Detailed reads of the prescription ingredients in the three commercial samples based on four barcodes are shown in **Supplementary Tables S5–S8**.

As for the adulterants of the labeled ingredients, *Ferula bungeana* Kitag. was detected in two of the commercial samples (WHS001 and WHS002) based on the ITS2 sequences. In addition to these labeled ingredients and their adulterants, several other potential impurities were found in the commercial Wuhu San samples. Based on the ITS2 sequences, *Scutellaria baicalensis* Georgi was detected in two commercial samples (WHS001 and WHS002), while *Salix* L. was detected in WHS002 and WHS003. Impurities, such as *Convolvulus arvensis* L., *Chenopodium album* L., and *Citrus* L., were also found in WHS003.

The Fungal Contamination of the Lab-Made and Commercial Wuhu San Samples Detected via ITS2

A total of 36 fungal OTUs were obtained based on ITS2 sequences, including 22 families and 24 genera. The reads number of the *Rhizopus* genus was the highest of the 24 detected genera, accounting for 94.33% of the total number of fungal reads. It was the predominant genera in the two lab-made mock samples and three commercial samples. Fungi belonging to the *Macrophomina*, *Fusarium*, *Aspergillus,* and *Alternaria* genera were the dominant abundant in this study. Most of these fungi were molds that were present during the storage of herbal medicines, while some were soil habitant fungi.

Here, 8, 7, 13, 16, and 20 fungal genera were detected in HSZY160, HSZY172, WHS001, WHS002, and WHS003, respectively. Fungi belonging to the *Fusarium*, *Rhizopus,* and *Alternaria* genera were detected in all five samples. In addition, the composition of the identified fungi in the two lab-made samples (HSZY160 and HSZY172) was similar at the genus level. The number of fungal species found in the commercial samples was significantly higher than in the mock samples, and some differences were evident between the fungal compositions of the three commercially available samples (**Figure 4**, **Supplementary Tables S9**). Besides the fungi belonging to genera found in all five samples, *Aspergillus*, *Penicillium*, *Geotrichum,* and *Mycocentrospora* were detected in all three commercial samples. WHS001 and WHS002 displayed a higher similarity in fungal composition than WHS003. Most of the fungal species were detected in WHS003, while *Aspergillus flavus,* which may produce aflatoxin that is harmful to human health, was detected in this sample (**Supplementary Figure S3**, **Supplementary Tables S10**).

DISCUSSION

The Feasibility of Shotgun Metabarcoding Technology in Authenticating the Herbal Ingredients of Wuhu San

DNA metabarcoding is currently the most widely used detection method for mixed biological samples. Many studies are available that involve the identification of biological ingredients of traditional herbal medicine based on DNA metabarcoding technology (Jia et al., 2017; Xin et al., 2018b; Shi et al., 2018; Zhang et al., 2020). However, the PCR amplification efficiency of universal DNA barcode primers is affected by the severe DNA degradation of traditional herbal medicine (Xin et al., 2018a). Furthermore, it may result in potential bias during PCR amplification using primers (Berry et al., 2011). Shotgun metabarcoding directly performs library construction and sequencing of the total DNA of mixed samples (Quince et al., 2017) and can obtain ITS2 sequences and multiple chloroplast DNA barcode sequences through the assembly for species identification (Xin et al., 2018a). This method has also been applied for studying clinical or complex environmental samples (Nielsen et al., 2014; Vangay et al., 2018). The shotgun metabarcoding method can reduce or eliminate the potential bias caused by PCR amplification and obtain a longer DNA barcode sequence interval than DNA metabarcoding. Several analytical biodiversity studies based on shotgun sequencing technology have shown that this technique can be used for biodiversity assessment. This method avoids the PCR amplification of particular gene markers to display species richness with high fidelity, while there is a significant correlation between the reads and biomass of most species (Zhou et al., 2013; Bista et al., 2018). However, this technology is more expensive, while the DNA quality requirements are also higher. Furthermore, the related bioinformatics analysis also presents a significant challenge.

This study shows that four barcode sequences can be successfully obtained in most of the medicinal materials

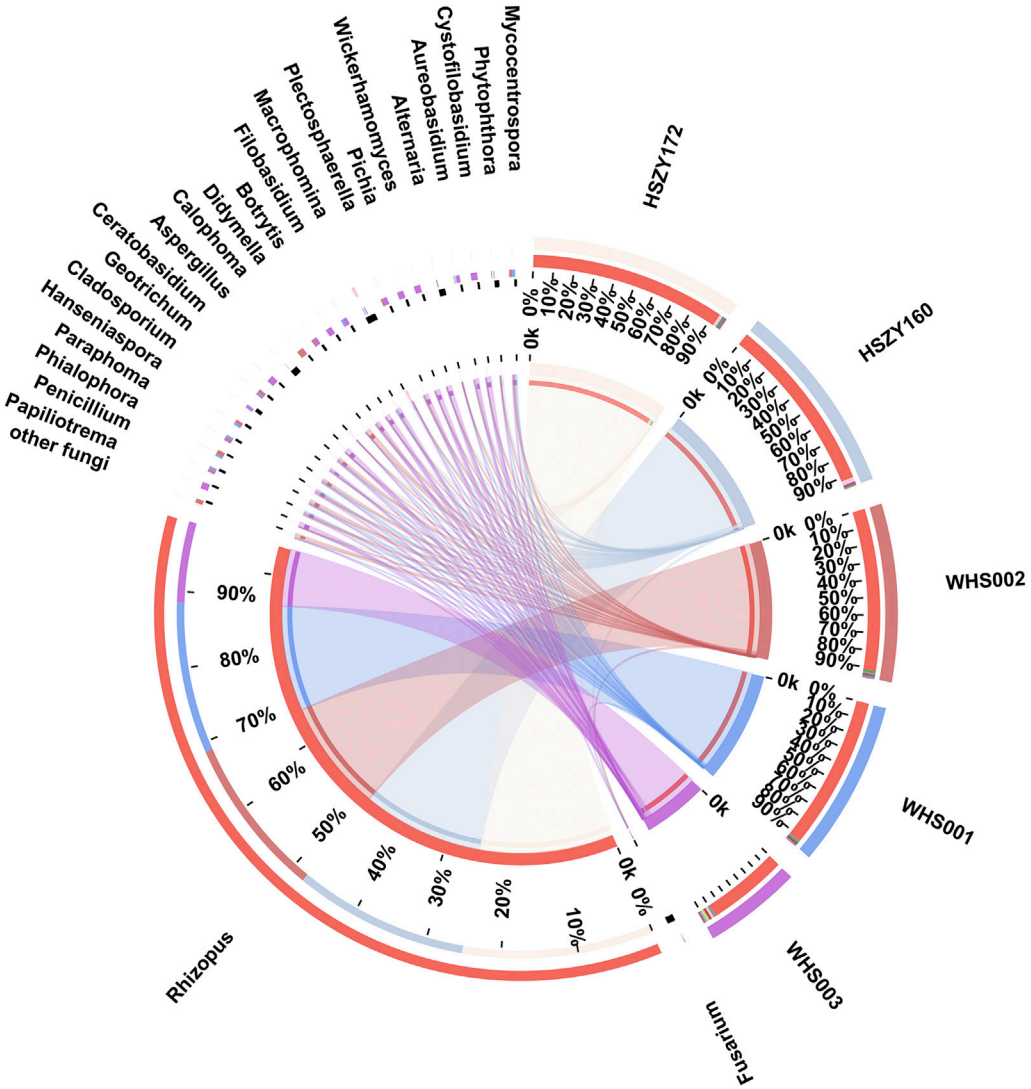

FIGURE 4 | Distribution of the fungi for each sample at the genus level. The data were visualized using Circos. The left half-circle indicates the distribution ratio of species in different samples at the genus level where the outer ribbon represents the species, the inner ribbon represents different groups, and the length represents the sample proportion of a particular genus. The right half-circle indicates the species composition in each sample where the color of the outer ribbon represents samples from different groups, the color of the inner ribbon represents the composition of different species in each sample, and the length of the ribbon represents the relative abundance of the corresponding species.

through shotgun metabarcoding technology in the lab-made mock samples. The mutual verification between the results obtained via different markers further confirmed the accuracy of the method. The results revealed that the *psbA-trnH* sequence of plants belonging to Araceae presented a low success rate via Sanger sequencing due to the adenine (A) and thymine (T) base content of over 70%, while their *psbA-trnH* sequences were difficult to obtain (Luo et al., 2009). It is speculated that the failure to obtain the *psbA-trnH* sequences of *Arisaema amurense* via shotgun metabarcoding technology is due to sequencing reads not being enriched enough for assembly or assembly failure caused by continuous AT repetition. Furthermore, the *Panax quinquefolius* barcode sequences were obtained in the HSZY172 sample, proving the sensitivity of the method for detecting prescription ingredient species. Arisaematis Rhizoma (Tiannanxing) was not detected in the three commercially available samples through any of the markers. It is speculated that this is due to the difference in the degree of processing of Arisaematis Rhizoma Preparatum (Zhitiannanxing) when preparing the lab-made samples and commercial products. The DNA degradation of Arisaematis Rhizoma Preparatum (Zhitiannanxing) in the commercial samples may be more severe. *Ferula bungeana*, a plant belonging to the Apiaceae, was detected in two of the commercial samples. Studies have shown that the dried roots of the *Ferula bungeana* are used as Saposhnikoviae Radix (Fangfeng) in the market (Yang, 2006; Chen and Chen, 2010). However, the efficacy of the two medicinal materials is quite different, and the identification of

the herbal materials should be enhanced to ensure the efficacy of traditional herbal patent medicine. Moreover, herbal impurities, such as *Salix* sp., *Chenopodium album*, *Convolvulus arvensis*, *Citrus* sp. and *Scutellaria baicalensis* were also detected. Their presence may be due to weeds that have been mixed in during harvesting (Geng et al., 2018) or accidental cross-contaminants from the same production line (Xin et al., 2018b). Furthermore, fungi were found in all the samples. During the planting, harvesting, transportation, and storage of herbal medicines, improper methods may cause fungal growth or the accumulation of mycotoxins (Ying et al., 2016), directly affecting the quality, efficacy, and safety of herbal medicines. This necessitates the examination of optimal storage conditions or preparation methods of herbal medicines contaminated by fungi (Wang, 2016). In summary, this indicated that shotgun metabarcoding could not only detect the adulteration of herbal materials in traditional herbal patent medicine, but it can also detect exogenous contamination, such as fungi and impurities. This proves the feasibility of shotgun metabarcoding for detecting biological ingredients in the traditional herbal patent medicine, Wuhu San.

The Challenges of the Current Shotgun Metabarcoding Method During Data Analysis

False Positives Caused by Reads Mapping in Conserved Regions

In this study, the ITS2 sequence of *Peucedanum japonicum* Thunb. was assembled from the lab-made mock samples. The coverage of the reads mapping was 100%, and the sequencing depth was 1,445.61. However, *Peucedanum japonicum* was not added to the lab-made mock samples. Visual reads mapping based on Codoncode Aligner indicated that the tail of the ITS2 sequence had a mapping depth exceeding 2000 ×, but only two mapping reads were present at the front end. Although the coverage of the fragments was uneven, the exceptionally high coverage of the tail significantly increased the overall coverage of the sequence, leading to the occurrence of false-positive sequences. Intercepting the tail segment, the NCBI BLAST analysis indicated that it was a 28S conserved sequence (**Supplementary Figure S4**). Further investigation revealed that the ITS2 sequence tail assembly was not accurate, preventing the ITS2 sequence annotation process from recognizing the 28S section, partially cutting it off. The 28S region is exceptionally conservative. Bowtie2 software randomly maps the reads to the reference genome with the same sequences during the mapping process (Langmead and Salzberg, 2012), resulting in an extremely high mapping depth for the conservative 28S region and a high average sequencing depth for the ITS2 sequences. This problem highlights the necessity to perform sequence annotation and primer removal accurately. Furthermore, CodonCode Aligner software can also verify the annotation results to reduce the occurrence of false-positive sequences.

The Accuracy of High Similarity Sequence Assembly

The assembly of high similarity sequences or low variability sequences is a challenge during shotgun metabarcoding data analysis (Quince et al., 2017). The optimized metagenomic data assembly software and more extensive k-mer parameters may overcome the assembly errors of lower similarity sequences to some extent. However, some difficulties remain when assembling the *matK* and *rbcL* sequences of some species in the same family, especially in the same genus. In this study, the ITS2 and *psbA-trnH* sequences of *Angelica dahurica* were obtained from the lab-made mock samples and commercial samples, but the *matK* and *rbcL* sequences of *Angelica dahurica* were not assembled. The prescriptions of Wuhu San contain *Angelica dahurica*, *Angelica sinensis,* and *Saposhnikovia divaricata* of the same family. The *matK* and *rbcL* sequences of the three species exhibited a similarity of more than 98%. It is speculated that the assembly of the three species may be incorrect due to insufficient assembly accuracy. The *matK* sequences of *Angelica dahurica*, *Angelica sinensis,* and *Saposhnikovia divaricata* obtained via Sanger sequencing were compared. There were six base differences between *Angelica dahurica* and *Saposhnikovia divaricata*, and 12 base differences between *Angelica dahurica* and *Angelica sinensis*. Furthermore, 14 base differences were evident between the *matK* sequences of *Saposhnikovia divaricata* and *Angelica sinensis* (**Supplementary Figure S5**). The *matK* sequence of *Angelica dahurica* displayed a higher similarity to that of *Saposhnikovia divaricata*, and differences were apparent in the bases at sites 42, 376, 415, 588, 722, and 758, indicating an average of 139 bp in a variant site. Analysis of the visual reads mapping results based on Codoncode Aligner showed that the base sites mentioned above have specific bases representing *Angelica dahurica* and *Saposhnikovia divaricata,* respectively (**Supplementary Figure S6**). Therefore, it is inferred that the assembly has not reached a high level of accuracy due to the small difference in sequence bases. The *matK* sequences of two species may be assembled into one sequence, representing the species sequence with more extensive sequencing depth. This is the same in the case of the *rbcL* sequence. Analysis performed via Sanger sequencing revealed that the bases of the *rbcL* sequences of *Angelica dahurica* and *Saposhnikovia divaricata* were T and A at the 270 base site, T and C at the 130 base site, and T and G at the 635 base site, respectively. The bases of the *rbcL* sequences of *Angelica dahurica* and *Angelica sinensis* at base sites 237, 270, and 475 were G/A, T/A, and C/T, respectively (**Supplementary Figure S7**). The base differences between the *rbcL* sequences of the three species were smaller. There were only five mutation sites among the *rbcL* sequences of *Angelica dahurica*, *Angelica sinensis,* and *Saposhnikovia divaricata*, that is, one variant site appeared on average 141 bp. The *rbcL* sequence mapping results of *Angelica dahurica* showed that different bases represented these three species at the various base positions (**Supplementary Figure S8**). The average length of a variation site in *matK* and *rbcL* sequences exceeds the length of commonly used k-mer (Quince et al., 2017). Using a more extended k-mer parameter may solve the problem of species distinction when the sequence

similarity exceeds 98%. However, the k-mer length may exceed the standard analysis length, requiring a massively distributed metagenome assembler, such as Ray, for *de novo* assembly to solve the computational time and memory challenge (Boisvert et al., 2012).

The Identity Threshold of DNA Barcodes for Constructing OTUs

This study initially conducted OTU sequence clustering according to 99% similarity to improve the efficiency and accuracy of the analysis. The results showed that *Angelica sinensis* was detected based on the ITS2, *psbA-trnH,* and *matK* regions, while the *Angelica sinensis* sequence was not detected based on the *rbcL* region. However, the *rbcL* sequence of *Saposhnikovia divaricata,* which belongs to the same family as *Angelica sinensis,* was detected. The Codoncode Aligner was used to further analyze the *rbcL* sequences of *Angelica sinensis* and *Saposhnikovia divaricata* obtained via Sanger sequencing. The results revealed that the *rbcL* sequences of the two species only displayed a 4-base difference. When the similarity was set to 99% for OTU clustering, the *rbcL* sequences of *Angelica sinensis* and *Saposhnikovia divaricata* was artificially divided into an OTU cluster. Therefore, the assembled *rbcL* sequences obtained via shotgun sequencing and the *rbcL* sequences of *Angelica sinensis* and *Saposhnikovia divaricata* acquired with Sanger sequencing were re-analyzed by building phylogenetic trees. Two sequences, namely "WHS001_rbcL_0189_k141_7" and "WHS002_rbcL_0047_k141_8" were found and grouped with the *rbcL* sequences of *Angelica sinensis* (HSYC2002 and HSYC2022) (**Supplementary Figure S9**). Therefore, the *Angelica sinensis* sequence could be detected by the *rbcL* region. This study further revealed that the *Angelica dahurica*, *Angelica sinensis,* and *Saposhnikovia divaricata* sequences were similar, especially those of *matK* and *rbcL*. Therefore, different levels of similarity should be set for OTU clustering when using DNA barcodes with varying species resolutions. For homologous species, the similarity should be further adjusted to 100% to avoid the undetectable phenomenon of sequences with similarities that are too high, which is also consistent with the current analysis strategy recommended by USEARCH.

The Species-Discriminating Power of the ITS2, *psbA-trnH*, *matK*, and *rbcL* DNA Barcodes

The four DNA barcodes displayed differences in the species discriminating power for the ingredients of Wuhu San prescriptions. All the ITS2 sequences obtained in this study can accurately identify species after BLAST. However, the *psbA-trnH* sequences of *Panax quinquefolius* and *Panax ginseng* did not have a variable site and could not be accurately distinguished. The regions of *rbcL* and *matK* exhibited certain limitations in identifying Apiaceae species in this study. Another study indicated that the efficiency of *rbcL* and *matK* sequences in identifying Apiaceae species was much lower than that of ITS2 sequences (Liu et al., 2014). Of the labeled ingredients in the Wuhu San prescription, *Angelica dahurica*, *Angelica sinensis,* and *Saposhnikovia divaricata* were all Apiaceae plants. In addition to a small base difference and insufficient assembly accuracy, the low discriminating power of the *rbcL* and *matK* sequences for Apiaceae may also be one reason for the failure to detect *Angelica dahurica* in Wuhu San samples based on these two regions.

The analytical results of this study indicated that the ITS2 sequences displayed the strongest species discriminating power, while that of *psbA-trnH* sequences was lower than the ITS2 sequences. The *matK* and *rbcL* sequences demonstrated the worst species discrimination. Although the discriminating efficiency of the chloroplast *psbA-trnH*, *matK,* and *rbcL* sequences in this study was lower than that of the nuclear ITS2 sequences, the sequences of the chloroplast genome also exhibited certain advantages. The chloroplast genome is mostly maternally inherited and represents single-copy sequences in plant cells (Chen, 2016; Daniell et al., 2016). Moreover, chloroplast DNA sequences can be used as a versatile tool for plant identification (Nock et al., 2011). Most plants contain a significant number of chloroplasts, making DNA easy to obtain. In addition, this study showed that the species obtained from the chloroplast sequences were relatively simple. The obtained sequences generally represented prescription ingredients or obvious adulterants. A combination of multiple DNA barcodes can improve the resolution and accuracy of species discrimination (Group et al., 2009; Arulandhu et al., 2017). In addition, the ITS2 sequences can also detect fungi, which can be used to monitor the potential risk of the fungal contamination of traditional herbal patent medicine (Sweeney and Dobson, 1998; Guo et al., 2020). Therefore, to take advantage of shotgun metabarcoding, combining multiple barcodes obtained via the technology can increase the reliability and applicability of the experimental results. It helps monitor the quality of traditional herbal patent medicine.

DATA AVAILABILITY STATEMENT

The high-throughput sequencing datasets presented in this study can be found in the National Center for Biotechnology Information (NCBI) SRA online repository. The accession number of the BioProject is PRJNA663116. The accession numbers of the BioSample specimens are SAMN16124456, SAMN16124457, SAMN16124458, SAMN16124459, and SAMN16124460. And the SRA accession numbers for the above five BioSample specimens are SRR12632599, SRR12632598, SRR12632597, SRR12632596, and SRR12632595, respectively.

The DNA barcoding sequences assembled from the Sanger sequencing datasets presented in this study can be found in the NCBI GenBank online repository. The accession numbers for these DNA barcoding sequences are MN727081, MN727076, MT821449, MT821451, MT821450, MT102865, MT994327, MT994328, MT994331, MT994330, MT994329, MN729559, MN729561, MW000338, MW000340, MW000339, MW000341, MN746764, MN746766, MW000334, MW000335, and MW000333.

AUTHOR CONTRIBUTIONS

LS and JL conceived and designed the study. WM, QZ, and HX performed the experiment. WM, LS, and MS analysed the data. WM, LS, and JL wrote the paper. LS and JL revised the paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.607200/full#supplementary-material.

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Chemical Authentication of Botanical Ingredients: A Review of Commercial Herbal Products

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Chemical methods are the most important and widely used traditional plant identification techniques recommended by national and international pharmacopoeias. We have reviewed the successful use of different chemical methods for the botanical authentication of 2,386 commercial herbal products, sold in 37 countries spread over six continents. The majority of the analyzed products were reported to be authentic (73%) but more than a quarter proved to be adulterated (27%). At a national level, the number of products and the adulteration proportions varied very widely. Yet, the adulteration reported for the four countries, from which more than 100 commercial products were purchased and their botanical ingredients chemically authenticated, was 37% (United Kingdom), 31% (Italy), 27% (United States), and 21% (China). Simple or hyphenated chemical analytical techniques have identified the total absence of labeled botanical ingredients, substitution with closely related or unrelated species, the use of biological filler material, and the hidden presence of regulated, forbidden or allergenic species. Additionally, affecting the safety and efficacy of the commercial herbal products, other low quality aspects were reported: considerable variability of the labeled metabolic profile and/or phytochemical content, significant product-to-product variation of botanical ingredients or even between batches by the same manufacturer, and misleading quality and quantity label claims. Choosing an appropriate chemical technique can be the only possibility for assessing the botanical authenticity of samples which have lost their diagnostic microscopic characteristics or were processed so that DNA cannot be adequately recovered.

Keywords: chemical marker, natural product, herbal product, food supplement, herbal medicine, authentication, adulteration, contamination

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INTRODUCTION

Herbal products are being sold under many and diverse commercial descriptions in the international marketplace, including herbal drugs, botanical drugs, botanicals, phytomedicines, traditional medicines (TMs), herbal medicines (HMs), traditional herbal medicines products (THMPs), natural health products (NHPs), dietary supplements (DSs), plant food supplements (PFSs), nutraceuticals (NCs) and food supplements (FSs) (Ichim, 2019), the differences being mainly due to the prevailing national legislation under which they are marketed (Simmler et al., 2018). Herbal products are commercialized as medicines or foods, according to their officially declared

intended final use by their manufacturers operating under various regulatory frameworks, and they are purchased, and subsequently used and consumed, for their medicinal claims (herbal medicines) or their expected health benefits (food supplements) (Thakkar et al., 2020). In the United Kingdom, for example, plant products are regulated under two main criteria, the first being what is claimed, i.e. if a manufacturer claims a medicinal effect, the product will automatically fall under medicines legislation; the second consideration being the activity of the plant *in vivo*, if it has shown to have a strong medicinal or pharmacological action then it is deemed a medicine regardless of the claims, the most notable plant in this category being *Hypericum perforatum* L. (St John's Wort). Whereas in the United States most plant products are regulated as food supplements (botanicals) and in Germany the majority are considered medicines. Unfortunately, these marketing differences, due to significant differences between the regulatory approaches across jurisdictions (Low et al., 2017), are further contributing to their poor regulation on the international market.

Accidental contamination or the deliberate use of filler or substitute species (Shanmughanandhan et al., 2016) leads inherently to non-authentic, adulterated products (Simmler et al., 2018). The adulteration of commercial herbal products is an internationally widespread problem, as it has been reported for many countries from all inhabited continents (Ichim, 2019; Ichim et al., 2020). Moreover, large percentages of adulterated products have been reviewed, irrespective of the formal category of herbal products, being affected food and dietary supplements and medicines altogether (Ichim and de Boer, 2021), including products used in centuries or even millennia-old Ayurveda (Revathy et al., 2012; Seethapathy et al., 2019) and Asian traditional medicine systems (Masada, 2016; Xu et al., 2019). The substantial proportion of adulterated commercial herbal products described appears to be independent of the methods used for their analysis, traditional pharmacopoeial methods being employed, such as macroscopic inspection (van der Valk et al., 2017), microscopy (Ichim et al., 2020), chemical techniques (Li et al., 2008; Upton et al., 2020), or even the more recently developed DNA-based ones, such as the rapidly technologically evolving DNA barcoding and metabarcoding (Ichim, 2019; Grazina et al., 2020).

On the global market, herbal products are sold in an extremely diverse variety of forms, from single ingredient, unprocessed, raw, whole plants to multi-species, highly processed extracts. Therefore, the successful authentication of commercial herbal products reported by peer reviewed studies are a valuable and useful source of information which provide the necessary practicalities, including their strengths and the limitations, of employing the right methods for a specific type of product along the length of its value chain (Booker et al., 2012). Such analyses of peer-reviewed authentication reports focused exclusively on commercial herbal products have concluded that, microscopy, a traditional pharmacopoeial identification method, is cost-efficient and can cope with mixtures and impurities but it has limited applicability for highly processed commercial samples e.g. extracts (Ichim et al., 2020). On the other hand, DNA-based identification, only recently adopted by the first two national Pharmacopoeias (Pharmacopoeia Committee of P. R. China, 2015; British Pharmacopoeia Commission, 2018), facilitate simultaneous multi-taxa identification by using the DNA of different origins extracted from complex mixtures and matrices but false-negatives can be expected if the DNA has been degraded or lost during post-harvest processing or manufacturing (Raclariu et al., 2018a; Ichim, 2019; Grazina et al., 2020). In this respect, our review adds the much needed peer-reviewed, systematically searched information, about the successful use of chemical identification for the authentication of commercial herbal products. While doing so, our review also provides some missing pieces of the commercial herbal products' authenticity puzzle.

METHODS

Databases

Search Strategy

Four databases were systematically searched for peer reviewed records following the PRISMA guidelines (Moher et al., 2009) using combinations of relevant keywords, Boolean operators and wildcards: [("herbal product" OR "herbal medicine" OR "traditional medicine" OR "food supplement" OR "dietary supplement" OR "herbal supplement" OR nutraceutical) AND (authentic* OR contaminat* OR substitut*)] for Web of Science, PubMed, Scopus, and [("herbal product" OR "herbal medicine" OR "food supplement" OR "dietary supplement" OR "herbal supplement" OR nutraceutical) AND (authentication OR contamination OR substitution)] for ScienceDirect. The option "search alert" was activated for all four databases, to receive weekly updates after the literature search was performed. Furthermore, we used cross-referencing to identify additional peer-reviewed publications.

Selection Process and Criteria

Identification: 10,497 records were identified through database searching (WoS = 1,317, PubMed = 3,253, Scopus = 5,446, and ScienceDirect = 481), and 196 additional records from cross-referencing and the weekly updates from the four databases. Screening: after the duplicates had been removed, 2,326 records were collected and their abstracts screened. After screening, 1,745 records were excluded for not reporting data relevant for the chemical authentication of herbal products. Eligibility: 581 full-text articles were assessed and screened based on the following eligibility criteria: 1) The reported products had to be "herbal products"; the full wide range of commercial names was searched for and accepted for being included in our analysis. 2) The analyzed products had to be "commercial"; keywords such as "purchased", "bought", were accepted. Our analysis excluded samples which were obtained "cost-free", a "gift" or "donated" by a person, institution or company. 3) The products had to be clearly allocated to a "country" or "territory" (e.g., European Union). 4) The conclusion "authentic"/"adulterated" had to be drawn by the authors of the analyzed studies. 5) The products had to be analyzed with a "chemical" method or techniques.

TABLE 1 | The authenticity of the chemically authenticated commercial herbal products at global level.

No. crt.	Country / territory	Products (details) / authenticated species	Products			Adulteration reported	Authentication method / marker (if reported)	Additional quality issues detected	Botanical/ chemical reference materials/ standards	Bibliographic reference
			total	authentic/ adulterated						
			no.	no.	no.					
1	Australia	grape seed extract products (capsules) from retail pharmacies, health stores / *Vitis vinifera*	9	4	5	complete substitution or heavy adulteration, possibly with peanut skin extract, *Pinus massoniana* (or other A-type procyanidin-containing species)	RP HPLC-UV-MS / catechin, epicatechin, procyanidin B2, procyanidin A2, rape seed oligomeric proanthocyanidins	not reported	*V. vinifera* (seeds, seed extracts), *A. hypogaea, P. massoniana, P. pinaster, V. macrocarpon, T. cacao* (extracts)	Govindaraghavan (2019)
	New Zeeland		6	6	0	n/a				
2	Australia	gingko products (capsule, tablets) from retail stores / *Ginkgo biloba*	6	3	3	adulteration with flavonol aglycones, likely with *Styphnolobium japonicum*	RP HPLC, LC-MS / flavonol aglycones (quercetin, kaempferol, isorhamnetin)	contained genistein, an isoflavone that does not occur in ginkgo leaf	authenticated samples of dried *Ginkgo biloba* leaf from commercial suppliers	Wohlmuth et al. (2014)
	Denmark		2	2	0	n/a				
3	Belgium	products (tablets and capsules) containing regulated plants / *Aristolochia fangchi*, *Ilex paraguariensis, Epimedium* spp., *Pausinystalia johimbe, Tribulus terrestris*	69	48	21	adulteration/ contamination with unlabeled ingredients: *A. fangchi* (forbidden), *I. paraguariensis, Epimedium* spp., *T. terrestris* (all should be notified to authorities), *P. johimbe*	FT-Mid-IR, HPLC-DAD, LC-MS	*P. yohimbe* or *T. terrestris* not identified in some products although claimed on the label	reference material of the five plant species (leaves, bark, fruits)	Deconinck et al. (2019)
4	Belgium	herbal products (capsules, tablets) from local pharmacy / *Passiflora edulis*	3	3	0	n/a	HPLC-DAD, HPLC-MS	not reported	commercial *P. edulis* (dry extract) (European Pharmacopoeia)	Deconinck et al. (2015)
5	Belgium	products containing three non-regulated herbs (capsule, tablets) from local pharmacy / *Frangula purshiana, Passiflora edulis, Crataegus monogyna*	3	3	0	n/a	HPLC-DAD–ELSD, HPLC-MS	not reported	commercial dry plant extracts of *F. purshiana, P. edulis, C. monogyna* (European Pharmacopoeia)	Deconinck et al. (2013)
6	Belgium	illegal products (tablets, capsules) containing regulated plant species / *Epimedium* spp., *Tribulus terrestris*	2	2	0	n/a	HPLC-PDA. HPLC-MS	adulteration with sildenafil	self-made triturations in three different botanical matrices from reference standards of *Epimedium* spp. leaves, *P. johimbe* bark, *T. terrestris* fruit	Custers et al. (2017)
7	Brazil	"carqueja" products (bags with pulverized plant material or parts of the plant) from commercial shops / *Baccharis trimera*	15	11	4	non-authentic	GC-FID / essential oil	intensity of the peaks in most of cases was different	authenticated samples of *B. trimera* (aerial parts, leaves) / standard oil of *B. trimera* (extracted)	De Ferrante et al. (2007)

(Continued on following page)

TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

No. crt.	Country / territory	Products (details) / authenticated species	Products			Adulteration reported	Authentication method / marker (if reported)	Additional quality issues detected	Botanical/ chemical reference materials/ standards	Bibliographic reference
			total	authentic/ adulterated						
			no.	no.	no.					
8	Brazil	"sarsaparilla" products from drugstores / *Smilax goyazana, S. rufescens, S. brasiliensis, S. campestris, S. cissoides, S. fluminensis, S. oblongifolia, S. polyantha*	15	0	15	different from the reference *Smillax* sp.	TLC / flavonoids, saponins, terpenoids, steroids, catechins	n/a	authenticated reference material (roots) of *S. brasiliensis, S. campestris, S. cissoides, S. fluminensis, S. goyazana, S. oblongifolia, S. rufescens, S. polyantha*	Martins et al. (2014)
9	Brazil	"copaiba" oil-resin products from local markets / *Copaifera multijuga*	12	3	9	substitution and adulteration with soybean oil	TLC	not reported	reference *C. multijuga* oil-resins, prepared mixtures of soybean oil and copaiba oil resin	Barbosa et al. (2009)
10	Brazil	"carqueja" products from herbal shops, pharmacies / *Baccharis trimera*	12	12	0	n/a	TLC / 3-o-methyl-quercetin	large variations in the percentage of flavonoids (quercetin)	*B. trimera* reference samples / Brazilian Pharmacopoeia (BP)	Beltrame et al. (2009)
11	Brazil	"janaguba" milk products from local market / *Himatanthus drasticus*	10	4	6	complete substitution or adulteration with *Hancornia speciosa*	TLC	not reported	authentic samples of "janaguba" latex, mango tree latex sample	Soares et al. (2016)
12	Brazil	"Bauhinia spp." products (ground dry leaves) from drugstores, local market / *Bauhinia forficata* ssp.	9	2	7	not containing claimed B. forficata	HPLC-UV/PDA, MCR-ALS/PCA	not reported	*B. forficata, B. f.* var. *longifolia* authenticated leaves	Ardila et al. (2015)
13	Brazil	"jatoba" sap products / *Hymenaea stigonocarpa, Hymenaea martiana*	6	0	6	probably achieved by a decoction of the stem bark or other sources	HPLC-MS / flavonoids, procyanidins	n/a	*H. stigonocarpa, H. martiana* authenticated sap and stem bark samples	De Souza Farias et al. (2017)
14	Brazil	herbal products from commercial shops / *Maytenus ilicifolia*	3	1	2	possible substitution with plants from the same family and/or contamination due to addition of similar other plants parts to the commercial one	FTIR, 1H NMR	not reported	*M. ilicifolia* control sample from the open market, in the selected natural form, recognized by ''herbal trackers''	Preto et al. (2013)
15	Brazil	herbal products (raw material) from different suppliers / *Echinodorus grandiflorus*	3	3	0	n/a	TLC / caffeic acid, isoorientin and swertiajaponin, o-hydroxycinnamic acid derivatives	variable quantity of some marker compounds	Brazilian Pharmacopoeia (BP) 5th edition	Dias et al. (2013)

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TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

No. crt.	Country / territory	Products (details) / authenticated species	Products			Adulteration reported	Authentication method / marker (if reported)	Additional quality issues detected	Botanical/ chemical reference materials/ standards	Bibliographic reference
			total	authentic/ adulterated						
			no.	no.	no.					
16	Canada	*Smilax ornata*, organic *Sarsaparilla* root, *Hemidesmus indicus* products from online store / *Hemidesmus indicus*, *Periploca indicus*	3	0	3	adulteration with *Decalepis hamiltonii* and *Pteridium aquilinum*	1H-NMR/HCA	not reported	reference samples of known provenance of *P. aquilinum, Smilax aristolochiifolia, D. hamiltonii, H. indicus*	Kesanakurti et al. (2020)
17	China	"Tong-guanteng" products from medicine markets, drug stores / *Marsdenia tenacissima*	62	61	1	substitution with *Tinospora sinensis*	TLC, HPLC / tenacissoside H	TS-H contents (0.39-1.09%) larger than that regulated in the Chinese Pharmacopoeia (0.12%)	genuine *M. tenacissima* herb	Yu et al. (2018)
18	China	ginseng products (pills, bag, injections, capsules, tablets, powders, dripping pills) from drugstores / *Panax ginseng, P. quinquefolius, P. notoginseng*	40	38	2	*P. ginseng* products adulterated (weak chromatographic peaks, and several marker compounds were not detected)	LC–MS / ginsenosides	in few products markers for PG not detected, signals for PN (ginsenoside Rf) very weak	authenticated ginseng crude drug samples	Yang et al. (2016)
19	China	Pinelliae rhizoma products from herbal medicine markets / *Pinellia ternata*	39	12	27	substitution with *Pinellia pedatisecta*	HPLC-DAD, HPLC-MS, LC-MS / triglochinic acid	not reported	authenticated batches of Pinelliae rhizoma and Pinelliae pedatisectae rhizoma / extracted and purified triglochinic acid	Jing et al. (2019)
20	China	"Wuweizi" (Schisandrae Chinensis Fructus) and "Nan-wuweizi" (Schisandrae Sphenantherae Fructus) products from pharmaceutical manufacturers, pharmacies / *Schisandra chinensis, S. sphenanthera*	36	34	2	substitution with *S. aphenanthera*	LC-DAD-MS, TLC, HPLC / schisandrin, anwulignan	not reported	authenticated batches of batches of Wuweizi and Nan-wuweizi, reference crude drugs, in-house prepared mixtures	Jiang et al. (2016)
21	China	American or Asian ginseng root products from stores / *Panax ginseng, P. quinquefolius*	31	28	3	adulteration and substitution of wild with cultivated ginseng	1H NMR-PCA / sucrose, glucose, arginine, choline, 2-oxoglutarate, malate, ginsenosides	not reported	n/a	Zhao et al. (2015)

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TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

No. crt.	Country / territory	Products (details) / authenticated species	Products			Adulteration reported	Authentication method / marker (if reported)	Additional quality issues detected	Botanical/ chemical reference materials/ standards	Bibliographic reference
			total	authentic/ adulterated						
			no.	no.	no.					
22	China	"Chaihu" (Bupleuri Radix) products from major herbal distribution centres / *Bupleurum chinense, B. scorzonerifolium*	31	20	11	substitution with *B. longiradiatum, B. bicaule, B. falcatum, B. marginatum* var. *stenophyllum*	HPLC-ELSD, HPTLC / saikosaponins	great variation in the content of the major saikosaponins	authenticated samples of *B. chinense, B. scorzonerifolium, B. falcatum, B. longiradiatum, B. bicaule, B. marginatum* var. *stenophyllum*	Tian et al. (2009)
23	China	red yeast rice (RYR) commercial raw materials from supplement manufacturers / *Monascus purpureus* - fermented rice	31	21	10	did not show the presence of any monacolins analyzed	UHPLC–DAD–QToF-MS / monacolins, citrinin	n/a	RYR authenticated samples	Avula et al. (2014)
	United States	RYR-containing products from online retailers / *Monascus purpureus* - fermented rice	14	14	0	n/a		large variations (20-40 fold) in quantity and quality of monacolin K		
24	China	Asian and American ginseng products from	31	23	8	adulteration with *P. ginseng*	UPLC/Q-TOF-MS / ginsenoside Rf, 24 (R)-pseudoginsenoside F11	not reported	self-prepared samples with different contents (spiking the Asian ginseng powder into the American ginseng powder)	Li et al. (2010)
	Canada	herbal markets, local	5	5	0	n/a				
	United States	drug stores / *Panax ginseng, P. quinquefolius*	4	4	0	n/a				
25	China	"Gou-Teng" batches of (Uncariae Rammulus Cum Uncis) from markets / *Uncaria macrophylla, U. hirsuta, U. sinensis, U. sessilifructus*	20	16	4	substitution with other *Uncaria* sp. or unlabelled mixtures with the five officially accepted *Uncaria* sp.	UPLC/Q-TOF MS / alkaloids	not reported	authenticated batches of five *Uncaria* sp. (stems with hooks) / isolated and identified alkaloids	Pan et al. (2020)
26	China	Chaenomelis Fructus (raw) products from manufacturers, herbal markets / *Chaenomeles speciosa*	20	19	1	the source plant is not *C. speciosa*	HPLC–DAD / quinic acid, malic acid, protocatechuic acid, shikimic acid, chlorogenic acid	the relative contents of each component may vary in some of the samples	n/a	Zhu et al. (2019)

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TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

No. crt.	Country / territory	Products (details) / authenticated species	Products			Adulteration reported	Authentication method / marker (if reported)	Additional quality issues detected	Botanical/ chemical reference materials/ standards	Bibliographic reference
			total	authentic/ adulterated						
			no.	no.	no.					
27	China	"Beimu" (Fritillariae Bulbus) products from drugstores / *Fritillaria taipaiensis, F. unibracteata var. wabuensis, F. delavayi, F. unibracteata, F. przewalskii, F. cirrhosa, F. ussuriensis, F. thunbergii*	16	11	5	substitution or adulteration with unlabeled *F. ussuriensis*	UPLC-QTOF-MS / steroidal alkaloids	loss of specific features, possibly resulted from different processes of different manufacturers	authenticated batches of *Fritilaria* sp.	Liu et al. (2020)
28	China	Menispermi Rhizoma products (dried rhizomes, pills, capsules) from drug stores / *Menispermum dauricum*	16	15	1	counterfeit (most of the important marker alkaloids could not be detected)	UPLC-DAD-MS / alkaloids	discrepancies among the samples of different origins (the contents of the nine alkaloids varied greatly)	authenticated MR batches from various drug stores / separated and purified (from MR) alkaloids	Liu et al. (2013a)
29	China	batches of "Shuxiong" tablets from manufacturers, drugstores / *Panax notoginseng, Carthamus tinctorius, Ligusticum striatum*	12	12	0	n/a	UPLC/QDa-SIM / (saponins, quinochalcone C-glycosides, 16 O-glycoside, phenolic acid, pathalides	low content of some markers in a few products possibly caused by different preparation process or use of poor-quality drug materials	crude drug reference materials Notoginseng Radix et Rhizoma, Carthami Flos, Chuanxiong Rhizoma	Yao et al. (2016)
30	China	"Huangqi" (Radix Astragali) products from wholesale TCM markets, city pharmacies / *Astragalus prompiquus*	12	11	1	substitution with *Astragalus tongonlensis*	HPLC-UV / isoflavonoids	total isoflavonoids content varies considerably	n/a	Wu et al. (2005)
31	China	"ci-wu-jia" tea products (leaf, leaf powder) from local stores / *Eleutherococcus senticosus*	11	8	3	adulteration with green tea (*Camellia sinensis*)	UHPLC-UV-MS/MS / organic acid derivatives, flavonoids, triterpene saponins	not reported	*E. senticosus* leaf samples collected from China / in-house UNIFI library of *Eleutherococcus* genus and green tea extracts	Wang et al. (2019)
32	China	*Panax ginseng* and *P. quinquefolius* products (bolus, tea, tablet, drink) from local pharmacies / *P. ginseng, P. quinquefolius*	11	10	1	substitution or adulteration with *P. ginseng*	UHPLC-TOF/MS/ OPLS-DA / ginsenosides	n/a	34 white ginsengs, 23 red ginsengs, 30 *P. notoginseng* and 21 *P. quinquefolius* collected samples	Wu et al. (2020)

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TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

No. crt.	Country / territory	Products (details) / authenticated species	Products			Adulteration reported	Authentication method / marker (if reported)	Additional quality issues detected	Botanical/ chemical reference materials/ standards	Bibliographic reference
			total	authentic/ adulterated						
			no.	no.	no.					
33	China	*Panax notoginseng* powder products from drug stores, CHM manufacturers / *P. notoginseng*	10	9	1	adulteration, possibly with flower material of *P. notoginseng*	UPLC/Qtof MS/ PCA / notoginsenosides, ginsenosides, 20S-ginsenoside Rh1, gypenoside XVII	not reported	authenticated *P. notoginseng* powder samples	Liu et al. (2015)
34	China	"Xihuangcao" (Isodonis lophanthoidis herba) from herbal markets / *Isodon lophanthoides*	9	7	2	substitution with *I. lophanthoides* var. *gerardianus*	HPTLC / 2α-O-β-D-glucoside-12-en-28-ursolic acid, 2α,19α-dihydroxy-12-en-28-ursolic acid, 2α-hydroxy-12-en-28-ursolic acid, ursolic acid	not reported	collected batches of *I. lophanthoides*	Lin et al. (2019)
35	China	*Panax ginseng* products from local drug stores / *P. ginseng*	8	5	3	substitution with *P. quinquefolius, Platycodon grandiflorus, Physochlaina infundibularis, Phytolacca acinosa*	FT-NIR	not reported	authenticated *P. ginseng* samples	Dong et al. (2020)
36	China	"Xihuangcao" products (tea bags) from retail stores / *Isodon lophanthoides, I. serra*	8	0	8	no *Isodon* sp. material, adulteration and substitution with unlabeled plant species	UPLC-ESI-QTOF-MS	n/a	authenticated *I. lophanthoides* and *I. serra* plant material / reference teas of many plant species	Wan et al. (2016)
37	China	gingko leaf product and health foods (tea, tablets, soft gels) from drug store, local stores / *Gingko biloba*	6	5	1	adulteration (the rutin content was uncharacteristically high)	HPLC(EIS)/MS / flavonol glycosides, terpene trilactones, flavonol aglycones, biflavones	not reported	*G. biloba* leaves collected from different habitats	Song et al. (2010)
38	China Bulgaria Greece Chile United Kingdom	St. John's Worth products (loose material) from herbal markets, pharmacies and producer's cultivation / *Hypericum perforatum*	5 2 2 1 1	5 2 2 1 1	0 0 0 0 0	n/a	HPTLC, 1H-NMR/PCA	low content of typical H.p. compounds apparently due to higher amount of woody material	authenticated *Hypericum* sp. samples	Scotti et al. (2019)
39	China	Aquilariae Lignum Resinatum (ALR) products from market / *Aquilaria sinensis*	3	0	3	little or different resin components	FT-IR, SD-IR, 2D-IR	not reported	standard ALR (the resin-rich wood of *A. sinensis*	Qu et al. (2016)
40	China	Aquilariae Lignum Resinatum (ALR) products from market / *Aquilaria sinensis*	3	0	3	Adulteration with other kind of wood (possibly *Gonystylus* spp.), and by adding cheap resin (e.g. rosin)	FT-IR, 2D–IR	n/a	reference *A. sinensis* samples, no-resin wood of *A. sinensis*, authentic ALR samples	Qu et al. (2017)

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TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

No. crt.	Country / territory	Products (details) / authenticated species	Products			Adulteration reported	Authentication method / marker (if reported)	Additional quality issues detected	Botanical/ chemical reference materials/ standards	Bibliographic reference
			total	authentic/ adulterated						
			no.	no.	no.					
41	China	*Ophiocordyceps sinensis* products from TCM market / *O. sinensis*	2	1	1	substitution with lepidopteran larvae infected by *Metacordyceps taii*.	HPLC / cordycepin, adenosine and other nucleosides	not reported	authenticated *O. sinensis* specimens collected in Tibet	Wen et al. (2016)
42	Croatia	gingko products (GBEs, food supplements / capsules, tablets, powder) / *Ginkgo biloba*	10	8	2	substitution with *Sophora japonica* extracts	HPLC / quercetin/ kaempferol ratio, ginkgo flavone glycosides (quercetin, kaempferol, isorhamnetin)	not reported	n/a	Budeč et al. (2019)
43	Denmark	St. John's Worth products (tablets, capsules) from commercial suppliers / *Hypericum perforatum*	10	10	0	n/a	1H-NMR/PCA	considerable differences in the products composition (e.g. flavonoids), inter-product and inter-batch variation	n/a	Rasmussen et al. (2006)
44	Egypt	herbal products (teas) from market / chamomile, marjoram, licorice, fennel, dill, caraway, basil, lemon grass, anise, chicory, achillea, verbascum, hibiscus, vine	3	0	3	adulterated with other species, some labeled species missing those of the formula	GC-MS, HPLC / essential oil, polyphenols, flavonoids	some of the herbs used are exhausted	reference herbal teas prepared from herbs purchased from the market	Kamal et al. (2017)
45	Egypt	herbal products (tea) / chicory, marjoram, nettle and senna leaves, liquorices roots, celery fruits, calendula flowers and fennel, senna and chicory	2	2	0	n/a	HPLC, GC-MS / sennoside A, esculetin, scopoletin. volatile oil	not reported	prepared standard herbal mixtures	Abdel Kawy et al. (2012)
46	European Union	*Panax ginseng* products (herb, root extracts, stem/leaf	12	6	6	*P. ginseng* leaf or other plant parts, *P. quinquefolius* roots	HPTLC, HPLC / ginsenosides	not reported	bulk crude *P. ginseng* dried root samples, *P. ginseng* leaf and stem	Govindaraghavan (2017)
	Australia	extract, berry extract) (capsules, tablets) / *P. ginseng*	4	1	3	*P. ginseng* leaf or other plant parts				
	China		1	0	1	leaf/stem				
47	European Union	food supplements	10	2	8	adulteration	HPLC-UV, LC-MS/MS / flavonoids and terpenes lactones (ginkgolides, bilobalide)	n/a	*G. biloba* herbal medicinal product (control)	Czigle et al. (2018)
	Greece	containing ginkgo dry extract or ginkgo leaf (tablets, soft and hard capsules) from local community pharmacies / *Ginkgo biloba*	1	0	1					

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TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

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			total	authentic/ adulterated						
			no.	no.	no.					
48	India	"Asoka" raw herbal products from shops / *Saraca asoca*	25	3	22	substitution	1D/2D NMR/PCA	not reported	taxonomically authenticated samples of *S. asoca* (bark, flower, stem)	Urumarudappa et al. (2016)
49	India Norway Romania Sweden United States	Garcinia products (capsules, tablets) from pharmacies, internet / *Garcinia gummi-gutta, G. indica*	5 1 1 1 2	5 1 1 1 2	0 0 0 0 0	n/a	1H NMR / (–)-hydroxycitric acid, (–)-hydroxycitric acid lactone	large variation in the content of (-)-hydroxycitric acid; only one product contained quantifiable amounts of (–)-hydroxycitric acid lactone	authenticated BRM from eleven species of *Garcinia* L.	Seethapathy et al. (2018)
50	India	licorice products (raw material) from local shops / *Glycyrrhiza glabra, G. uralensis, G. inflata*	2	2	0	n/a	HPTLC, HPLC / 18β-glycyrrhizic acid	not reported	vouchered, botanically confirmed sample, raw materials (whole, chopped, or powdered) of licorice root / United States Pharmacopeia (USP)	Frommenwiler et al. (2017)
51	Italy	bilberry products (extracts) from different producers / *Vaccinium myrtillus*	71	65	6	adulteration with anthocyanins extracted from other berries (black mulberry, chokeberry, blackberry)	HPLC-DAD, FT-NIR/PCA / anthocyanins and the respective aglycones	the amount of anthocyanins in the bilberry extracts in the range 18–34%	refined and standardized dry extract from the bilberry fruit	Gardana et al. (2018)
52	Italy	cranberry products (extracts) from herbal shops, local markets / *Vaccinium macrocarpon*	24	5	19	misidentification of the raw material	HPLC-UV/Vis, Orbitrap LC-MS / anthocyanins	only one product complied the criteria of good preparation, respected their uniformity of dosage, and contained V. macrocarpon	European Pharmacopeia	Mannino et al. (2020)
53	Italy	cranberry products (extracts) from herbal shops, local markets / *Vaccinium macrocarpon*	10	4	6	adulteration with *Morus nigra* extract	UPLC-DAD-Orbitrap-MS-PCA / anthocyanin, epicatechin/catechin, procyanidin A2/total procyanidin, procyanidin/ anthocyanin ratios	only one product provided the daily dose deemed effective for treating a urinary tract infection	fruits and extract of possible adulterants	Gardana et al. (2020)
54	Italy	sweet fenel pre-packaged teabags and instant tea products (freeze-dried powders) from local pharmacies, grocery stores / *Foeniculum vulgare*	5	5	0	n/a	GC–MS / constituents of volatile oil	possible presence of bitter fennel or, for the powdered material, the presence of other parts of fennel	commercial reference samples of fruits of *F. vulgare* / European Pharmacopoeia (1997) monograph	Bilia et al. (2002)

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TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

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			total	authentic/ adulterated						
			no.	no.	no.					
55	Italy	herbal product (liquid preparations containing four species) from herbalist shop / *Olea europaea, Crataegus rhipidophylla, Fumaria officinalis, Capsella bursa-pastoris*	2	0	2	adulteration with a root extract from a *Rauvolfia* sp. (indole alkaloids)	HPLC-DAD–MS, HPLC–MS, NMR	n/a	purchased herbal products and collected plant material	Karioti et al. (2014)
56	Italy	herbal product (liquid preparations containing five species) / *Olea europaea, Crataegus rhipidophylla, Fumaria officinalis, Capsella bursa-pastoris*	1	0	1	adulteration with an extract from a *Rauvolfia* sp (indole alkaloids)	HPLC-ESI-ITMS, NMR	n/a	n/a	Gallo et al. (2012)
57	Japan United States Italy Malaysia	bilberry products (extracts) from the marketplace (tablets, hard and soft gel caps) / *Vaccinium myrtillus*	20 15 4 1	20 7 2 0	0 8 2 1	n/a substitution with berries different from *V. myrtillus*	LC-MS / anthocyanins	marked composition differences	*V. myrtillus* reference dry extract	Cassinese et al. (2007)
58	Japan	herbal products (crude drug extracts) (soft capsules, hard capsules, sugarcoated tablets) from internet / *Poria sclerotium, Ophiopogonis* tuber, *Rheum emodi*	14	1	13	mislabeling, adulteration	HPLC-PDA / sennoside A, aloe-emodin, emodin, rhein, chrysophanol	illegal adulteration with sibutramine	authenticated rhubarb rhizome	Yoshida et al. (2015)
59	Japan	chasteberry extracts (granules, tablets, soft and hard capsules) purchased via internet / *Vitex agnus-castus*	11	8	3	adulteration, contaminated with *V. negundo*	HPLC-PCA, quantitative determination of chemical marker compounds / agnuside, casticin	poor formulation quality	reference standard of *V. agnus-castus* fruit dry extract	Sogame et al. (2019)
60	Japan	herbal products (tea bags, granules, tablets) containing senna stems / *Cassia alexandrina*	8	5	3	adulteration with senna leaves and midribs	TLC, HPLC / sennoside A, sennoside B	the amount of sennosides ranged from 0.2-11 mg	reference raw senna materials (stems, leaves)	Kojima et al. (2000)

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TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

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			total	authentic/ adulterated						
			no.	no.	no.					
61	Japan	Siberian ginseng products (capsules, teas) from internet / *Eleutherococcus senticosus*	4	3	1	substitution with *Panax ginseng.*	HPLC-DAD / eleutheroside B, eleutheroside E, isofraxidin	not reported	specimens of *E. senticosus*, *E. sessiliflorus* and congeneric species, crude drugs from markets / chemical standards isolated from an authenticated commercial SG sample	Zhu et al. (2011)
62	Malaysia	"Tongkat Ali" products from pharmacies, night markets, jamu shops, food courts, on-line stores / *Eurycoma longifolia*	46	20	26	substitution	HPLC, 2DE / protein marker (A), eurycomanone	the amount of the markers detected varies among the products	purified *E. longifolia* crude extract	Vejayan et al. (2018)
63	Malaysia	'Tongkat Ali' products (capsules, spherical tablets) from pharmacies, drug stores / *Eurycoma longifolia*	29	18	11	substitution	2DE / protein markers (A, B) (~14kDa)	not reported	standardized *E. longifolia* root extracts	Vejayan et al. (2013)
64	Malaysia	"Tongkat Ali" products (capsules, tea, tablet) from retail shops / *Eurycoma longifolia*	7	3	4	substitution	HPLC-DAD / eurycomanone	none of the products met the officially required minimum concentration of eurycomanone	authenticated *E. longifolia* plant and five-year-old root sample	Abubakar et al. (2018)
65	Mexic	"Damiana" botanical products (extracts) from local markets / *Turnera diffusa*	6	3	3	substitution, adulteration	1H-NMR/PCA / hepatodamianol	differences in the chemical components	authenticated *T. diffusa* specimens / purified chemical reference standard (hepatodamianol)	Lucio-Gutiérrez et al. (2019)
66	Pakistan	crude drugs from local market / *Foeniculum vulgarae, Curcuma longa, Aloe vera, Plantago ovata, Zingiber officinale, Glycyrrhiza glabra*	6	6	0	n/a	TLC, spectrophotometry, FTIR / anethole, barbaloin, xylose, galactose, gingerol-1, gingerol-2, 6-gingerol, glycerrihitic acid, curcumin	all the samples of *Plantago ovata* do not comply with the pharmacopoeial standard	n/a	Fatima et al. (2020)
67	Pakistan	"guggul" gum resin product from herbal market / *Commiphora wightii*	1	0	1	adulteration with *Mangifera indica* gum	NMR	n/a	authenticated gum resin samples of *C. wightii* and *M. indica*	Ahmed et al. (2011)

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TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

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			total	authentic/ adulterated						
			no.	no.	no.					
68	Poland	chamomile samples (fragmented, granulated) from different manufacturers / *Matricaria chamomilla*	19	19	0	n/a	HPLC / phenolic acids (gallic, caffeic, syringic, p-coumaric, ferulic), flavonoids (rutin, myricetin, quercetin, kaempferol)	not reported	n/a	Viapiana et al. (2016)
69	Poland	ginkgo products (leaf extracts) (capsules, tablets) from local pharmacies, markets, online pharmacies / *Ginkgo biloba*	16	9	7	adulteration probably with *Sophora japonica* (fruit or flower extracts)	ATR-FTIR, iPLS-DA / rutin, quercetin, kaempferol	large amounts of quercetin and kaempferol	standardized (24/6) ginkgo extracts	Walkowiak et al. (2019)
70	Poland	herbal products containing sage ethanolic extract (capsules, tablets, ointments, tincture, finished product) / *Salvia officinalis*	6	5	1	substitution	TLC / rosmarinic acid	not reported	*S. officinalis* authenticated botanical extracts	Cieśla and Waksmundzka-Hajnos (2010)
71	Romania	St. John's Wort products (herbal teas, capsules, tablets, extracts) from pharmacies, herbal shops, supermarkets, internet / *Hypericum perforatum*	50	34	16	substitution with other *Hypericum* sp. or did not contain *Hypericum* species in detectable amounts	TLC, HPLC-MS / rutin, hyperoside, hyperforin, hypericin	not reported	authenticated reference plant material of *H. elegans, H. maculatum, H. olympicum, H. patulum, H. perforatum, H. polyphyllum*	Raclariu et al. (2017)
	Slovakia		3	1	2					
	Turkey		2	1	1					
	Austria		2	2	0	n/a				
	Czech Republic		1	1	0					
	France		1	1	0					
	Germany		4	4	0					
	Italy		1	1	0					
	Netherlands		1	1	0					
	Poland		4	4	0					
	Spain		2	2	0					
	Sweden		1	1	0					
	United Kingdom		2	2	0					
72	Romania	*Echinacea* products (teas, capsules, tablets, extracts) from retail stores, e-commerce / *Echinacea purpurea, E. angustifolia, E. pallida*	34	30	4	substitution or adulteration with unlabeled *Echinacea* sp.	HPTLC / echinacoside, cynarin, cichoric acid, chlorogenic acid, caffeic acid, caftaric acid	products totally devoided of any Echinacea sp. material	reference botanical standards: *E. purpurea, E. angustifolia, E. pallida* (UPS)	Raclariu et al. (2018b)
	Czech Republic		2	0	2					
	Germany		3	0	3					
	Italy		1	0	1					
	Poland		2	1	1					
	Spain		2	0	2					
	Austria		1	1	0	n/a				
	France		1	1	0					
	Norway		4	4	0					

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TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

No. crt.	Country / territory	Products (details) / authenticated species	Products			Adulteration reported	Authentication method / marker (if reported)	Additional quality issues detected	Botanical/ chemical reference materials/ standards	Bibliographic reference
			total	authentic/ adulterated						
			no.	no.	no.					
73	South Korea	*Panax ginseng* (decoctions, beverages, capsules, tablets), *Platycodon grandiflorus* (decoctions, beverages), *Codonopsis lanceolata* (decoctions, beverages), *Pueraria montana* var. *lobata* (beverages) from local markets / *P. ginseng, P. grandiflorum, C. lanceolata, P. montana* var. *lobata*	81	81	0	n/a	HPLC, UPLC–DAD–ESI-IT-TOF-MS / lobetyolin, ononin	not reported	raw plant material of *P. ginseng, P. grandiflorum, C. lanceolata, P. montana* var. *lobata*	Choi et al. (2018)
74	South Korea	"Malabar tamarind" products from local market / *Garcinia gummi-gutta*	11	11	0	n/a	HPLC / cyanidin-3-O-sambubioside, cyanidin-3-O-glucoside	not reported	collected fruit rinds of *G. gummi-gutta*, purchased *G. indica* fruit samples	Jamila et al. (2016)
75	Taiwan	"myrobalan" (Fructus Chebulae) products from local herbal markets / *Terminalia chebula, Terminalia chebula* var. *tomentella*	28	20	8	substitution *with T. chebula* var. *parviflora*	HPLC / tannin-related constituents	not reported	reference standards, including some isolated previously from *T. chebula*	Juang and Sheu (2005)
76	Taiwan	herbal materials of Fritillariae Thunbergii Bulbus from local markets / *Fritillaria thunbergii*	12	12	0	n/a	HPLC-UV / peimine, peiminine	product with low total content of peimine (not to be used clinically)	n/a	(Lin et al., 2015)
77	Taiwan	white ginseng products (radix sliced material, powder, capsules) / *Panax ginseng*	8	7	1	not composed of 6 years old ginseng radix only	1H-NMR/PCA/CA	not reported	authenticated, one to six year-old, fresh white ginseng radix (*P. ginseng*)	Lin et al. (2010)
78	Taiwan China	5:1 concentrated extract products (prepared from dried roots) from different companies / *Scutellaria baicalensis*	6 4	6 4	0 0	n/a	HPLC / baicalin, baicalein	significant product-to-product and batch-to-batch variation of the marker compounds	n/a	Ye et al. (2004)
79	Thailand	white "Kwao Krua" products from Thai local markets, drugstores / *Pueraria candollei*	7	7	0	n/a	HPLC / isoflavone glycosides (puerarin, daidzin, genistin), isoflavones (daidzein, genistein)	not reported	authenticated *P. candollei*, *Mucuna macrocarpa*, *Butea superba* plant material, Kwao Krua crude drugs	Intharuksa et al. (2020)

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TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

No. crt.	Country / territory	Products (details) / authenticated species	Products			Adulteration reported	Authentication method / marker (if reported)	Additional quality issues detected	Botanical/ chemical reference materials/ standards	Bibliographic reference
			total	authentic/ adulterated						
			no.	no.	no.					
80	Thailand	*Garcinia atroviridis* products (capsules) from market / *G. atroviridis*	5	4	1	substitution	CZE / hydroxycitric acid and hydroxycitric acid lactone	not reported	n/a	Muensritharam et al. (2008)
81	Thailand	"Ya dok khao" smoking cessation tea product from local market / *Cyanthillium cinereum*	1	1	0	n/a	HPTLC / triterpenoid compounds (ß-amyrin, taraxasterol, lupeol, betulin)	not reported	*C. cinereum, E. sonchifolia* collected samples, raw *C. cinereum* materials	Thongkhao et al. (2020)
82	Turkey	chamomile products (tea bags, bulk or packaged crude flowers) from food stores, bazaar / *Matricaria chamomilla*	16	5	11	adulteration (possibly with *Anthemis* spp., *Tanacetum* sp. and *Chrysanthemum* sp.)	HPLC, HPTLC - PCA, HCA / apigenin 7-O-glucoside	A7G content in different tea brands ranged from 0.43-0.80 mg/g	wild and cultivated varieties of chamomiles, chamomile-like flowers (*Anthemis* L., *Bellis* L., *Tanacetum* L., *Chrysanthemum* L.)	Guzelmeric et al. (2017)
83	Turkey	Ginkgo products (extracts) from local pharmacy, local markets / *Ginkgo biloba*	13	13	0	n/a	LC-MS, HPLC-DAD / ginkgolides, flavonoid aglycones	total flavonoids and ginkgolides higher in medicinal products, no or very little flavonoids in food supplements	chemical reference standards (ginkgolides A, B, C, J), quercetin, kaempferol, rutin (isolated), isorhamnetin (prepared by acidic hydrolysis)	Demirezer et al. (2014)
84	Turkey	"okaliptus" products (leaves, essential oils) from herbal shops / *Eucalyptus globulus*	13	0	13	substitution with *E. camaldulensis*	TLC / essential oils	n/a	*E. camaldulensis, E. globulus, E. grandis* reference plant material / essential oils extracted from the reference plant material	Tombul et al. (2012)
85	United Kingdom Germany United States	turmeric products (capsules, tablets, soft gels, powder, extracts) from stores, internet / *Curcuma longa*	50	48	2	absence of *C. longa*	1H-NMR/ PCA, HPTLC / curcumin , piperine, (S)-ar-Turmerone	significant quality variation between samples	n/a	Chatzinasiou et al. (2019)
86	United Kingdom United States Germany	St John's Wort products (tablets, capsules, powder) from internet, pharmacies, stores / *Hypericum perforatum*	22 17 8	14 8 7	8 9 1	adulteration (possibly with other *Hypericum* sp. obtained from China or use of chemically distinct *H. perforatum* cultivars or chemotypes)	HPTLC, 1H-NMR/ PCA	significant compositional variation among commercial finished products, adulteration with food dyes	SJW registered and quantified products, SJW EP Reference Standard	Booker et al. (2018)
87	United Kingdom	*Sedum roseum* products (root and rhizome powders) (hard capsules, soft gel capsules, tables) from retail outlets, internet / *S. roseum*	39	32	7	substitution, adulteration with other *Rhodiola* sp. (e.g. *R. crenulata*)	HPTLC, MS, 1H NMR / rosavin, salidroside	lower rosavin content, substitution with 5-hydroxytryptophan	*S. roseum* crude drug material, *R. crenulata* aqueous extracts	Booker et al. (2016b)

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TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

No. crt.	Country / territory	Products (details) / authenticated species	Products			Adulteration reported	Authentication method / marker (if reported)	Additional quality issues detected	Botanical/ chemical reference materials/ standards	Bibliographic reference
			total	authentic/ adulterated						
			no.	no.	no.					
88	United Kingdom	Ginkgo food supplements (tablets, hard capsules, caplets) from health food stores, supermarkets, pharmacies, internet/ *Ginkgo biloba*	33	5	28	adulteration (not in compliance with their label specification)	1H NMR/ PCA, HPTLC / flavonoids, terpene lactones	variable quality (different from that described in pharmacopoeias)	quantified and licensed Ginkgo extracts, *G. biloba* leaf samples	Booker et al. (2016a)
89	United Kingdom	American ginseng, white Asian ginseng, sanchi ginseng samples from importing companies / *Panax ginseng, P. quinquefolius, P. notoginseng*	8	8	0	n/a	LC/MS/MS / malonyl-ginsenosides	not reported	authentic root samples of *P. ginseng, P. quinquefolius, P. notoginseng*	Kite et al. (2003)
90	United Kingdom	herbal tinctures from health shop / *Echinacea purpurea, Hypericum perforatum, Ginkgo biloba, Valeriana officinalis*	4	4	0	n/a	1H-NMR, MS / hyperforin, hypericin, ginkgolic acids, terpene lactones ginkgolides A, B, and C	not reported	n/a	Politi et al. (2009)
91	United Kingdom	herbal product (capsules) / *Equisetum arvense*	3	1	2	no *Equisetum* sp. material (no TLC chromatogram)	TLC / kaempferol glucosides	not reported	material deposited in herbarium / characters used in the European Pharmacopoeia to identify *Equisetum* sp.	Saslis-Lagoudakis et al. (2015)
	Bulgaria	herbal product (tea) / *E. arvense*	1	0	1	adulterated with *E. palustre*				
	Germany	herbal product (tea) / *E. arvense*	1	1	0	n/a				
92	United States	bitter orange products (tablets, capsules, gel-containing capsules, drink powders) from online / *Citrus aurantium*	59	59	0	n/a	LC–MS/MS / phenethylamines (synephrine, octopamine, tyramine, N-methyltyramine, hordenine)	very few appear to meet claims for their label concentration declarations	n/a	Pawar et al. (2020)
93	United States	*Echinacea* preparations (tablet, caplet, capsule, liquid, powder, granule) from health food, drug, and grocery stores / *E. purpurea, E. angustifolia, E. pallida*	49	31	18	adulteration, substitution with unlabeled *Echinacea* sp., no measurable *Echinacea*	TLC / cichoric acid, echinacoside	variability in chemical composition	n/a	Gilroy et al. (2003)

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TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

No. crt.	Country / territory	Products (details) / authenticated species	Products			Adulteration reported	Authentication method / marker (if reported)	Additional quality issues detected	Botanical/ chemical reference materials/ standards	Bibliographic reference
			total	authentic/ adulterated						
			no.	no.	no.					
94	United States	herbal supplements (loose powders, capsules, tablets, liquid extracts, dried fruit forms) to contain cranberry, lingonberry, bilberry, or blueberry from local stores or internet / *Vaccinium macrocarpon, V. vitis-idaea, V. myrtillus, V. corymbosum*	41	27	14	adulteration and substitution with *Vaccinium* sp.	HPLC/DAD / anthocyanins (cyanidin-3-glucoside)	wide variation of the anthocyanin content	verified authentic fruit with known anthocyanin profiles, anthocyanin profiles of small authenticated fruit samples	Lee (2016)
95	United States	goldenseal products (dried material, extract, freeze-dried material) (capsules, tinctures, powdered bulk materials, tea bags) from online / *Hydrastis canadensis*	35	32	3	adulteration with *Berberis. vulgaris, B. aquifolium, Coptis. chinensis*	LC-MS/PCA / berberine, hydrastine, canadine	not reported	reference materials (*H. canadensis, C. chinensis, B. aquifolium, B. vulgaris*) / canadine reference (isolated and purified from *H. canadensis*)	Wallace et al. (2018)
96	United States	black cohosh products (powder, dried extract, liquid extract) (capsules, tablets, soft gels, drops) from local stores or Internet / *Actaea racemosa*	33	19	14	not containing *A. racemosa* material	UPLC-PDA, UPLC-MRM / V9c and V9a markers, caffeic acid, ferulic acid, isoferulic acid	not containing the full spectrum of plant chemicals after preparation process	authenticated rhizome/ root materials from different *Actaea* sp.	Geng et al. (2019)
97	United States	ginkgo products (tablets, capsules, caplet) from health food stores, supermarkets / *Ginkgo biloba*	27	27	0	n/a	HPLC / flavone glycosides, terpene lactones, ginkgolic acids	relevant compositional differences, particularly with regard to the content of ginkgolic acids	EGb 761 extract	Kressmann et al. (2002)
98	United States	"'buchu" products (whole leaves, powders, capsules, tea bag) / *Agathosma betulina*	27	16	11	not containing labeled *A. betulina* or *A. crenulata*	HPTLC / rutin, chlorogenic acid, kaempferol	not reported	*A. betulina, A. crenulata* plant reference material	Raman et al. (2015)
99	United States	yohimbe products (powder, caplet, capsules, liquid, powdered drink mix) from retail health food outlets / *Pausinystalia johimbe*	26	17	9	not containing yohimbe material	GC/MS / yohimbine HCl, ajmaline, corynanthine	containing only trace amounts of yohimbine, largely devoid of the other alkaloids, possible presence of undeclared diluents	authenticated johimbe bark	Betz et al. (1995)

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TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

No. crt.	Country / territory	Products (details) / authenticated species	Products			Adulteration reported	Authentication method / marker (if reported)	Additional quality issues detected	Botanical/ chemical reference materials/ standards	Bibliographic reference
			total	authentic/ adulterated						
			no.	no.	no.					
100	United States	ginseng preparations from the genera *Panax* or *Eleutherococcus* from local health food store / *P. ginseng, P. quinquefolius, P. notoginseng, E. senticosus*	25	25	0	n/a	LC-MS, HPLC / ginsenoside (Rb1, Rb2, Rc, Rd, Re, Rf, Rg1), eleutheroside (B and E)	product-to-product variability in the amount of ginsenosides or eleutherosides present	n/a	Harkey et al. (2001)
101	United States	German chamomile, Roman chamomile and Juhua products (crude drugs, capsules, tea bags, crude drugs	24	20	4	substitution (not containing the labeled chamomille species) did not contain any detectable volatile components	GC/MS, PLS-DA / volatile compounds (b-Farnesene, a-bisabolol oxide A, B)	not reported	authenticated *C. nobile, M. chamomilla, C. morifolium* samples / essential oil samples obtained from the	Wang et al. (2014a)
	China	mixed with other plant materials, powder, extracts) from supermarkets, local retail pharmacies, online / *Matricaria chamomilla, Chamaemelum nobile, Chrysanthemum morifolium*	11	11	0	n/a			authenticated plant materials	
102	United States	grape seed powder products (capsules) from vitamin supplement retailers, supermarkets, online / *Vitis vinifera*	21	12	9	adulteration with peanut skin extract	HPLC/UV/MS, LC–MS, TLC / proanthocyanidin B-type dimers	wide degree of variability in chemical composition	authenticated grape seed extract, peanut skin extract, pine bark extract	Villani et al. (2015)
103	United States	gingko products (leaf extracts) from food supermarkets, local retail pharmacies, online / *Ginkgo biloba*	21	21	0	n/a	GC/MS, LC/MS, UHPLC/ MS / ginkgolic acids, terpene trilactones, flavonol glycosides	not reported	*G. biloba* authenticated and commercial plant samples (leaves, seeds, leaf extracts, sarcotesta)	Wang et al. (2014b)
104	United States	American and Korean ginseng products (fresh or dried roots) (powders, capsules, tablets) from local and national herbal health care stores / *Panax ginseng, P. quinquefolius*	20	18	2	devoid of ginseng material	RP-HPLC / ginsenosides (Rf, Rb1, Rc)	not reported	n/a	Mihalov et al. (2000)
	China		2	2	0	n/a				

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TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

No. crt.	Country / territory	Products (details) / authenticated species	Products			Adulteration reported	Authentication method / marker (if reported)	Additional quality issues detected	Botanical/ chemical reference materials/ standards	Bibliographic reference
			total	authentic/ adulterated						
			no.	no.	no.					
105	United States	black raspberry products (freeze-dried whole and pre-ground powders) (capsules, extract, liquid) form internet / *Rubus occidentalis*	19	12	7	possible substitution with blackberry (*Rubus* spp.)	HPLC/DAD/MS / anthocyanins (cyanidin-3-glucoside)	wide range of anthocyanin concentration	n/a	Lee (2014)
106	United States	milk thistle products (capsules with dried, oil-based extracts) from market / *Silybum marianum*	19	19	0	n/a	U-HPLC-HRMS / silymarin flavonoids, flavonolignans	marked differences in the content of individual flavonoids/ flavonolignans, even within different batches by the same manufacturers	reference dried milk thistle extract	Fenclova et al. (2019)
	Czech Rep		7	7	0					
107	United States	black cohosh products (dry extracts, powdered plant material)	19	7	12	subtitution and adulteration with *C. dhurica, C. foetida*	LC-MS/MS / actein, 23-epi-26-deoxyactein	not reported	Cimicifuga Rhizome (JP16) samples from different companies	Masada-Atsumi et al. (2014)
	Germany	(capsules, tablets) from	5	5	0	n/a				
	Switzerland	pharmacies, internet / *Actaea racemosa*	1	1	0					
108	United States	*Aloe vera* products / *Aloe vera*	18	18	0	n/a	1H-NMR / nicotinamide	differences among products (possible deacetylation)	authenticated *A. vera* samples (inner leaf powder, decolorized whole leaf freezing dried powder), Aloe acetylated polysaccharides reference standard	Jiao et al. (2010)
109	United States	*Tinospora* products from internet (capsules, caplets, granule, powder) / *T. crispa, T. sinensis*	17	15	2	substitution with *T. sinensis*	UHPLC-PDA-MS / flavonoid, alkaloids, amid, diterpenoids	not reported	reference plant samples of *T. crispa, T.sinensis, T. baenzigeri*	Parveen et al. (2020)
110	United States	skullcap and Chinese skullcap based dietary supplements from internet / *Scutellaria lateriflora, S. baicalensis*	15	6	9	substitution with *S. baicalensis* or *Teucrium canadense*	FI/MS/PCA / baicalin, verbascoside	very low *S. lateriflora* concentration	authenticated samples of *S. lateriflora* (aerial parts)	Sun and Chen (2011)
111	United States	"guarana" products (dried seeds, dried paste, seed powders, tablets, capsule) from local health food outlets, manufacturers, internet / *Paullinia cupana*	14	7	7	substitution (devoid of *P. cupana* material)	LC / theobromine, theophylline, caffeine, catechin, epicatechin	possible fortification with synthetic caffeine and dilution with inert ingredients	authenticated guarana seeds, dried paste	Carlson and Thompson (1998)

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TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

No. crt.	Country / territory	Products (details) / authenticated species	Products			Adulteration reported	Authentication method / marker (if reported)	Additional quality issues detected	Botanical/ chemical reference materials/ standards	Bibliographic reference
			total	authentic/ adulterated						
			no.	no.	no.					
112	United States	*Hoodia gordonii* products (gels, capsules, tablets, sprays, teas, snack bars, powders, juices) / *H. gordonii*	13	2	11	substitution (no *H. gordonii* detected, other botanicals present)	HPTLC / pregnane glycosides (hoodigosides, P57)	not reported	various *Hoodia* sp. / isolated chemical reference standards	Rumalla et al. (2008)
113	United States United Kingdom Canada Netherlands Switzerland Spain South Korea Finland Germany	saw palmetto products (soft and hard gel capsules, tablets, tinctures) from retail outlets, pharmacies / *Serenoa repens*	13 11 7 7 6 5 4 1 1	13 11 7 7 6 5 4 1 1	0 0 0 0 0 0 0 0 0	n/a	GC, 1H-NMR/PCA / quantification of fatty acids	inaccurate labeling of fatty acid content	n/a	Booker et al. (2014)
114	United States	St. John's Wort (herb/ aerial parts, extracts) products from market, online / *Hypericum perforatum*	12	6	6	adulteration (possible mixtures with *H. undulatum*)	HPTLC / rutin, hypericin, pseudohypericin	not reported	*H. perforatum* extract standard, *H. undulatum, H. montanum, H. tetrapterum*, and *H. hirsutum* samples	Frommenwiler et al. (2016)
115	United States	goldenseal products (capsules, raw, tea bag, liquid extract) from local retailers or internet / *Hydrastis canadensis*	12	12	0	n/a	HPLC / berberine chloride, (ÿ)-b-hydrastine	wide range of content variation for hydrastine (0.00–2.51%) and berberine (0.00–4.35%)	authenticated crude goldenseal powder	Abourashed and Khan (2001)
116	United States	"yohimbe" products (bark cut and sifted pieces, powders) from online / *Pausinystalia johimbe*	12	8	4	adulterated, yohimbine not detected	UPLC-UV-MS / yohimbine	products range widely in yohimbine content (0.1–0.91%)	authenticated *P. johimbe* bark samples	Raman et al. (2013)
117	United States	black cohosh products (extracts, powdered plant material) (tablets, capsules) from stores / *Actaea racemosa*	11	7	4	substitution and contamination with Asian *Actaea* species	TLC, HPLC, LC-MS / triterpene glycosides, phenolics	significant product-to-product variability in the amounts of the selected triterpene glycosides and phenolic constituents	authenticated plant material of *Actaea cimicifuga, Actaea dahurica, Actaea yunnanensis*	Jiang et al. (2006)
118	United States	pure *Hoodia gordonii* producs from the market / *H. gordonii*	10	1	9	substitution with *H. parviflora*, contamination	1H NMR / P57, hoodigoside L	not reported	authenticated samples of *H. gordonii, H. parviflora, H. ruschii, H. currorii* / isolated chemical reference standards	Zhao et al. (2011)

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TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

No. crt.	Country / territory	Products (details) / authenticated species	Products			Adulteration reported	Authentication method / marker (if reported)	Additional quality issues detected	Botanical/ chemical reference materials/ standards	Bibliographic reference
			total	authentic/ adulterated						
			no.	no.	no.					
119	United States	goldenseal products (root/rhizome) (capsules) from internet / *Hydrastis canadensis*	10	10	0	n/a	LC-UV, LC-MS / berberine, canadine, hydrastine, coptisine, palmatine, jatrorrhizine, dihydrocoptisine	not reported	reference samples (dried powders) of *H. canadensis* (root), *Coptis chinensis* (root)	Wallace et al. (2020)
120	United States	cranberry products (powders, concentrate, fruit solids) from common vendors or internet / *Vaccinium macrocarpon*	9	3	6	adulteration (with extracts from other plant species)	1H-NMR / triterpenoids, organic acids, total proanthocyanidins and anthocyanins	substantially variation of the metabolic profile, slightly lower PAC content may be caused by removal during manufacturing	*V. macrocarpon* freeze dried fruit powder, whole cranberry fruits of different cultivars	Turbitt et al. (2020)
121	United States	"ma-huang" products from local retailers, internet / *Ephedra sinica*	9	9	0	n/a	HPLC / ephedrine-type alkaloids	considerable variability in alkaloid content (EPH 1.08–13.54 mg) and lot-to-lot variations in EPH of 137%.	unprocessed *E. lematolepis*	Gurley (1998)
122	United States	standardized (24/6) ginkgo products (leaf extracts) from suppliers / *Ginkgo biloba*	8	5	3	adulteration (possibly with sophora extracts)	HPLC-DAD / flavone glycosides	high levels of quercetin and kaempferol	certified ginkgo extract 24/6, commercial extracts of *Styphnolobium japonicum*	Chandra et al. (2011)
123	United States	*Vangueria agrestis* products (extracts) / *V. agrestis*	7	4	3	adulteration	HPTLC / saponins, flavonoids, phenolics, iridoid	not reported	authenticated *V. agrestis* samples (twigs with intact leaves, stems, roots)	Raman et al. (2018)
124	United States	American ginseng products from supermarkets / *Panax quinquefolius*	6	4	2	substitution with *P. ginseng*	HPLC/HCA/PCA / ginseng saponins	not reported	standard *P. ginseng, P. notoginseng* samples, *P. quinquefolius* samples from USA, Canada, China	Yu et al. (2014)
125	United States	African mango products from internet / *Irvingia gabonensis*	5	1	4	substitution (do not contain detectable amount of authentic material)	UHPLC-PDA-HRMS / ellagic acid, mono-, di-, tri-O-methyl-ellagic acids and their glycosides	trace constituents of regular mango seeds	*M. indica* samples	Sun and Chen (2012)
126	United States	*Echinacea* products (tablets, capsules, powder) / *Echinacea purpurea*	5	1	4	adulteration	HPLC-CAD	not reported	*Echinacea* sp. (extracts, root, herb)	Waidyanatha et al. (2020)
127	United States	plantain products (tablets) / *Plantago major*	5	4	1	contamination with *Digitalis lanata*	Kedde reaction, TLC, LC-MS / cardiac glycosides (lanatosides A, B, C, digoxin, digitoxin)	not reported	n/a	Slifman et al. (1998)

(Continued on following page)

TABLE 1 | (*Continued*) The authenticity of the chemically authenticated commercial herbal products at global level.

No. crt.	Country / territory	Products (details) / authenticated species	Products			Adulteration reported	Authentication method / marker (if reported)	Additional quality issues detected	Botanical/ chemical reference materials/ standards	Bibliographic reference
			total	authentic/ adulterated						
			no.	no.	no.					
128	United States	black cohosh products from health store, marketplace / *Actaea racemosa*	4	3	1	substitution with *Cimicifuga foetida*	HPLC-PDA/MS/ELSD / (triterpene glycosides, phenolic compounds)	product inadequately manufactured (overheating)	*Actaea* sp. plant material / authentic *Cimicifuga* chemical reference standards	He et al. (2006)
129	United States	passion flower products (capsules) from online / *Passiflora edulis*	4	4	0	n/a	UPLC-UV-MS, HPTLC / flavonoids, harmane-carboline alkaloids	not reported	authenticated aerial parts of *P. edulis, P. violacea, P. suberosa, P. morifolia, P. quadrangularis*, seeds of *Peganum harmala*	Avula et al. (2012)
130	United States	feverfew extracts (capsules, drops) / *Tanacetum parthenium*	3	3	0	n/a	LC-UV/LC-MS / parthenolide	not reported	*T. parthenium* and *T. vulgare* plant material	Avula et al. (2006)
131	United States	herbal products (tea, capsules) / *Equisetum arvense*	3	3	0	n/a	TLC / kaempferol glucosides	not reported	material deposited in herbarium / characters used in the European Pharmacopoeia to identify *Equisetum* sp.	Saslis-Lagoudakis et al. (2015)
132	United States	goldenseal products (root powder) from bulk suppliers / *Hydrastis canadensis*	3	2	1	adulteration, possibly with *Coptis* root or barberry bark	LC-MS / alkaloids (berberine, hydrastine, canadine)	not reported	*Coptis japonica* root powder, *Berberis aquifolium* root powder, *Chelidonium majus* herb, *Berberis vulgaris* bark powder	Weber et al. (2003)
133	United States	ginseng products (liquid extract, capsules) from a local nutritional store / *Panax quinquefolius, P. ginseng, P. notoginseng*	2	2	0	n/a	UPLC/QTOF-MS/PCA / (ginsenosides, pseudoginsenosides, gypenosides, notoginsenosides)	not reported	authenticated ginseng roots (*P. quinquefolius, P. ginseng, P. notoginseng*)	Yuk et al. (2016)
134	United States	African mango sample (powdered seeds) / *Irvingia gabonensis*	1	0	1	contamination or adulteration with goji berry (*Lycium barbarum*)	HPLC-PDA, LC-IT-MS, 1H NMR / pyrrole alkaloid	n/a	authentic sample of African mango seed powder, goji berries	Li et al. (2014)
135	United States	American skullcap (freeze-dried) product / *Scutellaria lateriflora*	1	1	0	n/a	HPLC / flavonoids (baicalin, baicalein, wogonin)	not reported	*S. lateriflora* (aerial parts) reference material	Brock et al. (2013)
	Total		**2,386**	**1,734**	**652**					

TABLE 2 | The distribution and authenticity of the chemically authenticated commercial herbal products at national level.

Country/Territory	Products	Authentic products		Adulterated products	
	no.	no.	%[a]	no.	%[b]
United States	746	548	73	198	27
China	491	388	79	103	21
United Kingdom	123	78	63	45	37
Italy	119	82	69	37	31
South Korea	96	96	100	0	0
Brazil	85	36	42	49	58
Romania	85	65	76	20	24
Malaysia	83	41	49	42	51
Belgium	77	56	73	21	27
Japan	57	37	65	20	35
Taiwan	54	45	83	9	17
Poland	47	38	81	9	19
Turkey	44	19	43	25	57
India	32	10	31	22	69
Germany	22	18	82	4	18
European Union[b]	22	8	36	14	64
Australia	19	8	42	11	58
Canada	15	12	80	3	20
Thailand	13	12	92	1	8
Denmark	12	12	100	0	0
Croatia	10	8	80	2	20
Czech Republic	10	8	80	2	20
Spain	9	7	78	2	22
Netherlands	8	8	100	0	0
Pakistan	7	6	86	1	14
Switzerland	7	7	100	0	0
Mexico	6	3	50	3	50
New Zeeland	6	6	100	0	0
Egypt	5	2	40	3	60
Norway	5	5	100	0	0
Austria	3	3	100	0	0
Bulgaria	3	2	67	1	33
Greece	3	2	67	1	33
Slovakia	3	1	33	2	67
France	2	2	100	0	0
Sweden	2	2	100	0	0
Chile	1	1	100	0	0
Finland	1	1	100	0	0

[a]*The percentage values were rounded to the nearest whole number.*
[b]*Not reported by the authors the exact EU country.*

The set of retrieved full-text articles was further reduced by 446 that did not meet all eligibility criteria. Included: 135 records.

RESULTS

Different chemical methods have been successfully employed for the botanical authentication of 2,386 commercial herbal products, sold in 37 countries spread on six continents. The majority of the analyzed products were reported to be authentic (73%) but more than a quarter proved to be adulterated (27%), when the botanical identity of their content was compared with the label stated ingredients (**Table 1**).

The herbal products were purchased from 37 countries scattered over six continents: Europe (n = 20), Asia (n = 9), North America (n = 3), Australia (n = 2), South America (n = 2), and Africa (n = 1) (**Supplementary Table S1**). The numbers of reported samples were geographically heterogeneous, at continental level the highest number of commercial herbal products was reported for Asia (n = 877), North America (n = 767), Europe (n = 573), followed distantly by South America (n = 86), Australia (n = 25) and Africa (n = 5). The proportion of adulterated products varies significantly among continents, being highest in Africa (60%), South America (57%), Australia (44%), and lower in Europe (28%), North America (27%), and Asia (25%). The adulteration percentage of the last three continents enumerated is close to the global one (27%) which can be influenced also by the significantly higher number of commercial products analyzed and reported, compared with the samples analyzed from the other three continents.

The distribution of commercial samples among the 37 countries is highly heterogeneous as well (**Table 2**). More than 100 commercial products were reported for four countries, i.e. United States (n = 746), China (n = 491) followed distantly by

United Kingdom ($n = 123$) and Italy ($n = 119$). Another seventeen countries are well represented ($n \geq 10$) by the successfully analyzed samples, while the other sixteen countries have even fewer ($n < 10$) products reported.

In twelve countries, out of the total of thirty-seven, all the analyzed commercial herbal products (100%) were reported as authentic, albeit, for eight of them, less than 10 samples were reported. Notably, the botanical identity of the samples purchased from South Korea ($n = 96$) and Denmark ($n = 12$) matched the labeled information. The adulterated proportion in the remaining twenty-five countries varied widely, from 8% up to as much as 80%. From the countries where more than 10 samples from their marketplace have been chemically authenticated and non-authenticated products have been reported, the majority of the commercial products was adulterated, being the highest in India (69%), followed closely by Australia (58%), Brazil (58%), Turkey (57%) and Malaysia (51%). Noticeably, the adulteration percentage of the four countries with more than 100 commercial products reported is 37% (United Kingdom), 31% (Italy), 27% (United States) and the lowest is reported for China (21%).

Sampling Heterogeneity and Unavoidable Bias

The authentication raw data were all retrieved from peer-reviewed articles, the vast majority of them after they were indexed in the four major international databases which were systematically searched for while some other few articles were identified after cross-referencing. Although no limiting criteria (e.g. publication year, or language) was used, the authentication data reported in journals with limited-impact and international visibility might be underrepresented in the retrieved data. Moreover, the access of researchers from the economically depressed economies to high-impact journals, and especially to the OA journals, is a further limiting factor for publicly communicating the authentication results relevant for a certain country. On the other hand, as it was previously mentioned as possible bias, also the countries with a functional consumer safety system might be underrepresented as the authentication results of the commercial samples screened by the respective institutions will be published in internal bulletins or protocols, rather than in peer-reviewed journals (Ichim et al., 2020).

DISCUSSION

The chemical identification methods have confirmed that a substantial proportion (27%) of herbal products from the international market place is adulterated: on average, more than one in each four products sold in the 37 countries included in our analysis was proved to be non-authentic regarding their botanical identity. This adulteration percentage, revealed by employing many and very diverse chemical analytical methods, almost matches the figure obtained after the use of DNA-based techniques were assessed for their use for the authentication of commercial herbal products in a comparable number of countries: 27% (Ichim, 2019). Indeed, this percentage was obtained after almost a triple number of commercial herbal products ($n = 5,957$) were analyzed and their results reviewed recently. Notably, the microscopic authentication of commercial herbal products have reported a much higher adulteration rate (41%) but the number of analyzed samples was considerably much smaller ($n = 508$) which can be a possible bias of this finding (Ichim et al., 2020).

As it was previously reported by many peer-reviewed reports (Hoban et al., 2018; Seethapathy et al., 2019; Amritha et al., 2020; Anthoons et al., 2021; Palhares et al., 2021), irrespective of the authentication method, adulterated commercial HPs are geographically present across all continents (**Supplementary Table S1**). Moreover, this highly relevant category of commercial products was found to not comply with the labeled botanical ingredients in proportions almost identical ($26 \pm 2\%$), irrespective if they are traditionally used as herbal medicines, as commonly found in Asia, or overwhelmingly consumed as food supplements as in Europe or North America. These two main categories of herbal products commercialized in the global marketplace have many types of value chains (Booker et al., 2012), with some different stakeholders and entities along their shorter or more complex trade chains. Nevertheless, the end-users of both systems seem to be equally affected by non-authentic, accidental contamination or fraudulent substitution of labeled botanical ingredients and even the addition of compounds in an attempt to fool quality control testing e.g. as in adding food dyes to *H. perforatum* in order to achieve higher UV spectroscopy readings (Booker et al., 2018). Indeed, although monographs for herbal raw materials (e.g., Ph. Eur, USP) allow a minor presence of foreign organic matter (Parveen et al., 2016), the adulteration patters documented by employing different chemical methods, are very diverse and most of them are made possible only by the intentional, economically motivated and fraudulent actions of onerous producers or traders.

The total absence of labeled botanical ingredients and/or their extracts from the commercial herbal products tested was detected by using chemical methods. Commercial samples devoid of labeled botanical ingredient species (Carlson and Thompson, 1998; Ardila et al., 2015; Geng et al., 2019; Zhu et al., 2019) or not even substituted with their related species (Wan et al., 2016). An easy way to increase the profit margin of the products was the use of cheaper plant material as it was the use of other plant parts than the ones recommended, labeled and expected by the product's users, senna (*Senna alexandrina* Mill.) stems substituted with leaves and midribs (Kojima et al., 2000), *Panax ginseng* C.A.Mey roots with other plant parts (leaf or stem) (Govindaraghavan, 2017), or *Panax notoginseng* Burkill F.H.Chen roots with flowers (Liu et al., 2015). Another similar deceptive adulteration strategy was the reported use of extracts obtained from plant parts other than the recommended ones, such as the decoction of the stem bark to substitute the genuine "jatoba" sap products (*Hymenaea stigonocarpa* Hayne, *Hymenaea martiana* Hayne) and the adulteration of Aquilariae Lignum Resinatum (*Aquilaria sinensis* (Lour.) Spreng) products with cheap resin (e.g. rosin) (Qu et al., 2017).

The economically motivated adulteration includes also the use of unlabeled filler species as the DNA of species such as rice (*Oryza sativa* L.), soybean (*Glycine max* (L.) Merr.) and wheat (*Triticum* spp.) was previously identified in commercial herbal products (Newmaster et al., 2013; Ivanova et al., 2016). Yet, the TLC alone was able to detect the fraudulent use of soybean oil as filler in "copaiba" (*Copaifera multijuga* Hayne) oil-resin products (Barbosa et al., 2009).

The detection of unlabeled species with allergenic potential and known or suspected toxicity was previously reported by the use of DNA-based authentication techniques (Newmaster et al., 2013; Speranskaya et al., 2018). The same potential was shown by the phytochemical analyses which have been able to unmask the presence of unwanted and hazardous botanic ingredients, such as species that should have been notified to authorities (e.g. *Ilex paraguariensis* A. St-Hil., *Epimedium* spp., *Tribulus terrestris* L.), or forbidden toxic plants (e.g. *Aristolochia fangchi* Y.C.Wu exL.D.Chow and S.M.Hwang) (Deconinck et al., 2019) or even health hazardous contaminations, with *Digitalis lanata* Ehrh. added to plantain (*Plantago major* L.) products (Slifman et al., 1998). Moreover, as peanut allergy is a major public health concern and can be severe or even life-threatening (Gray, 2020), chemical methods have proved able to detect adulteration with the peanut skin extract of grape seed-containing herbal products (*Vitis vinifera* L.) from Australia (Govindaraghavan, 2019) and United States (Villani et al., 2015).

All the intentional adulteration practices documented and reported repeatedly till now (Li et al., 2008; Ichim, 2019; Xu et al., 2019; Ichim et al., 2020; Upton et al., 2020) can be evidenced by peer-reviewed reports referring to the top selling herbal products containing highly valued or widely used medicinal species across countries and cultures. The prices of ginseng herbal medicines and supplements vary widely based on the species, quality, and purity of the ginseng, and this provides a strong driver for intentional adulteration (Ichim and de Boer, 2021). Indeed, several chemical methods were able to identify ginseng products totally or partially devoid of the labeled *P. ginseng* plant material (Mihalov et al., 2000; Yang et al., 2016) and prove that, in most cases, labeled *Panax* species were substituted with other *Panax* species (Li et al., 2010; Yu et al., 2014; Dong et al., 2020), but also the substitution of ginseng root with leaves, stems or flowers (Liu et al., 2015; Govindaraghavan, 2017). Notably, chemical analysis was even able to detect the adulteration and substitution of wild with cultivated ginseng (Zhao et al., 2015) as well as a white ginseng products (*P. ginseng*) not composed of 6 years old ginseng radix only (Li et al., 2010).

Studies carried out at UCL School of Pharmacy, London have consistently shown that product adulteration is commonplace, with 25–40% of products typically being found to be of poor quality or adulterated, and especially with products obtained via the internet. Although with products that have been registered as Traditional Herbal Medicines under the Traditional Herbal Medicinal Products Directive (THMPD), no adulteration has so far been found and these products have shown to be of acceptable quality (Booker et al., 2016a; Booker et al., 2016b; Booker et al., 2018). This does not necessarily mean that all non-registered products (e.g. food supplements) are of poor quality but the problem being that it is difficult for the general public to be able to reliably discern high quality products from inferior ones. Organic certification provides some assurances regarding traceability, including origin, cultivation methods and manufacturing practices and so until more formal regulations are introduced for these food supplement products, buying organic may be the best option.

The many cases of substituted or adulterated herbal products purchased from a very high number of national marketplaces, where the labeled botanical ingredients did not match the chemically identified ones are, unfortunately, accompanied by other low-quality issues which additionally affect the safety and potential efficacy of commercial herbal products. As many as forty-one peer reviewed research articles, which have reported a case of adulteration among analyzed commercial samples, have also reported other quality issues which further lower the overall quality expected by their users and consumers. Additionally, another nineteen studies reported quality issues of the tested products without identifying any proof for their botanical identity adulteration. For the majority of herbal products reported, considerable variability of their labeled metabolic profile and/or content, such as the alkaloid content of "ma-huang" (*Ephedra sinica* Stapf) products (Gurley, 1998) or Menispermi Rhizoma (*Menispermum dauricum* DC) products (Liu et al., 2013b), selected triterpene glycosides and phenolic constituents in black cohosh (*A. racemosa*) products (Jiang et al., 2006) or the PAC content of cranberry products (Turbitt et al., 2020). Furthermore, aside of significant product-to-product variability, the marked differences of the content of individual flavonoids/flavonolignans in milk thistle (*Silybum marianum* (L.) Gaertn.) products have revealed quality difference also between different batches by the same manufacturers (Fenclova et al., 2019).

The peer-reviewed authentication results and the methods which were successfully employed to analyze commercial herbal products and significantly contribute to a better understanding of authenticity issues affecting the herbal industry and provides an as close-to-reality possible picture of the commercial herbal products' authenticity as well as examples of techniques to be efficiently and accurately used for their authentication.

It is clear that chemical analysis alone can only identify existing problems. In order to prevent these problems from arising in the first place, better governance needs to be implemented along all stages of the supply chain. Regulation can help with this process but resources are scarce and real progress on quality is more achievable through having closer and more focused co-operation between the regulators and the producers, manufacturers and retailers of herbal products.

AUTHOR CONTRIBUTIONS

MI performed the literature systematic search and analyzed the results. MI and AB wrote the manuscript together.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.666850/full#supplementary-material.

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REVIEW
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New Text

Mitochondrial Function as a Potential Tool for Assessing Function, Quality and Adulteration in Medicinal Herbal Teas

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Quality control has been a significant issue in herbal medicine since herbs became widely used to heal. Modern technologies have improved the methods of evaluating the quality of medicinal herbs but the methods of adulterating them have also grown in sophistication. In this paper we undertook a comprehensive literature search to identify the key analytical techniques used in the quality control of herbal medicine, reviewing their uses and limitations. We also present a new tool, based on mitochondrial profiling, that can be used to measure medicinal herbal quality. Besides being fundamental to the energy metabolism required for most cellular activities, mitochondria play a direct role in cellular signalling, apoptosis, stress responses, inflammation, cancer, ageing, and neurological function, mirroring some of the most common reasons people take herbal medicines. A fingerprint of the specific mitochondrial effects of medicinal herbs can be documented in order to assess their potential efficacy, detect adulterations that modulate these effects and determine the relative potency of batches. Furthermore, through this method it will be possible to assess whole herbs or complex formulas thus avoiding the issues inherent in identifying active ingredients which may be complex or unknown. Thus, while current analytical methods focus on determining the chemical quality of herbal medicines, including adulteration and contamination, mitochondrial functional analysis offers a new way of determining the quality of plant derived products that is more closely linked to the biological activity of a product and its potential clinical effectiveness.

Keywords: herbal tea, herbal medicine, quality control, mitochondria, functional analysis, potency

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INTRODUCTION

Quality control of medicinal herbs have been an issue for as long as humans have been using plants to heal. Around 50 BCE, in Dioscorides' *De Materia Medica*, one of the earliest written examples of a systematic pharmacopoeia, the author acknowledged the issue of adulteration though both accidental and fraudulent practices, and includes 40 examples of specific tests on how to detect them (Riddle, 1985). The majority were organoleptic, detecting adulteration through the senses, but several employed chemico-physical tests such as the ability of balsam (*Commiphora opobalsamum* (L.) Engl. *Burseraceae*) to be washed clean from a woolen cloth. A similar trend arose in Asia where

the first *materia medica*, the *Shen Nong Ben Cao Jing*, written c.a. the first century CE, described the tastes, qualities and growing regions of each drug so that it could be positively identified.

Today the issue of quality control is even more pertinent, with the global herb trade being worth over US$60 billion in 2017 involving 29,000 herbal substances and growing by 15% each year (Srirama et al., 2017). Of these, herbal teas are one of the most popular methods of consuming plant materials and account for a significant proportion of this market, estimated at US$4.2 billion by 2025 (Market Research Future, 2020). This does not include the US$22.7 billion market in conventional tea (*Camellia sinensis (L.) Kuntze Theaceae*) (Market Research Future, 2019) with water-based infusions and decoctions being some of the oldest and most popular methods of extracting the medicinal properties from herbs (The Herbarium, 2009). Both of these groups will be considered under the term "medicinal herbal teas" for the purpose of this review. They differ only in that decoctions simmer the water while the herbs are being soaked and infusions are made by pouring freshly boiled water over the herbs. Infusions make up the majority of the use in the western world due to their convenience and familiarity while decoctions remain popular in Asia, although the increasing pace of life is leading many companies to develop convenient instant powders and granules from pre-decocted herbs. Alcoholic tinctures, popular among professional western herbalists for their ability to extract specific less polar active compounds (Bone, 2003) but also with a history in the east (Flaws, 1994; Sionneau, 1995), as well as pills made from raw powdered herbs or extracts, will not be considered here.

Methods for detecting adulteration have grown in sophistication as the technology to examine them has developed (Fitzgerald et al., 2020). Visual identification has been enhanced by microscopic inspection and the simple chemico-physical analyses, described by Dioscorides, has been replaced by more advanced chemometric testing in the forms of chromatography and spectroscopy developed during the twentieth century. These methods can isolate and analyse the full chemical composition of plants and prove particularly useful in detecting adulterations with drugs or contaminants that may be invisible to the human eye and undetectable to the senses of taste and smell. High performance chromatographic methods have enabled specific fingerprints of each plant's chemical compositions, launching the development of a chromatographic atlas of herbal medicines (HPTLC Association, 2020). Recent advances in genetics have also enabled deoxyribonucleic acid (DNA) fingerprinting to be used in a similar fashion (Lou et al., 2010; Wong et al., 2018) making it possible to accurately identify a species as well as detect adulteration with plants that may look identical, even under the microscope, and with comparable chemical profiles.

Unfortunately, as the ability to detect adulteration has improved, so has the technology to adulterate herbal medicines. While much of it appears to be accidental, at the level of foraging or purchasing at a market, there is evidence to suggest that it is also deliberate, including the addition of drugs to enhance the effects of supposedly 'natural' supplements (Booker et al., 2016), dyes that make the color of vibrant herbs look more potent (Müller-Maatsch et al., 2016; Booker et al., 2018) and the substitution of herbal material which may have comparable chemical profiles and so evade detection by all but the most sophisticated tests (Booker et al., 2018; Frommenwiler et al., 2019).

Mitochondria stand at the center of a plethora of cellular activities, including controlling energy metabolism, the production of reactive oxygen species (ROS), apoptosis, cell division, immune signaling, and even at the very evolution of complex multicellular life (Lane, 2018). This suggests that every bioactive substance will have some effect on mitochondrial function. Many medicinal herbal teas are marketed in an overly simplistic manner for their antioxidant benefits (Paur et al., 2011) although it remains unproven and unclear whether antioxidant consumption improves health outcomes *in vivo* (Berger et al., 2012). However, the fact that herbal and traditional plant medicines have some of the highest levels of antioxidants of any foodstuffs (Carlsen et al., 2010) suggest that many of their purported therapeutic properties would involve the regulation of mitochondrial systems. We suggest that by profiling these effects, it may be possible to develop a library of mitochondrial fingerprints for individual herbs. This library, adjunct to those that exist for high performance thin layer chromatography (HPTLC) and DNA profiling databases, would be a powerful tool, not only to understand herbal function, but also determine adulteration and potency, something currently lacking in standard tests.

The great advantage of measuring biological activity directly, rather than by analysing composition, is that the aim of medicinal herbs is to modulate biological systems and in some cases, this may not be due to the presence of any single active ingredient. Chemical and DNA profiles may therefore be misleading, especially when there are multi-herb blends as commonly observed with commercial teas and medicinal formulas. In contrast, if the mitochondrial action of a formula can be mapped, in conjunction with chemical and DNA profiles, then it will be possible to compare batches and formulations against standardised mitochondrial fingerprints. Once a unique pattern has been detailed, the potency of each batch can be ascertained.

METHODS

This study was conducted in accordance with PRISMA guidelines for systematic reviews.

Search Strategy

We searched PubMed and Google Scholar for literature on the existing methods of examining herbal teas for quality control. The search terms used were:

PubMed: ((chromatography) OR (spectrometry) OR (spectroscopy) OR (NMR) OR (ultraviolet) OR (infrared) OR ((DNA) OR (genetic) barcoding) OR (bioassay)) AND ((herbal tea) OR (tisane) OR (decoction)) AND ((adulteration) OR (quality) OR (contamination)). With the filters "Full Text" and "English" language applied, obtaining 363 results.

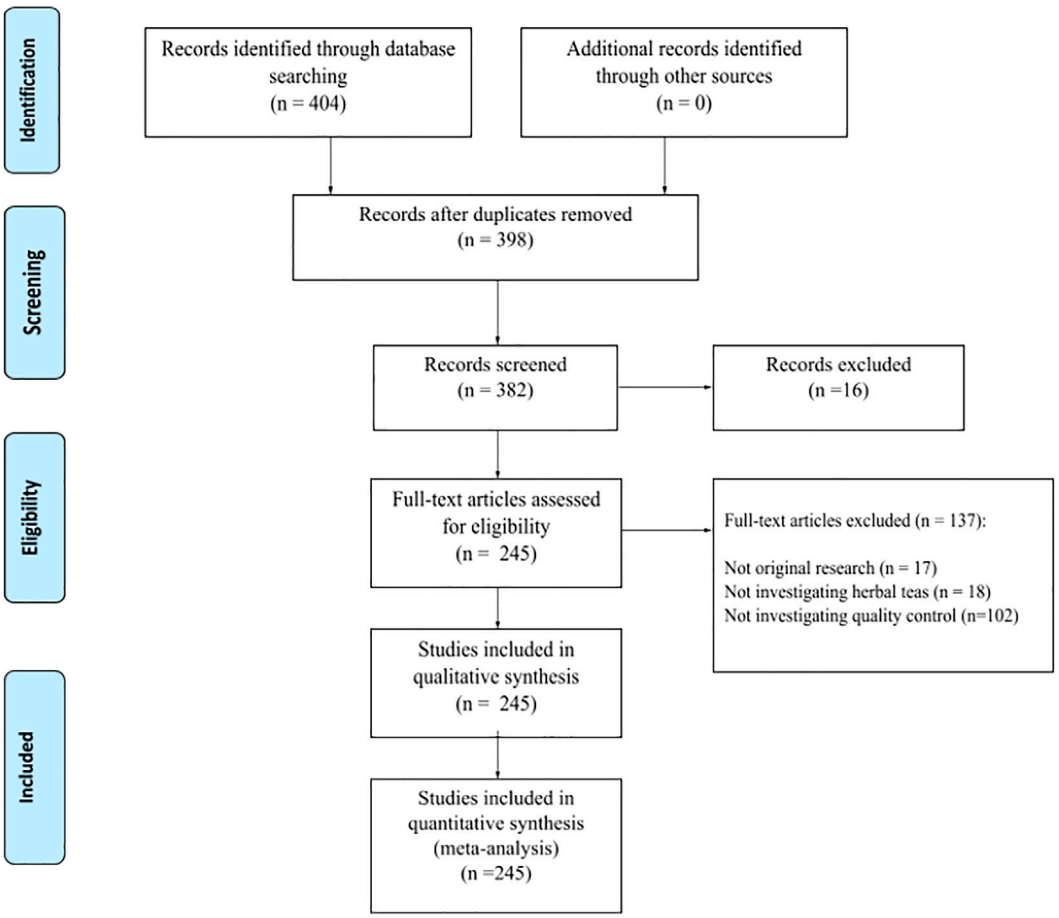

FIGURE 1 | PRISMA flow diagram of the literature review process. Adapted from Moher et al (2009).

Google Scholar: allintitle: "herbal tea" (chromatography OR HPLC OR HPTLC OR GC OR spectrometry OR spectroscopy OR MS OR NMR OR ultraviolet OR infrared OR "DNA barcoding" OR "genetic barcoding" OR bioassay) obtaining 41 results. Six duplicates were removed before screening commenced.

Inclusion and Exclusion Criteria

Exclusion criteria for screening was that publications must contain author, title, date of publication, abstract and that the full text was available in English. Inclusion criteria for eligibility was that the publications were peer reviewed papers, investigating teas, decoctions or water extractions and investigating quality control.

RESULTS

Included Studies

A total of 398 papers were initially identified after a preliminary search of the databases, 245 papers were included in the final analysis, with a total of 153 excluded based on the above described criteria (**Figure 1**). A complete table of all included papers, their methods and objectives can be found in the **Supplementary Information (Table S1)**.

TABLE 1 | Occurrence of methods used in quality control of herbal teas

Method	Number of studies	Percentage
Liquid chromatography (LC)	178	72%
Gas chromatography (GC)	18	7%
Thin layer chromatography (TLC)	11	4%
Mass spectrometry (MS)	129	53%
Nuclear magnetic resonance (NMR)	13	5%
Ultraviolet-visible spectroscopy (UV-Vis)	57	23%
Infrared spectroscopy (IR)	8	3%
Optical emission spectroscopy (OES)	4	2%
DNA barcoding	15	6%
Biological assays	31	13%

By providing a breakdown of the methods and their application (**Table 1**) it can be seen that chromatography is the most popular technique, with liquid chromatography (LC)

being the most favored method overall, followed by mass spectrometry (MS). This is mainly because both of these techniques can be used to separate as well as analyse herbal extracts, which is an essential endpoint when assessing complex mixtures as normally found in herbal tea blends. Each individual method shall now be described with its advantages and disadvantages, including a summary of what the literature revealed.

Chromatography

The literature review revealed chromatographic methods as the most popular for identifying adulteration of herbs. This is due to the fact that they can separate complex compounds into their unique components creating a 'chemical fingerprint' which can be used qualitatively and quantitatively (Hansen, 2015). All chromatographic methods achieve this through the universal principle of separating mixtures by distributing its components between two phases: a mobile phase, which carries the components through a medium, and a stationary phase that remains fixed causing the various constituents to separate as they migrate at different speeds (Liu, 2011). The chemical fingerprint that arises can then be compared against a reference standard or used as a preparatory step toward further analysis of the individual compounds using spectroscopic methods to identify unknown compounds. The combination of a separation technique and one or more spectroscopic detection methods is known as a hyphenated technique with almost every published chromatographic study utilising at least one spectroscopic technique. This reveals one of the main limitations of all chromatographic methods, as well as their strength, for while its analytical capacities are limited to observation of analyte peaks, it excels in separating individual components which can act as an initial preparatory step to further analysis (Braithwaite and Smith, 1999).

There are many different types of chromatographic techniques, usually named after one of their phases or their method of interaction. Of the types used in the analysis of herbal teas, liquid and gas chromatography (GC) are named after the mobile phase while thin layer chromatography (TLC) refers to the type of stationary phase used (Coskun, 2016). Of particular interest are "high performance" varieties of LC and TLC which use automated techniques to provide more accurate, reproducible readings. Each technique has been shown to have its own advantages and disadvantages.

Liquid Chromatography (LC)

LC is one the most popular techniques found in the literature with a total of 177 papers included in the review. While LC simply refers to the state of the mobile phase, the majority of papers analysing herbs used "High Performance Liquid Chromatography" (HPLC) or "Ultra-High Performance Liquid Chromatography" (UPLC). Here the liquid mobile phase is pumped through a solid adsorbent stationary phase in a column under high pressure enabling sufficient resolution to be used quantitatively, while LC is primarily used as a preparative technique for other forms of analysis. The main difference between the HPLC and UPLC is the relative pressure used and the resolution achieved, with the latter having superior resolution and speed (Dyad Labs, 2018). HPLC and UPLC account for 150 of the 178 LC papers (84%), with only 35 (20%) using the standard form (7 using both). These methods are especially useful for quality control because of the process being largely automated which makes the results highly reproducible. The results are usually presented as a line graph where the peaks can be compared to show the presence of various compounds and their relative quantity making it ideal for generating a chemical 'fingerprint' against which other samples can be compared. This is reflected in the overwhelming majority of the papers using LC to identify quality markers in order to standardise market products.

TABLE 2 | Uses of liquid chromatography in the analysis of herbal teas

Uses of liquid chromatography	Number of studies
To identify quality markers	132
To detect mycotoxins	11
To detect pyrrolizidine alkaloids	10
To detect pesticide residues	8
To detect adulterant species	7
To detect polycyclic aromatic hydrocarbons	3
To detect adulterant drugs	3
To detect tropane alkaloids	3
To detect heavy metals	2

Table 2 lists the uses of all forms of LC in the analysis of herbal teas included in the review. The primary use of LC is to separate out compounds for further analysis with spectroscopic techniques but HPLC features ultraviolet detection as an integral part of the system to detect when different compounds are eluting from the column. However, 106 (60%) of the studies included in the review using HPLC also hyphenated it with another spectrometric or spectroscopic technique such as MS, nuclear magnetic resonance spectroscopy (NMR) or infrared spectroscopy (IR). MS was the most popular with 102 papers combining it with LC while only eight used NMR and six of those used it in conjunction with MS. Only two papers used IR, neither in conjunction with any other technique.

LC appears to be the primary choice in the analysis of herbal teas since they are naturally delivered in liquid form. It therefore does not require the samples to be subjected to additional processing, preserving less stable compounds. However, it is less suited in the analysis of volatile compounds for which GC is the technique of choice, provided that they are unchanged by heat (Ng, 2017). LC is especially useful for analysing samples containing salts or carrying a charge which cannot be analysed with GC (Painter, 2018).

These limitations are illustrated by Kokalj et al. (2014) who claimed to provide the first detailed report of the chemical composition of *Tiliae flos* (*Tilia cordata* Miller, *T. platyphyllos* Scop. and *T. x vulgaris* Heyne, *Tiliaceae*) based on their procyanidin content. However, their analysis disregarded the volatile content, despite acknowledging that it was also important for quality control. Yap et al. (2007) also encountered a similar problem while attempting to develop a method of quality control for *Dang Gui Bu Xue Tang* (a combination of *Astragalus membranaceus* (Fisch.) Bge.,

TABLE 3 | Uses of gas chromatography in the analysis of herbal teas

Uses of gas chromatography	Number of studies
To identify quality markers	7
To detect pesticide residues	7
To detect heavy metals	3
To detect polycyclic aromatic hydrocarbons	2
To detect adulterant drugs	1

Fabaceae and *Angelica sinensis* (oliv.) Diels, *Apiaceae*), reporting difficulties in detecting the components carvacrol and Z-butylidenephthalide due to their volatility, despite the volatile oils of *A. sinensis* being important for many of its bioactive effects.

Gas Chromatography (GC)

GC works along the same principles as LC except that the sample is vaporised and carried by an inert gas (the mobile phase) into the stationary phase, under controlled temperature (Turner, 2020). The stationary phase is usually a liquid coated on a solid support contained within a glass column which elutes the components of the mobile phase at different rates, depending on their different interactions with the stationary phase. The resulting extraction is then passed to a detector which displays the results as a chromatogram. One of the great advantages of GC is the ease with which it can be connected to MS by using a mass analyser as the detector. Of the 18 studies included in the review that used GC, 15 (83%) were associated with MS.

One of the most notable trends that emerged from the GC literature was its use in detecting pesticides (**Table 3**). Seven out of 12 papers (58%) that searched for pesticides used GC, with a relatively high number detecting polycyclic aromatic hydrocarbons (2 out of 5, the other 3 using LC). This popularity is because it can be both selective and sensitive with simultaneous detection of many residues at lower concentrations compared with other techniques (van der Hoff and van Zoonen, 1999) giving it an important specialist role in the quality control of herbal teas, especially in the analysis of volatile compounds, including many environmental pollutants. However, it is not well suited to analysing any compounds which are affected by heat, contain salts or carry a charge for which LC is the technique of choice (Ng, 2017; Painter, 2018).

One interesting use of GC was in providing a chemometric revision of the aromatic sensory descriptions of honeybush (*Cyclopia* spp. Vent., *Fabaceae*) and rooibos (*Aspalathus linearis* (Burm.f.) R. Dahlgren, *Fabaceae*) tea traditionally used to assess quality (Du Preez et al., 2019). While the authors' claim that this improves our understanding of aroma descriptions is true, it could also be used to artificially modulate the scent of low quality herbs.

Thin Layer Chromatography (TLC)

TLC uses a thin layer of material made of small particles for the stationary phase. This causes the mobile phase to rise along the surface via capillary action at a constant rate and the compounds adhere to the vacant spaces (termed adsorption) at differing rates depending on their relative solubility in the mobile phase and their affinity for the stationary phase. These then remain in place after the mobile phase has evaporated and can be derivatized by various treatments to make all the analytes detectible (CAMAG, 2020). It is a less popular method of analysing herbal teas than LC with only 11 papers included in the review utilising this method but deserves a thorough examination because its use for analysing herbal materials is expanding.

Two thirds (7) of the papers included in the review used TLC to find identifying quality markers to confirm a correct species; of the others, the uses were as varied as detecting adulterant species (Shen et al., 2012; Lam et al., 2016; Guzelmeric et al., 2017), adulterant drugs (Miller and Stripp, 2007) or moulds and mycotoxins (Halt, 1998). These show the potential for TLC to be almost as varied as for LC.

As can be seen from these papers, a high performance variety of TLC has been developed similar to the high performance version of LC. It is a relatively new technique evidenced by the fact that far fewer papers have been written using it, with only five included in the present search, all being written in the last 8 years. However, its high throughput speed, low cost and options for analysing the results visually or by computer readout means that it is rapidly becoming the technique of choice for verifying species and detecting adulteration in herbal medicines (Omicron, 2018; Frommenwiler et al., 2019). The number of studies found in the present search belies the popularity and potential of HPTLC in herbal medicines research due the fact that it was restricted to teas while most studies investigate supplements and raw herbs using other solvents. If the search is modified to only "(HPTLC) and (herbal medicine) and [(adulteration) or (quality) or (contamination)]" then 45 results are returned. There is no reason why HPTLC cannot be used with water extractions and Guzelmeric et al. (2017) even found that it surpassed HPLC in detecting species adulteration of chamomile (*Matricaria chamomilla* L., *Asteraceae*) herbal teas, so the literature using this method is certain to expand.

One of the reasons for HPTLC becoming the technique of choice for analysing herbal medicines is that the results can be readily displayed as the mobile phase travels up the plate which in turn can be easily compared to reference standards or adulteration markers. This also facilitates a high throughput with faster analysis than HPTLC due to being able to perform several analyses in parallel (Bairy, 2015) and the small size of the mobile phase means that it is relatively inexpensive on materials (Loescher et al., 2014). This has led to a rapid growth of an HPTLC atlas of plants (HPTLC Association, 2020) which is being used as a reference compendium for all herbal products. One limitation of HPTLC is that it is predominantly a qualitative or semi-quantitative technique, however, as with other chromatography methods, it can be linked to quantitative analytical hardware, e.g. MS, where higher precision is desired.

Spectroscopy/Spectrometry

Spectroscopy is the study of how radiated energy and matter interact; spectrometry is the measurement of this interaction

TABLE 4 | Uses of mass spectrometry in the analysis of herbal teas

Uses of mass spectrometry	Number of studies
To identify compounds as quality markers	81
To detect pyrrolizidine alkaloids	13
To detect heavy metals	10
To detect adulterant species	8
To detect mycotoxins	8
To detect pesticide residues	5
To detect adulterant drugs	2
To detect polycyclic aromatic hydrocarbons	2

(ATA Scientific, 2020). Some form of energy is projected at the matter which absorbs it creating an excited state. This then generates some form of electromagnetic waves which can be observed and measured.

Mass Spectrometry (MS)

Through the use of mass-to-charge ratio (m/Q) of ions MS can reveal the elemental or isotopic signature of a molecule or compound and enable its quantification. First, the substance under investigation is ionised in order to make it susceptible to influence by a magnetic field. Then, the charged ions are accelerated to a known speed and deflected using a magnetic field (Clark, 2019). The degree to which the magnetic field deflects the ionised molecules from their course is assessed and used to determine its molecular weight. This process can be further refined by adding an additional mass analyser, known as tandem mass spectrometry, MS/MS or MS^2, (Mittal, 2015). "Time of flight" (TOF) is one of the most common forms of tandem mass spectrometry based on the principle that heavier ions travel more slowly than lighter ions (Gilhaus, 2005). Another is the use of four cylindrical rods (a quadrupole) that generate oscillating electrical fields selecting specific ions based on the stability of their trajectories as they pass through. Quadrupoles are often set up in a triplicate formation, called a "triple quadrupole" (QqQ), where the first quadrupole is used to select certain ions, the central quadrupole acts as a collision chamber to fragment them and the last selects the fragments to be analysed (Schreiber, 2017). These setups are commonly used in biomolecular research of complex molecules.

MS is by far the most popular spectroscopic method utilised in the analysis of herbal teas. A total of 129 papers included in the review using some form of MS were identified. This is probably due to its ability to identify and characterise unknown compounds, which gives it an enormous advantage over chromatographic techniques when analysing newly identified plant compounds or forensically analysing adulteration of herbal preparations. However, 113 (88%) of these studies combined MS with some form of chromatography in order to separate the compounds before analysis, with LC being the most common (102, or 90%), followed by GC (15, or 13%) and less frequently with TLC (3, or 3%). This preparatory step can have an enormous influence on the results as can be seen by the MS studies searching for mycotoxins in tea (*C. sinensis*). Reinholds et al. (2020) found that in 140 different samples almost 97% of black teas, 88% of green teas and 100% of oolongs included in their study contained quantifiable levels of fungus with all Puerh (Chinese fermented tea) samples containing mycotoxins despite having the lowest levels of fungal contamination. Conversely, Monbaliu et al. (2010) analysed 91 teas and found only one sample of Ceylon melange to be contaminated with no mycotoxins in the drinkable products despite also testing Puerh. This is significant as Puerh is a fermented variety known to contain high concentrations of mould (Sedova et al., 2018). Both used UPLC systems to prepare the samples so unless their storage conditions prior to analysis were dramatically different, these two studies show that despite the detection capabilities of MS, the results can be easily swayed by even small variations in the preparatory steps.

From the distribution of uses for MS (**Table 4**) we can see a similar spread of uses to LC where the main usage is to identify and characterise marker compounds for future quality control. One of the main differences is that many of these studies are characterising a plant and identifying markers for the first time, or where a forensic analysis of herbs was being undertaken without prior knowledge of what the researchers were searching for. An important consideration when using MS, despite its outstanding sensitivity, is its relatively high cost, approximately US$500,000 capital cost, with US$250,000 yearly maintenance fees (El-Khoury, 2018) making them the domain of research laboratories or larger QC departments.

Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR is one of the preeminent techniques for determining the structure of organic compounds (Aryal, 2018). By placing molecules in a strong magnetic field and exciting its basic nuclear constituents (eg ^{1}H, ^{13}C, ^{31}P), structural and quantitative information can be readily obtained (Emery Pharma, 2018; Zinkel, 2019).

Although NMR is renowned in its capability to determine the molecular structure of any unknown organic compound, it suffers from some considerable limitations in relation to quantitative analysis. These include its relative insensitivity requiring 10–100 times the concentration of sample (>1 μM) compared to MS, LC or GC (10–100 nM), making it unsuitable for detection of trace metabolites (Emwas et al., 2019). Another drawback is the expense of the equipment with initial capital costs often exceeding US$1 million, although smaller, lower resolution benchtop versions are available for under US$100,000 (Reisch, 2015). These limitations are reflected in the literature with only 13 papers utilising NMR with almost half of these (6) also using MS and often adding NMR to acquire additional information when MS was not specific enough. Interestingly, only one study explored the idea of using high resolution ^{1}H NMR spectroscopy with multivariate statistical analysis as a method to identify the composition of complex mixtures of herbs without the use of prior separative techniques (Marchetti et al., 2020) building on the work of Booker et al. (2014) who used the same technique to analyse chemical variability of turmeric (*Curcuma longa* L. *Zingiberaceae*) along value chains. This highlights one of its

considerable advantages, to be able to provide a herbal 'fingerprint' for a wide range of compounds, of differing polarity, without the need to separate them using chromatographic methods. Given the relative expense generally involved with NMR, this technique is an unlikely method to be routinely used in the herbal tea industry. However, once the equipment is available, sample running costs are comparatively low and so there are some opportunities for contract analysis e.g. through universities.

Ultraviolet-Visible Spectrophotometry (UV–Vis)

Works by passing wavelengths of light in the ultraviolet (100–400 nm) and visible (400–700 nm) regions of the spectrum through a sample and measuring the light transmitted on the other side (Raja and Barron, 2020). The wavelengths not transmitted indicate the photons that have exactly matched the energy band gap required to promote a molecule from its ground state to an excited state have been absorbed. This provides an absorbance spectrum readout that can be compared to published literature for qualitative identification or quantitatively assessed, measuring the concentration of a sample using Beer's Law if the absorptivity of a substance is known, or can be calculated (Venton, 2017). The light source is usually a deuterium lamp that emits light in the 170–375 nm ultraviolet spectrum, and a tungsten filament lamp, which produces light from 350 to 2,500 nm for the visible range, which is then filtered for specific wavelengths. Alternatively a diode-array which can measure a whole spectrum of light in a single run can also be used (Dong and Wysocki, 2019).

Of the 58 studies included in the current review, 56 (97%) coupled UV-Vis with HPLC. This is a common combination and an integral addition to HPLC kits (Cross, 2016) meaning that the other HPLC studies will have also used UV-Vis but did not discuss the results in their papers.

The reliance of UV-Vis on published literature demonstrates one of its major limitations. While most of the studies (55 or 95%) looked at assessing herbal medicines by comparing their analytes against known constituents, Zhao et al. (2012) proposed UV-Vis as a method of detecting adulteration of Chinese medicines with drugs. While they were able to successfully identify 11 antihypertensive drugs that had been mixed into herbal medicines, their only samples were preparations that they had spiked themselves and therefore had the references to hand. When herbal medicines are adulterated with drugs, they are increasingly using designer analogues in order to avoid detection making techniques such as LC-MS and NMR necessary for their identification (Haneef et al., 2013; Patel et al., 2014).

Infrared Spectroscopy (IR)

IR, similarly to UV-Vis utilises specific wavelengths of radiation. The infrared light interacts with the bonds of molecules causing them to stretch or bend in symmetric or asymmetric ways when energy is absorbed from a very particular wavelength which in turn will depend on the functional groups within the molecule (Reusch, 2013). Troughs in transmittance in the region above 6.5 μm gives information about functional groups, while the region below 6.5 μm is known as the fingerprint region and gives a very intricate pattern that can be used to determine the chemical composition of compounds. IR can take place in the near-, mid- or far-infrared regions, or use a Fourier Transform method to simultaneously beam many frequencies, repeated in bursts of different combinations over a short time and then calculate the absorbance at each wavelength. The near range, from 780–2,526 nm is the most commonly used in quality control due to having higher energy and penetration capacity and producing less heat (Zeng et al., 2011) while the mid-infrared spectrum (2.5–25 μm) is superior for identifying structure and functional groups (Liang et al., 2011).

A total of eight papers included in the review used IR despite its versatility. Kokalj et al. (2014) found it to be a suitable low cost, rapid and simple method of quality control for both single herbs and mixtures in routinely tested samples. Ma et al. (2017) found IR could successfully determine adulteration of Chinese yam (*Dioscorea polystachya* Turcz., *Dioscoreaceae*) powder with cheaper corn and wheat starches while Yap et al. (2007) found it capable of differentiating Asian from American ginseng (*Panax ginseng* C.A. Meyer and *P. quinquefolius* L., *Araliaceae*). Lee et al. (2014) found IR outperformed GC when identifying caffeine and catechin content of tea (*C. sinensis*), while Chen et al. (2019) found IR to be of equal effectiveness to HPLC in evaluating the quality of *Rhizoma Atractylodis* (*Atractylodes macrocephala* DC., *Asteraceae*) decoction pieces. Cebi et al. (2017) also found IR could detect sibutramine in tea and coffee samples, although like UV-Vis, only spiked samples were used and analogues may be more problematic. Despite the broad range of applications which would seem to suit small herbal tea companies in Europe, the literature is largely dominated by MS and NMR data from universities, pharmaceutical corporations and the Asian herbal medicine market which is often government backed and far more integrated into conventional healthcare than in Europe or the US (Liu and Salmon, 2010). These organisations already have access to MS and NMR equipment and most smaller labs have UV-Vis fitted as standard equipment on their chromatography devices. This means an extra expense to install an IR device, train staff in its use and to develop the reference models for which there are few available in English and whose development requires time and expertise in chemometrics resulting in its low popularity as a method of analysing herbal teas (Zeng et al., 2011).

Optical Emission Spectroscopy

This is one of the less common forms of spectroscopy used in the quality evaluation of herbal teas, only appearing in five papers but worth mentioning because it has a very specific application for detecting metal content. This is because it functions by generating a spark between an electrode and a metal sample while in a high energy inductively coupled plasma state and using a spectroscope to detect the unique spectrum specific to each element (Shimadzu, 2020). All five papers utilising this method were using it to evaluate potentially toxic levels of metals in herbal teas with Malik et al. (2013) and Rubio et al. (2012) finding levels of aluminum high enough to warrant imposing consumption

TABLE 5 | Results of genetic analysis on teas searching for adulteration.

Author	Aim	Result
Osathanunkul (2018)	Find adulterants in Soursop (*Annona muricata* L. *Annonaceae*) teas	Three out of eleven (27%) samples contained incorrect species
Omelchenko et al. (2019)	Examine 6 herbal teas, 6 herbal medicines & 6 spices for adulteration	Twelve (67%) products contained different materials to those labelled. 6 likely to be economically motivated
De Castro et al. (2017)	Examine 32 herbal teas	Two (6%) found to be adulterated
Olivar et al. (2016)	5 *Vitex negundo* L. *Lamiaceae* samples, often used as herbal tea in the Philippines	Only one satisfied the database criteria for genetic authenticity
Xin et al. (2015)	Authenticate 90 commercial *Rhodiola crenulata* (Hook.f. & Thomson) H. Ohba *Crassulaceae* products from hospitals and drug stores	Only 36 (40%) contained the correct species. 35 (38.9%) contained *R. serrata* H. Ohba and 9 (10%) *R. rosea* L. Remaining 10 (11.1%) were 3 other R. species
Duan et al. (2017)	Identify species in *Radix Clerodendrum* tea samples used in the Dai ethnic group's medicine	Of 27 samples, only 1 (3.7%) was authentic *Clerodendrum japonicum* (Thunb.) *Lamiaceae*. Most were another medicinal species but 4 were potentially toxic *Lantana camara* L. *Verbenaceae*
Wang et al. (2016)	To find a DNA signature region which can be used to identify *Angelica sinensis* (Oliv.) Diels. *Apiaceae* in decoction powders	Of 9 decoction powders, 7 (78%) were identified as *Angelica pubescens* Maxim. *Apiaceae*

limits in hibiscus (*Hibiscus sabdariffa* L., *Malvaceae*) and mint (*Mentha sp.* L., *Lamiaceae*) respectively.

Biological Methods

Biological methods differ from those above in that they use biological systems to test for authenticity and quality in herbs. The most widespread method is certainly genetic testing but there are also several examples utilising biological assays to assess the effects of herbs and extracts on cell cultures and even *in vivo* preclinical models.

DNA Barcoding and Genetic Analysis

DNA barcoding takes a small section of genetic code from an unidentified organism and compares it to a reference library of DNA sections such as the Barcode of Life Data (BOLD) systems database (iBOL, 2020). It is often quicker and more precise than traditional taxonomic classification but the reliability of data is only as good as the database and, in the rush to categorise as many species as possible, there are many errors that may be present (Lathe et al., 2008). Countries, where there is a strong vested interest in herbal medicine, have created large herbal medicine genetic databases such as the Medicinal Materials DNA Barcode database of Traditional Chinese Medicines (Lou et al., 2010; Wong et al., 2018) but where there is less of a vested interest in employing curators to error check and correct herbal plant materials, the number of vouchered species is likely to be less and the database of poorer quality.

Fifteen studies included in the review used genetic based methods. As expected, all were being used to identify the correct species, half (7) to check for adulterants and identify them, the rest to confirm the species when using another technique for identification. Some of these papers provide a disquieting glance of how frequently tea adulteration takes place in the commercial arena (**Table 5**).

One of the complications in the use of DNA barcoding in the taxonomy of traditional medicines is that these methods were not utilised by the original practitioners when describing the original materials. Therefore a number of species can fit their description and we are now forcing their morphological and ecological descriptions to fit a narrow DNA profile. This is evident in the way that many traditional medical books have multiple species assigned a single entry (Bensky et al., 2004). Hence, when analysing the medicine of a traditional ethnic group such as Duan et al. (2017) undertook of the Dai people's medicinal plant Ha-Bin-Liang and found that only one of the 27 samples matched *Clerodendrum japonicum* (Thunb.) Sweet, *Lamiaceae* listed as the correct species in the pharmacopoeia, they assume a priority position of DNA barcoding over the practitioners who defined the medicinal material in the first place.

Bioassays

The use of bioassays presents an interesting opportunity for determining quality of herbs by directly measuring their effects on a biological system. It could be argued that this is the most crucial factor in any form of quality control, since functionality is at the core of herbal teas and medicinal plant consumption. With most herbs the identity of the component(s) responsible for a given effect are often uncertain or completely unknown. Moreover, given that it is recognised by herbal practitioners that rare, unavailable or ethically suspect herbs can be substituted with a taxonomically distinct species that has comparable effects (Selvam, 2010; Fang et al., 2013; Hall, 2019), measuring the effect of a herb or blend directly should be central in assessing quality control.

A total of 29 papers included in this review were identified as using some form of biological assay to assess the quality of herbal teas. With the exception of Wu et al. (2018), the reported biological assays were accompanied by a variety of adjunct methodologies to examine the chemical composition of the herbs. A plethora of assays were used for different purposes, some of which are outlined in **Supplementary Table S2**.

The most common assay used was the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) antioxidant assay, employed by 12 of the 29 papers (41%) included in this review, followed by the Ferric Reducing Antioxidant Power (FRAP) assay (5 papers, 17%) and various assays to measure the total phenolic content (4 papers,

14%). Since oxidative stress is a byproduct of mitochondrial activity, these studies suggest that mitochondrial function plays an important role in determining the functional quality of herbs. Despite this none of the studies looked at mitochondria directly.

Bioassays tend to be used to find a biological mechanism of action behind specific compounds which can then be selected for quality control and measured with chromatographic or spectroscopic methods. They are rarely a method of quality control in themselves. This reveals a distinct pharmaceutical bias in the fields of quality control of herbal teas, where the search for a 'single active ingredient' has become the main driving force in basic research, despite the fact that the use of isolated active compounds frequently leads to ineffective or less effective outcomes (eg. Lu et al., 2011). Of late, this has led modern drug discovery to look at synergies between multiple compounds and complex effects using -omics analysis (Thomford et al., 2018).

Mitochondria in Quality Control of Medicinal Herbal Teas

The existing methods of quality control in herbal teas focus heavily on chemometric testing for specific marker compounds. When there is attention to biological activity, it is largely to test novel compounds in order to determine which ones are biologically active and can potentially be developed into drugs. These often become the primary quality markers (eg., Qin et al., 2019), followed by compounds unique to the species and then specific levels of others in relation to other species. This reveals a distinct tendency toward the pharmaceuticalisation of herbal medicine and represents a problem when dealing with complex mixtures.

One area that remains problematic for detection of herbal adulteration is the practice of mixing old herbs that may be losing their potency, with small amounts of fresh herbs. These 'mixtures' will share the same relative chemical and DNA profile, albeit with some reduction in certain active ingredients and only a fully quantitative methodology would be able to detect any potential 'adulteration'.

One approach to overcome this issue would be a method that tests a herbal blend functionally, based on what it does to a complex biological system when viewed as a whole rather than the sum of its parts. Several attempts to use bioassays to ascertain the effectiveness of herbs on their intended condition have the potential to achieve this. For example, Shen et al. (2008) used a 48/80-induced histamine test on rat peritoneal cells to assess which species of magnolia flowers (*Magnolia* L. *Magnoliaceae*) should be considered the medicinal variety for rhinitis and Wu et al. (2019) used a xylene-induced ear oedema model in mice to determine the best *Polygonum chinense* L., *Polygonaceae* species to use for its anti-inflammatory effects in Liang Cha (Chinese cool tea). If a batch of herbs could reproduce these activities it may be considered functionally correct. However, in both of these cases animals had to be sacrificed making these methods unethical for routine testing in herbal quality control and most suitable for determining the best species which are then identified chemometrically. Currently there is no universal tool that can directly profile the biological effects of herbal medicines on the body.

Functional mitochondrial testing presents a unique opportunity to tackle this issue as these organelles are integral to most biological functions, providing the energy necessary to perform most tasks as well as generating important signaling mechanisms both within (Quirós et al. (2016) and between cells (Liu and Ho, 2018; Picard et al., 2018) through DNA, redox, hormonal, neurological and immune mediated pathways.

Here we discuss the potential of using a plethora of mitochondrial functions as the basis for a functional pipeline for the testing of herbal teas. As well as describing the different techniques required to achieve this, we discuss its potential use in both *in vitro* and *in vivo* conditions. The proposed methodology can be used in isolation or as an adjunct to standard laboratory methods for quality control.

The Relevance of Mitochondria to Herbal Function

Mitochondria are at the center of multicellular life. They have been known to be the site of respiration since 1949 (Kennedy and Lehninger, 1949) which already made them indispensable to most cellular functions and the very existence of multicellular life. For many years this was considered their sole responsibility with the research into mitochondria being focused on explaining the mechanisms of how this is achieved. With the discovery of the chemiosmotic gradient that drives oxidative phosphorylation (OXPHOS) to generate adenosine triphosphate (ATP) (Mitchell, 1961) their function was largely thought to be described. Recent new discoveries have demonstrated their involvement in a host of other functions necessary for multicellular life including cellular signaling, stress responses, apoptosis, cancer, ageing, inflammatory response and even mental and neurological function (Nunnari and Suomalainen, 2012; Kramer and Bressan, 2018; Annesley and Fisher, 2019). These mirror some of the main reasons why people utilise herbs (Benzie and Wachtel-Galor, 2011) leading to the possibility that mitochondria may underpin some traditional herbal concepts such as "Qi" (loosely translated as vital force or energy) which is central to the traditional descriptions of the actions of herbs (Wallace, 2008).

One of the most popular classes of herbs are adaptogens which aim to enhance our non-specific responses to stress, improving energy levels, boosting the immune system and enhancing mental functioning (Panossian and Wikman, 2010). However, their multi-target effects have made it difficult to determine any singular mechanisms by which they can be measured (Gerontakos et al., 2020). Since all of these functions can in part be attributed to mitochondria, they make an ideal group to begin analysing and profiling their effects on these organelles in order to determine the underlying mechanisms.

As well as adding to the general knowledge on these herbs, potentially providing new avenues for treatments to be

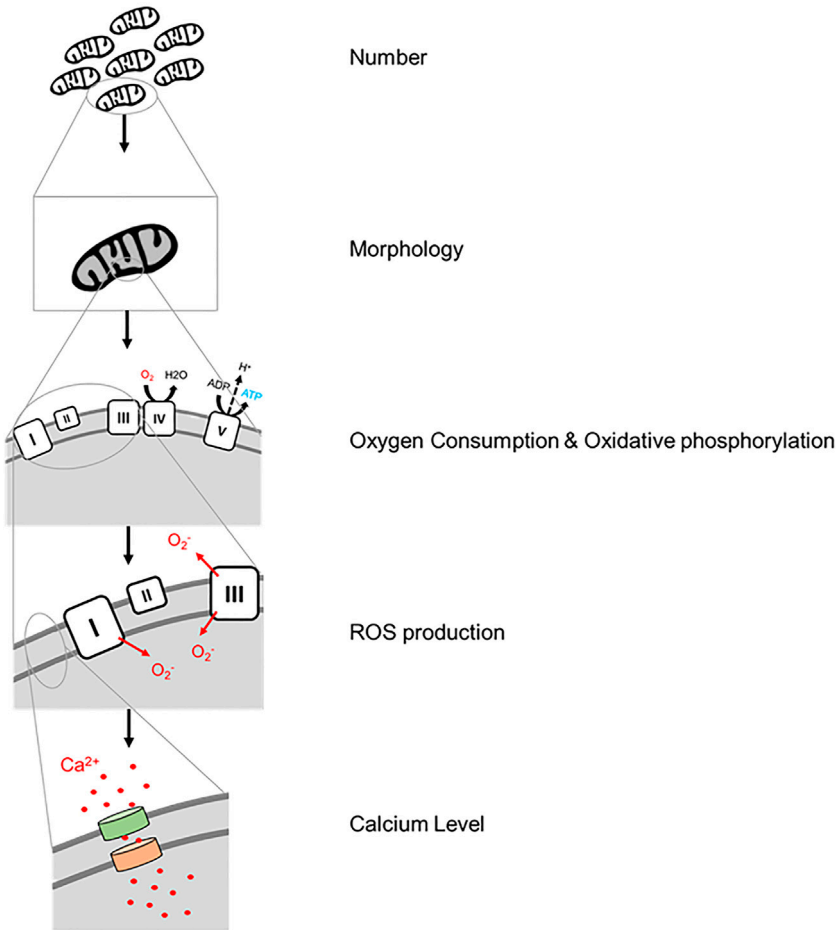

FIGURE 2 | Graphical representation of the levels of mitochondrial testing that are possible today.

developed, we suggest that mitochondria may provide a means of evaluating the quality of a herbal blend, by observing their activity on these essential organelles and comparing inter-batch variability. The strength of a system such as this is that the profile can be applied to a single herb with complex ingredients such as adaptogens, or to a blend of herbs which may also have multiple targets and mechanisms. The initial research may require some pilot work to find the ideal cell lines and specific assays to use but once profiled and recorded, future batches can be tested against the same standard. Potency can also be measured by comparing the degree to which the herb or blend creates the known effect. The advantage of using biological assays instead of chemical testing alone is that knowledge of the active ingredients or their proportion, which may be numerous and have complex interactions with each other, is not essential. Instead the effect of the herbal tea on its target tissues is measured directly and a statement of quality based on effects rather than the current methods of purported quantities of specific compounds. To discuss how this is being carried out, each specific test will be explained in turn along with its principles of operation and limitations.

METHODS OF MITOCHONDRIAL ANALYSIS

Mitochondrial function can be measured by a series of assays to assess their various functions. These include their quantity which reveals the balance between their biogenesis and mitophagy; their dynamic morphology into group formations; the OXPHOS cycle and its byproducts; and the signaling methods employed by mitochondria, especially their use of calcium (**Figure 2**).

Mitochondrial Biogenesis and Mitophagy

Mitochondrial homeostasis is preserved by two opposing processes: biogenesis, in which new mitochondria are generated from the existing ones, and the removal of damaged mitochondria though mitophagy (Yoo and Jung, 2018; Popov, 2020). Both processes are controlled by signaling proteins that either instruct the mitochondria to self-renew, or enable autophagosomes to recognise damaged mitochondria and deliver them to lysosomes for degradation. The first step to determining the balance between these two processes is to measure the number of mitochondria present. This can be achieved by tagging the organelles with a fluorescent dye and

using live cell fluorescent microscopy imaging techniques such as epifluorescence, laser-scanning confocal and spinning disk confocal microscopy, and analysing the resulting images with computer software (Viana et al., 2015). Any herbs which can be found to reliably alter the quantity of mitochondria can be assumed to be affecting their biogenesis or mitophagy. However, the number of mitochondria does not necessarily correlate with their health as disruptions in both biogenesis and mitophagy have been reported in senescence, ageing, metabolic diseases, neurodegeneration, cancer and kidney disease so further investigations into the functional capacity of the mitochondria and their behaviour in between biogenesis and mitophagy is necessary to fully understand their functioning.

Mitochondrial Morphology and Dynamics

In contrast to the traditional depiction of mitochondria as discrete bean-shaped organelles, recent findings have revealed them to be a dynamic network that extends throughout the cell (Bereiter-Hahn and Vöth, 1994). This enables them to employ a variety of strategies to maintain their integrity under stress before becoming dysfunctional and being signaled for mitophagy. The main mechanism behind these morphological changes are the fusion and fission of their inner and outer membranes in response to metabolic stimuli. These are often related to nutrient availability and the metabolic state of the cell with nutrient withdrawal and mild stress inducing fusion to become a hyperfused network with increased OXPHOS while nutrient excess and severe stress induces fission with impaired OXPHOS and increased fragmentation (Wai and Langer, 2016). Fusion is achieved by a three step process of tethering, docking and finally fusion of their outer membranes by guanosine triphosphate (GTP) hydrolysis and may enable sharing of matrix components and protection against engulfment by phagosomes (Tilokani et al., 2018). In fission the mitochondria are encircled by a dynamin related protein (Drp1) leading to a narrowing of the membrane which is then enhanced by GTP hydrolysis marking the site of future scission. These fragmented mitochondria can then become targets for mitophagy which is important for mitochondrial quality control but excessive fragmentation and mitophagy is associated with cell death and several diseases including many neurodegenerative disorders (Pickles et al., 2018).

Mitochondrial morphology and dynamics can also be captured using imaging techniques combined with fluorescent markers and computer analysis (Harwig et al., 2018). If herbal teas can show a tendency toward fusion they may indicate increased resistance to stress, whereas a tendency toward fission will suggest increased mitophagy. In healthy cells increased mitophagy may indicate a reduced ability to adapt to stress but in damaged or diseased cells it may be part of an essential quality control mechanism. Since the quantity and morphology of mitochondria can only tell so much about the functional state of mitochondria, the next level of detail is to measure their level of functioning with their OXPHOS capacity.

Oxidative Phosphorylation (OXPHOS)

OXPHOS is one of the main functions of the mitochondria, involving the pumping of protons into the intermembrane space and using a chain of carriers to generate an electrochemical proton gradient which is used to drive the production of ATP from adenosine diphosphate (ADP) + phosphate (P) in the presence of oxygen (O_2) (Alberts et al., 2002). This stored energy can then be broken down again to drive the majority of reactions in eukaryotic cells. Therefore, another way of measuring mitochondrial function is to assess the concentration of ATP being generated in the cell, the mitochondrial membrane potential ($\Delta\Psi m$) being generated by the proton pumps in Complexes I, III and IV of the electron transport chain (ETC), and the oxygen consumption rate (OCR) of the mitochondria.

Adenosine Triphosphate (ATP) Synthesis

Since the final product of mitochondrial respiration is ATP synthesis, the most obvious method of assessing mitochondrial function is to measure ATP concentration. While several methods exist to measure ATP concentration (Rajendran et al., 2016), one of the most sensitive and reliable techniques is the bioluminescent luciferin–luciferase reaction (Morciano et al., 2017; Morciano et al., 2019). The enzyme luciferase, derived from the North American firefly *Photinus pyralis* L. *Lampyridae*, generates a flash of yellowish-green light proportional to the amount of ATP present as a byproduct of the oxidation of the substrate D-luciferin into oxyluciferin in the presence of magnesium ions. This light has a peak emission at 560 nm and so can be detected with luminescence detectors. Herbs which increase ATP production can be said to increase the energy reserves within our cells. The limitation of this technique is that it does not differentiate between the ATP of the cells and those from other substances such as those that may have been introduced from the herbal materials, some of which may also have their own luminescence (AIB Staff, 2013).

Mitochondrial Membrane Potential ($\Delta\Psi m$)

The $\Delta\Psi m$ is generated by the proton pumps of complexes I, III and IV of the ETC which create an electrochemical gradient that can be harnessed to generate ATP. High $\Delta\Psi m$ leads to the production of reactive oxygen species (ROS) and oxidative stress but a sustained drop in $\Delta\Psi m$ may also be harmful due to lack of ATP production and too low levels of ROS which may create a state of reductive stress that is just as harmful to cells as levels that are too high (Zorova et al., 2018). Herbs which increase $\Delta\Psi m$ may be behind ROS induced apoptotic signaling and may indicate anti-cancer effects if seen only in the cancerous cell lines and not their non-cancerous analogues. Herbs that keep $\Delta\Psi m$ at an optimum level, even in senescent cells, may have adaptogenic effects against ageing.

Several fluorescent lipophilic cationic dyes can be used to measure $\Delta\Psi m$ including:

- TMRM & TMRE (tetramethylrhodamine methyl and ethyl ester)
- Rhod123 (Rhodamine 123)
- DiOC6(3) (3,3′-dihexyloxacarbocyanine iodide)
- JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide)

The basic principle of all of these is the following: they accumulate within the mitochondria in inverse proportion to ΔΨm making more polarised mitochondria accumulate more dye and depolarised mitochondria accumulate less (Perry et al., 2011). This can then be detected by measuring the fluorescence or imaged with a camera. Each dye has its particular advantages and disadvantages but the overall limitations of these methods are that changes in mitochondrial morphology, localisation, or mass might also affect fluorescence measurements. Therefore controls that assess whether these changes are also happening are advisable to conduct alongside the ΔΨm probes, although these tests are also not without their limitations and may affect ΔΨm and respiration themselves Buckman et al. (2001).

Oxygen Consumption Rate (OCR)

Oxygen plays a critical role in the generation of energy through the OXPHOS chain which is the primary source of energy in complex biological systems. Therefore, measuring the extracellular OCR in real time can reveal how much mitochondrial activity is taking place within a culture of cells. This can be measured using an extracellular flux analyser by isolating a monolayer of cells covered with an extremely small volume of media (about 2 μL) and placing a probe 200 microns above the monolayer (Agilent Technologies, 2020). The probe measures the concentrations of dissolved oxygen in the transient microchamber every 2–5 min and calculates the OCR. It then lifts allowing the media to mix with the microchamber, restoring cell values to the baseline.

Further modifications to this assay enable the determination of exactly how much oxygen is being utilised by the mitochondria for ATP synthesis, what their spare capacity is and how much is being used by other biological processes (Agilent Technologies, 2019). This is achieved by injecting up to four drugs into the sample at user specified times which affect specific complexes in the ETC. Oligomycin inhibits ATP synthase decreasing electron flow through the ETC resulting in a drop in mitochondrial respiration and OCR. By measuring the difference between normal respiration and the oligomycin adjusted OCR, the amount of O_2 used for cellular ATP production can be calculated. Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), an uncoupling agent that disrupts the ΔΨm and collapses the proton gradient, disinhibits electron flow through the ETC and causes OCR to reach its maximum. This corresponds to the ability of the cell to respond to increased energy demand under stress by enabling its spare respiratory capacity. Finally, a mixture of rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor, shuts down mitochondrial respiration completely enabling the detection of non-mitochondrial respiration by other processes such as that consumed by oxidase and oxygenase enzymes (Halliwell and Gutteridge, 2015). This can be deducted from the other measurements to acquire an accurate calculation of mitochondrial respiration. Each of these responses can then be used to acquire a detailed description of a herb's effects.

Reactive Oxygen Species (ROS)

Reactive Oxygen Species (ROS) are highly reactive by-products of the mitochondrial respiration process, formed when the electrons being carried by the ETC leak and combine with oxygen to form superoxide (O_2^-) and/or hydrogen peroxide (H_2O_2), which can cause irreversible cell damage and even death (Zhao et al., 2019). Under normal physiological conditions this leak is estimated to be around 0.2–2% and serves several important functions including apoptosis, autophagy, differentiation, adaptive responses to endoplasmic reticulum (ER) stress and hypoxia, hormetic antioxidant defence and innate immunity. However, under abnormal conditions, they have been implicated in pathological states including alcohol induced and non-alcoholic liver disease, ageing, hearing loss, atherosclerosis, cardiomyopathy, ischemia/reperfusion injury, cancer, diabetes, epilepsy, Huntington's, Alzheimer's and Parkinson's disease (Brand, 2016). Seeing how many of the biggest killers and causes of long term morbidity are on this list (WHO, 2018), it is not surprising that many herbal supplements and teas have based their advertising on the antioxidant effect of various compounds found in plants, despite the fact that these results have not been reproduced *in vivo* (Berger et al., 2012) and could actually be causing detrimental effects on health by affecting the regular physiological functions of ROS such as reducing the hormetic effects of exercise (Pingitore et al., 2015). Some herbs may even be useful because they increase ROS; for example, by priming cancerous cells for apoptosis making them more susceptible to chemotherapeutic agents (Henley et al., 2017).

Dichlorodihydrofluorescein diacetate (DCFDA) is one of the most widely used techniques for directly measuring the redox state of a cell (Eruslanov and Kusmartsev, 2010). It works through intracellular esterases which cleave the two ester bonds from the original molecule to produce H_2DCF that then accumulates intracellularly and oxidises to form highly fluorescent dichlorofluorescein (DCF). This can be measured by detecting the increase in fluorescence at 530 nm when excited at 485 nm to provide a measure of generalised oxidative stress. It is unable to provide a direct measure of any particular reactive species since neither H_2O_2 nor O_2^- can oxidise H_2DCF directly but must be decomposed to radicals while other substances in the cell may also produce the reaction including other radicals as well as cytochrome c, responsible for activating the caspase cascade that initiates apoptosis, making assessment of ROS during apoptosis using DCFDA especially problematic (Lawrence et al., 2003).

MitoSOX is another assay used for detecting ROS, especially O_2^-. It contains a positively charged derivative of dihydroethidium (HE) that rapidly accumulates in mitochondria, where it is oxidised by reactive species to become 2-hydroxyethidium (2-OH-E^+) that then binds to DNA producing a red fluorescence (Kauffman et al., 2016). This is more specific than DCFDA but it can still suffer from overlapping fluorescence from ethidium (E^+) which is not formed from O_2^- and may be generated in larger quantities than the radicalised form (Zielonka and Kalyanaraman, 2010; Nazarewicz et al., 2013).

Although both assays have their drawbacks, a critical approach to measuring ROS combined with data from other tests and controls can give some valuable insights into the degree of

oxidative stress that a cell culture is undergoing and how exposure to herbal teas may affect this.

Calcium (Ca^{2+}) Levels

Ca^{2+} has emerged as an important signaling molecule that facilitates communication between the mitochondria and the ER enabling the mitochondria to respond to the energy demands of the cell (Rossi et al., 2019). In response to ER stress, Ca^{2+} stores are released through inositol 1,4,5-trisphosphate receptors located in numerous places where the mitochondria are in close contact with the ER. These enable the mitochondria to be exposed to far higher concentrations of Ca^{2+} than in the rest of the general cytosol where it is transported into the mitochondria by voltage-dependent anion channels located on the outer mitochondrial membrane and then taken inside the mitochondria through a channel in the inner mitochondrial membrane called the mitochondrial calcium uniporter. Moderate levels activate several enzymes of the citric acid cycle, boosting ATP synthesis and enabling mitochondrial adaptation to the cells' metabolic needs, while high levels sensitise the mitochondria to pro-apoptotic stimuli, promoting the opening of the mitochondrial permeability transition pores, initiating cell death (Bravo-Sagua et al., 2017). This apoptotic mechanism has been implicated in several diseases including ischemia-reperfusion injury, liver and muscle diseases including cardiomyopathy, cancer, and neurodegenerative disorders (Britti et al., 2018; Romero-Garcia et al., 2019).

Ca^{2+} levels can be detected inside mitochondria using indicators like Rhod-2 acetoxymethyl (AM) ester which increases in fluorescence when it binds with Ca^{2+}, excitable at 557 nm and emitting a signal at 581 nm (Cayman Chemical, 2021). This can be applied to many cell types but it is not mitochondria-specific so a second spectrally distinct dye such as MitoTracker Green may be used prior to imaging to ensure that mitochondrial calcium can be differentiated from other sources (Maxwell et al., 2018). These readings can then provide additional information on the mechanism behind increased ATP synthesis or apoptosis to determine how a herbal tea achieves an effect.

CONCLUSION

Various chromatographic and spectroscopic techniques have been applied to the quality control of herbal medicines in addition to organoleptic and wet chemistry methods. Advances in analytical hardware have led to more and more detailed analysis of plant products and particularly licensed herbal medicines, which are required to conform to the standards of the national pharmacopoeias. Moreover, plant products have been shown to have a history of adulteration and contamination. Adulteration may be accidental or may result from a desire to increase profits through using cheaper ingredients. Chemical analysis methods are essential tools in the fight against poor quality and adulteration. However, although chemical purity can be readily determined, this gives little information regarding the biological activity of a given substance as herbs consist of many different compounds and it is not typically known which the active ingredient is, and current quality testing mainly relies on the analysis of marker compounds that may or may not contribute to the desired clinical effectiveness of a herbal medicine. Mitochondrial analysis presents us with a unique opportunity to address this deficiency and to develop methods that can give us a broader perspective on herbal quality and one that takes into consideration how herbs can affect biological processes. Not least for the manufacturers of such products, this offers another avenue for value addition and the means to distribute products under an entirely new marketing strategy but also for consumers, it provides the knowledge that the herbal products produced have been tested on complex biological systems and proven to be of good chemical quality.

Current analytical methods focus on determining the chemical quality of medicinal herbal teas, including adulteration and contamination. Mitochondrial analysis and associated methods propose a new way of determining the quality of plant derived products that is more closely linked to the biological activity of a product and its potential effectiveness. Future work should focus on generating data that will validate this testing methodology.

AUTHOR CONTRIBUTIONS

Conceptualization: SW, AB, and JB. SW performed the literature review and wrote the main body of the paper. IK created the graphic illustration and provided critical feedback. MSA, RM, AB, and JB read and provided critical feedback.

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Danshen (*Salvia miltiorrhiza*) on the Global Market: What Are the Implications for Products' Quality?

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Background: Danshen (Radix et rhizoma *Salviae miltiorrhizae*; *Salvia miltiorrhiza* Bunge, Lamiaceae) is commonly used in Asia, including China, Japan, and Korea with markets in America and Europe growing substantially. It is included in multiple pharmacopeias and salvianolic acid B and tanshinone IIA are used as quality markers. However, on the markets, substitutes and different processing methods often are a concern. a concern regarding patients' safety and expected outcomes.

Aims: This study aims at understanding the quality of Danshen-derived products on the market, and the relationship between the chemistry, biological activity and the processing and storage methods.

Methods: For heavy metal analysis, inductively coupled plasma optical emission spectrometry was used. High performance thin-layer chromatography and proton nuclear magnetic resonance coupled with principal component analysis were used to understand the variation of metabolite composition. MTT assay and LPS induced NO production assay were used to evaluate the cytotoxicity effect and anti-inflammatory activity, respectively.

Result and Discussion: Six out of sixty samples exceed the limits of cadmium according to the Chinese or United States Pharmacopoeia. Arsenic, lead and copper contents are all below pharmacopoeial thresholds. With more complex processing procedure, the risk of

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Abbreviations: °C, degree Celsius; ; 1H-NMR, proton nuclear magnetic resonance; As, arsenic; BP, British pharmacopoeia; Cd, cadmium; CFDA, china food and drug administration; CHCl3, chloroform; ChP, Pharmacopoeia of the people's republic of China; CNS, central nervous system; Cu, copper; 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; HCA, hierarchical cluster analysis; HKCMMS, Hong Kong Chinese materia medica standards; HPLC, high performance liquid chromatography; HPTLC, high performance thin layer chromatography; ICP-OES, inductively coupled plasma—optical emission spectrometry; IL, interleukin; ISO, international organization for standardization; JP, Japanese pharmacopoeia; LPS, lipopolysaccharide; 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 center; NMPA, national medical products administration; NO, nitric oxide; Par, Pareto scaling; Pb, lead; PBS, phosphate buffer saline; PCA, principal component analysis; Ph. Eur, european pharmacopoeia; PLS-DA, partial least squares discriminant analysis; Q^2, the estimate of goodness of prediction; R^2, the estimate of goodness of fit; Rf, retardation factor; rpm, round per minute; *Salvia*, *S.*; Sample no., sample number; SEM, standard error of mean; SFDA, state food and drug administration; TCM, traditional Chinese medicine; THP, Taiwan herbal pharmacopeia; TLC, thin layer chromatography; TNF-α, tumor 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 organization.

heavy metal contamination increases, especially with arsenic and cadmium. The metabolite compositions show a variability linked to processing and storage methods. Authenticated samples and Vietnamese primary samples contain higher salvianolic acid B, and their chemical compositions are more consistent compared to Chinese online store samples. Overall, a significant chemical variation can be observed in Danshen products directly linked to processing and storage method. In the MTT assay, fourteen samples show cytotoxicity while seven samples increase the proliferation of RAW264.7. In the LPS induced NO production of RAW 264.7, only seven samples show significant inhibitory effects.

Conclusion: This is the first interdisciplinary investigation focusing on understanding the current market and the quality of Danshen. The quality of Danshen products on the high street are inferior to the authenticated samples. The results of the bioassays selected is not useful to differentiate the quality and composition according to the current definition in the pharmacopoeias. Overall, this approach highlights the tremendous variability of the products linked to processing and the need for more systematic and stringent quality assurance.

Keywords: metabolomics (OMICS), H-NMR, HPTLC, ICP-OES (coupled plasma optical emission spectrometer), danshen (Salvia miltiorrhiza bunge.), quality assessement, MTT assay, griess assay

INTRODUCTION

Radix *et* rhizoma *Salviae miltiorrhizae* (Danshen, from *Salvia miltiorrhiza* Bunge, Lamiaceae) has been used commonly as a traditional Chinese medicine in many Asian countries such as China, Japan, and Korea. It is one of the most important botanical drugs in modern TCM. Based on traditional Chinese medicine theory, Danshen has the properties of removing blood stasis and promoting blood circulation; clearing menstruation and relieving pain and swelling (Zhou et al., 2005). Between 200 and 300 B.C., "Shen Nong's Classic of the Materia Medica"—Shennong Ben Cao Jin (*Anonymous*) cited Danshen as a better-class medicine. In 2015, the total demand of Danshen was estimated to be 18.4 thousand tons according to a market report (Zhiyan Consulting Group, 2017).

The common medical indications for Danshen include menstrual disorders, cardiovascular diseases, chronic pain and thoracic obstruction. Currently, more than thirty clinical studies related to using Danshen to treat different cardiovascular diseases have been conducted, and they have shown promising evidence for improving the clinical symptoms (Wang et al., 2017a). Furthermore, some scientists study other potential medical indications of Danshen including diabetes (Jia et al., 2019), stroke (Bonaccini et al., 2015; Kim et al., 2018) and cancer (Chen et al., 2013; Wang et al., 2017b). The species is known well in terms of its chemistry. Until now, more than 40 abietane diterpenes (tanshinones) and 50 phenolic acids (salvianolic acids) have been identified (Jiang et al., 2005; Zhang et al., 2012; Chen et al., 2014). These are essential for understanding the botanical drug's pharmacological activity.

In the current understanding, tanshinone IIA inhibits the oxidization of the low-density lipid oxidization in RAW 264.7 macrophage via NF-κβ signaling (Jang et al., 2006; Chen et al., 2007). The possible anti-inflammatory effect is linked to inhibiting the expression of IL-1, IL-6, and TNF-α in NF-κβ pathway (Jang et al., 2003) as well as moderating IKK, ERK, and JNK in MAPKs and Nrf2 pathway (Zhang and Wang, 2007; Li et al., 2010; Bi et al., 2012). Several studies show that salvianolic acid B also inhibits the oxidization of low-density lipids (Zhao et al., 2008; Ho and Hong, 2011; Yang et al., 2012) and protects vascular endothelial cells by regulating VCAM-1, ICAM-1, TNF-α, etc. (Du et al., 1995; Chen et al., 2001; Lay et al., 2003; Pan et al., 2011). These pathways are linked to NO production releases from different cells including macrophages and endothelial cells (Black and Garbutt, 2002; Berg and Scherer, 2005; Moore and Tabas, 2011; Mangge, 2014) and closely related to cardiovascular diseases.

Many pharmacopoeias have recognized Danshen (British Pharmacopoeia, 2015; Chinese Pharmacopoeia, 2015; Japanese pharmacopoeia, 2017; United States Pharmacopeial Convention, 2016). The quality of Danshen is defined on the basis of salvianolic acid B and tanshinone IIA as markers, but their standard levels vary in the different regulatory systems (**Table 1**). However, Pang et al., 2016 argues that the current quality control from pharmacopoeias using salvianolic acid B and tanshinone IIA is inadequate as more metabolites in Danshen, such as cryptotanshinone, rosmarinic acid and tanshinone I, are of importance for the pharmacological activities. Different phytochemical analytical methods for Danshen such as HPTLC (Hu et al., 2005), UV (Wang et al., 2014; Yan et al., 2016), MS (Liang et al., 2017; Ni et al., 2019) and NMR (Jiang et al., 2014) have been developed. The current quality standard of Danshen does reflect these the scientific advances.

In addition, the variability of Danshen materia prima available on the markets complicates attempts for consistent quality control. Historically, the production of Danshen was limited to

Shandong, Henan, Hubei and Sichuan. One of the earliest investigations on tanshinones showed the tanshinone IIA contents varying from 0.02 to 0.32% depending on the origins. 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% (Huang et al., 1980a; Huang et al., 1980b). Today, dried roots are cultivated commercially all over China except in Hainan, Inner Mongolia, Jilin, Heilongjiang, Tibet and Xinjiang, but the processing and cultivation methods vary. Some countries in Asia, including Taiwan, Japan and Vietnam, have small production areas, but the majority of the Danshen used is imported.

Another report (Zhang et al., 2018b) states the substitute species and the increase of new cultivars extravagate the quality problem particular in Danshen. At least 19 other *Salvia* species have been used traditionally as Danshen (Xu et al., 2018) in some local areas, and of these, *S. bowleyana* Dunn. and *S. przewalskii* Maxim are the most common substitutes. Both have a chemical composition similar to *S. miltiorrhiza*, including salvianolic acids and tanshinones. It is common for farmers to cross-pollinate and cultivate better disease-resistant hybrid cultivars. Two cultivars, *S. miltiorriza* var. *miltiorrhiza* and var. *charbommellii*, are documented under the taxa of *S. miltiorrhiza* in the flora of China (Li and Ian, 2015). These practices impact on the quality of Danshen and it is difficult to identify these cultivars as they have similar chemical compositions.

In the 2015 reports of NMPA (Zhang et al., 2018a and b), 39.7% of Danshen (defined as Radix *S. miltiorrhiza*) did not pass the tests of macroscopic characteristics, the extract, water or biomarker content. It had one of the highest failure rates among of the TCM materials. This raises two issues: 1) What are the factors of the consistency of the quality in Danshen products? and 2) What is the definition of Danshen "quality" regarding safety and therapeutic effects? The current standard criteria of TCM uses major secondary metabolites or characteristic metabolites to define the quality. Nevertheless, quality is an essential basis for safety and potential bioactivities. Therefore, an integrated approach to chemical composition and bioactivity is needed (Wang et al., 2019).

AIMS AND OBJECTIVES

Clearly, better ways for a consistent high-quality supply of the primary material and finished products are needed. Ways to ascertain this require an understanding of what problems actually cause poor quality. Moreover, there is a lack of understanding of the quality of Danshen derived products. Authenticity and quality assessment need to be understood as a consequence of an entire specific value chain. This project aims to understand the quality of plant derivative medicinal products using a phytochemical-metabolomic approach combined with a bioassay and an assessment of one class of potential contaminants—heavy metals.

MATERIALS AND METHODS

Sample Collection

Authenticated *S. przewalskii* Maxim, *S. bowleyana* Dunn and *S. miltiorrhiza* Bunge samples were obtained from NIFDC, Kew Gardens, America Herbal Pharmacopoeia, Brion, Lfl and YuFu biotek. Authenticated samples were collected by botanists and were processed straight after the harvest.

Commercial samples were collected in Phố Lãn Ông (Vietnam) or on "Taobao" (a Chinese online store platform). The reason for examining Phố Lãn Ông is that the traceability was low and the quality of the herbal materials in Vietnam was not well regulated.

The biggest Chinese e-commerce platform Taobao which covering accredited online stores and typical online sellers was chosen as one of the channels of collecting samples. During May 2017, "丹参" (the simplified Chinese word of Danshen), and "丹参粉" (Danshen powder) were used as keywords.

All samples are deposited at the School of Pharmacy's collection of commercial and pharmacognostic specimens, with the intention of a longer term integration into the collection of the Uebersee-Museum Bremen (BREM). The sample list with the sample information, such as origin, price, material form, is attached in the **Supplementary Document**.

Sample Preparation

Three types of samples were collected: 1) crude dried roots or rhizomes, 2) dried root or rhizome powder and 3) concentrated extracts. The method of sample preparation was developed based on Kim et al. (2010). Crude roots or rhizomes samples were ground into fine powder by a mechanic grinder (GT203840, Tefal, United Kingdom), and passed through a sieve, resulting in particles of less than 1 mm size. All samples were stored with silica gel packs to avoid humidity.

All samples were accurately weighed with less than 0.5% error and extracted in 75% methanol with 1: 20 (g/ml) drug-solvent ratio followed by 30 s of vortexing and 30 min of ultra-sonication. The extracted samples were centrifuged under 1,400 rpm at 15°C for 10 min.

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, United States), ethyl acetate, toluene and glacial acetic acid (HPLC grade, Fisher Scientific, United Kingdom), formic acid (ACS reagent, 98%, BDH Chemicals Ltd., United Kingdom), DMSOd6, ≥99.0% (Cambridge Isotope Laboratories, Inc., United States), PBS buffer pH 7.4, D-MEM, penicillin-streptomycin 10,000 U/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, United States), H_2O_2 (HPLC grade, Sigma-Aldrich, United States/analytical grade, Carl Roth, Germany), HNO_3 (analytical grade, Carl Roth, Germany), HCl (analytical grade, Carl Roth, Germany), phosphoric acid (≥98%, ACROS Organic,

TABLE 1 | Comparison of Radix et Rhizoma Salvia miltiorrhiza quality criteria based on different regulatory bodies. Gray boxes–no quality parameter specified.

		ChP 2015	Thp 2nd	USP 40th	Jp 17th	Ph. Eur 9th	BP 2015	Hkcmms	WHO	ISO
Water-soluble extractives		50%	35%	35%				57%		
Alcohol-soluble extractives		46%	15%	15%	42%			52%		
Tanshinones		0.25%		0.20%						
Tanshinone IIA			0.20%	0.10%		0.12%	0.12%	0.12%		
Salvianolic acid B		3%		3%		3%	3%	4%		
Rosmarinic acid								0.17%		
Loss of drying		13%	15%	13%		10%	10%	12%		
Ash insoluble		10%	10%	10%		10%	10%	8%		
Ash insoluble in HCl		3%		3%		3%	3%	2%		
Heavy metal (mg/kg)	Pb	5	5	5	10	5	5	5	10	10
	Cd	0.3	0.3	0.3		0.2	1	1	0.3	4
	As	2	2	2	5		5	2		2
	Hg	0.2	0.2	0.2		0.1	0.1	0.2		3
	Cu	20	20							

United States), RotiStar ICP-standard matrix: 5% HNO_3 (Carl Roth, Germany).

Cell Line

RAW 264.7 (American Type Culture Collection, United States).

Chemical Standards

DSS (NMR grade, ≥99.9%, Sigma-Aldrich, United States), sulfanilamide ≥99%, N-1-naphthylethelene (ACS reagent, ≥98%, Sigma Aldrich, United States), MTT ≥ 97.5% HPLC grade, LPS from *Escherichia coli* (Sigma Aldrich, United States).

Salvianolic acid A ≥ 98%, salvianolic acid B ≥ 95%, danshensu ≥ 98%, rosmarinic acid ≥ 98%, caffeic acid ≥ 98%, tanshinone IIA ≥ 98%, tanshinone I ≥ 96%, cryptotanshinone ≥ 98%, and dihydrotanshinone I ≥ 96% (Tauto Biotech, China).

Instruments

Grinder (GT203840, Tefal, United Kingdom), Rotamixer (Hook and Tucker Instruments Ltd., United Kingdom), Grant XB22 ultrasonic bath (Grant Instruments, United Kingdom), Centrifugator (Centrifuge 5804 R, Eppendorf, Germany), electronic balance (Sartorius CP64, Sartorius AG, Germany), Freeze dryer (ModulyoD Freeze Dryer, Thermo Fisher Scientific, United Kingdom), NMR tube (VWR international Ltd., United States), Bruker Advance 500 MHz spectrometer (Bruker, Germany), HPTLC plates silica gel 60 F 254 (Merck, Germany), Linomat 5 (CAMAG, Switzerland) coupled with a 100 µL syringe (CAMAG, Switzerland) and compressed air with 60–90 psi., Automatic Developing Chamber ADC 2 (CAMAG, Switzerland), TLC Visualizer (CAMAG, Switzerland) Microwell plate Nunclon 96 well (Thermo Scientific Nunc, United Kingdom), GalaxyB CO_2 incubator (Scientific Laboratory Supplier Ltd., United Kingdom), 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, United States), VisionCATS (CAMAG, Switzerland), Smart Analyser Vision February 5, 0937 (SPECTRO, Germany).

High Performance Thin Layer Chromatography

The HPTLC method was developed from Booker et al. (2014) and the "Hong Kong Material Medica - Radix Salviae Miltiorrhizae Monograph" Chinese Medicine Division of the Department of Health (2005). 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 min and pre-conditioned for 5 min. The condition of the tank was held at the humidity of 33% and a temperature of 23°C. The process stopped once the developing solvent reached the plate to 80 mm migration distance. After 5 min plate drying, the plates were visualized under white light, 254 and 366 nm wavelength. VisionCATS, the HPTLC workflow and analytical software, was used in plate analysis and graphic editing.

Heavy Metal Analysis

The method of heavy metal analysis was developed based on Cooper et al. (2007), Olesik et al. (1995). 300 mg of powdered sample was weighed accurately in the microwave digestion tube. 1 ml of H_2O_2 for 10 min, 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 h were added to each sample. Before microwave digestion started, samples 7.5 ml of 35% HCl was added. The tubes were sealed and placed in the microwave digestion system. After digestion, the digestates were diluted into 50 ml milli-Q water and the samples were analyzed by ICP-OES. The programs of the microwave digestion and the

operation system and the limitation of the detection were listed in **Supplementary Document**.

Sample Preparation for Bioassay and ^{1}H-NMR

The methanol content of the supernatant was evaporated at 60°C dry heat plate under a fume hood for 1.5 h and freeze-dried. All extracts were stored in −80°C freezers and defrosted as needed.

Maintenance of RAW 264.7 Cell Line

The RAW 264.7 cell line, a macrophage transformed from Abelson murine leukemia virus, was cultured in D-MEM supplemented with 10% heat-inactivated FBS (Hyclone, Utah, Logan, United States), 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.

Cytotoxicity in RAW 264.7 Cells

A variant of the MTT assay developed from Funk et al. (1993) was used for evaluating cytotoxicity. It aimed at understanding the biological effect of extract to macrophage cells. 200 μL of RAW 264.7 was seeded at a density of 3×10^4 cells/μl in 96-well plate. The cells were incubated at 37°C and 5% CO_2 supplied. After 24 h, the cells were treated with the sample at the concentrations of 100, 50, and 25 μg/ml, as well as the blank and the negative control for 24 h. All treatments were adjusted to the final concentration of 0.05% DMSO. After treatment, the cells were incubated with 100 μL 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 h. After the removal of the MTT solution, 100 μL of DMSO was added into each well, and the plate was shaken horizontally for 1 min. Cell viability was measured at 570 nm using a microplate reader.

Inhibition of LPS Induced NO Production

The Griess assay (Jin et al., 2009) aimed at understanding the anti-inflammatory activity of different Danshen products was used. 200 μL of RAW 264.7 was seeded at the density of 2.5×10^5 cells/μl in the 96-well plate. The cells were incubated at 37°C and 5% CO_2. After 24 h, the cells were treated with the sample at the concentrations of 100, 50, and 25 μg/ml, as well as the blank, negative control and positive control which was 20 μL 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 the LPS for 24 h. Griess solution A and B (solution A: 4% sulfanilamide in 10% phosphoric acid; solution B: 0.4% N-1-naphthylethelene) were freshly prepared within an hour before the next step. 100 μL of cell culture medium was mixed with 25 μL of solution B, and 25 μL of solution A. 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 μM $NaNO_2$ 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 min after. The absorbance was measured at 550 nm wavelength in the microplate reader. The calculation of nitrate/nitrite concentration was calculated as follows:

$$\text{Nitrate/nitrite concentration} = \frac{[\text{Ab} - \text{Ab}_{\text{blank}}]}{\text{Slope of NaNO}_2\text{ standard curve}},$$

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

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

Nuclear Magnetic Resonance and Metabolomic Profiling

The method for NMR analysis was developed from Li et al. (2015). This experiment aimed at understanding the chemical variation of market samples and differentiate the samples using multivariate analysis. 275 μL 200 mg/ml of each sample dissolved in $DMSO_{d6}$ was mixed with 275 μL of 0.2% DSS dissolved in $DMSO_{d6}$. All the samples were analyzed by 500 MHz ^{1}H-NMR spectrometry with 256 scans which required 20 min 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 μsec and relaxation delay (RD) = 1.0 s. 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 optimized 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 normalized 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 data were analyzed by the multivariate analysis software, SIMCA.

Multivariate Analysis (PCA)

All data, such as product information, NMR binned spectra, cytotoxicity MTT 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 were 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 categorizing samples with a similar pattern.

RESULTS AND DISCUSSION

In order to understand the quality issues, we used a range of established and non-standard techniques. As a baseline analysis, we looked at the heavy metal levels and the content of biomarker using HPTLC as described in the Chinese Pharmacopoeia. HPTLC and heavy metal analysis are the typical quality evaluation of herbal medicine. However, the content of heavy metals does not have therapeutic effects, and HPTLC does not provide a full spectrum of chemical composition. Hence, in addition, two more broad-spectrum methods ^{1}H-NMR profiling and an *in vitro* test were employed. It will help to understand the suitability of identifying quality marker using these models.

The Difference Between Samples Collected From Different Channels

Seventy-one samples were studied and information on geographic origin, additives and processing method was collected where possible. The samples from Chinese online stores have most of the product information listed on the website, but the physical store retailers can only provide a general geographical origin of their sample. It implies that physical retailers do not pay adequate attention to product information, and it shows low traceability of Danshen raw materials.

Of the fifteen samples sourced in Vietnam, four samples could not be traced to a geographical origin due to the lack of information provided by the retailer. Another four samples were cultivated in Vietnam while seven samples were imported from China. According to the field observation on the Vietnamese herbal market and the communication with the retailers, they weekly sundry their Danshen dried roots. This processing of Danshen materials is not often seen in other countries.

Chinese online store samples represent typical processing and storage method in China. They are generally dried in bulk size, and the retailers stored the materials under cool and dry condition. Authenticated samples are processed in a standardized operation. The roots were harvested carefully to avoid harming the materials and dried immediately after harvest. The dried materials are kept in a sealed, cool and dry condition.

Heavy Metal Levels in Danshen Derived Products

In the heavy metal analysis (**Figure 1**), none of the samples exceeds the limits of As, Cu or Pb as set in any pharmacopoeia. All samples are below the limits for arsenic based on the ChP or other pharmacopoeias (less than 2 mg/kg). Only six samples from authenticated, Vietnamese and individual samples obtain detectable arsenic content and most of them are lower than 0.2 mg/kg. The highest As content is from E17 (a concentrated extract), which has 1.33 mg/kg. The lowest limit of lead among the pharmacopoeia is 5 mg/kg, and all samples are under 1.5 mg/kg (**Figure 1**).

The sample with the highest copper content (C11 with 19 mg/kg) is just under the 20 mg/kg limit of pharmacopoeias. The average copper content of the raw materials and the powdered material samples (9.64 mg/kg) are at least eight times higher than of the concentrated extracts (1.12 mg/kg) (**Figure 1**). One of the possible reasons is that the industrial extraction process of commercial *S. miltiorrhiza* extract would not dissolve all the copper in the roots of Danshen, but the heavy metal analysis extraction completely digests the materials. As the concentrated extracts are secondary products, the content of copper will be lower than raw materials. However, it needs further investigation to conclude.

However, six out of sixty samples exceed cadmium limits according to ChP or USP, including four commercial raw materials and two concentrated extracts (**Figure 1**). None of these samples is sourced from Vietnam or authenticated samples, and most of these show undetectable levels. Our result also shows that the more processing of the product has, the higher possibility of heavy metal contamination, especially in arsenic and cadmium.

To summarize, the results from ICP-OSE reflect that *S. miltiorrhiza* derived products do not have a severe excess of heavy metals. It is generally safe but with the occasional exception of cadmium. It also shows that the chance of heavy metal contamination increases with the increase of processing procedure. The study from Yan et al., 2012 shows a similar result in Cd level, but it also stated the excessive Cu in *S. miltiorrhiza* should be common. Furthermore, the study shows that heavy metal content does not have a strong relationship with its soil quality. However, not many heavy metal studies in *S. miltiorrhiza* products regarding the relationship between cultivation and processing methods have been carried out.

Chemical Composition Variation of Danshen Products on the Market

To understand the variation of quality in terms of chemical composition in Danshen products, HPTLC and ^{1}H-NMR-PCA were used. HPTLC is a typical analytical method in pharmacopoeias that is quick, easily accessed, and reasonably priced. However, as it is a chromatographic technique, the result depends on the combination of the stationary phase and the mobile phase. Hence it is not easy to obtain the whole spectrum of metabolites in the samples, for instance, polysaccharides.

In the result of HPTLC (**Figure 2**), the authenticated samples have a higher level of major secondary metabolites of *S. miltiorrhiza*, for instance, salvianolic acid A, B, rosmarinic acid, caffeic acid, tanshinone IIA, cryptotanshinone and dihydrotanshinone. The samples of A8 to A12 come from the same source in Taiwan but are dried using different processing methods. The contents of salvianolic acids in these samples are similar, but A9, A10, and A12 have recognizably higher levels of tanshinone IIA and crytotanshinone in HPTLC. Compared to

A11, they were dried at low temperature after cutting. A8 was dried at high temperature to imitate the farmers' practice during the bad weather. It indicates that the degradation of secondary metabolites in *S. miltiorrhiza* relates to water retention time, drying temperature and cutting.

In addition, *S. bowleyana* and *S. przewalskii* are both distinct from authenticated *S. miltiorrhiza* in regard to the ratio of metabolites. *S. bowleyana* (A16) has very similar hydrophilic metabolites such as caffeic acid, salvianolic acid B and rosmarinic acid, but the contents of tanshinones are lower than authenticated *S. miltiorrhiza* samples in general. On the other hand, *S. przewalskii* (A6 and A17) has more tanshinones, including tanshinone IIA cryptotanshinone and perhaps other tanshinones compared to *S. miltiorrhiza* samples. The contents of salvianolic acid B and rosmarinic acid are merely undetectable, however, one of the *S. przewalskii* samples (A6) has an exceptionally high level of caffeic acid. This result matches with the results from other studies on *S. bowleyana* and *S. przewalskii* (Huang et al., 1980b; Li et al., 1993).

Vietnamese samples also show high levels of salvianolic acid B and slightly lower levels of caffeic acid and rosmarinic acid (**Figure 2**). However, the tanshinone levels in Vietnamese samples are significantly lower than the authenticated samples. The chemical compositions of Vietnamese samples are relatively consistent. These samples were sourced from a farm in Northern Vietnam and Chinese supplier(s), which include at least two different sources, this consistent result of HPTLC may be due to a similar supply chain, including processing and storage.

The HPTLC results of Chinese online store samples show that the qualities for these commercial products are highly diverse, and there is a chance of adulteration in particular in the case of C11 (**Figure 2**). C11 has a high level of caffeic acid and some prominent bands on tanshinone IIA and other tanshinones, but it has a low level of salvianolic acid B and rosmarinic acid. This pattern is similar to A17 and A6, which are authenticated *S. przewalski* Maxim. C12 and C14 also have a low level of salvianolic acid B, which are possible to be poor quality or adulteration as well (**Figure 2**).

On the other hand, ^{1}H-NMR is a comprehensive chemical analysis, but less sensitive compared to HPTLC. It identifies and quantifies the shield of hydrogens which can be applied to most of the organic compounds. Hence, it helps to compensate for the disadvantages of HPTLC adding more information, such as structures, chemical class, amount of chemicals and more, of the metabolites in the samples.

The ^{1}H-NMR results are not quantified as the most of the $R^2 >$ 0.9 but not >0.997, resulting in a poor predictability in quantitative terms. The ^{1}H-NMR-PCA result shows that the best correlation with the chemical variation is the sales channels (**Figure 3**). The estimate of goodness of fit (R^2) and the estimate of goodness of prediction (Q^2) are 0.572 and 0.49, respectively. 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 (Broadhurst et al., 2006; Worley and Powers, 2013). **Figure 3A** demonstrates the samples' chemical similarity according to ^{1}H-NMR results. R^2 and Q^2 are expected to be smaller than other metabolomics models as the model has a high level of diversity in the sample collection. Hierarchical Clustering Analysis (HCA) is used to explore the classification of samples via the similarity of their chemical compositions and shown in **Figure 3B**.

All authenticated *S. miltiorrhiza* samples are classified in group 1 including samples cultivated in America (A1–A5), Germany (A13 and A14), Taiwan (A8–A12) and China (A7, A15, and A18). Apart from V1 and V8 (samples originated from China), the Vietnamese samples with unknown geographical origin (V4, V6, V14, and V15), imported from China (V5, V7, V10, V11, and V13) and cultivated in Vietnam (V2, V3, V9, and V12) are classified in group 2 as well as A16 (*S. bowleyana*), which indicates they have similar chemical composition. All the Chinese online store samples are categorized as group 3 with A6 and A17 (*S. przewalskii*) as well as V1 and V8. According to the HCA and PCA results in **Figure 3**, the authenticated *S. miltiorrhiza* samples from China are differentiated from Vietnamese samples imported from China or Chinese online store samples. Also, the authenticated *S. miltiorrhiza* samples from different geographical origins show high level of similarity in their chemical composition. Hence, the geographical origin does not show a direct relationship with the chemical composition in the PCA.

On the other hand, the HCA categorization highly matches with the sale channels and the processing and storage method of the samples. As mentioned, the samples group 1 are all authenticated *S. miltiorrhiza* samples and are processed and storage with a standard operation. The samples in group 2 are all from Vietnamese herbal market which the samples were sundried regularly. The samples in group 3 are either *S. przewalskii* or represent typical processing and storage method in the market. Hence, it is highly possible that there is a direct or indirect relationship between the chemical composition of Danshen samples and their processing and storage condition.

Figures 3C,D are the same model but show the VIP difference of component 1 and 2. VIP determines the contribution of plots, which are chemical shifts here, to the model. **Figure 3C** shows that principal component 1 differentiates samples by polysaccharides and **Figure 3D** shows that principal component 2 differentiates samples by chemical standards 1, 4, 6, and 9 which are salvianolic acid B, A, rosmarinic acid and caffeic acid respectively. All authenticated *S. miltiorrhiza* and Vietnamese samples are situated at the positive part of component 2, which has a high contribution from caffeic acid, salvianolic acid B and rosmarinic acid NMR binned region compared to Chinese online store samples.

While the PCA in **Figure 3** is a general categorization statistical analysis, and the PLS-DA in **Figures 4**, **5** further investigates the chemical difference among samples. **Figure 4** shows the chemical composition difference between Chinese online store samples and Vietnamese samples. The cumulative R^2X, cumulative R^2Y and Q^2Y of this PLS-DA are 0.652, 0.86, and 0.78, respectively, and the permutation test shows a huge distinction between the random and actual model. These show the model is valid and good in fitness and prediction. Only one sample (V7) is the outlier of this model which means this sample is independent, and no samples are similar

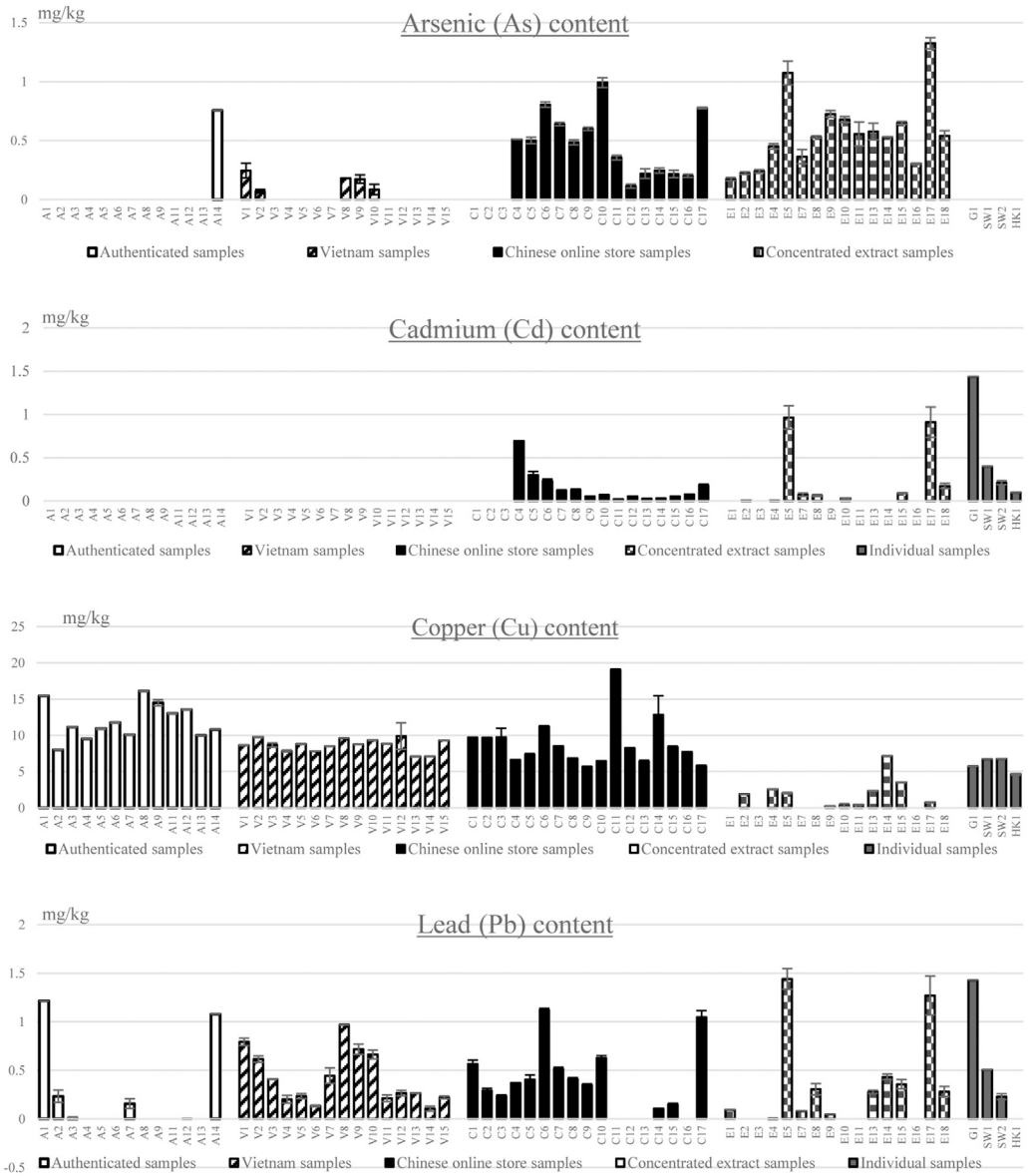

FIGURE 1 | Heavy metal analysis of authenticated samples (A1–A14), Vietnamese samples (V1–V15), Chinese online stores samples (C1–C17) and concentrated extracts (E1–E5, E7–E11, and E13–E18) as well as some individual samples (G1, SW1, SW2, and HK1).

to it. **Figure 4B** shows that the Chinese online store samples have a higher contribution from tanshinone IIA, cryptotanshinone, tanshinone I and dihydrotanshinone I regions (δ 1.30–1.27 ppm and δ 7.6–7.0 ppm). Vietnamese samples have a higher contribution from salvianolic acids region (δ 6.8–6.0 ppm) and the primary metabolite region (δ 3.1–2.5 ppm), and it is significant to the model.

Similarly, **Figure 5** shows the PLS-DA of the NMR results of authenticated samples against Vietnamese samples. The cumulative R^2X, cumulative R^2Y and Q^2Y are 0.699, 0.903, and 0.867 respectively, and the permutation test shows a considerable distinction between the random and actual model. No samples are the outlier according to the hotelling T2 range. The authenticated samples have a higher contribution from tanshinone IIA, cryptotanshinone, tanshinone I and dihydrotanshinone I regions (δ 1.30–1.27 ppm and δ 7.6–7.0 ppm). Vietnamese samples have a higher contribution from the salvianolic acids region (δ 6.8–6.0 ppm) and the primary metabolite region (δ 3.1–2.5 ppm). Also, the region of δ 3.1–2.5 ppm contributes the most to the model.

Overall, these PCA and PLS-DA models demonstrate that Vietnamese samples have higher levels of salvianolic acids than authenticated samples than Chinese online store samples. Authenticated samples have higher tanshinones than Chinese online store samples than Vietnamese samples. Vietnamese samples also have significantly stronger signals in the primary metabolite region (δ 3.1–2.5 ppm). The PCA model also shows that Vietnamese samples and authenticated samples differ from

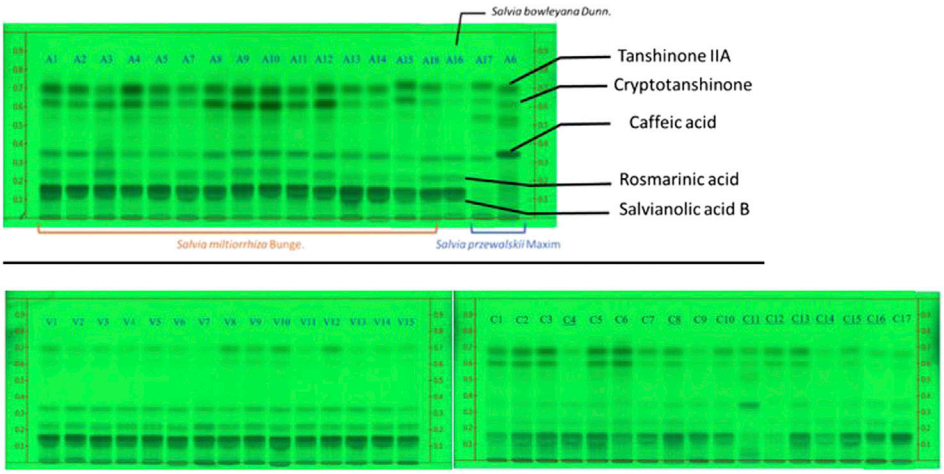

FIGURE 2 | HPTLC results of authenticated samples, Vietnamese samples and Chinese online store samples. The HPTLC is performed under 254 nm developed with solvent system (toluene: chloroform: ethyl acetate: methanol: formic acid (v/v) = 2: 3: 4: 0.2: 2). A1–A18 are authenticated samples including *S. miltiorrhiza*, *S. bowleyana*, and *S. przewalski*. V1–V15 are Vietnamese samples and C1–C17 are Chinese online store samples. The samples underlined were sold as grounded powder while others were dried roots.

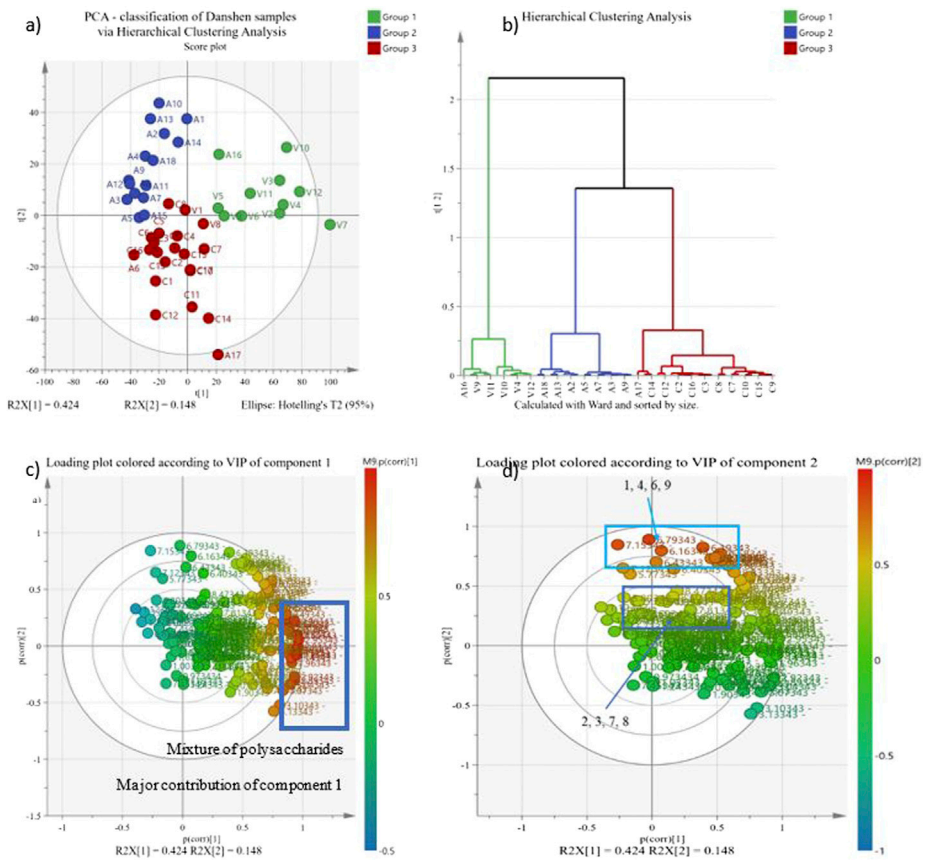

FIGURE 3 | PCA of all Danshen samples. The PCA-X corresponds to the binned regions of all ^{1}H-NMR spectra of Danshen samples as X variables. All X variables are scaled by Pareto scaling. In **(A)** PCA scatter score plot and **(B)** Hierarchical Clustering Analysis for PCA, Figure **(C)** and **(D)** are the loading plots of figure **(A)**. The colours of the loading plots are referring the p value of the X variable contribute to the principal component 1 in figure **(C)**, and the component 2 in figure **(D)**. A, authenticated samples; C, Chinese online store samples; V, Vietnamese samples

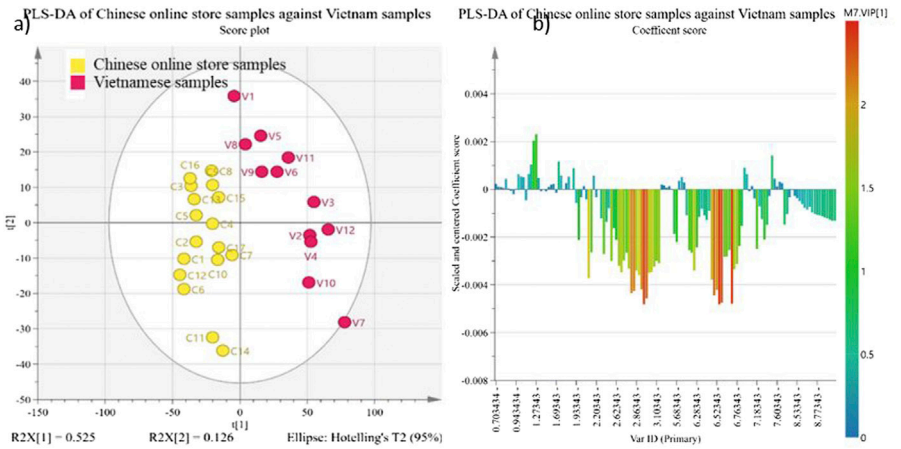

FIGURE 4 | PLS-DA of Chinese online store samples against Vietnamese samples. The PLS-DA corresponds to the binned regions of ^{1}H-NMR spectra of Chinese online store samples (C1–C17) and Vietnamese samples (V1–V12) as X variables and the value chains of samples as the Y variable. All X variables are scaled by Pareto scaling. The yellow colour in **(A)** PLS-DA scatter score plot represents the Chinese online store samples, and the pink represents Vietnamese samples. The colours of the samples in figure **(B)** loading plot are referring the VIP value of the X variable contribute to the principal component 1.

Chinese online store samples. It indicates that the processing method and preservation are more critical to the chemical composition of *S. miltiorrhiza* derived products than geographical origin.

Can a Bioassay Be Used to Assess the Quality of Danshen Preparations?

The MTT assay is widely used to understand the cytotoxicity and proliferative effects of samples. Here is employed to understand the variation in bioactivity safety, and it is a basis for conducting the Griess Assay. This LPS induced NO assay provides data on the anti-inflammatory activity of the samples, relevant since Danshen is used for pain-related and inflammatory vascular diseases. The activity of Danshen is closely related to NO signaling and NF-κβ pathway. These two assays aim to understand the bioactivity of different Danshen samples and whether it is possible to differentiate the quality of Danshen. **Supplementary Table S6** shows the samples with cytotoxic effects and proliferative effect to RAW 264.7. The cytotoxicity activities of all samples are attached in the **Supplementary Document**.

In the MTT assay, twenty-one out of fifty-six samples tested shows considerable differences to the control group after applying

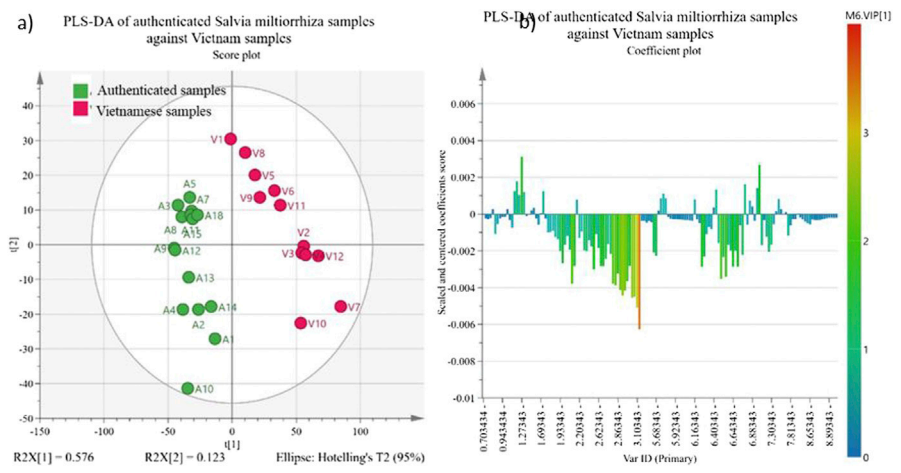

FIGURE 5 | PLS-DA of authenticated *S. miltiorrhiza* samples against Vietnamese samples. The PLS-DA corresponds to the binned regions of ^{1}H-NMR spectra of authenticated Salvia miltiorrhiza samples (A1–A18, except A6, A16, and A17) and Vietnamese samples (V1–V12) as X variables and the sales channel of samples as the Y variable. All X variables are scaled by Pareto scaling. The green colour in **(A)** PLS-DA scatter score plot and represents the authenticated Salvia miltiorrhiza samples and the pink represents Vietnamese samples. The colours of the samples in figure **(B)** loading plot are referring the VIP value of the X variable contribute to principal component 1.

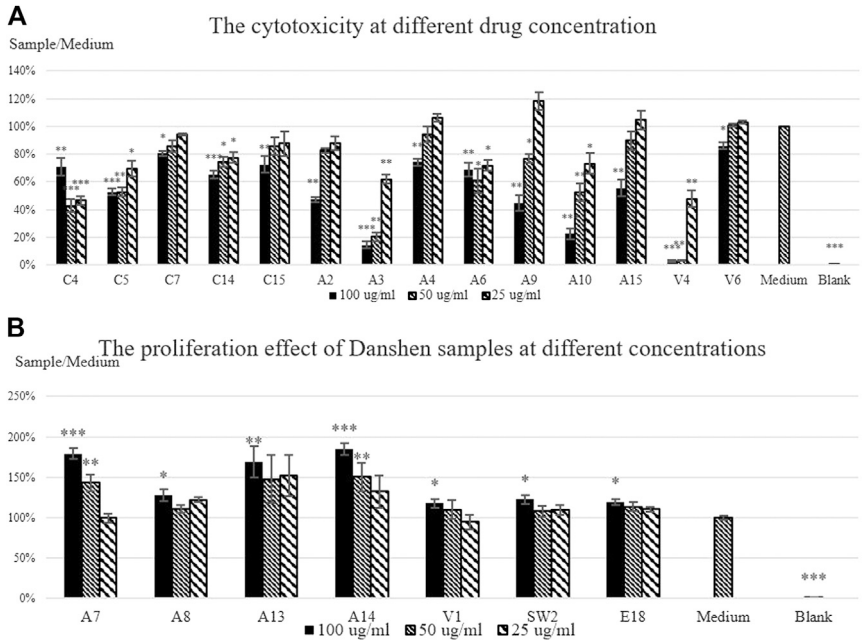

FIGURE 6 | The cell viability results of Danshen sample extracts in RAW 264.7. 200 μl of RAW 264.7 with the density of 3×10^4 cells/μl is treated with 100, 50, and 25 μg/ml of sample for 24 h. **(A)** the results of the samples showing the significant cytotoxic effect to RAW 264.7 **(B)** the results of the samples showing a proliferative effect in RAW 264.7. 0.05% DMSO was used as the control of the experiment. The cell viability percentage is 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.

Danshen sample extract at the initial concentration of 100 μg/ml. Fourteen samples show cytotoxic activity on the cells, including seven authenticated samples, five Chinese online store samples, and two Vietnamese samples. Apart from C4, the effects of these samples on the cells show a dose-response relationship. While A3 and A10 have the highest level of salvianolic acid B and tanshinone IIA, the cell viabilities of A3 and A10 drop to less than one fourth compared to the control. In general, there is a considerable variation in the level of activity of the different samples.

As mentioned, the samples of A8 to A12 come from the same source but were processed differently, showing the extreme differences among samples. A9 and A10 show the cytotoxicity, while A8 shows the increase of cell viability. Also, A8, which shows proliferation in cell viability, is dried in an oven at high temperature while A9 and A10,

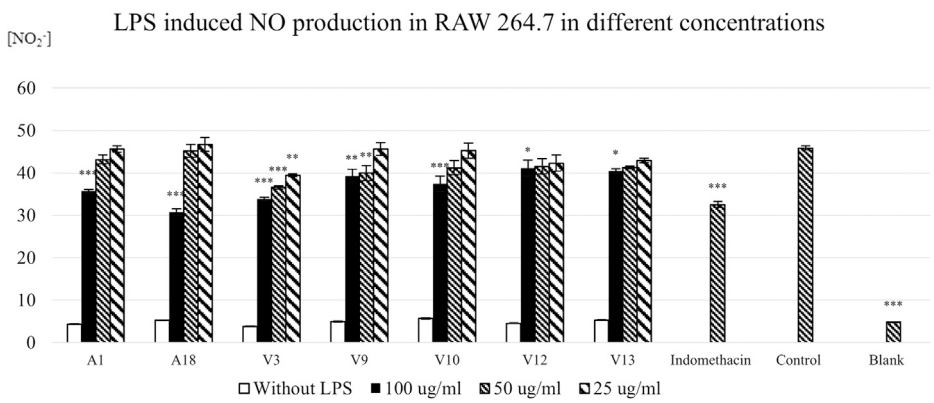

FIGURE 7 | The effect of Danshen extracts on LPS induced NO production in RAW 264.7. 200 μl of the RAW 264.7 cells with the density of 2.5×10^5 cells/μl is treated with 100, 50, and 25 μg/ml of sample for 24 h. 20 μg/ml of indomethacin is used as the positive control. The results of the samples that show significant inhibition on LPS induced NO production on the RAW 264.7. 0.05% DMSO was used as the control of the experiment. The NO concentration is 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.

which are dried in an oven at low temperature and freeze-dried, respectively, show cytotoxicity in RAW 264.7. As expected, different processing results in changes to the biological activity.

On the other hand, seven samples show increases of cell viability, including four authenticated samples, one Vietnamese sample, one Swiss sample, and one Danshen concentrated extract. Of these, A7, A13, and A14 show an increase of more than 50% compared to the control. A13 and A14 are cultivated on the same farm with the same method and processed in the same way but in a different year. The time difference here does not significantly change the biological activities of the product, but further study needs to be done, for instance, more systematic sample collection and recording weather changes and soil condition.

The high level of bioactivity variation in MTT assay shows the bioactivity of Danshen varies from sample to sample and no specific pattern or relationship is found in the samples' cytotoxicity or proliferation activity using PCA. Interestingly, Danshen samples on the market can lead both to cytotoxicity and proliferation on RAW 264.7 and all the samples show dose-response relationship expect C4, A8, and A13.

In the Griess assay, LPS triggers immunoresponse which increases the NO level; hence the model indicates the anti-inflammatory activity. Taken together differences in cell viability of RAW 264.7 after treatment with Danshen (**Figure 6**) in the LPS induced NO production (**Figure 7**; after exclusion of samples with cytotoxicity in RAW 264.7) demonstrate the variability in the biological effects of this set of Danshen samples. Within the non-cytotoxic Danshen samples, only seven samples, including two authenticated samples and five Vietnamese samples, show significant inhibitory dose-response dependent effects on the LPS induced NO production of RAW 264.7. Very few samples show anti-inflammatory effects in the Griess assay indicating that the chemical variation on the market significantly affects the biological activities of herbal products. V2, V9, and V12 are claimed to be cultivated in Vietnam, and two of them show NO inhibitions while all the Chinese online store samples do not inhibit NO signals. The anti-inflammatory activity of Vietnamese samples may also be linked to the storage practice of Danshen materials in the Vietnamese TCM herbal pharmacies.

Clearly, in the case of Danshen and the use of these bioassays, there is a correlation between processing and their bioactivities, but these assays are not useful to differentiate the quality and composition according to the current definition in the pharmacopoeia. Most likely, this is linked to the Danshen containing at least two classes of metabolites, which contribute to the activity and to the lack of comprehensive phytochemical profiling, with some minor compounds potentially having a substantial effect on these pharmacological targets. Nonetheless, in the standard of pharmacopoeia, a lot of samples are not identified as "poor quality" in chemical composition. Nevertheless, not many samples suggest anti-inflammatory activity and fourteen samples show a potential cytotoxicity to RAW 264.7 which may raise safety and quality issues. It indicates that the current chemical standards of *S. miltiorrhiza* in most of the pharmacopoeias are inadequate to assess biological activities of the product and to a certain extent, the quality as well.

CONCLUSION

This study focuses on understanding the correlation of the phytochemistry, biological activity as well as the processing and storage methods of Danshen (*S. miltiorrhiza* Bunge.). It is also the first interdisciplinary study to evaluate the possibility of using trace metals analysis, HPTLC, ^{1}H-NMR, with a combination of bioassays such as cytotoxicity and anti-inflammatory activity in a metabolomic approach. Clearly other quality parameters could have been included like the concentration of herbicides and pesticides, but this was not feasible within this project. This study also compares different authenticated samples from different geographical origins as well as marketed samples. It reveals a detailed picture of Danshen and its derivatives' quality.

This study demonstrates that the quality of Danshen varies among different sales channels. No major problems were identified with regards to heavy metal content, but the variability, especially of the processed material, remains a problem. The variation in heavy metal content and the chemical compositions are directly linked to the processing of the product but not the geographical origin. The bioassay approach selected clearly resulted in ambivalent results. As the levels of the current chemical standards of Danshen are not the primary factors of the cytotoxicity and anti-inflammatory effects, it implies that the current chemical standards in pharmacopoeia do not represent the entire picture of the anti-inflammatory activity in Danshen. A future option would be to explore other pharmacological *in vitro* assays especially ones associated with potential cardiovascular targets.

It is evident that the Danshen products on the market show huge variations in chemical composition and biological activities. The chemical compositions of the authenticated samples and Vietnamese samples from the early stages of a value chain are more consistent than Chinese online store samples. This is similar to previous findings from our group, e.g. on St. John's wort and roseroot (Booker et al., 2016; Scotti et al., 2019) where in general, the main chemical variability was shown to be due to differences in the value chains of the products. It shows that it is worth investigating the possibility of cultivating herbal materials globally combined with an appropriate and sustainable processing method, instead of cultivating in the "traditionally authenticated" herbal materials region. Further investigations using a metabolomics approach to understand the relationship between phytochemistry and biological activity are needed.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KK carried out the experiment and wrote the manuscript with the supervision of MH. RK and RL helped designing heavy metal analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.621169/full#supplementary-material.

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Precise Species Detection in Traditional Herbal Patent Medicine, Qingguo Wan, Using Shotgun Metabarcoding

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As one of the high-incidence diseases in the world, pharyngitis seriously affects the lives of those with the condition. Qingguo Wan is a herbal medicine used for treating pharyngitis, and its quality evaluation is currently only accomplished via traditional identification. However, precise identification becomes challenging with fake products on the market or fungal contamination during the production process. This study used the Illumina NovaSeq platform for targeting the ITS2, *psbA-trnH*, *matK*, and *rbcL* sequences to survey the species composition of lab-made and commercial samples. The results showed that a total of 34.56 Gb of raw data that was obtained represented more than 0.23 billion reads. After assembly, annotation, and operational taxonomic unit clustering, 103, 12, 10, and 12 OTUs were obtained, which belonged to the ITS2, *psbA-trnH*, *matK*, and *rbcL* sequences of the mock lab-made and commercial samples. The analytical results indicated that the sequences of all the prescription ingredients were successfully obtained in the two lab-made samples. The positive control medicinal *Panax quinquefolius* L. sequence was obtained in HSZY175, while *Scutellaria baicalensis* Georgi, *Lonicera japonica* Thunb. *Menispermum dauricum* DC. and *Paeonia lactiflora* Pall. were detected in the three commercial samples. The detection results of the other four herbs in different fragments were not all the same. In addition, a total of 28 fungi OTUs, representing 19 families and 20 genera, were obtained from both the commercial and mock lab-made samples. *Aspergillus, Cladosporium,* and *Penicillium* dominated among the 20 genera. This study demonstrated that the shotgun metabarcoding method is a powerful tool for the molecular identification of the biological ingredients in Qingguo Wan. It can be used to effectively supplement traditional methods while providing a new technique for the quality evaluation of Qingguo Wan.

Keywords: shotgun metabarcoding, Qingguo Wan, identification, pharyngitis, traditional patent medicine

INTRODUCTION

Pharyngitis is caused by the direct infection of the pharynx and is one of the most common diseases in the world. Continuous industrial development has severely affected the air quality, climate, and environment in recent years. Frequent hazy weather conditions result in an increase in the incidence of pharyngitis and the number of patients requiring treatment. In 2012, Zhang et al. (Hong et al., 2012) reported that about one-third of patients who routinely suffer from pharyngeal discomfort and visit otolaryngology clinics, were displaying chronic pharyngitis. An American medical survey has shown that the number of pharyngitis cases has reached tens of millions every year (Kalra et al., 2016). The inhalation of too much dust and harmful gases, or the frequent consumption of irritating food increases the probability of pharyngitis (Pearce et al., 2020). If the condition is serious, it affects not only the normal functioning of the respiratory system but may also impact the patient's quality of life. Therefore, it is essential to prevent potentially life-threatening complications. Qingguo Wan is a water honey pill commonly used as a traditional medicine preparation for the treatment of pharyngitis. Qinguo Wan is made from eight kinds of medicinal materials, namely Canarii Fructus (Qingguo), Lonicerae Japonicae Flos (Jinyinhua), Scutellariae Radix (Huangqin), Menispermi Rhizoma (Beidougen), Ophiopogonis Radix (Maidong), Scrophulariae Radix (Xuanshen), Paeoniae Radix Alba (Baishao), and Platycodonis Radix (Jiegeng). It eliminates pharyngeal pain, reducing swelling and relieving pain (Commission, 2020). Several studies have shown that the chemical ingredients in Qingguo Wan prescriptions exhibit excellent anti-inflammatory effects (Shen et al., 2003; Geng et al., 2011; Zhang, 2012; Jia et al., 2016; Nikzad-Langerodi et al., 2017). Of these, Canarii Fructus represents the primary curative ingredient in relieving throat pain, while displaying various pharmacological activities that include antibacterial and anti-inflammatory properties. Furthermore, it has an excellent therapeutic effect on acute pharyngitis (Geng et al., 2011; Jia et al., 2016). Baicalin is a compound obtained from Scutellariae Radix, denoting the main anti-inflammatory ingredient (Shen et al., 2003; Zhang, 2012). Furthermore, the Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) evaluation of the Lonicerae Japonicae Flos extract also showed comparatively good anti-inflammatory activity (Nikzad-Langerodi et al., 2017). The three medicinal herbs mentioned above are used for treating pharyngitis to achieve an optimal therapeutic effect. The remaining five medicinal herbs can increase the efficacy according to the monarch and the minister of traditional Chinese medicine.

Traditional methods, including microscopic identification, thin layer chromatography (TLC) identification, and high-performance liquid chromatography (HPLC) identification have been adopted to detect the seven medicinal ingredients in Qingguo Wan according to the Chinese Pharmacopoeia (Commission, 2020), while the method for identifying Platycodonis Radix has not been described. The microscopic identification method was applied to identify Canarii Fructus, Lonicerae Japonicae Flos, Scutellariae Radix, Scrophulariae Radix, and Paeoniae Radix Alba. TLC was used to identify chlorogenic acid (CGA), paeoniflorin, baicalin, Menispermi Rhizoma, Ophiopogonis Radix, and Scrophulariae Radix. Moreover, the baicalin (Yicun, 2014; Commission, 2020), paeoniflorin (Lei and Futang, 2004), gallic acid (Huowang et al., 2016), and CGA (Yuexin, 2013) content were determined using HPLC technology. Although the above mentioned methods can provide valuable information for the quality control of Qingguo Wan, it is difficult to identify these ingredients through a universal approach and many taxa are difficult to be discriminated among closely related species even if their chemical content tests were qualified (Zhang et al., 2020). Furthermore, most of the traditional methods are depending on human expertize, time-consuming, and they are not appropriate for large-scale computer processing due to the lack of a standardized database (Chen et al., 2014). To overcome such limitations, several methods depending on DNA markers and high throughput sequencing technology have been proposed for evaluation (Lo and Shaw, 2019). In 2018, Xin et al. used HPLC and TLC to identify the active ingredients in Longdan Xiegan Wan (Xin et al., 2018a), and found that the detection results were meeting the Chinese Pharmacopoeia standards. However, the molecular identification technology detected the substitution of Akebiae Caulis (Mutong), Alismatis Rhizoma (Zexie), and the non-prescribed species *Bupleurum marginatum* Wall. ex DC. in the commercial samples. Furthermore, fungi belonging to *Aspergillus* were also revealed by molecular identification technology. The similar results have been found that even though TLC and HPLC tests verified that the products adhered to the existing quality standards, a study by Yufeng Ningxin indicated that substitution with *Pueraria montana* var. *thomsonii* (Benth.) M.R.Almeida and fungal contamination with *Aspergillus* were detected in three herbal products via further DNA metabarcoding (Zhang et al., 2020). An integrated method including macro- and microscopic, chemical and genetic authentication strategies was used to differentiate *Cyanthillium cinereum* from its adulterant *Emilia sonchifolia* (Thongkhao et al., 2020). In addition, some medicinal materials have similar chemical components, and new adulteration based on this phenomenon was found. Han et al. (Zitong et al., 2017) found that the herbal material, Lonicerae Japonicae Flos, was artificially adulterated by Eucommiae Folium in 7% of tested Chinese patent medicines since both Lonicerae Japonicae Flos and Eucommiae Folium contained CGA. It can be seen from the above research that the quality of traditional medicinal materials has always been an issue of global concern. A comprehensive and systematic method is required to accurately identify the original sources of all medicinal materials to ensure the safety and efficacy of clinical medicines.

With the development of high-throughput sequencing technology, the shotgun sequencing approach is a molecular technology mainly used in microbiology. It can be used to analyze mixed samples by randomly breaking genomic DNA, constructing libraries, and non-target sequencing (Quince et al., 2017). Xie et al. (Xie et al., 2013) used shotgun sequencing to study the microbial community structure in different environments during the fermentation process of Shaoxing

rice wine, and showed that the community structure and gene function composition changed significantly at different time points. Recent studies by Yang et al. (Xiang et al., 2016) and Arıkan et al. (Arıkan et al., 2020) successfully used this technique to detect changes in the microbial communities in food or beverages. In addition, shotgun sequencing is also widely used in the study of human microbial communities. A randomized clinical study of diabetic patients showed that dietary fiber can specifically increase the beneficial intestinal flora in the human intestine, improving the clinical symptoms of type 2 diabetes while using this technology (Zhao et al., 2018). A study on the influence of American immigration on human intestinal microbiota found that immigration from non-Western countries to Western countries shifted the individual's microbiome to a more westernized state by using this technology (Vangay et al., 2018). Oh et al. (Oh et al., 2014) successfully applied this technology to the study of human skin microbiota. These studies have shown the feasibility of shotgun technology in identifying complex mixed samples. Xin et al. (Xin et al., 2018a) first used this technology in 2018 by obtaining a small amount of DNA from a sample and analyzing multiple fragments to identify the species in the traditional patent medicine, Longdan Xiegan Wan. The successful application of this technology provides new methods and avenues for identifying the biological ingredients of traditional patent medicine. In 2021, a new approaches combining DNA barcoding and shotgun sequencing was employed for the species identification of Wuhu San and the results showed that this method was potential to effectively complement traditional identification methods (Liu et al., 2021).

In this study, the biological ingredients of Qingguo Wan are identified using shotgun metabarcoding technology. The feasibility of the method is examined using lab-made samples, while the applicability of the technique used for identifying traditional patent medicines is verified with commercial samples, providing a new method for evaluating the quality of Qingguo Wan.

MATERIALS AND METHODS

Sample Collection

Eight medicinal materials including Canarii Fructus (Qingguo, HSYC2064), Lonicerae Japonicae Flos (Jinyinhua, HSYC2058), Scutellariae Radix (Huangqin, HSYC2017), Menispermi Rhizoma (Beidougen, HSYC2053), Ophiopogonis Radix (Maidong, HSYC2047), Scrophulariae Radix (Xuanshen, HSYC2057), Paeoniae Radix Alba (Baishao, HSYC2036), and Platycodonis Radix (Jiegeng, HSYC2048) were collected from Tongrentang Pharmacy and were morphologically authenticated according to the floras of China and the Chinese Pharmacopoeia (Commission, 2020). And they were deposited in the Institute of Medicinal Plant Development herbarium (herbarium code "IMD", NYBG: https://www.nybg.org/). The eight medicinal materials are shown in **Figure 1**.

Based on the prescription described in the Chinese Pharmacopoeia, two mock samples of Qingguo Wan were prepared as follows: 1) Eight herbal materials were crushed into powder. 2) The powder was sieved and evenly mixed at the dosage and ratio described in **Table 1**, and this sample was marked as HSZY163. 3) The Panacis Quinquefolii Radix powder was added to the mixed powder prepared in step 2 as a positive control with the same amount of Qingguo, and this sample was marked as HSZY175. 4) These two powder samples were mixed with honey and water, after which it was molded into pills.

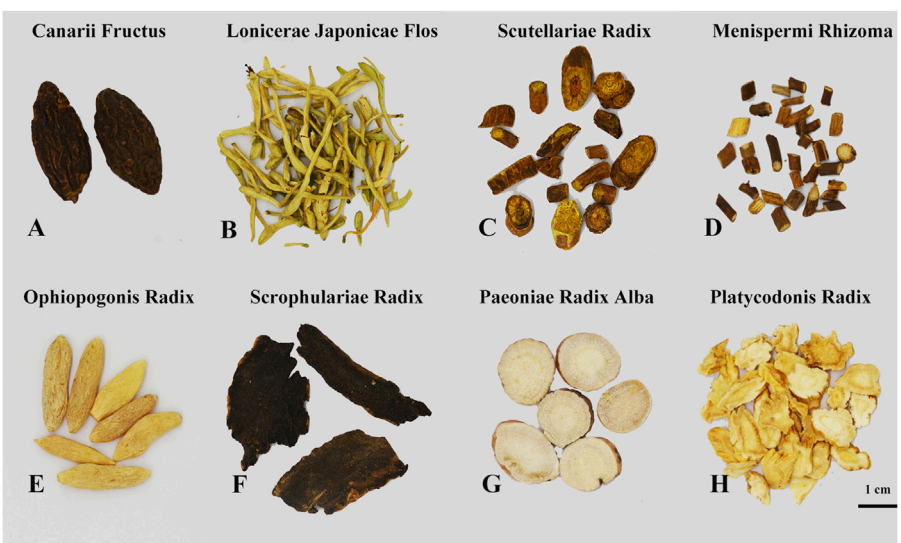

FIGURE 1 | The morphological characteristics of the eight herbal materials found in Qingguo Wan. **(A)** Canarii Fructus (Qingguo), **(B)** Lonicerae Japonicae Flos (Jinyinhua), **(C)** Scutellariae Radix (Huangqin), **(D)** Menispermi Rhizoma (Beidougen), **(E)** Ophiopogonis Radix (Maidong), **(F)** Scrophulariae Radix (Xuanshen), **(G)** Paeoniae Radix Alba (Baishao), and **(H)** Platycodonis Radix (Jiegeng).

TABLE 1 | The proportion and dosage of herbal materials listed in the prescription of lab-made and commercial Qingguo Wan according to the Chinese Pharmacopoeia.

Medicinal herbs	Original species	Dosage(g)	Proportion (%)
Canarii Fructus (Qingguo)	*Canarium album* (Lour.) DC.	100	12.5
Lonicerae Japonicae Flos (Jinyinhua)	*Lonicera japonica* Thunb.	100	12.5
Scutellariae Radix (Huangqin)	*Scutellaria baicalensis* Georgi.	100	12.5
Menispermi Rhizoma (Beidougen)	*Menispermum dauricum* DC.	100	12.5
Ophiopogonis Radix (Maidong)	*Ophiopogon japonicus* (Thunb.) Ker Gawl.	100	12.5
Scrophulariae Radix (Xuanshen)	*Scrophularia ningpoensis* Hemsl.	100	12.5
Paeoniae Radix Alba (Baishao)	*Paeonia lactiflora* Pall.	100	12.5
Platycodonis Radix (Jiegeng)	*Platycodon grandiflorus* (Jacq.) A.DC.	100	12.5

In addition, three commercial Qingguo Wan samples were obtained from Tongrentang herbal store and designated as A19 (Lot no. 16035178), HSZY146 (Lot no. 18499001), and HSZY150 (Lot no. 16035178) to examine the applicability of the method in commercial samples.

DNA Extraction, High-Throughput Sequencing, and Data Analysis

The meta-genomic DNA of traditional herbal patent medicine was extracted according to the previously published protocols of the CTAB-based method (Chunsong et al., 2015) with some changes in the pretreatment steps described by Xin et al. (Xin et al., 2018a). The meta-genomic DNA was tested for integrity via gel electrophoresis and quantified using the NanoDrop ONE ultra-micro spectrophotometer (Thermo Fisher Scientific Inc. USA). Finally, the meta-genomic DNA was sheared into fragments and sequenced using the Illumina NovaSeq platform.

Total DNA was sequenced using the Illumina NovaSeq platform via the shotgun metabarcoding approach. The sequencing adapter and low-quality reads were filtered using Trimmonmatic v0.38 (Bolger et al., 2014). The paired-end reads belonging to ITS2, *psbA-trnH*, *matK,* and *rbcL* were enriched using the local python scripts described by Shi et al. (Shi et al., 2019). The enriched reads belonging to the above four DNA barcoding regions were assembled using MEGAHIT v1.2.9 (Li et al., 2015) and MetaSPAdes v3.13.2 (Nurk et al., 2017) with a value range of k-mer 31–127. The contigs obtained with the two types of software were merged, and duplicates were removed with cd-hit (Li and Godzik, 2006) at 100% identity. The traditional DNA barcoding region of *psbA-trnH*, *matK,* and *rbcL* was acquired by removing the primer sequences using Cutadapt v2.10 (Martin, 2011). The ITS2 regions were determined using a hidden Markov model (HMM)-based annotation methods (Keller et al., 2009). Chimera detection for annotated contigs was performed using UCHIME v4.2 (Edgar et al., 2011). The sequences belonging to each marker were clustered into OTUs at 99% identity using Usearch v11 (https://www.drive5.com/usearch/), and the representative sequences of each OTU were selected for further analysis. The shotgun paired-end reads were mapped to the OTU representative sequences using bowtie2 v2.4.1 (Langmead and Salzberg, 2012), while the sequencing depth and coverage values were calculated using samtools v1.10 (Heng et al., 2009). Poor quality OTUs were removed when its representative sequences displayed a sequencing depth ≤3 and/or coverage ≤95%. The remaining high-quality OTUs were used for species assignment by searching the barcode of traditional Chinese herbal medicine data system (TCM-BOL) (Shilin et al., 2014), the barcode of life data system (BOLD) (Ratnasingham and Hebert, 2007), and GenBank (Clark et al., 2016) databases using the basic local alignment search tool (BLAST) (Camacho et al., 2009). Finally, MEGAN v6.18.9 (Huson et al., 2007) was used for statistics and the taxonomic visualization of the species composition of the traditional herbal patent medicine.

RESULTS

High-Throughput Sequencing and Shotgun Metabarcoding Data Assembly

The total DNA of lab-made and commercial samples were sequenced according to a shotgun metabarcoding strategy, and a total of 34.56 Gb of raw data, including more than 230 million paired-end reads, were obtained, which was an average of 6.91 Gb of raw data for each sample. One of the lab-made samples, HSZY163, displayed the most significant amount of sequencing data at 10.81 Gb, while the commercial sample, HSZY150, exhibited the smallest amount of sequencing data at 3.68 Gb. For the five samples, there were 145,688, 2,025,476, 36,135, and 72,051 paired-end reads that were enriched for ITS2, *psbA-trnH, matK,* and *rbcL*, respectively. HSZY175 had the most abundant ITS2 sequencing reads, while HSZY163 had the most paired-end reads belonging to the three chloroplast markers (**Table 2**).

A total of 4,607 contigs were assembled using MEGAHIT, while 6,450 contigs were assembled using MetaSPAdes. All contigs obtained by the two types of software were combined, and a total of 7,694 unique contigs were acquired after removing redundant sequences. Furthermore, 318 OTUs of traditional ITS2, *psbA-trnH, matK,* and *rbcL* DNA barcoding regions were obtained after removing primers and annotations. A total of 137 OTUs were generated after clustering at a 99% similarity level, of which 103 OTUs belonged to ITS2. The number of OTUs produced by the nuclear ITS2 was approximately three times that of chloroplast *psbA-trnH, matK,* and *rbcL* (**Table 3**). The number of specific and shared prescription ingredients in Qingguo Wan identified based on ITS2, *psbA-trnH*, *matK,* and *rbcL,* is shown in **Figure 2** and **Supplementary Tables S1, S2**.

TABLE 2 | A summary of the high-throughput sequencing data and the number of reads enriched for ITS2, *psbA-trnH*, *matK*, and *rbcL*.

Sample id	Bases (Gb)	Total reads	The number of enriched reads for each DNA barcode				
			ITS2	*psbA-trnH*	*matK*	*rbcL*	Total
A19	5.9	19,683,174	26,961	292,081	3,859	7,954	330,855
HSZY146	6.19	20,634,727	26,023	401,180	6,494	11,711	445,408
HSZY150	3.68	12,277,349	18,273	281,522	3,782	8,714	312,291
HSZY163	10.81	36,046,154	29,459	546,745	14,280	26,052	616,536
HSZY175	7.98	26,615,513	44,972	503,948	7,720	17,620	574,260

TABLE 3 | The number of contigs of four DNA barcodes in the Qingguo Wan samples.

Parameter	ITS2	*psbA-trnH*	*matK*	*rbcL*
Number of unique contig	417	7102	58	117
Number of DNA barcodes after annotation and chimera detection	196	47	33	42
Number of OTUs	103	12	10	12
Average length (bp)	197.6	477.5	863.1	703
GC%	58.6	29.8	34.5	42.9

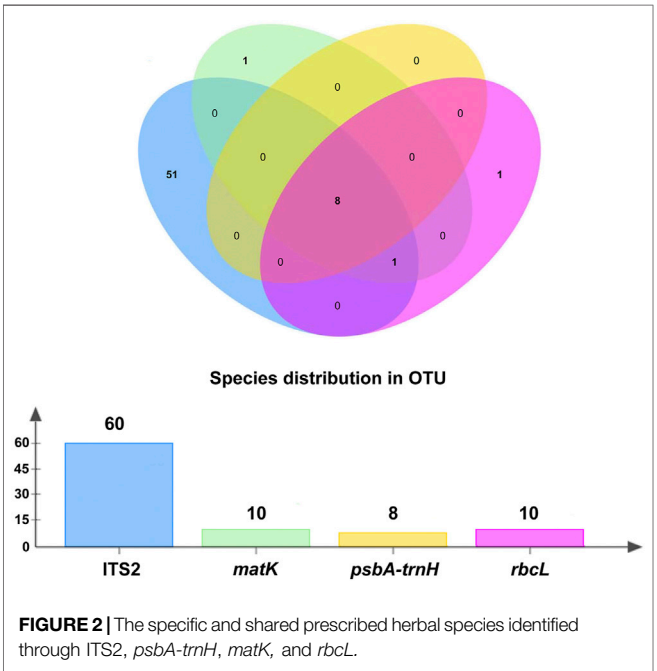

FIGURE 2 | The specific and shared prescribed herbal species identified through ITS2, *psbA-trnH*, *matK*, and *rbcL*.

The Species Detection Ability of the Shotgun Metabarcoding for Two Lab-Made Mock Samples

A total of 104 OTUs were obtained for the ITS2, *psbA-trnH*, *matK*, and *rbcL* regions from the two lab-made samples (HSZY163 and HSZY175). For the ITS2 region, there were 20 common OTUs in the two lab-made mock samples, which could be identified to the original species of the eight prescribed herbal ingredients including *Canarium album* (Lour.) DC., *Lonicera japonica* Thunb., *Scutellaria baicalensis* Georgi, *Menispermum dauricum* DC., *Ophiopogon japonicus* (Thunb.) Ker Gawl., *Scrophularia ningpoensis* Hemsl., *Paeonia lactiflora* Pall., and *Platycodon grandiflorus* (Jacq.) A. DC. In addition, it also included some common fungi species, which are described in detail below. For *psbA-trnH*, the same eight OTUs were obtained in both lab-made samples, and their sequences were consistent with the original species of the five ingredients labeled in the prescription. *Lonicera japonica*, *Scrophularia ningpoensis*, and *Paeonia lactiflora* were represented by two OTUs, but that of *Ophiopogon japonicus* was not obtained. Reassessment of the sequence data revealed that the *Ophiopogon japonicus* sequences were present in both samples, but partial sequences were assembled. For *matK*, the same eight OTUs were obtained in both lab-made samples, and their sequences were consistent with the original species of eight ingredients labeled in the prescription. For *rbcL*, the two lab-made samples displayed the same nine OTUs, which all belonged to the original species of the eight prescription ingredients, with *Scrophularia ningpoensis* represented by two OTUs. However, some differences remained between the two lab-made samples, HSZY163 and HSZY175. For example, the *psbA-trnH* OTUs of *Canarium album* and *Scutellaria baicalensis* were present in HSZY163 but could not be obtained in HSZY175. Finally, the ITS2, *psbA-trnH*, *matK*, and *rbcL* for the positive control *Panax quinquefolius* were successfully obtained from HSZY175 (**Table 4**).

The Species Composition of the Commercial Qingguo Wan Samples as Detected by Shotgun Metabarcoding

Three commercial samples were analyzed using the same method as with the lab-made mock samples. Except for *Platycodon grandiflorus*, which was not detected in sample A19, all the original species were detected in all three commercial samples according to the described ingredients for ITS2 and included *Canarium album*, *Lonicera japonica*, *Scutellaria baicalensis*,

TABLE 4 | The species detection of the ITS2, *psbA-trnH*, *matK,* and *rbcL* DNA barcoding regions obtained *via* shotgun metabarcoding.

Species	HSZY163				HSZY175			
	ITS2	*psbA-trnH*	*matK*	*rbcL*	ITS2	*psbA-trnH*	*matK*	*rbcL*
Canarium album	√	√	√	√	√	—	√	√
Lonicera japonica	√	√	√	√	√	√	√	√
Scutellaria baicalensis	√	√	√	√	√	—	√	√
Menispermum dauricum	√	√	√	√	√	√	√	√
Ophiopogon japonicus	√	√	√	√	√	√	√	√
Scrophularia ningpoensis	√	√	√	√	√	√	√	√
Paeonia lactiflora	√	√	√	√	√	√	√	√
Platycodon grandiflorus	√	√	√	√	√	√	√	√
Panax quinquefolius	/	/	/	/	√	√	√	√

Note: "√" indicates that the corresponding DNA barcode of this species was obtained, and "—" indicates that the corresponding DNA barcode of this species cannot be obtained, and "/" indicates that this herbal material was not added to the sample.

Menispermum dauricum, *Ophiopogon japonicus*, *Scrophularia ningpoensis,* and *Paeonia lactiflora*. Some plant species not listed in the prescription were also detected in the three commercial samples. For example, a total of 140 paired-end reads belonging to *Eleutherococcus sessiliflorus* (Rupr. and Maxim.) S.Y.Hu were detected in A19, accounting for 0.7% of the total paired-end reads of ITS2. One OTU with 71 mapping paired-end reads were detected in sample HSZY146 and was identified as *Elymus tsukushiensis* Honda. A total of 41 *Liriope muscari* (Decne.) L.H.Bailey paired-end reads were detected in the HSZY150 sample, while the sequencing depth was 15.78. The detailed taxonomical content of the three commercial samples detected for ITS2 is shown in **Figure 3** and **Supplementary Table S3**.

When *psbA-trnH* was used for species detection, *Lonicera japonica*, *Scutellaria baicalensis*, *Menispermum dauricum*, and *Paeonia lactiflora* were detected in the three commercial samples. *Canarium album*, *Scrophularia ningpoensis,* and *Platycodon grandiflorus* were detected in HSZY146 and HSZY150. *Ophiopogon japonicus* was not detected in any of the three commercial samples (**Figure 4** and **Supplementary Table S4**).

For *matK* and *rbcL*, *Canarium album*, *Lonicera japonica*, *Scutellaria baicalensis*, *Menispermum dauricum,* and *Paeonia lactiflora* were detected in the three commercial samples. *Platycodon grandiflorus* and Scrophulariaceae were detected in HSZY146 and HSZY150. *Elymus sibiricus* L. was detected in HSZY146, accounting for 1.7% of the total reads of *matK*. *Thinopyrum elongatum* (Host) D.R.Dewey can be detected in HSZY146, accounting for 1.4% of the total *rbcL* reads. *Ophiopogon japonicus* was not detected in A19 and HSZY150. *Scrophularia ningpoensis* and *Platycodon grandiflorus* were not detected in A19 (**Supplementary Figures S1, S2** and **Supplementary Tables S5, S6**).

The Fungal Contamination of the Lab-Made Mock and Commercial Samples Detected by ITS2

A total of 28 fungi OTUs, representing 19 families and 20 genera, were obtained from the two lab-made mock samples and three commercial samples. Taxonomical assignment demonstrated that Aspergillaceae, Cladosporiaceae, and Pleosporaceae represented the most abundant families in the Qingguo Wan samples. Further taxonomical assignment at the genus level indicated that *Aspergillus*, *Cladosporium,* and *Penicillium* were the predominant of the 20 genera. Fungi belonging to the *Aspergillus* and *Penicillium* genera were detected in all five samples. *Aspergillus* was the most abundant genera in A19, HSZY146, and HSZY150, while *Cladosporium* dominated in HSZY163 and HSZY175 (**Figure 5** and **Supplementary Table S7**).

DISCUSSION

The Feasibility of Shotgun Metabarcoding for Detecting Biological Ingredients in Qingguo Wan

In this study, the optimized DNA extraction method can be used successfully to obtain DNA that meets the standard requirements, facilitating effortless shotgun sequencing. The two lab-made and three commercial samples provided the DNA barcode sequences of eight ingredients, obtaining several assembled sequences, which indicated that the shotgun metabarcoding method could be used for species detection in Qingguo Wan. However, there are differences in the identification ability of each marker. In 2010, seven plant DNA barcodes (*psbA-trnH*, *matK*, *rbcL*, *rpoC1*, *ycf5*, ITS2, and ITS) were deeply compared (Chen et al., 2010), the data indicated that ITS2 presented the most suitable region for DNA barcode applications. ITS2, *psbA-trnH,* and *matK* were used to study Longdan Xiegan Wan (Xin et al., 2018a). The results showed that the ITS2 locus exhibited higher identification efficiency, while the *psbA-trnH* region was less effective. In a study to explore DNA barcodes suitable for the identification of Apiaceae, the ITS/ITS2+*psbA-trnH* combination was found to hold considerable potential value (Liu et al., 2014). According to the research mentioned above, ITS2 displayed the strongest distinguishing ability, followed by *psbA-trnH*. And the *rbcL* and *matK* proposed by CBOL Plant

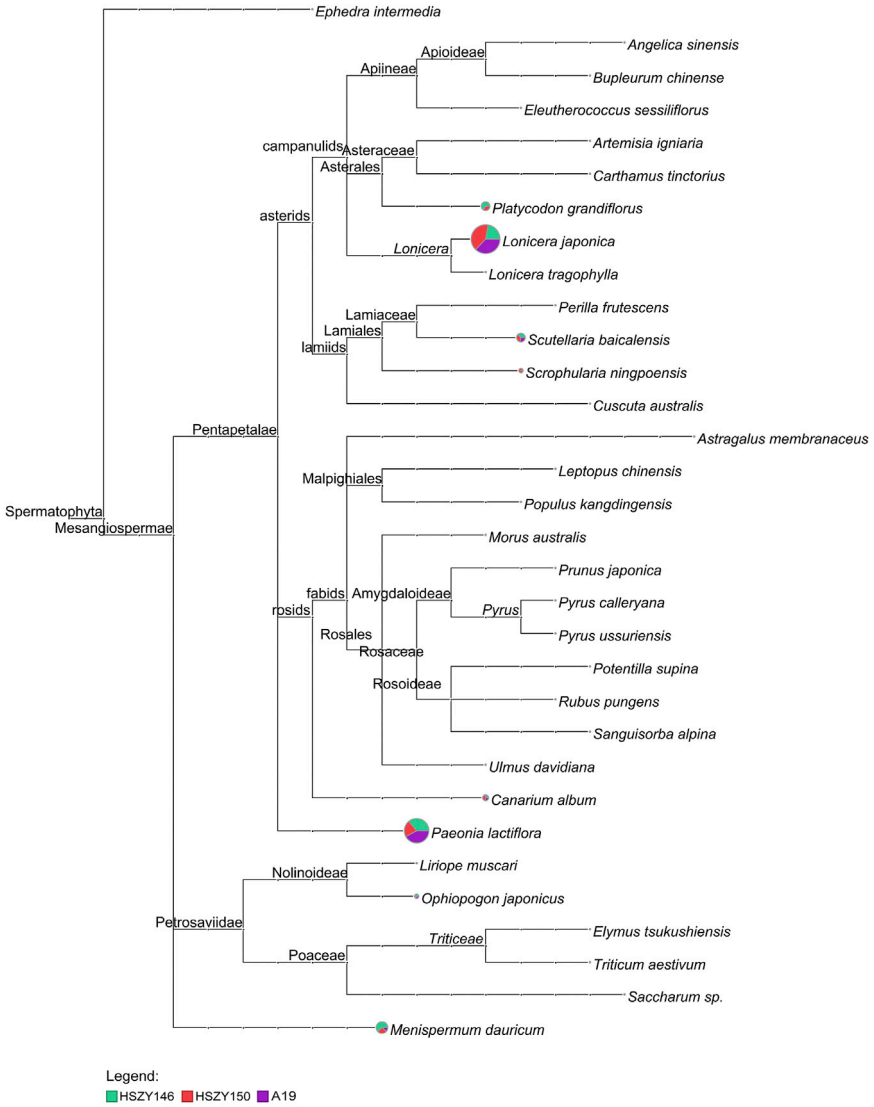

FIGURE 3 | MEGAN6 analysis result for the three samples detected by reads belonging to ITS2 against the NCBI-NT and TCM-BOL database. Each taxonomic node is drawn as a pie chart indicating the proportion of each species in the taxon for each sample.

Working Group should be adopted for the routine use of DNA barcoding (CBOL Plant Working Group, 2009). A recent study on the identification the TCM preparations, including Bazhen Yimu Wan, Da Huoluo Wan, Niuhuang Jiangya Wan, and Yougui Wan, used ITS2 and *trnL* as targets for herbal materials assessment. The results based on ITS2 showed a higher level of reliability than those of *trnL* at the species level, while the integration of both biomarkers provided higher sensitivity and reliability (Yao et al., 2020). Here, multiple barcode markers were used to improve the results obtained using a single marker for the shotgun metabarcoding analysis. Therefore, this study uses multi-barcode joint identification, which can compensate for the shortcomings of single barcode identification capability, and the results can be verified by different markers.

Some non-prescription ingredients and fungi were found in commercial samples based on ITS2 sequences. The possible reasons for this phenomenon are divided into the following categories: 1) A specific ingredient of another traditional herbal product was accidentally mixed into the Qingguo Wan due to a shared production line. For example, a few sequences belonging to the *Ephedra* genus was detected in the HSZY146 commercial sample. Further analysis showed that the prescription of a herbal product produced by the same company contained medicinal materials derived from the *Ephedra* genus, and accidental cross-contamination was found in a previous report (Xin et al., 2018b). 2) Fungal contamination was introduced during the processes of planting, pre-processing, storage, and transportation. The contamination with fungal species belonging to the genus *Aspergillus*, *Penicillium,* and *Cladosporium* are common in plants, which may produce mycotoxins

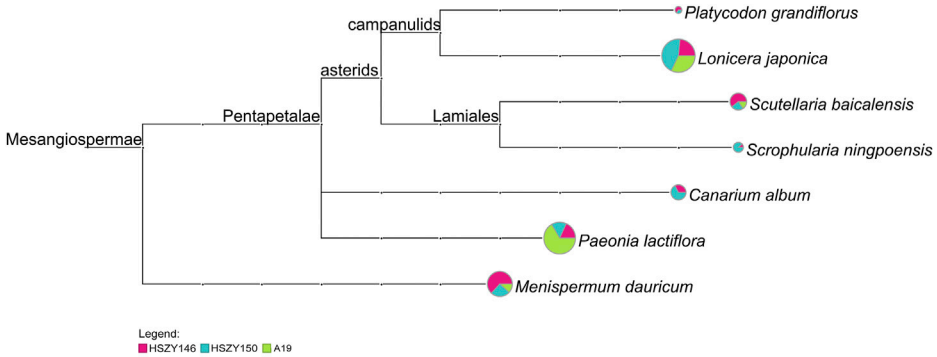

FIGURE 4 | MEGAN6 analysis result for the three samples detected by reads belonging to *psbA-trnH* against the NCBI-NT and TCM-BOL database. Each taxonomic node is drawn as a pie chart indicating the proportion of each species in the taxon for each sample.

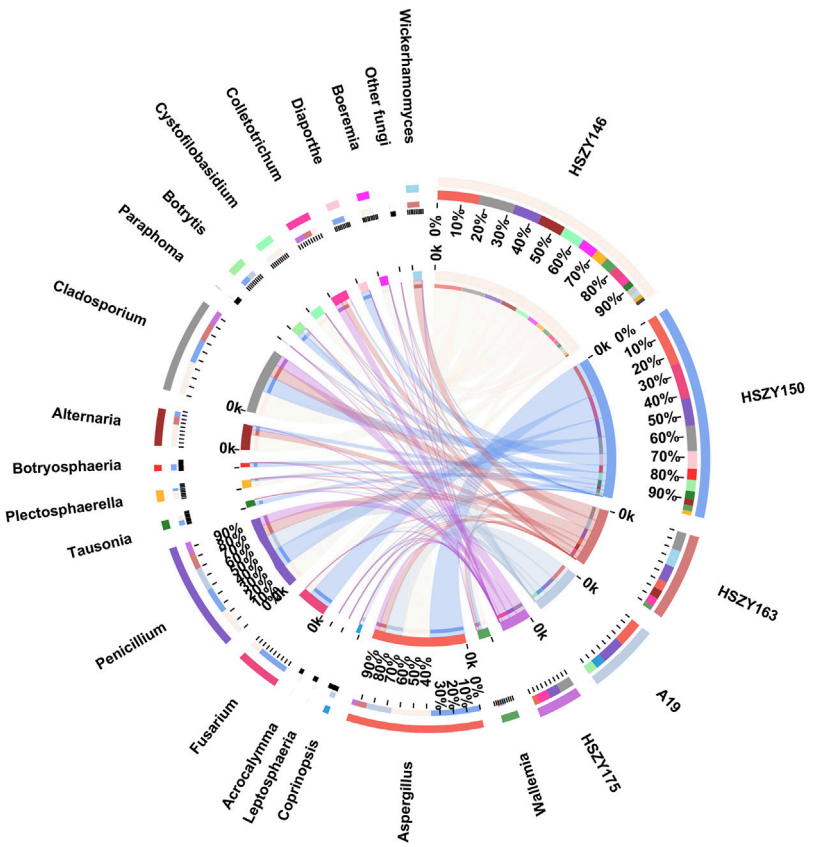

FIGURE 5 | Distribution of the fungi for each sample at the genus level. The data were visualized by Circos. The left half-circle indicates the distribution ratio of species in different samples at the genus level: the outer ribbon represents the species; the inner ribbon represents different groups, and the length represents the sample proportion of a particular genus. The right half-circle indicates the species composition in each sample: the color of the outer ribbon represents samples from different groups; the color of the inner ribbon represents the composition of different species in each sample, and the length of the ribbon represents the relative abundance of the corresponding species (Guo et al., 2020).

and have an impact on the efficacy and safety of traditional herbal products (Guo et al., 2020; He et al., 2020; Jiang et al., 2020).

Difficulties and Challenges Faced by Shotgun Metabarcoding Technology During the Biological Detection of Qingguo Wan

The successful application of shotgun sequencing technology is based on the extraction of high-quality DNA. Traditional medicinal materials contain many ingredients, such as polysaccharides, polyphenols, and various secondary metabolites (Jun et al., 2007; Fanglin and Shulian, 2010; Guanming et al., 2010; Anyu and Wenqin, 2015; Ruilian et al., 2019). In addition, different medicinal parts contain different amounts of DNA, while the types and quantities of chemical substances also vary. These factors affect the quality of the extracted DNA (Kun et al., 2012) and may present significant obstacles when using shotgun sequencing technology to authenticate the ingredients in traditional herbal patent medicine. In this study, high-quality DNA was obtained by optimizing the DNA extraction method of traditional patent medicine, Qingguo Wan (**Supplementary Table S8**). In addition, there are some variations in the sequencing dataset (e.g. 10.81 Gb for HSZY163 *vs.* 3.68 Gb for HSZY150) due to the problem of uneven mixing in the preparation of the PCR-free library, however, it did not have much impact on the detection results especially for the labeled ingredients as the results of the abundant copies of chloroplast and ITS2 sequences in the plant cells.

Several steps in the bioinformatics pipeline are critical during the process of sequence analysis. For example, correct annotation and primer removal have a significant impact on the results. The *psbA-trnH* sequence coverage of *Scutellaria baicalensis* and *Canarium album* did not reach the 95% threshold in HSZY175. The mapping results indicated that the coverage was unevenly distributed, leading to a certain degree of unreliability in the identification results. As shown in **Supplementary Figure S3**, the BLAST results of the high coverage region represent the *trnH* conserved sequence, which may be attributed to an error in the annotation or assembly process, failing to remove the *trnH* sequence and producing poor overall mapping results. Therefore, the *Scrophularia ningpoensis* sequence with good mapping results was selected for comparison. As shown in **Supplementary Figure S4**, the coverage is evenly distributed and continuously extended with high credibility. It is this high sequence coverage that has ensured highly accurate species recovery (Xin et al., 2013). As mentioned above, if errors occur during the annotation process, which is typically caused by a poorly assembled contig, the species identification results will be unreliable. The correct annotation can produce reliable results, as shown in **Supplementary Figure S4**. Based on this problem, a python script was developed to automate annotation and primer removal, following a manual inspection to ensure the accuracy of the results. It is suggested that future research ensures that the sequence is strictly and accurately annotated and that primers be removed before analysis to eliminate the possibility of false-positive sequences. Analysis problems such as the above mentioned are mainly due to the short-read length generated by second-generation sequencing, which makes sequence assembly and annotation more difficult. The major advantage of third-generation sequencing is the extreme long read length, which can overcome the difficulties of second-generation sequencing. However, the throughput of the third-generation sequencing is low and with high cost. Recently, long-read shotgun metagenomics has been used for studying oral phageome, which revealed that the power in uncovering bacteriophages with enhanced scaffolding, characteristics of their genes, and their interaction with host bacterial immunity (Yahara et al., 2021). It can be seen that long-read metagenomics has broad application prospects if the cost of long-read sequencing can be dropped and sequencing accuracy can be improved.

CONCLUSION

This study used shotgun metabarcoding approach to authenticate the biological ingredients of Qingguo Wan, and the results showed that all of the labeled ingredients can be successfully detected by the combination of four frequently used DNA barcodes such as ITS2, *psbA-trnH*, *rbcL* and *matK*. The current study further confirmed that there were differences in the identification efficiency of the four DNA barcodes, and a multi-barcode approach was essential for improving the ability of detecting the species composition in complex herbal products. And, the fungal contamination can be found both in mock and commercial samples with the analysis of ITS2 barcodes obtained from the shotgun sequencing data. Finally, this study firmly showed that shotgun metabarcoding is not only valuable for the quality control of Qingguo Wan, but also can be used for the identification of other traditional herbal products as long as its DNA can be successfully obtained.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository and accession numbers can be found below: NCBI's Sequence Read Archive (SRA) and SRR12629050, SRR12629049, SRR12629048, SRR12629047, SRR12629046.

AUTHOR CONTRIBUTIONS

LS and JL conceived and designed the study. BL collected samples. QZ and WM performed the experiment. LS and MS analyzed the data. LS and MS wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.607210/full#supplementary-material

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Biological Ingredient Analysis of Traditional Herbal Patent Medicine Fuke Desheng Wan Using the Shotgun Metabarcoding Approach

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With the widespread use of traditional medicine around the world, the safety and efficacy of traditional herbal patent medicine have become an increasing concern to the public. However, it is difficult to supervise the authenticity of herbal materials in mixed herbal products according to the current quality standards, especially for traditional herbal patent medicine, with a distinct variance in the dosage of herbal materials. This study utilized the shotgun metabarcoding approach to analyze the biological ingredients of Fuke Desheng Wan (FKDSW), which is an effective traditional herbal product for the treatment of dysmenorrhea. Six herbal materials were collected, and a lab-made mock FKDSW sample was produced to establish a method for the authentication assessment of biological ingredients in traditional herbal patent medicine based on shotgun metabarcoding. Furthermore, four commercial FKDSW samples were collected to verify the practicality of the shotgun metabarcoding approach. Then, a total of 52.16 Gb raw data for 174 million paired-end reads was generated using the Illumina NovaSeq sequencing platform. Meanwhile, 228, 23, and 14 operational taxonomic units (OTUs) were obtained for the ITS2, *matK*, and *rbcL* regions, respectively, after bioinformatic analysis. Moreover, no differences were evident between the assembly sequences obtained *via* shotgun metabarcoding and their corresponding reference sequences of the same species obtained *via* Sanger sequencing, except for part of the ITS2 and *matK* assembly sequences of *Paeonia lactiflora* Pall., *Saussurea costus* (Falc.) Lipsch. and *Bupleurum chinense* DC. with 1–6 different bases. The identification results showed that all six prescribed ingredients were successfully detected and that the non-authentic ingredient of Bupleuri Radix (Chaihu, *Bupleurum chinense* DC. or *Bupleurum scorzonerifolium* Willd.) was found in all the commercial samples, namely *Bupleurum falcatum* L. Here, 25 weed species representing 16 genera of ten families were detected. Moreover, 26 fungal genera belonging to 17 families were found in both lab-made and commercial FKDSW samples. This study demonstrated that the shotgun metabarcoding approach could overcome the biased PCR amplification and authenticate the biological ingredients of traditional herbal patent medicine with a distinct variance in the dosage of the herbal materials. Therefore, this provides an appropriate evaluation method for improving the safety and efficacy of traditional herbal patent medicine.

Keywords: shotgun metabarcoding, traditional herbal patent medicine, biological ingredients, weeds, fungi

INTRODUCTION

Dysmenorrhea is a common gynecological disease that can harm the health, work status, and quality of life of women. Research was conducted in various countries to determine the number of adolescents and young women suffering from dysmenorrhea, the results of which indicated that the prevalence rate of this condition ranged from 34% (Egypt) to 94% (Oman) (De Sanctis et al., 2016). Although the prescribed first-line therapy for dysmenorrhea is non-steroidal anti-inflammatory drugs, which usually alleviate menstrual pain by inhibiting peripheral, systemic prostaglandins, and their corresponding downstream effects, there are still approximately 18% of women who continue to suffer from the condition who display a distinct resistance to this treatment (Iacovides et al., 2015; Oladosu et al., 2018). As a result, traditional herbal medicines have been proposed as alternative therapies for dysmenorrhea (Oladosu et al., 2018). Fuke Desheng Wan (FKDSW) has been recommended as a gynecological medicine for curing dysmenorrhea caused by liver depression or the stagnancy of both blood and qi (Commission, 1994). This treatment exhibits a unique curative effect in relieving dysmenorrhea, regulating abnormal menstruation, and improving complications (Commission, 1994). FKDSW is a honeyed pill consisting of six herbal materials, including Angelicae Sinensis Radix (Danggui), Paeoniae Radix Alba (Baishao), Aucklandiae Radix (Muxiang), Notopterygii Rhizoma et Radix (Qianghuo), Leonuri Herba (Yimucao), and Bupleuri Radix (Chaihu), at significantly different and very precise dosages. Leonuri Herba (Yimucao) is the primary ingredient in FKDSW at a level of 59.26%, whereas the content of Aucklandiae Radix (Muxiang), Notopterygii Rhizoma et Radix (Qianghuo), and Bupleuri Radix (Chaihu) displayed the lowest dosage level at 3.70%. The quality control and biological ingredient assessment of FKDSW are challenging compared to other traditional herbal patent medicines with similar dosage levels of each ingredient. In this case, the characteristics of medicinal materials with low content will be overwhelmed by the characteristics of medicinal materials with high content. Although the thin-layer chromatography identification method used for FKDSW, involving Angelicae Sinensis Radix (Danggui) and Paeoniae Radix Alba (Baishao), is recorded in the current standard (Commission, 1994), it is not enough to authenticate all of its labeled ingredients due to the current methods don't cover all the ingredients. For example, an authenticity survey revealed that approximately 35.3% of Bupleuri Radix (Chaihu) samples in herbal markets were identified to be adulterants in 85 samples of Bupleuri Radix (Chaihu) (Wang et al., 2017). Moreover, there are lots of researches proved that the widespread adulteration of commercial herbal products have been found throughout the global market (van der Valk et al., 2017; Ichim, 2019; Ichim et al., 2020). For example, *Clematis armandii*, an adulterant of Akebiae Caulis (Mutong, *Akebia trifoliata*), was detected in the commercial herbal product of Longdan Xiegan Wan (Xin et al., 2018a). And there are 7% of herbal products of the Lonicerae japonicae Flos contained both of two adulterants Eucommiae Folium and Lonicerae Flos (Gao et al., 2017). Ichim (2019) and Ichim et al. (2020) found that herbal products containing undeclared contaminant, substitute, and filler species, or none of the labeled species were distributed across all continents and regions.

Compared to traditional pharmacopoeial identification methods including macroscopic, microscopic, chemical authentication approaches, DNA-based technology is more universal and accurate and can discriminate different species based on specific DNA fragments or even complete genomic information without the influence of environmental modification or the limitation of experiences, in spite of DNA-based methods do not provide any quantitative nor qualitative information of the active compounds in the herbal materials or the herbal preparations (Chen et al., 2012; Chen et al., 2018; Raclariu et al., 2018; Grazina et al., 2020). The protocol of DNA-based species identification methods like DNA metabarcoding mainly includes three steps: 1) total DNA extraction, 2) PCR amplification of the target DNA regions with universal primers, and 3) the identification and biodiversity assessment of multiple species in complex herbal preparations and products using high-throughput sequencing technology (Taberlet et al., 2012; Coghlan et al., 2015; Arulandhu et al., 2017; Gao et al., 2017; Song et al., 2017). However, the dependence of all these methods on PCR amplification limits their application in identifying the biological ingredients in traditional herbal patent medicine. For example, there are a large number of substances in traditional herbal materials or herbal products, such as polysaccharides, polyphenols, or excipients, which may inhibit PCR amplification and even lead to false-negative PCR results (Schrader et al., 2012). Furthermore, PCR bias, due to the differential binding of PCR primers to DNA templates, may lead to the loss of target sequences of some taxa or introduce chimeric sequences and other errors (Berry et al., 2011; Porter and Hajibabaei, 2018).

Shotgun metagenomics is capable of untargeted sequencing of all biological genomes from a single bulk sample without PCR amplification (Quince et al., 2017), which has been recognized as an unbiased method for investigating multiple species in various environmental or clinical samples (Bovo et al., 2018; Vijayvargiya et al., 2019; Ye et al., 2019). For example, Bovo et al. (2018) analyzed the environmental DNA samples of honey bees using shotgun metagenomic sequencing and reported five major biological groups in the two samples, including arthropods, plants, fungi, bacteria, and viruses, indicating that this method could be applied in large-scale experiments. Moreover, shotgun metagenomic sequencing holds additional benefits, such as species detection, the discovery of novel species, exploring the potential functions and relative abundance of the species of an organism, while reducing the reliance on independent cultures and PCR amplification approaches (Jovel et al., 2016; Ranjan et al., 2016; Porter and Hajibabaei, 2018; Ye et al., 2019). Although shotgun metagenomic sequencing has the potential of becoming a powerful analytical tool and has been applied to monitor the biological ingredients of traditional herbal patent medicine (Xin et al., 2018a), it presents two inherent difficulties that need to be overcome, namely the considerable amount of data that is generated and the limited number of whole genomic reference sequences. Since the purpose of the traditional herbal patent medicine identification is only to determine the species

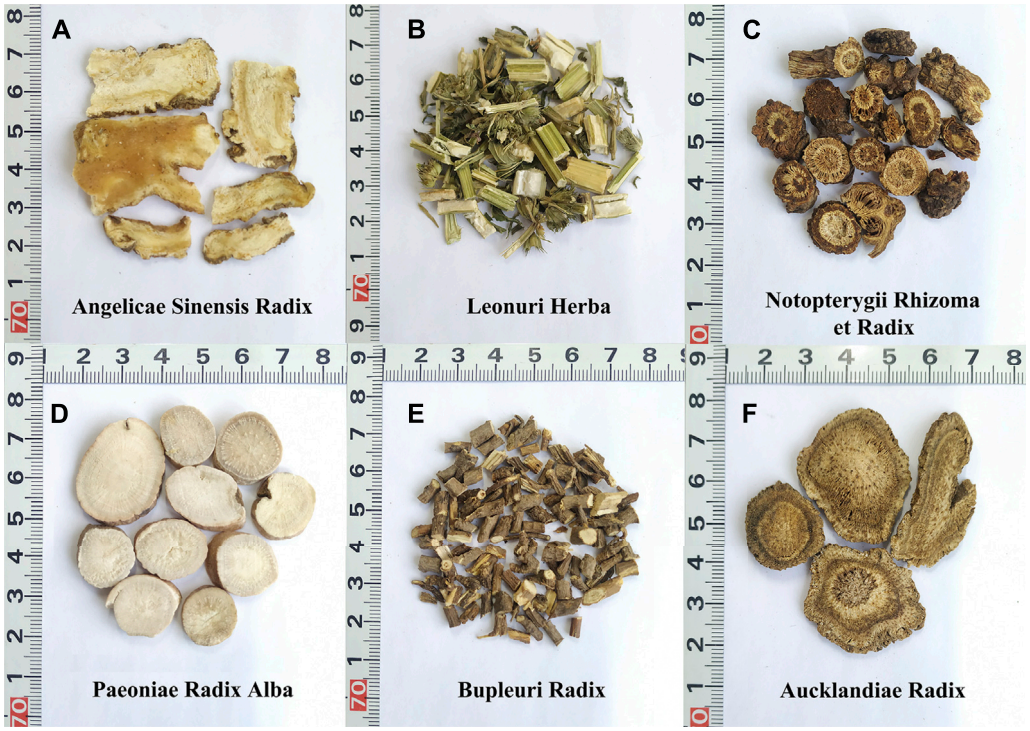

FIGURE 1 | The morphological characteristics of the six herbal materials in FKDSW. **(A)** Angelicae Sinensis Radix (Danggui). **(B)** Leonuri Herba (Yimucao). **(C)** Notopterygii Rhizoma et Radix (Qianghuo). **(D)** Paeoniae Radix Alba (Baishao). **(E)** Bupleuri Radix (Chaihu). **(F)** Aucklandiae Radix (Muxiang).

composition of biological ingredients, selecting the phylogenetic or DNA barcoding markers may provide an alternative, fast, accurate, and lightweight approach (Segata et al., 2012; Sunagawa et al., 2013). With the development of high-throughput sequencing and bioinformatics, shotgun metabarcoding is expected to become a highly effective regulatory approach for the authentication assessment of biological ingredients in herbal product mixtures.

FKDSW is selected as the research object in this study. Shotgun metagenomic sequencing is used in conjunction with DNA barcoding (defined as "shotgun metabarcoding" in the present research) to determine the species composition in FKDSW and discuss the feasibility of this approach for monitoring the biological ingredients in traditional herbal patent medicine with distinct variance in the dosage of herbal materials. Meanwhile, this study combines multiple materials evidence to make its strategies and techniques for clearly and efficiently authenticating the herbal materials of FKDSW, effectively supplementing traditional identification methods, which will be useful for improving the holistic quality control of traditional herbal patent medicine.

MATERIALS AND METHODS

Sample Collection

Six herbal materials were purchased from the Beijing Tongrentang herbal store to prepare the mock FKDSW sample and included Angelicae Sinensis Radix (Danggui, HSYC 2002), Paeoniae Radix Alba (Baishao, HSYC 2036), Aucklandiae Radix (Muxiang, HSYC 2037), Notopterygii Rhizoma et Radix (Qianghuo, HSYC 2038), Leonuri Herba (Yimucao, HSYC 2039), and Bupleuri Radix (Chaihu, HSYC 2078). Then, these materials were authenticated *via* morphological and DNA barcoding identification. In particular, the morphological identification was based on the herbal materials of form, shape and size, color, external markings and texture, organoleptic characters (odor, taste, and mouthfeel) recorded in the Chinese Pharmacopoeia (Commission, 2020). And these materials are deposited in the Institute of Medicinal Plant Development herbarium (herbarium code "IMD", NYBG: https://www.nybg.org/). The six medicinal materials and their prescribed proportions are shown in **Figure 1**, **Table 1** and **Supplementary Table S1**. To verify the feasibility and accuracy of the shotgun metabarcoding approach, the mock FKDSW sample was handcrafted in the laboratory according to the preparation method recorded in the pharmaceutical standard (Volume 9, WS3-B-1743-94) (Commission, 1994), and named HSZY167. Four batches of commercial FKDSW samples were purchased from drug stores to test the applicability of shotgun metabarcoding and numbered as HSZY003, HSZY140, HSZY153, and HSZY154.

DNA Extraction and Quantification

The DNA was extracted from the herbal materials following the previously published DNA barcoding protocol (Liu et al., 2017)

TABLE 1 | The prescribed proportion of each herbal ingredient that was labeled in FKDSW.

Herbal material Latin name (pinyin name)	Prescribed proportion (%)
Angelicae Sinensis Radix (Danggui)	14.81
Paeoniae Radix Alba (Baishao)	14.81
Aucklandiae Radix (Muxiang)	3.70
Notopterygii Rhizoma et Radix (Qianghuo)	3.70
Leonuri Herba (Yimucao)	59.26
Bupleuri Radix (Chaihu)	3.70

and principles for traditional Chinese herbal medicine (Chen et al., 2014). Detailed steps were carried out according to the protocol supplied by the manufacturer of the plant genomic DNA extraction kit [Tiangen Biochemical Technology (Beijing) Co., Ltd, China]. Then, the extracted DNA was amplified *via* PCR with primer sets of ITS2, *matK*, and *rbcL* regions using 2 × Taq master mix (AidLab Biotechnologies Co., Ltd., China). The primer selection and PCR conditions adhered to the barcode of traditional Chinese herbal medicine data system (TCM-BOL) (Chen et al., 2014), the consortium for the barcode of life (CBOL) plant working group (Group, 2009), and the barcode of life data system (BOLD) (Ratnasingham and Hebert, 2007). Then the PCR products were electrophoresed on 1.0% agarose gel using GelRed nucleic acid gel stain (Biotium Biotechnologies Co., Ltd., United States) to determine the integrity.

The metagenomic DNA was extracted from the traditional herbal patent medicine samples according to a method reported (Cheng et al., 2015), with some minor modifications during the pretreatment steps (Xin et al., 2018a). Finally, the concentration and quality of the DNA in all the samples of the herbal materials and traditional herbal patent medicine were determined using a NanoDrop ONE ultra-micro spectrophotometer (Thermo Fisher Scientific, Inc., United States).

Sequencing, Bioinformatic Analysis, and Species Identification

The PCR products of ITS2, *matK*, and *rbcL* were bi-directionally sequenced using an ABI 3730xL DNA Analyzer (Thermofisher Co., Ltd., United States). Then, the sequencing output files generated *via* Sanger sequencing were aligned and assembled using Codoncode aligner v 9.0.1 (CodonCode Corp., Dedham, MA, United States).

The Illumina NovaSeq platform was used for the shotgun sequencing of the traditional herbal patent medicine samples after a meta-genomic DNA paired-end library was constructed with sheared fragments. The sequencing output files generated by high-throughput sequencing were used to remove the adapters, and low quality reads using Trimmonmatic v0.38 (Bolger et al., 2014). The ITS2, *matK*, and *rbcL* sequences were downloaded from the National Center for Biotechnology Information (NCBI) to construct the reference database. And the reads after quality control were aligned to the reference database using basic local alignment search tool (BLAST), then the targeted paired-end reads of ITS2, *matK*, and *rbcL* were enriched by local python scripts (Shi et al., 2019). The enriched reads were assembled with MEGAHIT v1.2.9 and MetaSPAdes v3.13.2 (Li et al., 2015; Nurk et al., 2017), while the k-mer values ranged from 31 to 127. The assembled contigs were clustered, and redundant data were removed using cd-hit with 100% identity (Li and Godzik, 2006). Cutadapt v2.10 was employed to remove the primer sequences of the conventional DNA barcoding *matK* and *rbcL* regions (Martin, 2011), while the ITS2 region was annotated using the hidden Markov model (HMM)-based annotation method (Keller et al., 2009). Chimeric sequences were removed by UCHIME v4.2 (Edgar et al., 2011). The remaining sequences were clustered into operational taxonomic units (OTUs) at 100% sequence similarity using Usearch v11 (https://www.drive5.com/usearch/). The OTUs of the representative sequences were further processed with Bowtie2 v2.4.1 and samtools v1.10, where the former was used to map the shotgun paired-end reads to the representative sequences, and the latter was employed to calculate the sequencing depth and coverage values (Langmead and Salzberg, 2012; Etherington et al., 2015). Then, the OTUs were manually removed, satisfying the following parameters: sequencing depth ≤3 and/or coverage ≤95%. The BLAST search (Camacho et al., 2009) was applied according to the information in the GenBank (Benson et al., 2014), TCM-BOL (Chen et al., 2014), and BOLD (Ratnasingham and Hebert, 2007) databases to obtain the remaining OTUs and facilitate species identification. All sequences acquired *via* Sanger sequencing were deposited in the GenBank database. The GenBank accession numbers are shown in **Table 2**. Finally, the resultant BLAST files were imported into MEGAN v6.18.9 for taxonomic analysis and to obtain the statistics regarding the species composition of the traditional herbal patent medicine (Huson et al., 2016).

RESULTS

The Authentication of the Six Herbal Materials in FKDSW and Their Reference ITS2, *matK*, and *rbcL* DNA Barcodes

The authentication of the six herbal materials was further verified *via* DNA barcoding to ensure their accuracy. All the ITS2 sequences of the six herbal ingredients used for producing the lab-made mock FKDSW sample were successfully obtained and assigned to species by blasting to the TCM-BOL database. The *matK* sequences of Paeoniae Radix Alba (Baishao) and Notopterygii Rhizoma et Radix (Qianghuo), and the *rbcL* sequences of Paeoniae Radix Alba (Baishao), Notopterygii Rhizoma et Radix (Qianghuo), and Bupleuri Radix (Chaihu) failed to be amplified by the universal primers, while only the *matK* and *rbcL* sequences of the remaining herbal materials were used for species assignment by blasting to the BOLD and GenBank nt databases. The comprehensive species identification results of three DNA barcodes showed that the six herbal materials were authentic species recorded in the Chinese Pharmacopoeia, which were consistent with the morphological identification results. Finally, the original

TABLE 2 | The species of herbal materials assigned by classic DNA barcoding and their GenBank accession numbers.

Sample ID	Species	GenBank accession number		
		ITS2	*matK*	*rbcL*
HSYC2002	*Angelica sinensis* (Oliv.) Diels	MN712234	MN746764	MN729559
HSYC2036	*Paeonia lactiflora* Pall.	MN712235	—	—
HSYC2037	*Saussurea costus* (Falc.) Lipsch.	MN712236	MW000342	MW000336
HSYC2038	*Hansenia weberbaueriana* (Fedde ex H.Wolff) Pimenov & Kljuykov	MN712237	—	—
HSYC2039	*Leonurus japonicus* Houtt.	MN712238	MW000343	MW000337
HSYC2078	*Bupleurum chinense* DC.	MN712239	MW000344	—

Note: "—" indicates that the corresponding reference sequence was not obtained.

species of Angelicae Sinensis Radix (Danggui), Paeoniae Radix Alba (Baishao), Aucklandiae Radix (Muxiang), Notopterygii Rhizoma et Radix (Qianghuo), Leonuri Herba (Yimucao), and Bupleuri Radix (Chaihu) were verified to be *Angelica sinensis* (Oliv.) Diels, *Paeonia lactiflora* Pall., *Saussurea costus* (Falc.) Lipsch. (synonym: *Aucklandia costus* Falc.), *Hansenia weberbaueriana* (Fedde ex H.Wolff) Pimenov & Kljuykov, *Leonurus japonicus* Houtt., and *Bupleurum chinense* DC. The quantity and quality of the extracted DNA are shown in **Supplementary Table S2**. The identification results and GenBank accession numbers are presented in **Table 2**.

An Overview of the High-Throughput Sequencing Data and Shotgun Metabarcoding Assembly

The average extracted DNA concentration from the five FKDSW samples was 110.5 ng/μL, while the A_{260}/A_{280} values ranged from 1.8 to 1.9, indicating that the quantity and quality of the metagenomic DNA extracted from FKDSW adequately met the requirements of high throughput sequencing (**Supplementary Table S3**). A total of 52.16 Gb of clean data was generated from about 174 million paired-end reads using the Illumina NovaSeq sequencing platform. On average, 10.43 Gb of clean data was acquired for each FKDSW sample. The commercial HSZY003 sample displayed the largest quantity of sequencing data at 18.14 Gb, while HSZY140 had the least at 6.6 Gb. Furthermore, 371,553 paired-end reads were enriched for ITS2, *matK*, and *rbcL* from the clean data, while the number of paired-end reads belonging to the ITS2, *matK*, and *rbcL* regions was 217,085, 52,642, and 101,826, respectively. Further analysis showed that the number of reads enriched for each marker was consistent with the clean data of its corresponding sample (**Supplementary Table S4**). In total, 1,248 unique contigs were generated, in which the number of unique contigs of ITS2, *matK*, and *rbcL* was 888, 150, and 210, respectively. Moreover, 466 DNA barcodes were obtained from ITS2, *matK*, and *rbcL* after conventional DNA barcoding region annotation and chimera detection. Subsequently, these DNA barcodes in the conventional DNA barcoding region were clustered into 265 OTUs comprising 228, 23, and 14 OTUs for ITS2, *matK*, and *rbcL*, respectively (**Table 3**). Considering the potential sequencing or assembly errors, further accuracy verification was performed for each OTU *via* read mapping. Finally, 133 reliable OTUs were obtained, including 98 ITS2 OTUs, 21 *matK* OTUs, and 14 *rbcL* OTUs.

The Species Detection of in the Lab-Made Mock Sample and the Verification of the Assembly Results

A total of 58 reliable OTUs were obtained from the ITS2, *matK*, and *rbcL* regions in the lab-made mock FKDSW sample, including 36 ITS2 OTUs, 12 *matK* OTUs, and 10 *rbcL* OTUs. The species assignment results showed that all the original species of the six prescribed herbal ingredients were successfully detected and included *Angelica sinensis*, *Paeonia lactiflora*, *Saussurea costus*, *Hansenia weberbaueriana*, *Leonurus japonicas,* and *Bupleurum chinense* (**Table 4**). Of these OTUs in the ITS2, *matK*, and *rbcL* regions, seven were identified as *Bupleurum chinense*, which exhibits the most significant number of OTUs among the six labeled species. Although there are only three OTUs belonging to *Leonurus japonicas*, which contains the largest number of mapping reads. And 4,528, 9,906, and 10,263 reads could be mapped to the ITS2, *matK*, and *rbcL* OTUs of *Leonurus japonicas*, respectively.

To determine the assembly accuracy of ITS2, *matK*, and *rbcL* from the shotgun sequencing data, this study compared these sequences (called "assembly sequences" here) with their corresponding reference sequences of the same species obtained *via* Sanger sequencing. For the ITS2 region, all assembly sequences and reference sequences of the original species belonging to six prescribed herbal ingredients were obtained *via* the two sequencing methods, respectively (**Supplementary Figure S1**). The assembly sequences obtained from *Angelica sinensis*, *Hansenia weberbaueriana*, and *Leonurus japonicas* were absolutely consistent with the reference sequences obtained *via* Sanger sequencing. Three types of ITS2 assembly sequences were evident for *Paeonia lactiflora*, one of which was consistent with the reference sequence, while the others were 1–2 base substitutions for the reference sequences. Two types of haplotypes of ITS2 assembly sequences were obtained from *Saussurea costus* and *Bupleurum chinense*, respectively, with 0–4 base difference from the reference sequences. For the *matK* sequences, four species shared sequences that were generated *via* the two sequencing methods. The assembly sequences from the *matK* region of *Angelica sinensis*, *Saussurea costus,* and *Leonurus japonicas* were identical to the

TABLE 3 | The assembly results of three DNA barcodes in the FKDSW samples.

	ITS2	*matK*	*rbcL*
Number of unique contigs	888	150	210
Number of DNA barcodes after annotation and chimera detection	386	47	33
Number of OTUs	228	23	14
Average length (bp)	210.5	844.2	703
GC content (%)	59	33.2	42.8

TABLE 4 | Species detection of the ITS2, *matK*, and *rbcL* DNA barcoding regions obtained *via* shotgun metabarcoding in the lab-made mock FKDSW sample.

Herbal ingredients	Original species	ITS2	*matK*	*rbcL*
Angelicae Sinensis Radix (Danggui)	*Angelica sinensis*	√	√	√
Paeoniae Radix Alba (Baishao)	*Paeonia lactiflora*	√	√	√
Aucklandiae Radix (Muxiang)	*Saussurea costus*	√	√	√
Notopterygii Rhizoma et Radix (Qianghuo)	*Hansenia weberbaueriana*	√	√	√
Leonuri Herba (Yimucao)	*Leonurus japonicus*	√	√	√
Bupleuri Radix (Chaihu)	*Bupleurum chinense*	√	√	√

Note: "√" means that the assembly sequence of this species was obtained.

reference sequences. As for the three *matK* assembly sequences of *Bupleurum chinense*, there were 0, 1, and 6 base differences in these assembly sequences compared with its reference sequences. The assembly sequences obtained *via* shotgun metabarcoding were identical to their corresponding reference sequences of the same species obtained *via* Sanger sequencing for *Angelica sinensis*, *Saussurea costus,* and *Leonurus japonicas* in the *rbcL* region.

The Authentic and Non-Authentic Ingredients Detected in the Commercial Samples

In total, 98 reliable ITS2 OTUs were obtained from the commercial samples, of which 11 OTUs were identified as the original plant species of six prescribed herbal ingredients. The BLAST results demonstrated that *Angelica sinensis*, *Paeonia lactiflora*, *Saussurea costus*, *Hansenia weberbaueriana*, *Leonurus japonicus,* and *Bupleurum chinense* were detected based on the ITS2 sequences in four commercial samples. *Hansenia forbesii* (H.Boissieu) Pimenov & Kljuykov, which is another botanical source species for Notopterygii Rhizoma et Radix (Qianghuo), was also detected in the HSZY003, HSZY153, and HSZY154 samples. In addition, *Bupleurum falcatum* L. was detected in four commercial samples and is a non-authentic ingredient for Bupleuri Radix (Chaihu), as shown in **Figure 2** and **Supplementary Table S5**.

Among the obtained 21 *matK* OTUs and 14 *rbcL* OTUs, *Paeonia lactiflora* and *Leonurus japonicus* were detected in the four commercial FKDSW samples. The original plant species of Angelicae Sinensis Radix (Danggui) and Bupleuri Radix (Chaihu) were determined at the genus level in the commercial FKDSW samples. For example, one OTU obtained from the *matK* region was identified as several species belonging to the genus *Angelica* in the four commercial FKDSW samples, but could not be assigned to a specific species. Moreover, five OTUs obtained from the *matK* and *rbcL* regions belonged to *Bupleurum* in the three commercial FKDSW samples, of which three were shared by HSZY003, HSZY153, and HSZY154. In addition, there are other contaminating species were found in the commercial FKDSW samples, which are described in detail in **Supplementary Figures S2, S3**, and **Supplementary Table S5**.

In general, six prescribed ingredients in the four commercial FKDSW samples were successfully detected with the combination of the ITS2, *matK*, and *rbcL* sequences. The ITS2 region displayed higher identification efficiency at the species level, while it was difficult to determine some Asteraceae and Apiaceae at the species level using the *matK* and *rbcL* sequences.

Unlabeled Plant Species Detected in the Lab-Made Mock and Commercial Samples

The contaminating plant species found in this study were represented by 68 OTUs from the ITS2, *matK*, and *rbcL* regions, and were classified into 14 families that included 24 genera and 30 species, while the remainder could be resolved at the genera or family levels, as shown in **Supplementary Tables S6–S8**. Ten plant families with 16 genera that included 25 species denoted common field weeds, such as *Artemisia annua* L., *Artemisia argyi* H.Lév. & Vaniot, *Artemisia scoparia* Waldst. & Kit., and *Erigeron canadensis* L. from Asteraceae, *Humulus scandens* and *Cannabis sativa* L. from Cannabaceae, *Ipomoea purpurea* (L.) Roth and *Ipomoea nil* (L.) Roth from Convolvulaceae, *Setaria viridis* (L.) P.Beauv. from Poaceae, as well as *Abutilon theophrasti* Medik. and *Hibiscus trionum* L. from Malvaceae. Of these, *Artemisia*, *Humulus,* and *Ipomoea* signified the three most common plant genera of these potential species. For the ITS2 region, a total of 11 families with 22 genera that included 28 species were identified for the contaminating plant species. Additionally, six families with six genera that included six species, as well as four families with four genera that included three species, were detected for the *matK* and *rbcL* regions,

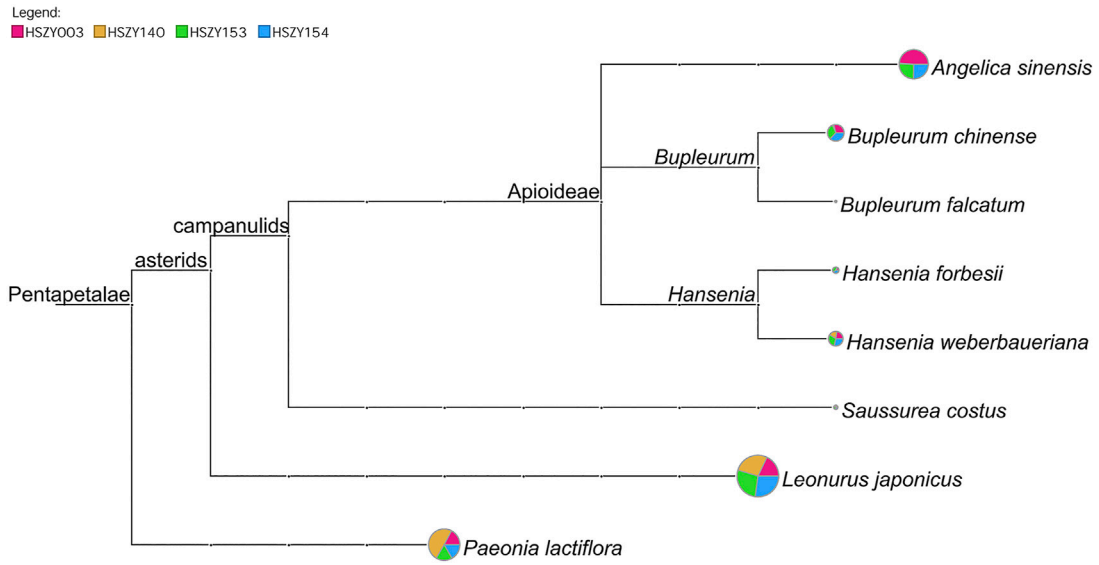

FIGURE 2 | A comparison of the taxonomic analytical results of the prescribed biological ingredients in four commercial FKDSW samples based on ITS2 sequences. Each taxonomic node represents a taxon in the NCBI taxonomy and is labeled according to its name. The pie chart shows the ratio of shotgun reads assigned to the corresponding taxon in four samples, while the size of the circle is scaled logarithmically to indicate the number of shotgun reads assigned to the taxon.

respectively. Furthermore, there were distinct differences in the relative species abundance of the contaminating plant species between the mock and four commercial FKDSW samples. For example, only four genera of botanical contamination were found in the mock FKDSW sample, including *Artemisia*, *Humulus*, *Ipomoea,* and *Erigeron*. However, all botanically contaminating species found in this study were detected in the commercial samples, containing a small number of other arbor species that included *Ulmus* of Ulmaceae, *Populus* of Salicaceae, *Robinia* of Fabaceae, and *Broussonetia* of Moraceae. The HSZY003 sample comprised a large proportion of botanical contamination, including 23 genera with 29 species.

The Fungal Communities in the Lab-Made Mock and Commercial Samples

This study analyzed the fungal communities in five FKDSW samples *via* ITS2. In total, 40 fungal ITS2 OTUs were obtained, and the BLAST results indicated that a total of 17 families that included 26 genera of fungal species were detected in all the FKDSW samples (**Supplementary Table S9**). Pleosporaceae represented the most abundant family, accounting for 17.54–41.29% of the fungal reads in five FKDSW samples, followed by Aspergillaceae, Cladosporiaceae, and Nectriaceae, as shown in **Figure 3**. Further taxonomical classification at the genus level demonstrated that *Alternaria*, *Aspergillus*, *Cladosporium,* and *Fusarium* were the most dominant genera among the 27 detected genera, with a relative abundance of 17.54–41.29%, 9.68–23.39%, 6.05–21.64%, and 7.56–19.30%, respectively, as shown in **Figure 4**. There were differences in fungal communities between the lab-made mock and four commercial FKDSW samples. *Alternaria* was the dominant genera in the four commercial samples, while *Aspergillus* was the most abundant genera in the lab-made mock sample. In addition, the fungal communities in the HSZY003, HSZY140, HSZY153, and HSZY154 commercial samples were composed of 21, 10, 12, and 8 genera, respectively, while the lab-made mock sample only contained seven genera, namely, *Alternaria*, *Aspergillus*, *Cladosporium*, *Colletotrichum*, *Didymella*, *Fusarium,* and *Plectosphaerella*.

DISCUSSION

Challenges and Biotechnical Advances of Shotgun Metabarcoding in Traditional Herbal Product Authentication

Shotgun metagenomics is widely used for the detection and characterization of microbial community structures and functions, including those found in the human intestinal tract (Laudadio et al., 2018), in soil (Khodakova et al., 2014), in food (Yang et al., 2016), and in marine water (Venter et al., 2004), as well as terrestrial arthropod communities (Zhou et al., 2013). Xin et al. (2018a) applied shotgun metagenomic sequencing for the first time to detect the biological ingredients in Longdan Xiegan Wan, verifying the feasibility of the method for monitoring the species composition in traditional herbal patent medicine. A significant difference was evident compared with Longdan Xiegan Wan since the content of the highest dosage of the all the herbal materials exceeded that of the lowest dosage in FKDSW more than 16 times. In this study, shotgun metabarcoding was performed directly to analyze the biological ingredients in lab-made mock and commercial

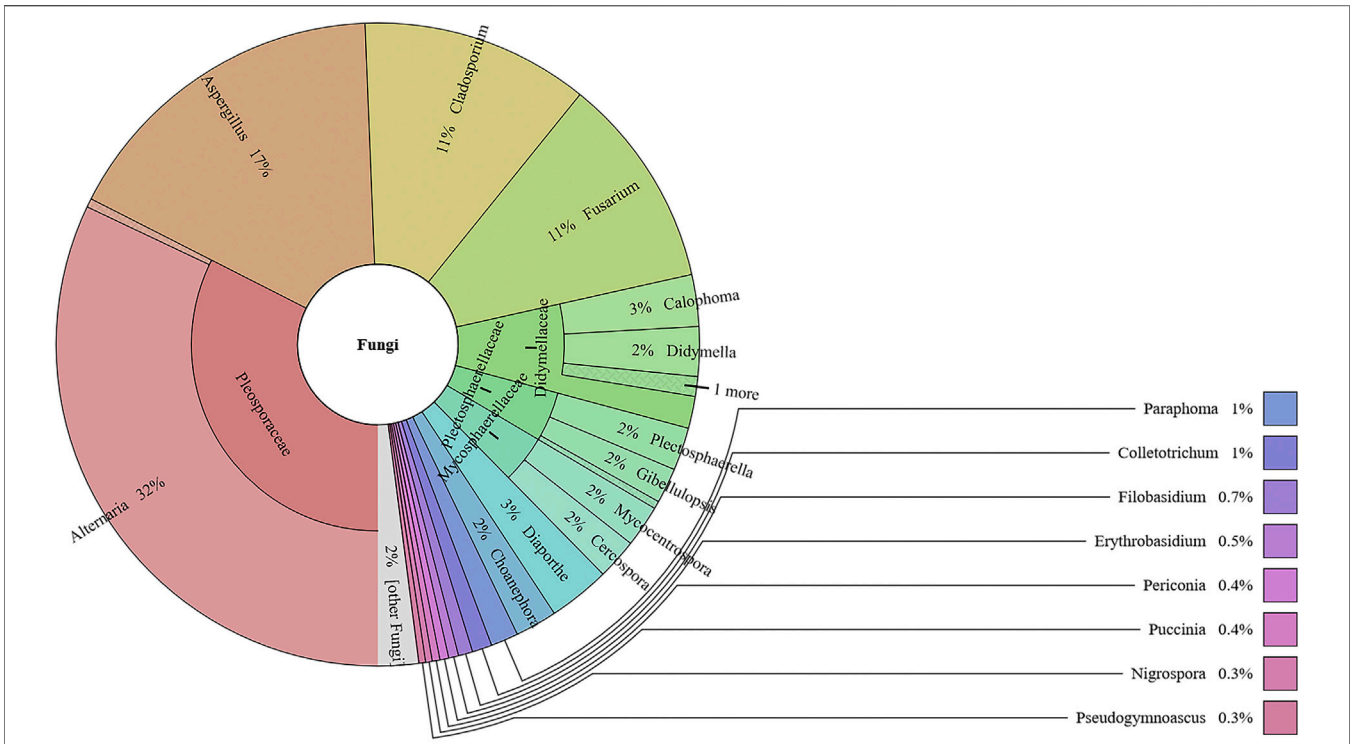

FIGURE 3 | The fungal composition detected in lab-made mock and commercial FKDSW samples at the family and genus levels.

FKDSW samples, while the raw data obtained were qualified for subsequent data processing, such as assembly, annotation, and alignment.

Thereafter, all the assembly sequences of the ITS2, *matK*, and *rbcL* barcodes were successfully obtained for the six prescribed herbal ingredients in the mock FKDSW sample. The obtained assembly sequences were compared with each reference sequence, and the results indicated that all the assembly sequences were identical to the corresponding reference sequences except for part of the ITS2 and *matK* assembly sequences denoting *Paeonia lactiflora*, *Saussurea costus,* and *Bupleurum chinense*. A possible reason for the base differences between the assembly sequences and the reference sequences may be the multiple ITS2 copies or individual differences among plants (Álvarez, 2003). Although there are frequently hundreds of ITS2 copies with potentially dozens of different sequences in each genome, all ITS2 variants are sufficient for species identification in most cases (Song et al., 2012). Furthermore, there are positive and significant correlations between the amount of content of each herbal material and the read depth of ITS2, *matK*, *rbcL* barcodes (Zhou et al., 2013; Bista et al., 2018), however, the change ratio of the read depth with the amount of content is not with a good linear relationship. Finally, the analytical results suggested that all the prescribed and non-prescribed ingredients in the four commercial samples were detected based on the combination of three DNA barcodes even at low level amount of content. This verified that shotgun metabarcoding can overcome the biased PCR amplification and is particularly suitable for authenticating the biological ingredients in traditional herbal patent medicine with distinct variances in the herbal material dosages.

A total of 51 species were identified at the species level based on the ITS2 sequences that were obtained, which included all the prescribed medicinal materials and most of the exogenous biological contamination. Nevertheless, only nine species were determined at the species level based on the *matK* and *rbcL* sequences. Some Asteraceae and Apiaceae species were resolved at the genera level or higher, such as the original plant species of Angelicae Sinensis Radix (Danggui) and Bupleuri Radix (Chaihu). For this phenomenon, it may be caused by the identification efficiency of different DNA barcodes (Gao et al., 2010; China Plant et al., 2011). In 2010, Chen et al. tested the performance of seven candidate DNA barcodes (*psbA-trnH*, *matK*, *rbcL*, *rpoC1*, *ycf5*, ITS2, and ITS) from medicinal plants. The results showed that the identification efficiency of the ITS2 region at the species level was 92.7%, and it was proposed as the standard DNA barcoding for medicinal plants (Chen et al., 2010). Compared to the ITS/ITS2 regions, the identification efficiency of the *matK* and *rbcL* DNA barcodes was too low to distinguish more species in Asteraceae and Apiaceae (Gao et al., 2010; Liu et al., 2014). Although the chloroplast gene in *matK* and *rbcL* did not recognize the species in some cases, they displayed an excellent ability to identify orchids (Lahaye et al., 2008) or discriminate among species in congeneric pair-wise comparisons (Newmaster et al., 2006). Moreover, chloroplast genomes have the advantage of maternal inheritance, avoiding genetic recombination, and the high-copy number of plastids per cell are accessible to extract genomic DNA (Twyford and Ness, 2017). Therefore, we recommend that *matK* and *rbcL* as the

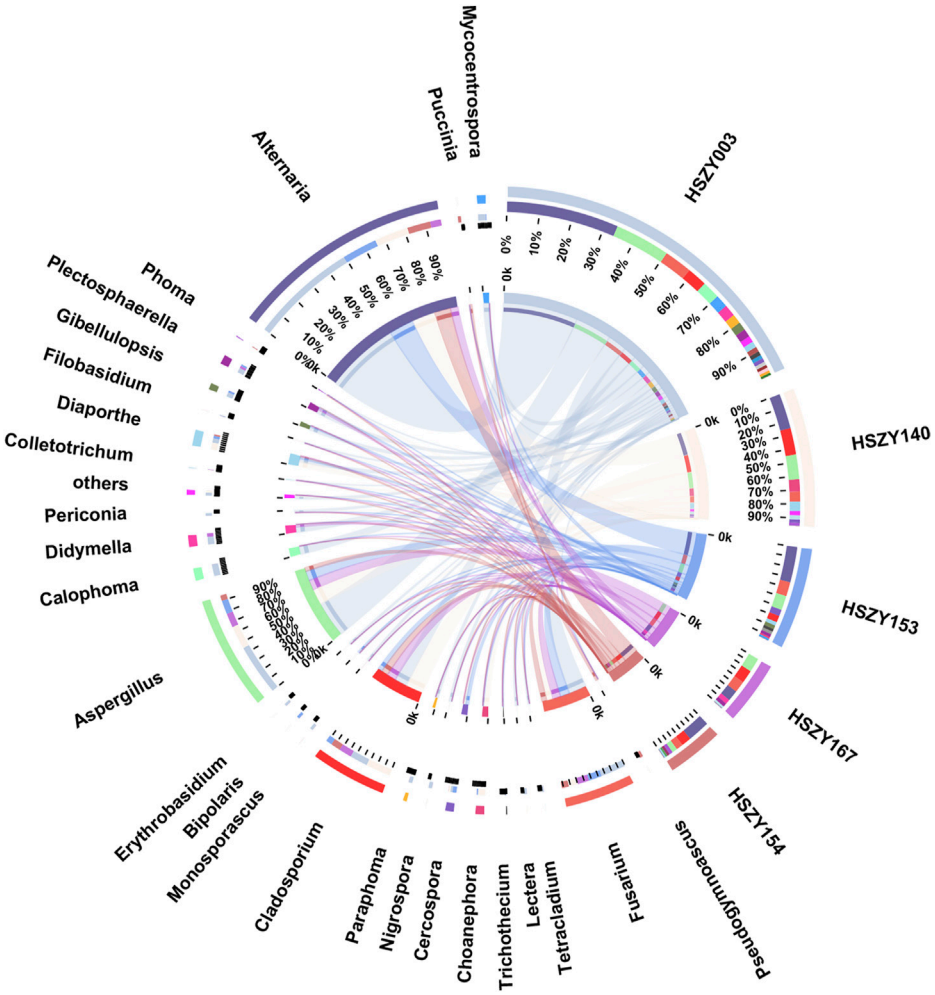

FIGURE 4 | The fungal community distribution for each sample at the genus level. The data were visualized using Circos. The left half-circle indicates the distribution ratio of species in different samples at the genus level: the outer ribbon represents the species, the inner color of the ribbon represents different groups, and the length represents the proportion of the sample for a particular genus. The right half-circle indicates the species composition in each sample: the color of the outer ribbon represents samples from different groups, the color of the inner ribbon represents the composition of different species in each sample, and the length of the ribbon represents the relative abundance of the corresponding species.

complementary barcodes to ITS2 for the identification of medicinal plants. To sum up, this study established a biological method for monitoring traditional herbal patent medicine *via* shotgun metabarcoding with ITS2 as the core barcode, and *matK* and *rbcL* as the supplementary barcodes, effectively supplementing traditional identification methods.

Shotgun Metabarcoding is a "Best Practice" Method for the Detection of Biological Contamination Like Non-Authentic Ingredient, Weed, and Fungi Species

In addition to the prescribed herbal medicines, this study identified some unlabeled biological species, such as *Bupleurum falcatum*, weeds, and several fungi species. Bupleuri Radix was officially derived from the dried root of the Asteraceae plants, *Bupleurum chinense,* and *Bupleurum scorzonerifolium* Willd., according to the Chinese Pharmacopeia (Commission, 2020). However, there are still more than 20 additional species of the genus *Bupleurum* that are habitually utilized in China as a non-authentic ingredient for Bupleuri Radix (Liu et al., 2011). *Bupleurum falcatum* was introduced from Japan into China in the early 1970s and has been recorded as the legal original species of Bupleuri Radix in Japanese and Korean Pharmacopoeia (Yuan et al., 2017; Yeom et al., 2018). In 2007, Zhu et al. (2017) analyzed the chemical profiles of *Bupleurum chinense*, *Bupleurum yinchowense* R.H.Shan & Y.Li, and *Bupleurum falcatum* using UHPLC-QTOF-MS. The results indicated that the chemical profiles of the root samples from the three *Bupleurum* species were similar, especially the characteristic saikosaponins. However, a study suggested that the extracts of *Bupleurum chinense*, *Bupleurum falcatum,* and *Bupleurum scorzonerifolium* exhibit different pharmacological activities (Yuan et al., 2017). Using the FKDSW with the dried root of *Bupleurum*

falcatum instead of the root of *Bupleurum chinense* or *Bupleurum scorzonerifolium* poses a potential threat to its clinical efficacy.

A total of 10 plant families with 16 genera that included 25 weed species were detected in five FKDSW samples, such as species from *Artemisia* (*Artemisia annua*), *Humulus* (*Humulus scandens*), and *Ipomoea* (*Ipomoea purpurea*), which are all common field weeds found on the farmland where traditional herbal materials are cultivated (Ya-ling et al., 2018). It is not unusual for weed species to be mixed into traditional herbal products (Xin et al., 2018b). Jia et al. (2017) detected a variety of non-listed plant species in the commercial Yimu Wan samples using single-molecule realtime sequencing and DNA barcoding. Of these, species from *Humulus*, *Ipomoea*, *Artemisia,* and *Amaranthus* are mostly denoted common weeds, and sequences belonging to these species accounted for a large proportion of the total sequences. Controlling field weeds is not only an essential factor that impacts the artificial standardized cultivation of traditional herbal materials but is also a significant problem affecting global agricultural production. In addition, there are strict standards about using pesticides and fertilizers during the cultivation process of traditional herbal materials (Sang, 2017). Therefore, weed control is challenging during the cultivation of traditional herbal materials because of strict requirements, high standards, and high labor costs. "Leonuri Herba (Yimucao)" is the dried ground part of *Leonurus japonicas*. And it easily mixes with the leaves of some *Artemisia* plants during the manufacturing process due to their overlapping morphological features. *Ipomoea purpurea*/*Ipomoea nil,* and *Humulus scandens* are twining herbs (Zhengyi et al., 2013) that twine around the stem of *Leonurus japonicus* while growing. This might be the reason why *Artemisia*, *Humulus,* and *Ipomoea* were the most common plant genera found in five FKDSW samples.

Similar to agricultural products, plants cultivated for herbal medicines are also vulnerable to fungal contamination during the process of cultivation, processing, transportation, and storage (Xing et al., 2016; Guo et al., 2020; He et al., 2020). This study identified a total of 17 families that included 26 genera of fungal species in five FKDSW samples, and there is a certain difference in fungal communities between the mock and the commercial FKDSW products. The reason for this difference in fungal communities may be due to that the herbal materials for making the mock sample were purchased from medicinal stores, while the raw herbal materials of the commercial products were collected by the FKDSW manufacturers (He et al., 2020). Furthermore, most of the detected fungi species were common pathogenic fungi typically present during the growth period of field plants. For example, *Cercospora canescens* is an important pathogen of Cercospora leaf spot that can lead to serious yield loss of Yardlong bean (Duangsong et al., 2018). It is a remarkable fact that the *Alternaria*, *Aspergillus,* and *Fusarium* genera represent the most common infective fungi in agricultural products, food, and herbal medicines among the dominant bacteria detected, and are potential mycotoxin-producing microbial flora (Xing et al., 2016; Alshannaq and Yu, 2017; Guo et al., 2020). Of these, aflatoxins (AFs) and ochratoxin A, produced by *Aspergillus,* denote the most important contaminants due to their strong carcinogenicity (Perrone and Gallo, 2017). Therefore, the 2020 edition of the Chinese Pharmacopoeia (Commission, 2020) expressly limit the content of AFs in Chinese medicinal materials, such as Jujubae Fructus (Dazao), Cassiae Semen (Juemingzi), and Hordei Fructus Germinatus (Maiya), which are moldy-prone Chinese medicinal materials, expressed as aflatoxin B_1 (AFB_1)≤ 5 μg/kg, and the total amount of aflatoxin G_1, aflatoxin G_2, AFB_1 and aflatoxin B_2 ≤ 10 μg/kg. Consequently, it is suggested that the quality control of the entire industrial chain should be reinforced using DNA barcoding technology during the cultivation, harvesting, processing, transportation, and storage processes of herbal medicines to ensure the safety and efficacy of traditional herbal medicines.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI Bioproject: PRJNA663116, BioSample: SAMN16132426, SAMN16132427, SAMN16132428, SAMN16132429, SAMN16132430. SRA accession numbers are: SRR12640731, SRR12640730, SRR12640729, SRR12640728, SRR12640727. The DNA barcoding sequences assembled from the Sanger sequencing datasets presented in this study can be found in the NCBI GenBank online repository. The accession numbers for these DNA barcoding sequences are MN712234, MN712235, MN712236, MN712237, MN712238, MN712239, MN746764, MW000342, MW000343, MW000344, MN729559, MW000336, MW000337.

AUTHOR CONTRIBUTIONS

LS and JL conceived and designed the study. CZ, WK, and BL collected samples. QZ, WM, and HX performed the experiment. LS, MS, and HX analysed the data. HX and QZ wrote the manuscript. JZ and JJ revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.607197/full#supplementary-material

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