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Perspective

Method establishment for upgrading chemical markers in pharmacopoeia to bioactive markers for biological standardization of traditional Chinese medicine

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ABSTRACT

Quality surveillance on authentication, safety and efficacy of proprietary Chinese medicines (pCm) are certainly the top priorities for the industries. Nowadays, the quality control system adopted is mainly chemical marker-oriented, concerning basically the correct use of raw material and safety issues, while the biological activities of the chemical marker(s) are seldom considered. Hence, there is an undefined relationship between the amount of chemical markers and the claimed pharmacological activities. In view of the need in identifying appropriate markers for biological standardization of pCm products, the present study aimed to establish a systematic methodology for verifying whether the chemical marker of a traditional Chinese medicine (TCM) listed in Chinese Pharmacopoeia could be upgraded to a bioactive marker with certain efficacy in treating a particular disease. Our proposed methodology included a series of work on extraction, quantification, literature search and *in vivo* pharmacological experiments, in which the water extractability, biological effects at theoretical dose and oral bioavailability of the candidate chemical markers were all taken into consideration. The feasibility and implication of this bioactive markers verification methodology were further elaborated. Our findings will serve as the foundation for further research and development of biological standardization of TCM.

1. Introduction

Traditional Chinese medicines (TCM) have long been used by Chinese populations around the world to alleviate ailments, replenish deficiencies, promote health, or even extend lifespans. TCM products can mainly be classified into i) prepared slices of crude drugs (飲片 yǐn piàn), ii) TCM food/supplements including herbal tea; and iii) proprietary Chinese medicines (pCm). In general practice, the chemical markers listed in the monographs of herbal pharmacopoeias, such as Pharmacopoeia of the People's Republic of China (CP) (中華人民共和國藥典, zhōng huá rén mín gòng hé guó yào diǎn),[1] Taiwan Herbal Pharmacopoeia (臺灣中藥典, tái wān zhōng yào diǎn), United States Pharmacopoeia (USP) and European Pharmacopoeia (EP), are usually employed by TCM industries as the characteristic constituents for quantification to reflect the product quality and for the subsequent pCm registration as stipulated by law. However, in certain cases, these chemical markers may not be present or the amount too low to be detected in pCm, nor they possess any particular biological activities. The case of Rehmanniae Radix (RR) may be taken as an example. As listed in CP, catalpol and verbascoside are two chemical markers for authentication and assay of RR. Our group has investigated and compared the fibroblast-proliferating activities of three different RR samples

Abbreviations: CP, Pharmacopoeia of the People's Republic of China; pCm, proprietary Chinese medicines; TCM, traditional Chinese medicine.

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(fresh RR, dried RR and steamed RR) and its chemical components using human normal fibroblast cells Hs27.[2] Results showed that catalpol did not possess any stimulatory effect on fibroblast proliferation, implying that even though its amount in the pCm product is high enough to comply with certain chemical requirements, the bioefficacy (in this case, wound healing potency) of the products cannot be guaranteed. On the other hand, we found that verbascoside could stimulate fibroblast proliferation in a dose-dependent manner. However, its amount was too minute to be detectable in aqueous or ethanolic RR crude extract using ultra-performance liquid chromatography-diode array detection (UPLC-DAD) analysis. Therefore, it was concluded that both CP's chemical markers for RR, catalpol and verbascoside, did not contribute to the fibroblast-proliferating activity of RR extracts.

As demonstrated in the cases of verbascoside and catalpol, low water extractability or weak biological activity of chemical markers may impede their application in the quality assurance of pCm. Ideally, a good marker should be TCM-specific, easily detectable, chemically stable, in reasonable quantity and biologically active so that its amount can reflect the actual chemical composition as well as biological activity of the pCm.

In view of the need in identifying appropriate markers for biological standardization of pCm products, this project was set up to establish a systematic methodology for verifying whether the chemical marker of a TCM listed in CP could be upgraded to a bioactive marker with certain efficacy in treating a particular disease. Here, "bioactive marker" refers to a water-soluble pure compound that should be detectable in the TCM aqueous extract, bioavailable, and also possess a desirable biological activity, whereas "biological standardization" is a means of quality control to assess TCM's quality by measuring the amount of bioactive marker in its aqueous extract. As a prototype, this study will focus on single herbs, but not on compound formulae. Once the bioactive markers of single herbs are identified, those of compound formulae may be inferred later.

2. Methodology

Fig. 1 illustrates a proposed workflow to verify whether a chemical marker listed in herbal pharmacopoeia (CP was adopted in this case) could be promoted to a bioactive marker for a particular TCM. It can be divided into the following steps.

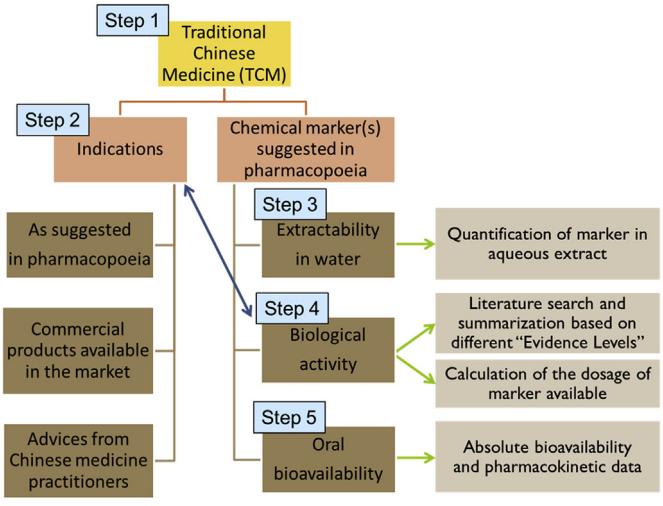

Fig. 1. A workflow for verifying whether a chemical marker listed in Chinese pharmacopoeia could be upgraded to a bioactive marker for a particular TCM.

2.1. Step 1: purchase of TCM samples

At least three samples of each TCM should be purchased from different renowned suppliers. Raw herbs should be authenticated using morphological characterization and thin layer chromatography (TLC) in accordance with procedures detailed in CP. Voucher specimens may be deposited at designated herbarium/ museum, if any.

2.2. Step 2: identification of TCM's key indication

In order to establish a bioactive marker, it is pivotal to firstly identify the key indication of the TCM. Besides the indications listed in CP, those of commercial products available in the market and advices from CM practitioners should be taken as additional information to determine the main pharmacological action of the TCM.

2.3. Step 3: quantification of the marker in aqueous extract

As TCM are always extracted in water and served in the form of decoction, the bioactive markers should be water soluble. In order to determine whether the candidate marker, i.e. the chemical marker(s) listed in CP for identification and assay, can be extracted in water, aqueous extracts of TCM are prepared by soaking the raw herb in 10-fold (w/v) of distilled water for 1 h and then boiling under reflux for one hour twice. The extracts are pooled, filtered and concentrated under reduced pressure at 60 °C. The concentrated extract is then lyophilized to dryness. The weight of dried extract collected is recorded and the extraction yield is calculated. Once the aqueous extract is prepared, quantification of the marker can be performed using high performance liquid chromatography (HPLC) as described in CP, if any.

2.4. Step 4: literature search for biological activities of the marker

Undoubtedly, the biological activity of the marker should be in coherence with TCM's indication. After TCM's indication is figured out, thorough literature search for the desired biological activities of the candidate marker should be conducted using updated and reliable data sources such as PubMed (MEDLINE), Web of Science, and China Academic Journals Full-text Database. For those scientific papers showing promising biological effect of the candidate marker, primary sorting according to level of evidence in the evidence pyramid (http://fgcu.libguides.com/EBP/levels) should be conducted. In brief, scientific data can be classified into the following nine levels of evidence:

Level 1: Evidence from systematic review or meta-analysis of all relevant randomized controlled double blind trials (RCT);
Level 2: Evidence obtained from at least one well-designed RCT;
Level 3: Evidence obtained from well-designed cohort studies;
Level 4: Evidence obtained from well-designed case control studies;
Level 5: Evidence obtained from case series;
Level 6: Evidence obtained from case reports;
Level 7: Evidence from the opinion of authorities and/or ideas, reports of expert committees;
Level 8: Evidence from animal researches;
Level 9: Evidence from *in vitro* researches.

After that, route of administration and the reported effective doses should be listed out. Then, it is crucial to calculate the amount of the marker present in TCM at CP's suggested daily dose and compare this "theoretical dose" with the reported effective dose to check whether the biological activity of the marker has been

proven or not.

2.5. Step 5: oral bioavailability of the marker

Since pCm are always consumed via oral route, it is important to check whether the bioactive marker can be absorbed from the gut or not for it to exert its biological activity in human bodies. Therefore, data on oral bioavailability of the marker should be searched.

With the above proposed methodology, Salviae Miltiorrhizae Radix et Rhizoma (丹參, dān shēn), the dried root and rhizome of *Salvia miltiorrhiza* Bge. (family Labiatae), was used as an example/case study below to illustrate the sophisticated concept of this project.

3. Results - case study using Danshen

3.1. Step 1: purchase of Danshen samples

In total, six samples of Danshen were purchased from five renowned TCM wholesalers in Hong Kong. Their voucher specimen numbers were assigned as 3501, 3497, 3502, 3535, 3536 and 3537. They were authenticated using morphological characterization and thin layer chromatography in accordance with procedures detailed in CP. The voucher specimens were deposited in the museum of Institute of Chinese Medicine, The Chinese University of Hong Kong.

Fig. 2 shows the TLC chromatogram of Danshen samples and chemical markers observed under visible light or UV 365 nm. As shown in the upper panel, tanshinone IIA was detected in all Danshen samples (Lanes 4–9). However, salvianolic acid B was found in samples 3497 and 3502 only (lower panel, Lanes 4 and 6). To ensure that all Danshen samples meet the CP standard, HPLC analysis was conducted to quantify the amounts of "cryptotanshinone + tanshinone I + tanshinone IIA" and "salvianolic acid B" in the raw herbs, and the results are listed in Table 1. Unexpectedly, none of the six Danshen samples contains more than 0.250% of "cryptotanshinone + tanshinone I + tanshinone IIA". Although sample 3497 contains the highest amount (0.152%), it still does not meet CP's standard on tanshinones. On the other hand, only sample 3497 contains more than 3.0% of salvianolic acid B. Taking the results of TLC and HPLC into account, Danshen sample 3497 was selected for further studies.

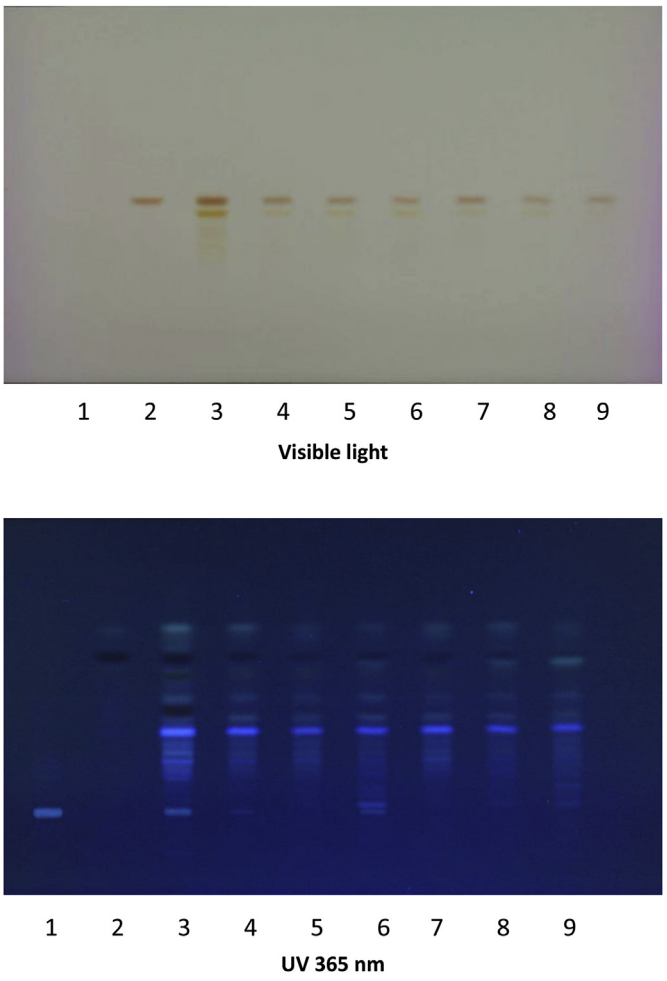

Fig. 2. TLC chromatogram of Danshen samples observed under visible light (upper panel) or UV 365 nm (lower panel). Stationary phase: TLC silica gel F_{254} plate (Merck, NJ, USA); mobile phase: chloroform-toluene- ethyl acetate-methanol- formic acid (6: 4: 8: 1: 4, v/v) and then petroleum ether (60–90 °C)- ethyl acetate (4: 1, v/v). Lane 1: Salvianolic acid B (Rf 0.28); Lane 2: Tanshinone IIA (Rf 0.62); Lane 3: Reference herb (National Institutes for Food and Drug Control, China); Lane 4: Sample 3497; Lane 5: Sample 3501; Lane 6: Sample 3502; Lane 7: Sample 3535; Lane 8: Sample 3536; Lane 9: Sample 3537.

3.2. Step 2: identification of Danshen's key indication

As recorded in CP, Danshen can remove blood stasis and relieve pain, promote the flow of blood and stimulate menstrual discharge, as well as ease the mind. It is commonly used for treating menstrual disorders, mass formation in the abdomen, pricking pain in the chest and abdomen, pain in acute arthritis and subcutaneous infection, fidgets and insomnia, hepatosplenomegaly, and angina pectoris.[1]

On the market, there are many Danshen pCm. Danshen Baoxin Cha (丹參保心茶 dān shēn bǎo xīn chá), composed of Danshen and green tea, can be used as a complementary medicine for blood stasis-induced chest pain and palpitations. Compound Danshen Dripping Pills (複方丹參滴丸 fù fang dān shēn dī wán), comprising Danshen, Sanqi and borneol, is a pCm for improving blood circulation, preventing/treating coronary heart disease and angina pectoris. Danshen Yixin Capsules (丹參益心膠囊 dān shēn yì xīn jiāo náng), contains Danshen, Sanqi and other TCM, is also used for relieving coronary heart disease and angina pectoris.

According to CM practitioners' experience and advices, Danshen is generally prescribed to alleviate those diseases related to blood circulation and heart function.

Taking these pieces of information into consideration, Danshen is a cardiovascular tonic herb for patients suffering from coronary heart disease and vascular disorders.

3.3. Step 3: quantification of chemical markers in aqueous extract of Danshen sample 3497

There are four chemical markers listed in CP for the identification and assay of Danshen. They are cryptotanshinone, tanshinone I, tanshinone IIA and salvianolic acid B. As listed in Table 2, only very minute amount of cryptotanshinone, tanshinone I and tanshinone IIA could be detected in the aqueous extract of Danshen sample 3497 (in abbreviation: Danshen 3497) using HPLC. Owing to their extremely low water extractability, these tanshinones was not pursued further to be bioactive markers. On the other hand, salvianolic acid B is water soluble, and it could be extracted and determined in Danshen 3497 aqueous extract (5.24%).

3.4. Step 4: literature search for biological activities of the marker

After identifying the key indication of Danshen and proving that

Table 1
Amount of "cryptotanshinone + tanshinone I + tanshinone IIA" and "salvianolic acid B" in the raw herb of different Danshen samples.

Danshen samples voucher specimen numbers	Cryptotanshinone + Tanshinone I + Tanshinone IIA (CP standard: ≥ 0.250%)	Salvianolic acid B (CP standard: ≥ 3.000%)
3497	0.152%	4.751%
3501	0.108%	1.010%
3502	0.118%	2.824%
3535	0.104%	1.556%
3536	0.086%	2.358%
3537	0.072%	0.888%

Table 2
Amount of chemical markers in Danshen 3497 aqueous extract.

Chemical markers	Amount in Danshen 3497 aqueous extract (w/w)
Cryptotanshinone	<0.0014%
Tanshinone I	0.0036%
Tanshinone IIA	<0.0014%
Salvianolic acid B	5.2410%

salvianolic acid B is a water-extractable chemical marker, the next step is to figure out whether salvianolic acid B possesses any pharmacological activities that are related to the cardiovascular tonic effect of Danshen.

Using updated and reliable databases such as PubMed (MEDLINE), Web of Science, and China Academic Journals Full-text Database, a thorough literature search for salvianolic acid B was conducted. Those scientific papers pertaining to cardiovascular tonic effect of salvianolic acid B were shortlisted and they were sorted according to their evidence levels. Moreover, route of administration, dosage and major findings were listed out in Table S1 (Supplementary Material). It has been reported that salvianolic acid B exhibits antiplatelet effects in patients with acute coronary syndrome (ACS). Also, it can protect the heart against ischemia-reperfusion injury and myocardial infarction as demonstrated in different animal models.

Although the cardiovascular tonic effects of salvianolic acid B are documented, it is still unclear whether its reported effective doses can be correlated with CP's suggested daily dose of the raw herb. Therefore, calculations should be performed in order to estimate the "theoretical dose" of salvianolic acid B that should be available when Danshen at CP's suggested daily dose is used.

3.4.1. "Theoretical dose" of salvianolic acid B calculated from CP-recommended daily dose of Danshen

Recommended daily dose of Danshen in CP = 10–15 g

Yield of water extraction of Danshen 3497 (w/w) = 61.42%

Yield of salvianolic acid B in water extract as determined by HPLC (w/w) = 5.241%

Content of salvianolic acid B in high daily dose of Danshen = 15 g × 61.42% x 5.241% = 483 mg

Human Equivalent Dose (HED) = 483 mg/ 60 kg = 8.05 mg/kg

Mouse dose of salvianolic acid B = 8.05 mg/kg x 12.3 = 99 mg/kg

Rat dose of salvianolic acid B = 8.05 mg/kg x 6.2 = 50 mg/kg

As shown above, if 15 g of Danshen raw herb is extracted by water and consumed, the theoretical amount of salvianolic acid B available will be 8.05 mg/kg for human or 50 mg/kg for rat. As reported by Qiao and colleagues, oral feeding of salvianolic acid B at 55 mg/kg into Wistar rats was effective to act against myocardial ischemia-reperfusion injury.[3] Moreover, Huang et al. demonstrated that intragastric administration of salvianolic acid B at 50 mg/kg could relieve acute myocardial ischemia in Sprague Dawley (SD) rats.[4] Wu's group also showed that intragastric administration of salvianolic acid B at dosages below 50 mg/kg for 35 days was able to treat hypertrophic cardiomyopathy in rats.[5] Therefore, the "theoretical dose" of salvianolic acid B (50 mg/kg for rat) was proven effective for cardiovascular diseases models in preclinical studies.

3.5. Step 5: oral bioavailability of salvianolic acid B

Pharmacokinetic studies conducted by Wu's group[6] and Zhou's group[7] demonstrated that the oral bioavailability of salvianolic acid B in SD rats was around 2.3–3.9%. Although its intestinal permeability is quite limited, salvianolic acid B can reach the systemic circulation after administration via oral route.

3.6. Summary for Salviae Miltiorrhizae Radix et Rhizoma (Danshen)

As recorded in CP, there are two kinds of chemical marker for quantification assay of Danshen. They are tanshinones and salvianolic acid B. As tanshinones cannot be extracted into aqueous extract of Danshen, they are not good candidates for bioactive marker of Danshen. On the other hand, salvianolic acid B is water soluble and can be extracted into aqueous extract of Danshen, proven effective in acting against cardiovascular diseases, and also orally bioavailable. Therefore, salvianolic acid B can be upgraded to a bioactive marker for Danshen in treating cardiovascular diseases.

4. Discussion

In the present study, we proposed a methodology to verify the biological activities of chemical markers so as to upgrade them to bioactive markers for quality control of pCm. While many scientists devote their research efforts to investigation on effectiveness or action mechanisms of the markers, we propose linking biological effect of the markers with pCm quality together. Through a series of work on extraction, quantification, literature search and animal experiments, the water extractability, biological effects at theoretical dose and oral bioavailability of the candidate chemical markers are revealed and linked steps by steps. In the course of literature search, systematic review or meta-analysis of all relevant randomized controlled double blind trials (RCT) represents the highest level of evidence. If clinical data are not available, results from animal experiments can be used. Since *in vitro* assays involve only direct cell-drug interaction but not taking absorption and systemic metabolism into account, data from cell studies are not powerful enough to prove the pharmacological effects of the chemical markers for verification in our methodology. Therefore, the biological activity of the chemical marker at theoretical dose should be investigated, at least, using animal models. Without considering its

amount in aqueous extract of TCM or its theoretical dose, it may be a hasty decision to conclude that a chemical marker exhibits certain biological activities even though there are numerous scientific papers reporting its pharmacological actions *in vitro*.

A new concept of "quality marker" (Q-marker) for quality assessment of Chinese medicines and their products was firstly proposed by Academician Liu Changxiao in 2016.[8,9] Liu's group commented that the quality control system adopted in CP is in general chemical marker-oriented and raised the concerns about the doubtful relationship between the chemical markers and the holistic efficacy.[10] Therefore, they developed a multi-disciplinary platform comprising analytical chemistry, bionics, chemometrics, pharmacology and pharmacodynamics to obtain bioactive chemical markers as the Q-markers and applied these Q-markers in Chinese medicine quality assurance.[11,12] While the key issue we wanted to address in the current study was similar to that of Liu's, we were more focusing on the importance of theoretical dose and emphasizing on the proof of *in vivo* pharmacological effect of the chemical marker.

Using Danshen as an example, we demonstrated how to promote one of its chemical markers into a bioactive marker. The case of salvianolic acid B is quite straightforward. From raw herb purchasing, water extraction, amount quantification to literature search for biological activity and oral bioavailability, all the relevant information of salvianolic acid B can be easily obtained through in-house experiments or retrieved from databases. However, the verification route may be rough in some cases, for instance, when the efficacy of the candidate bioactive marker at theoretical dose has not been scientifically proven or reported. This is the situation found in 6-gingerol which is the CP-suggested chemical marker for Zingiberis Rhizoma (乾薑, gān jiāng). We found that the key indication of Ganjiang is for alleviating epigastric pain, vomiting and diarrhea. 6-Gingerol could be detected in aqueous extract of Ganjiang [0.468% (w/w)] and its theoretical dose was calculated to be 2.5 mg/kg for mice. Although it has been shown that 6-gingerol at 100 or 200 mg/kg was effective in suppressing experimental dextran sulphate sodium (DSS)-induced ulcerative colitis in mice,[13] its potency at 2.5 mg/kg was still unknown. Therefore, in our laboratory, we repeated Ajayi's experiment[13] using low dosages (2.5 and 12.5 mg/kg) of 6-gingerol. Results showed that 6-gingerol treatment (2.5 or 12.5 mg/kg) could significantly restore the levels of superoxide dismutase, glutathione peroxidase and IL-1 beta in mice with DSS-induced colitis and such activities were comparable to 50, 100 or 200 mg/kg 6-gingerol.[13] However, low doses (2.5 or 12.5 mg/kg) of 6-gingerol did not significantly alter the levels of glutathione and malondialdehyde nor the activity of myeloperoxidase as high doses (50, 100 or 200 mg/kg) did. These data suggested that 6-gingerol at theoretical dose exhibited mild inhibitory effects on colonic inflammation. As a result, we concluded that 6-gingerol may act as bioactive marker for Ganjiang in treating ulcerative colitis. However, its effective dose is higher than the CP-recommended dose. The Ganjiang example illustrated that if the required information could not be obtained from literature, we then need to carry out the relevant experiments to generate the data.

The verified bioactive markers serve two purposes. On one hand, they can be used as references by TCM industries regarding the quality control of their pCm products. Instead of chemical markers, bioactive markers are more likely the indicators to reflect the pharmacological activities of the pCm products. The higher the amount of bioactive markers, the stronger the therapeutic effects (provided that the bioactive markers do not exert any toxicities). Accordingly, pCm manufacturers may refine the formulation or the extraction process so as to increase the amount of the bioactive markers in their products. Afterwards, the levels of bioactive markers in pCm products can be measured to check for batch-to-batch quality consistency. On the other hand, if those chemical markers can be upgraded to bioactive markers, it can be recorded as additional information in pharmacopoeias for regulatory purpose. We here propose to add a 'bracket' behind the bioactive marker to indicate its pharmacological activities. For example, "salvianolic acid B (cardiovascular tonic)" under the Danshen monograph indicates that salvianolic acid B is the active compound of Danshen in treating cardiovascular diseases. This may provide more insights for researchers in future drug development.

In conclusion, a systematic and comprehensive methodology has been established. It is feasible to use the proposed methodology to verify whether the chemical marker of a specific herb listed in the Chinese Pharmacopoeia can be promoted to a bioactive marker with certain efficacy in treating a particular disease. The bioactive marker identified thereof can then be used for biological standardization of Chinese medicines or proprietary Chinese medicines.

Conflicts of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2018.09.003.

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