Front. Med. 2015, 9(4): 457–467
DOI 10.1007/s11684-015-0417-8

RESEARCH ARTICLE

Evidence chain-based causality identification in herb-induced liver injury: exemplification of a well-known liver-restorative herb *Polygonum multiflorum*

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Abstract **Herbal medicines have recently been recognized as the second most common cause of drug-induced liver injury (DILI) in the United States. However, reliable methods to identify the DILI causality of some herbs, such as Heshouwu (dried root of *Polygonum multiflorum*), remain lacking. In this study, a total of 12 307 inpatients with liver dysfunction and 147 literature-reported cases of Heshouwu DILI were screened. A general algorithm indicated that only 22.5% (9/40) and 30.6% (45/147) of all hospitalization and literature case reports, respectively, demonstrate the high probability of DILI causality of Heshouwu. By contrast, 95% (19/20) of all cases prospectively investigated by pharmacognosy, phytochemistry, and metabolomic tests exhibited highly probable causality, including a patient who was previously incorrectly attributed and a case that was excluded from Heshouwu causality by pharmacognostic evidence. Toxin (heavy metals, pesticides, and mycotoxins) contamination was also excluded from Heshouwu DILI causality. The objectivity of these screening methods for Heshouwu DILI diagnosis addresses safety concerns regarding stilbene-containing herbal medicines and dietary supplements.**

Keywords *Polygonum multiflorum*; Chinese herbal medicine; drug-induced liver injury; pharmacognosy; metabolomics; stilbene

Introduction

As the global consumption of traditional Chinese medicines (TCM) and other herbal healthcare products has recently and rapidly expanded, reports on TCM-induced adverse reactions, especially drug-induced liver injury (DILI), have also steadily increased. The potential toxicity of TCMs and other herbal medicines is frequently neglected because natural herbal medicines are typically believed to exhibit beneficial health effects, including the ability to extend lifespans, promote good health, and cure diseases. Thus, herbal medicines are typically considered safe materials. However, cases of DILI attributed to TCMs and other herbal medicines are increasingly being reported. According to LiverTox, a website established by the United States National Library of Medicine, DILI is associated with over 30 types of herbal medicines, including Heshouwu (*Polygonum multiflorum* Thunb.), Leigongteng (*Tripterygium wilfordii* Hook. f.), Chaihu (*Bupleurum chinense* DC., a constituent of Sho-saiko-to), Yinyanghuo (*Epimedium brevicornu* Maxim.), milk thistle (*Silybum marianum*), senna (*Cassia acutifolio*), and

Received May 22, 2015; accepted July 7, 2015

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cascara (*Rhamnus purshiana*). According to the American College of Gastroenterology (ACG) [1], herbal medicines are recognized as the second most common cause of DILI in the United States.

DILI diagnosis is challenged by the lack of a definitive diagnostic test. The methods most commonly used to quantify the strength of the association between liver injury and the implicated medications include the Roussel Uclaf Causality Assessment Method (RUCAM) [2], the Maria and Victorino scale [3], and the structured expert opinion process proposed by the US Drug-Induced Liver Injury Network (DILIN) [4]. However, a retrospective investigation by the DILIN revealed rather low reliabilities when using the RUCAM scale, which features test-retest and inter-rater scores of 0.54 and 0.45, respectively [5]. Complete identification of all medications is a fundamental element in determining DILI causality. However, because of the increasing polypharmacy contributed by multiple providers, obtaining a high-level accuracy can be difficult when solely relying on patient recall [6]. This problem is compounded in cases of herbal medicine-related DILI because herbal medicines are generally used in polypharmacy and sometimes combined with western medicines. Even worse, according to the World Health Organization, herbal products available to consumers in the marketplace may be contaminated with hazardous materials (e.g., heavy metals, mycotoxins, and pesticides) or substituted with alternative plant species and fillers that are not listed on their labels [7]. Without exclusion of these confounding factors, misidentification of causality may occur and lead to confounding of the scientific diagnosis of herbal DILI, overdiagnosis, and overreporting [8]. Significant controversy surrounds the confounding variables of suspected hepatotoxicity of black cohosh [9,10] and *Pelargonium sidoides* [11]. Causality assessment quality is more important than the quantity of cases, as noted by Teschke [12]. An exclusion algorithm is always included in universal causality assessment methods (e.g., the RUCAM scale and ACG clinical guideline) to exclude other causes of liver injury. However, identification of the real source of herbal DILI has been minimally addressed.

We present herein a typical example of DILI causality determination of the popular herb Heshouwu (dried root of *P. multiflorum,* family Polygonaceae), which has been used for thousands of years in China as a tonic for liver and kidney conditioning. This herb has also recently been used to treat Alzheimer's disease, Parkinson's disease, hyperlipidemia, and liver injury. The most popular uses of Heshouwu include prevention of hair loss and graying, and its most attractive benefit is aging prevention and lifespan extension. The major component in Heshouwu, 2,3,5,4'-tetrahydroxy *trans*-stilbene-2-*O*-β-glucoside (TSG), is a structural analog of resveratrol (3,5,4'-trihydroxy *trans*-stilbene), which can prolong the lifespan of model organisms by activating Sirtuin 1 (Sirt1) [13,14], thereby resulting in autophagy stimulation [15]. However, Heshouwu-related DILIs have been reported in Hong Kong in 1996 and were announced by the Medicines and Healthcare Products Regulatory Agency in 2006. The increasing number of DILI reports related to Heshouwu have led to doubts regarding the usefulness and safety of the herb. Some researchers believe that Heshouwu is effective and safe because it is traditionally considered to exert liver-restorative effects on the TCM system and its major component, TSG, has been proven to exhibit hepatoprotective activity in modern experiments. Others practitioners, however, doubt the herb's safety based on DILI case reports. Nevertheless, the published case reports regarding Heshouwu leave room for debate because of the absence of excluding confounding DILI causality factors (e.g., heavy metals, pesticides, and fungal toxin contamination in the herb) and the lack of pharmacognostic identification of patients' digested materials.

In this paper, we attempt to identify the causality of DILI suspected to result from Heshouwu use by considering additional translational laboratory tests, such as pharmacognosy, phytochemistry, and metabolomics, to complete the evidence chain [16] based on general RUCAM assessment (Fig. 1 and Table 1). We propose that this translational research will dispel disputes regarding Heshouwu-caused DILI and bring attention to the scientific diagnosis and causality identification of herbal medicine-induced liver injury.

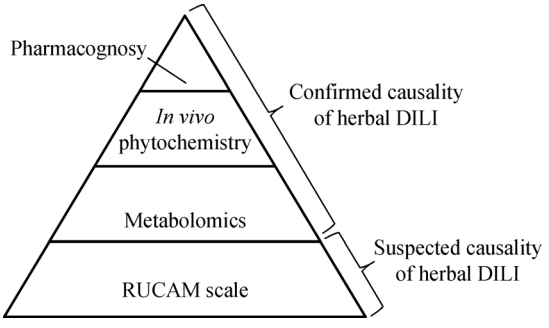

Fig. 1 Stepwise strategy for causality assessment of herbal DILI based on RUCAM.

Materials and methods

Patients

Patients (n = 20) diagnosed with Heshouwu-caused DILI or known to be using hepatotoxic drugs were enrolled in this study. Age- and sex-matched cases of autoimmune hepatitis (AIH) (n = 12) and hepatitis B virus (HBV) infection (n = 12) were enrolled in the study as control

groups. Diagnosis of the enrolled patients with DILI, AIH, and HBV was performed according to international codified criteria. The study protocol was approved by the ethics committee of the 302 Military Hospital, and written informed consent was obtained from each subject. Blood samples were collected from the patients during the first routine clinical examination of their hospitalization.

Metabolomic analysis and *in vivo* chemical identification

All of the blood plasma samples obtained from the patients were stored at −80 °C until analysis. Prior to analysis, the samples were thawed, diluted with acetonitrile at a ratio of 1:3 (v/v), and then centrifuged at 10 000 rpm for 10 min at 4 °C. The supernatant was filtered through a syringe filter (0.22 μm), and 3 μl of the supernatant was injected into a liquid chromatograph coupled with a high-resolution hybrid quadrupole time-of-flight mass spectrometer (LC-QTOF MS). The blood metabolomics of suspected Heshouwu DILI patients was determined [17] and *in vivo* metabolites were identified by LC-QTOF MS [18].

Pharmacognostic analyses

Microscopic powder characteristics were studied according to standard methods [19]. DNA barcoding was performed [20]. Phytochemistry analysis was performed by using an Agilent iFunnel 6550 QTOF mass spectrometer [17,21].

Heavy metal, mycotoxin, and pesticide determination

Heavy metals (Cu, As, Cd, Hg, and Pb) in the patients' ingested residual drugs were detected by inductively coupled plasma mass spectrometry according to the Chinese Pharmacopeia [22]. Mycotoxins (AFB1, AFB2, AFG1, AFG2, and OTA) were detected by LC-MS (AB SCIEX 5500 QTRAP mass spectrometer) according to the Chinese Pharmacopeia [22]. Sixteen organophosphates and 26 organochlorines were detected by a gas chromatograph in tandem with a mass spectrometer (Agilent 7890-5977 GC-MS) [23].

Results

Literature research on Heshouwu DILI

Most of the literature-reported cases of Heshouwu DILI were not assessed by using internationally adopted scaling methods. Thus, we reassessed the causality of these cases by using the RUCAM scale and found that only 30.6% of the reported cases indicated highly probable (RUCAM scale points > 8) causality by Heshouwu usage; another 58.5% of the reported cases showed a possible (RUCAM points > 3 and < 5) level of confidence (Table S1). Over half of the reported cases did not record details of the diagnosis or exclusion process.

Clinical research on Heshouwu DILI

Hospitalization records of 302 Military Hospital for the past 10 years showed 40 DILI cases suspected to have resulted from Heshouwu usage, accounting for 1.67% of the total 2391 DILI cases reported. Suspected cases were assessed by using the RUCAM scale, and 22.5% of the enrolled cases showed highly probable (RUCAM points $>$ 8) causality by Heshouwu usage; another 40.0% of the cases exhibited possible causality (Table S2). The most common confounding factor in diagnosis was the concurrent positive results of autoimmune antibodies (Table S3). The clinical features of Heshouwu-related DILI are summarized in Table S3. The median intake period of Heshouwu before liver injury was 1.5 months, and the median dosage was 13.8 g/d. The average hospitalization period of Heshouwu DILI cases was 35 days. After drug withdrawal and continuous treatment, 38 cases (95.0%) were healed by the time of the follow-up examination. One case (2.5%) progressed to liver failure with subsequent liver transplantation, and one patient (2.5%) died. Three cases of rechallenge were recorded when the patients were discharged from the hospital after recovery. In total, 13 cases were biopsied, and the most common pathological features observed were hepatocellular necrosis, lobular portal inflammation, enlargement and congestion in the sinusoid, as well as pigment granules phagocytized by Kupffer cells in the sinusoids (Table S3 and Fig. 2A). Except for the incidence of Kupffer cell-phagocytized pigment granules in the sinusoids, which was frequently observed in Heshouwu DILI (9/13), no special features with respect to sex, age, type of liver injury, or Heshouwu DILI biochemistry or histopathology were noted that could be used to discriminate the cause from other drugs.

Metabolomic analysis of Heshouwu DILI

Twenty cases of suspected Heshouwu DILI were prospectively analyzed (Table S4). Using metabolomic analysis of plasma, the suspected Heshouwu DILI cases were differentially diagnosed from other liver diseases, such as AIH and acute-on-chronic liver failure of HBV, and clustered with other DILI cases induced by other known drugs in the PLS-DA plot (Fig. 2B). Thus, the enrolled Heshouwu cases exhibited metabolomic features more similar to other DILI cases than to AIH and HBV. In these enrolled Heshouwu cases, eight cases were previously confounded by diagnosis as DILI or AIH because of a positive autoimmune antibody test on routine clinical

Table 1 Evidence Chain-based Causality Identification Algorithm (ECCIA) for herbal medicine-induced liver injury on the bases of RUCAM

Assessments	Methods/items	Score
	RUCAM	
1–7	Time of onset / Course / Risk factors / Concomitant drug(s) / Nondrug causes / Previous information / Rechallenge	−9 – 10
	ECCIA	
8	Metabolomics	
	Suggestive to DILI	+ 3
	Not suggestive to DILI	0
	Suggestive to AIH	−3
9	Pharmacognosy*	
	Authentic	3
	Not applicable	0
	Fraudulent or contaminated with toxins	−3
10	*In vivo* phytochemistry	
	Presence of characteristic metabolites	+ 3
	None presence	0

* At least two methods are recommended in pharmacognostic identification because not all methods are applicable to the test samples. The recommended methods include DNA barcoding, microscopy, and phytochemistry.

examination. Autoimmune liver disease was excluded for these eight cases based on the results of metabolomic differentiation. Reexamination after treatment indicated negative results of autoimmune antibodies, which confirms the diagnosis of DILI and the exclusion of AIH. The screened metabolomic biomarkers of high relativity comprised lysophosphatidylcholines, phosphatidylcholines, prostaglandins, and fatty acids (Table S4).

In vivo phytochemistry identification for Heshouwu DILI

Some metabolites derived from Heshouwu phytochemicals were detected by LC-QTOF MS of some of the patients' plasma and urine samples. The most commonly detected metabolite was glucuronic acid-conjugated phase II metabolite of TSG (Fig. 2C and 2D, $[M-H]^- m/z = 581$), as well emodin glucuronide (Fig. 2E and 2F, $[M-H]^- m/z = 445$). The identified metabolites are listed in Table S5. Identification of the metabolites provides key information to confirm digestion of Heshouwu in patients when the drugs were administered as a powdered mixture of unknown formulae and composition. Considering that anthraquinone metabolites are also associated with rhubarb and other herbs, TSG glucuronide is more informative than other metabolites in terms of the *in vivo* phytochemistry identification of Heshouwu DILI.

Pharmacognostic identification for Heshouwu DILI

Heshouwu pharmacognosy is historically unclear, and its origin has been incorrectly recorded in the well-known TCM classic, *Compendium of Materia Medica* (《本草纲目》), written by Shi-zhen Li in the Ming Dynasty of China. This text describes two origins, a male and a female Heshouwu (Fig. 3A). According to modern botany, Heshouwu has only one origin, *P. multiflorum* Thunb. (Fig. 3B), which is not a dioecious plant. The "male" form is verified to be *P. multiflorum*, and the "female" form is believed to be *Cynanchum bungei* or its affiliates. A fraudulent substitute of Heshouwu, the humanoid Heshouwu with male or female appearance, has frequently appeared in China in recent years. These frauds are identified as the root of *Musa basjioo* Sied. *et* Zucc. and are sculptured or cultivated in molds to form a man's or woman's appearance (Fig. 3C).

Using pharmacognosy to identify the six batches of drug materials collected from patients with suspected Heshouwu DILI, five batches were identified as authentic Heshouwu, and one batch was determined to be the fraudulent substitute. The patient who digested the fraudulent drug was excluded from causality by Heshouwu usage. The authentic Heshouwu samples were further identified as three batches of crude herb and two batches of processed herb by DNA barcoding (Fig. 3 D), microscopy (Fig. 3E), and LC-MS (Fig. 3F). Both crude and processed Heshouwu yielded three distinct characteristic components (Fig. 3G): TSG (chromatographic peak 1), emodin 8-*O* glucoside (peak 2), and emodin (peak 3). All chemicals identified from the clinically collected Heshouwu samples are listed in Table S6.

Exclusion of toxin contamination in Heshouwu

Determination of heavy metals, mycotoxins, and pesticides in the clinically collected Heshouwu samples revealed no

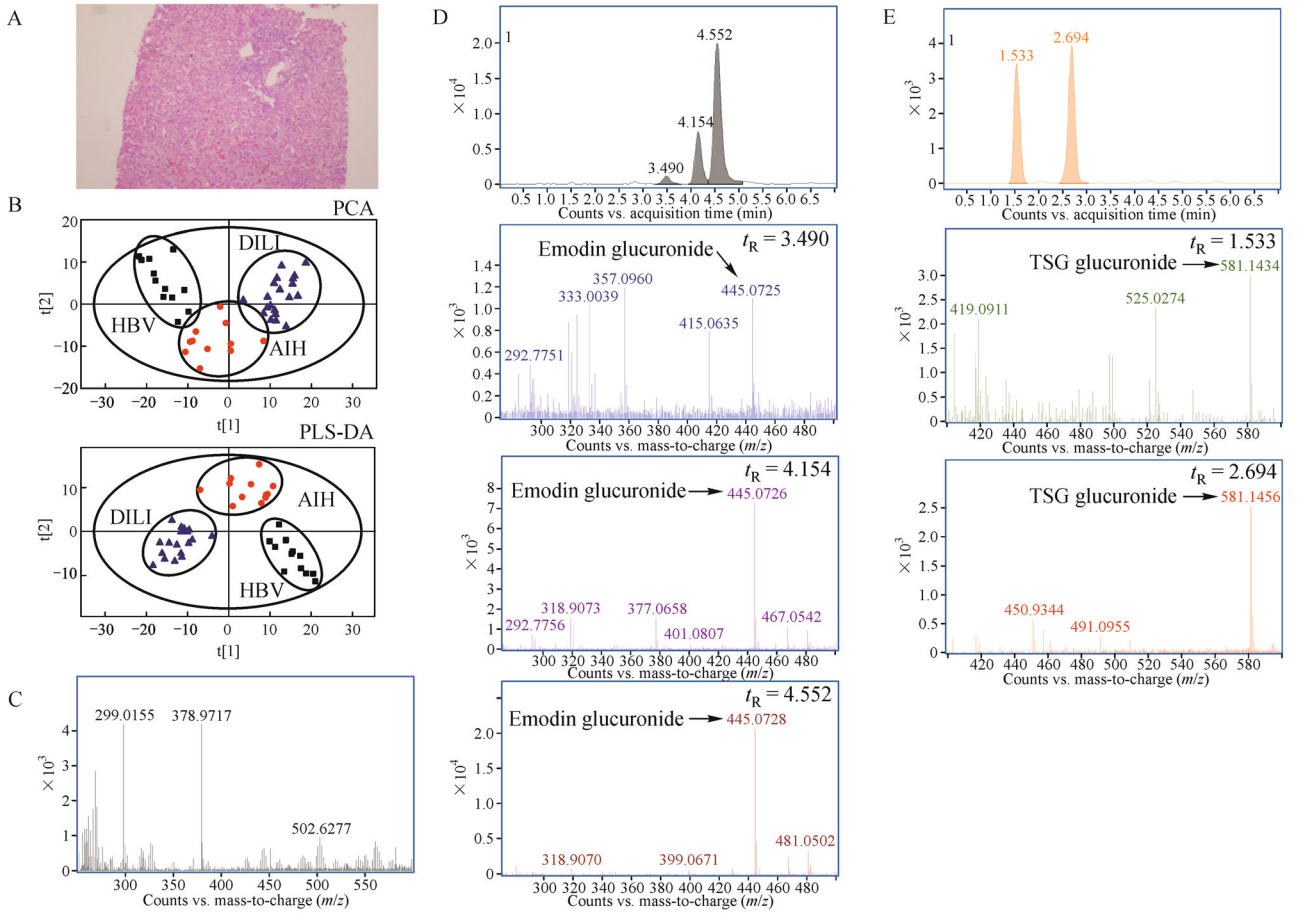

Fig. 2 Liver biopsy, metabolomic, and *in vivo* chemical identification analyses of Heshouwu-induced liver injury in patients. (A) Representative liver biopsy of a Heshouwu DILI patient with hepatocellular necrosis, lobular portal inflammation, and enlargement and congestion in the sinusoid. (B) PCA- and PLS-DA-based metabolomic differentiation of Heshouwu DILI from AIH and HBV. The Heshouwu DILI cases were separated clearly from AIH and clustered with other DILI cases induced by other known drugs. Blue triangles represent "DILI," red circles represent "AIH," and black boxes represent "HBV." (C) Background mass spectra of sera. (D) Chromatogram and mass spectra of emodin glucuronide in Heshouwu in patients' sera. (E) Chromatogram and mass spectra of TSG glucuronide in Heshouwu in patients' sera.

compounds exceeding the safety limits specified by the Chinese Pharmacopeia or European Union standards (Tables S7 – S9). The possibility that toxin contamination caused liver injury was excluded.

Evidence Chain-Based Causality Identification

An identification and exclusion flow diagram based on the Evidence Chain-Based Causality Identification Algorithm (ECCIA) is depicted in Fig. 4. In total, 12 307 patients who were hospitalized for liver dysfunction cases and 147 literature-reported cases of Heshouwu DILI were investigated. Forty cases of suspected Heshouwu DILI were screened from 2391 hospitalized DILI cases. By using the RUCAM general algorithm, only 22.5% (9/40) and 30.6% (45/147) of the cases achieved highly probable causality ($>$ 8 points) among the hospitalization and literature case reports, respectively. By contrast, 95% (19/20) of all prospectively investigated cases showed highly probable causality via ECCIA.

One of the most common confounding factors in RUCAM and the current guidelines is the overlap between DILI and autoimmune liver diseases. Approximately 40% (8/20) of the cases studied exhibited positive serum autoimmune antibodies, and 15.4% (2/13) of the cases exhibited histological features common to both DILI and AIH. Such overlaps restrict the valid diagnoses by RUCAM, although they can be differentiated by using a metabolomic biomarker assay.

Representative case of uncovering Heshouwu causality and rechallenge

One representative DILI case was diagnosed by the patient's physician; however, the DILI was not ascribed to a suspected drug because none of the drugs reported by

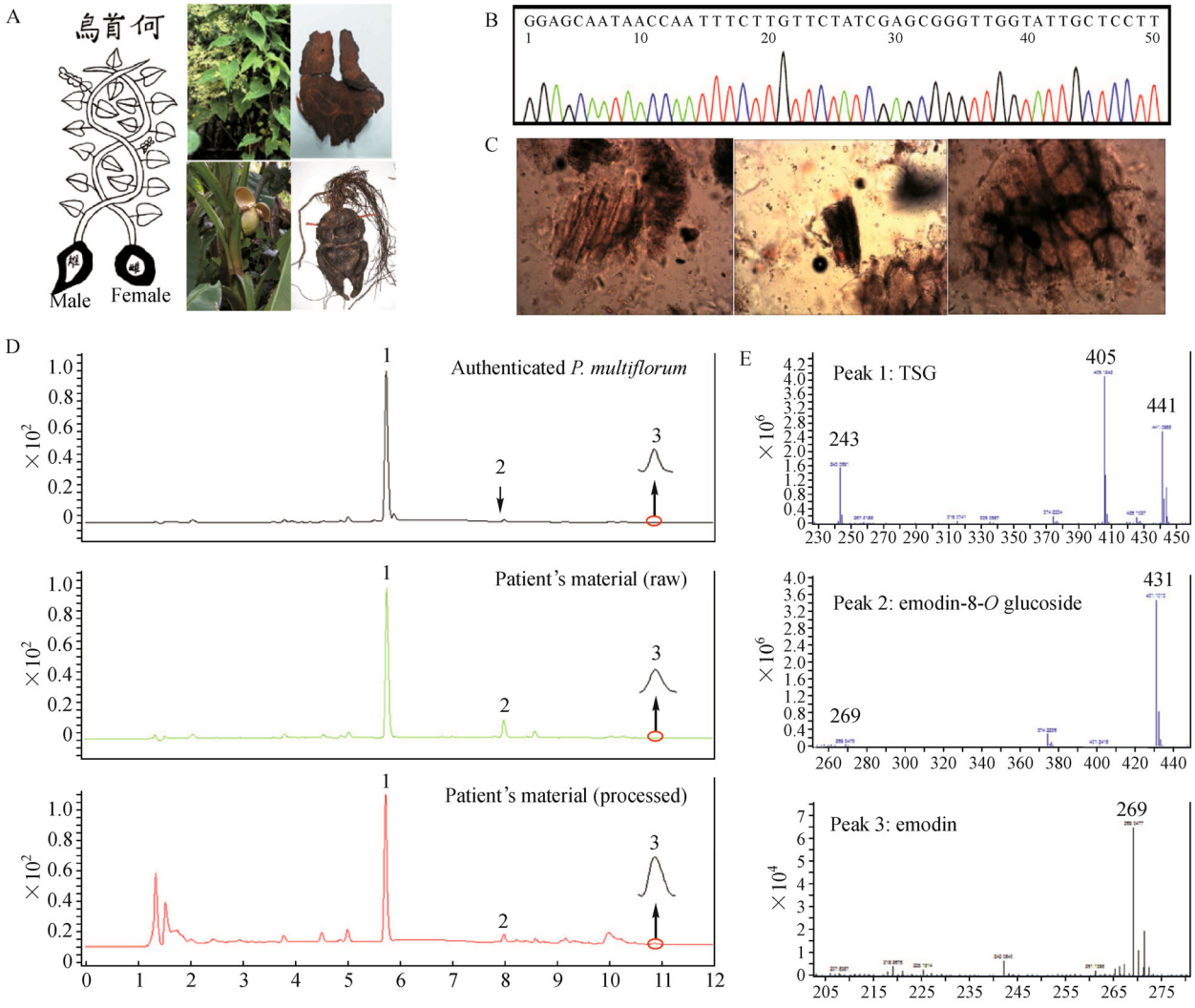

Fig. 3 Pharmacognostic authentication of the digested materials of patients. (A) Photographs of ancient record and the plant and sliced root of *P. multiflorum*. The left photograph describes the "male" and "female" forms of Heshouwu in the *Compendium of Materia Medica*, which was written by Shi-zhen Li in the Ming Dynasty of China. The top right photographs present the plant and sliced root of *P. multiflorum* used as authenticated Heshouwu in China. The lower right photograph shows the plant and sculptured root of *Musa basjioo* Sied. *et* Zucc., which is misused as fraudulent Heshouwu. (B) DNA barcoding authentication shows the *P. multiflorum* species in the patient's digested material. (C) Representative microscopic photographs of the patient's digested material show the characteristic features of heat-prepared *P. multiflorum*, e.g., fiber cells, brown cells, and vessel cells. (D) HPLC fingerprints show similar characteristic peaks between the patients' digested materials and authenticated *P. multiflorum*. Characteristic peaks were identified by the reference substances and mass spectrometry. (E) Mass spectra of the characteristic peaks.

the patient were known to cause livery injury. The patient's initial biochemical panel was as follows: ALT (282 U/L), AST (189 U/L), GGT (95 U/L), AP (128 U/L), total bile acids (68 μmol/L), direct bilirubin (10.7 μmol/L), and total bilirubin (22 μmol/L). Viral and autoimmune liver diseases were excluded. Abdominal ultrasound, CT, and MRI examinations revealed diffused liver parenchyma injury. Liver biopsy demonstrated drug- or toxin-induced chronic hepatitis. *In vivo* phytochemistry analysis identified specific Heshouwu-derived metabolites in the patient's plasma and urine (Fig. 2C – 2F). Thus, we inquired about the patient's use of Heshouwu medication. The patient admitted this oversight and explained that he had always thought of Heshouwu as a food rather than a drug. The drug residue formerly ingested was identified as *P. multiflorum* by pharmacognostic analyses.

This patient was particularly noteworthy because he developed liver injury upon rechallenge with *P. multiflorum* after discharge from the hospital. The patient received an herbal patent medicine called *Heluo Shugan*, which also includes *Heshouwu*, for one month. The patient experienced fatigue throughout the treatment, had yellow

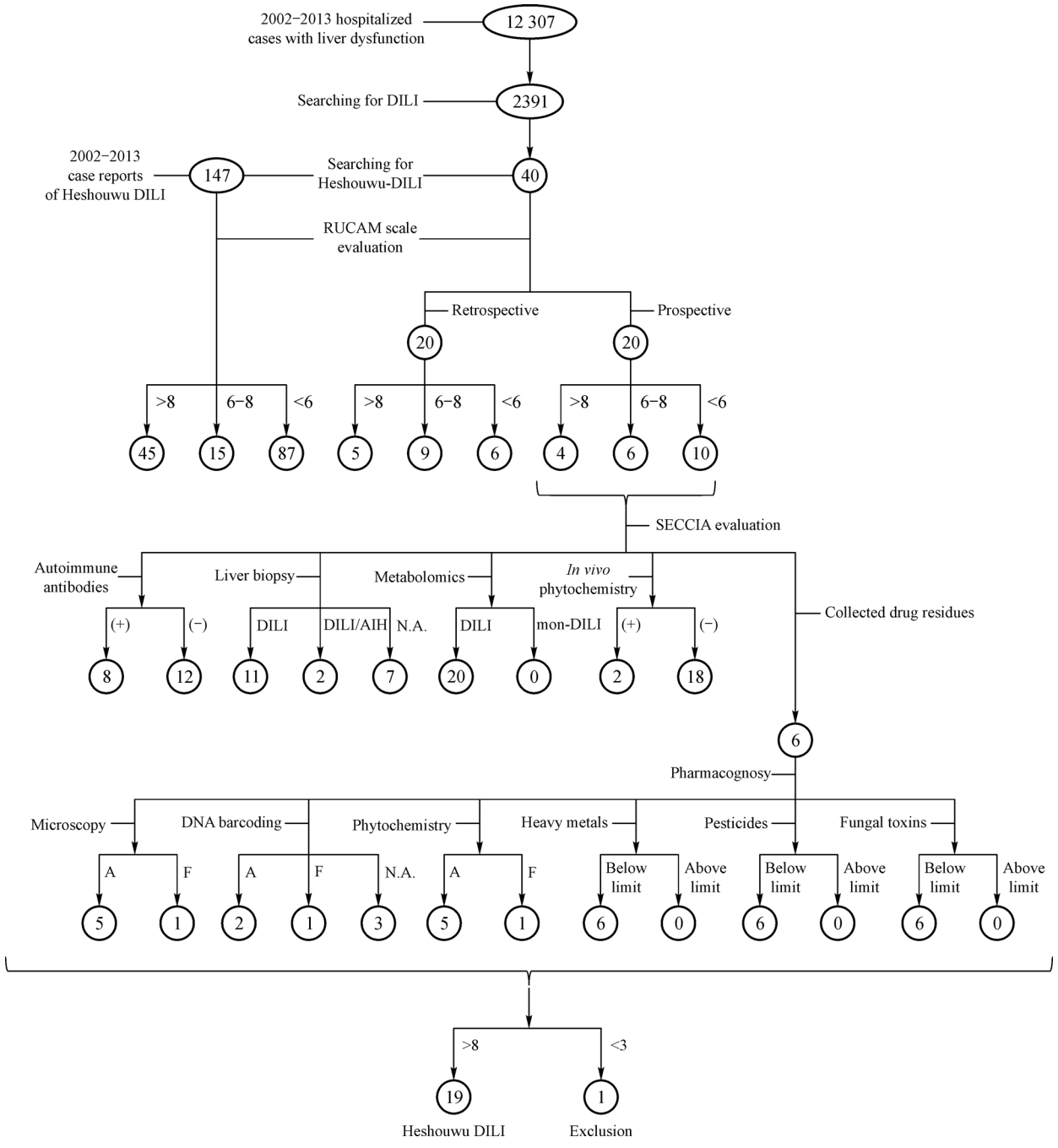

Fig. 4 Complete identification and exclusion flow of suspected Heshouwu DILI by ECCIA. N.A., not applicable; A, authentic; F, fraudulent.

urine, and showed increased ALT values exceeding 1000 U/L. After cessation of the TCM capsule and treatment in a local hospital for one month, the patient's liver enzymes steadily decreased to normal levels.

Representative case of exclusion of Heshouwu DILI

A 59-year-old woman developed pruritus and yellow urine followed by elevated liver enzymes and jaundice for over one month. The patient's initial biochemical panel was as follows: ALT (355 U/L), AST (415 U/L), GGT (461 U/L), AP (182 U/L), total bile acids (187 μmol/L), and direct (160.9 μmol/L) and total bilirubin (198.4 μmol/L). Viral and autoimmune liver diseases were excluded. Abdominal ultrasound, CT, and MRI examinations revealed diffused liver parenchyma injury. A liver biopsy suggested drug- or toxin-induced hepatitis with mixed inflammatory cell infiltration in the sinusoid. The patient claimed that she

received TCM powder named "*Heshouwu*" before hospitalization. Apparently, the direct cause of liver injury was Heshouwu. We detected residual drug powder but did not detect any *P. multiflorum* by DNA barcoding, microscopy, or LC-MS. The patient admitted that the TCM powder was not purchased from a legal medical institution. Therefore, DILI causality by Heshouwu was excluded in this case. The patient recalled taking antibiotics (amoxicillin and spiramycin) and nonsteroidal anti-inflammatory drugs (acetaminophen) to treat a "cold" for which she did not seek a professional medical opinion before her sickness. The patient recovered, and her liver enzymes normalized after four weeks of hospitalization and five weeks of follow-up medication.

Discussion

Heshouwu has been used for over 1000 years as a liver-restorative drug to treat liver diseases. However, its hepatotoxicity has always been argued by Chinese ethnopharmacologists, who assert that poor evidence has been published regarding DILI case reports. A major argument point is the insufficient exclusion of viral hepatitis E and AIH in the early literature. Acute hepatitis E accounts for up to 13% of all suspected DILI cases [24,25]. Confounding findings between DILI and AIH have been addressed by the literature [26]. One of the most confounding factors in RUCAM and current guidelines is the overlap between DILI and autoimmune liver diseases, which can be differentiated by a metabolomic biomarker assay. Metabolomics can provide effective differentiation between DILI and AIH [27]. We noted that a considerable number of Heshouwu-related case reports did not record the detailed process of exclusion, especially for hepatitis E and AIH, thereby restricting the accuracy of the causality assessment of Heshouwu DILI. In this paper, we evaluated 40 suspected Heshouwu DILI cases from 2391 hospitalized DILI patients. Twenty cases were prospectively investigated by comprehensive exclusion and diagnosis (Fig. 4), and 10 cases revealed only possible (RUCAM scale 3–5 points) causality because of difficulties encountered in setting a clear Heshouwu DILI diagnosis. The most common confounding factor was the concurrence of autoimmune antibodies in eight patients, although hepatitis E was excluded in all cases. Seven patients out of 20 cases did not undergo biopsies to attain histopathological evidence; two patients exhibited some AIH-like histopathological characteristics, as well DILI features, and this result probably indicates a tendency of progression from DILI into AIH (Fig. 4). DILI diagnosis in these two cases was difficult to perform because of the absence of biopsies and the presence of positive autoimmune antibodies. A metabolomic study was therefore performed and results revealed that all 20 patients were clearly differentiated from AIH and HBV (the control group) but clustered with DILI controls induced by known drugs (Fig. 2B). This result provides confirmative evidence of the diagnosis of DILI in these patients, including those who failed to obtain a clear diagnosis based on routine clinical tests alone.

Another point of argument is the insufficient pharmacognostic identification of the patients' ingested drugs in early reports of Heshouwu DILI. In a high-throughput sequencing study, the common occurrence of non-declared constituents or fraudulent species in the tested TCM products in Australia was reported [28]. An insufficient pharmacognostic identification example involves the previously reported nephrotoxicity of the Chinese herbal medicine Mutong, which was recognized later as the incorrect substitution of Chuan Mutong (*Clematidis armandii*) by Guan Mutong (*Aristolochiae manshuriensis*) [29]. Thus, full identification of the real origin of suspected herbs in a causality assessment is critical, even if the diagnosis of DILI is confirmative. Patients may neglect to report Heshouwu in their medication history because of their lack of awareness regarding the risk of using this herb, as the aforementioned example indicated, leading to difficulties in clarifying causality. Microscopic authentication identified a sample of digested material from a patient as a fraudulent form of *P. multiflorum*; another sample was identified as the powder of the crude material of *P. multiflorum*, and several other samples were identified as heat-processed materials of *P. multiflorum*. Such crude materials were confirmed as *P. multiflorum* by DNA barcoding (Fig. 3D), and the fraudulent sample was further identified as *Tribulus terrestris*. The heat-processed material of *P. multiflorum* produced no valid results in the DNA barcoding test because of DNA degradation attributed to the heat and steam applied in the process. Through chemical identification, the fraudulent sample was eventually differentiated from the other samples (Fig. 4).

A third point of argument involves toxin contamination (e.g., heavy metals, mycotoxins, and pesticides). Toxin contamination is a serious concern in herbal DILI causality assessment because high concentrations of these toxins may also cause liver injury after prolonged exposure. Researchers who doubt the hepatotoxicity of Heshouwu often blame toxin contamination as the real cause of Heshouwu-related DILI. In this paper, the results reveal three samples with detected mycotoxins (AFB_1 and OTA), although mycotoxin levels were less than the upper European Union safe limit (Table S7). Heavy metals and pesticides were detected in six and three samples, respectively, but no samples exceed upper European Union safe limits (Tables S8 and S9). Thus, toxin contamination may be excluded from the causality of Heshouwu DILI.

Hepatotoxic chemicals attributed to Heshouwu DILI remain in dispute. Some scholars believe that

anthraquinones, e.g., emodin, are the major hepatotoxins in Heshouwu. However, other Polygonaceae family herbs, e. g., *Rheum palmatum* and *P. cuspidatum*, contain the same anthraquinones at 5- to 20-fold higher levels but are seldom associated with DILI reports. A pharmacokinetic report described the delayed elimination of emodin influenced by TSG; however, no toxicity data to assess such an interaction has been presented [30]. Although TSG is a type of phytoalexin produced by plants to kill pathogenic organisms, it is usually considered a safe compound, and its analog, resveratrol, is a trendy chemical with outstanding development prospects. Nevertheless, a recent report named TSG as the major hepatotoxic component in Heshouwu [31], and a known hepatotoxin, diethylstilbestrol, has a *trans*-stilbene structure similar to TSG. The cytotoxicity of *trans*-stilbene has been reported [32]. *trans*-Stilbene can be transformed into *cis*-stilbene [33], which seems to have a stronger cytotoxicity than *trans*-stilbene. Combretastatin A-4 (CA-4), a natural *cis*-stilbene, possesses intense cytotoxicity, which makes it one of the strongest inhibitors of tubulin [34]. The derived analog of CA-4 exhibits a very low half maximal inhibitory concentration (IC_{50}) of 13.9 nmol/L to tumor cells [35]. Because the *trans*-TSG in Heshouwu can also be transformed into *cis*-TSG [36], the hepatotoxicity of *cis*-TSG must be evaluated to clarify whether it is the major toxin attributed to Heshouwu DILI.

DILI diagnosis requires careful investigation and a comprehensive differential diagnosis to conclude a clear causality [6]. Diagnosing herbal medicine-associated DILI involves specific difficulties because of the complexity of herbal products, toxin contamination, and unknown formula compositions in some cases. No single causality identification approach is perfectly suitable for herbal DILI [37]. The most frequently used DILI causality analysis tool, the RUCAM scale, is limited in general clinical practice [4]. As an important RUCAM component, the agent's labeled reactions and previous publication of hepatotoxicity are required. Unfortunately, the quality of previous information regarding herbal medicines is usually insufficient to fulfill this RUCAM component, which in turn leads to causality underestimation. In addition, 30% – 40% of all patients do not disclose the use of herbal medicines to their physicians [38], which can lead to incomplete or incorrect diagnosis. The structured expert opinion process proposed by the DILIN can provide higher agreement rates and likelihood scores than RUCAM [4]. However, the five-grade scale system used by either the RUCAM or DILIN leaves an uncertainty of diagnosis in some cases with a low confidence of evidence (e.g., acquired probable or possible grade of causality). Under these circumstances, the need to gather additional evidence to complete the evidence chain is evident. In general, metabolomics or other biomarkers assays can provide additional exclusive evidence from confounding liver diseases, pharmacognostic identification will be helpful in discovering the fraudulent species or non-declared constituents, and *in vivo* phytochemistry identification will provide further confidence of evidence, which are summarized in the ECCIA. Such additional analysis are not always applicable because drug metabolites present a specific life time in the circulation, and the patient's digested drug materials are sometimes unavailable. Nevertheless, more evidence acquired through ECCIA can yield higher evidence confidence for diagnosing herbal DILI in practice.

In summary, it is important in clinical practice when physicians encounter low-confidence evidence during herbal DILI assessment. In this study, we either excluded the causality of fraudulent herbs or discovered the causality of Heshouwu in some cases that had been previously incorrectly attributed. Causality was determined using laboratory tests, which highlights the importance of microscopy, DNA barcoding, chemical identification, and other pharmacognostic approaches in herbal DILI causality assessment. We also affirmed the clinical and pharmacognostic evidence of Heshouwu-induced liver injury and excluded the causality of toxin (e.g., heavy metals, mycotoxins, and pesticides) contamination for the first time.

The risk of liver injury by Heshouwu has not been stressed in the clinical community as much as other widely recognized hepatotoxic herbs; thus, the herb requires considerably more attention in clinical diagnosis and regulatory administration. Historical TCM books accorded great emphasis on the necessity of processing to reduce Heshouwu toxicity, and the toxicity-attenuating effect of processing has been proven [39]. However, the traditional time-consuming processing method of steaming and solarization repeated over nine cycles has not been maintained and the current practice involves steaming for only several hours. Insufficient processing may increase the toxic risk of Heshouwu in clinical usage. In recent years, toxicity concerns regarding stilbenes have increased [40,41] especially when a phase 2 clinical trial of the resveratrol-based drug, SRT501, was suspended because 5 of the 24 patients who participated in this trial developed cast nephropathy and even renal failure [42]. The objectivity of Heshouwu-induced liver injury raises new concerns regarding the safety of stilbene-containing herbal medicines and food supplements.

Acknowledgements

This work was supported by the National Key Technology R&D Program (No. 2015ZX09501-004-001-008), the National TCM Industry Science and Technology Program (No. 201507004-04), the National Natural Science Foundation of China (Nos. 81373984, 81503350, and 81403126), and the Beijing Natural Science Foundation (No. 7152142). The authors wish to acknowledge

Jiyan Chen for providing photographs of fraudulent Heshouwu, Yonghe Zhang for determining the pesticides, and Dongping Xu for his suggestion regarding the scale table of ECCIA.

Compliance with ethics guidelines

Jiabo Wang, Zhijie Ma, Ming Niu, Yun Zhu, Qingsheng Liang, Yanling Zhao, Jingyuan Song, Zhaofang Bai, Yaming Zhang, Ping Zhang, Na Li, Yakun Meng, Qi Li, Lushan Qin, Guangju Teng, Junling Cao, Baosen Li, Shilin Chen, Yonggang Li, Zhengsheng Zou, Honghao Zhou, and Xiaohe Xiao declare that they have no conflict of interest. All procedures followed were in accordance with the ethical standards of the Ethics Committee of the 302 Military Hospital and the *Helsinki Declaration* of 1975, as revised in 2000 (5). Informed consent was obtained from all patients involved in this study.

Electronic Supplementary Material Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s11684-015-0417-8 and is accessible for authorized users.

References

1. Chalasani NP, Hayashi PH, Bonkovsky HL, Navarro VJ, Lee WM, Fontana RJ; Practice Parameters Committee of the American College of Gastroenterology. ACG Clinical Guideline: the diagnosis and management of idiosyncratic drug-induced liver injury. Am J Gastroenterol 2014; 109(7): 950–966, quiz 967
2. Danan G, Benichou C. Causality assessment of adverse reactions to drugs—I. A novel method based on the conclusions of international consensus meetings: application to drug-induced liver injuries. J Clin Epidemiol 1993; 46(11): 1323–1330
3. Maria VAJ, Victorino RMM. Development and validation of a clinical scale for the diagnosis of drug-induced hepatitis. Hepatology 1997; 26(3): 664–669
4. Rockey DC, Seeff LB, Rochon J, Freston J, Chalasani N, Bonacini M, Fontana RJ, Hayashi PH; US Drug-Induced Liver Injury Network. Causality assessment in drug-induced liver injury using a structured expert opinion process: comparison to the Roussel-Uclaf causality assessment method. Hepatology 2010; 51(6): 2117–2126
5. Rochon J, Protiva P, Seeff LB, Fontana RJ, Liangpunsakul S, Watkins PB, Davern T, McHutchison JG; Drug-Induced Liver Injury Network (DILIN). Reliability of the Roussel Uclaf Causality Assessment Method for assessing causality in drug-induced liver injury. Hepatology 2008; 48(4): 1175–1183
6. Barritt AS, Lee J, Hayashi PH. Detective work in drug-induced liver injury: sometimes it is all about interviewing the right witness. Clin Gastroenterol Hepato, 2010, 8(7): 635–637
7. De Smet PA. Herbal medicine in Europe—relaxing regulatory standards. N Engl J Med 2005; 352(12): 1176–1178
8. Teschke R, Eickhoff A, Wolff A, Frenzel C, Schulze J. Herbal hepatotoxicity and WHO global introspection method. Ann Hepatol 2013; 12(1): 11–21
9. Teschke R, Bahre R, Genthner A, Fuchs J, Schmidt-Taenzer W, Wolff A. Suspected black cohosh hepatotoxicity—challenges and pitfalls of causality assessment. Maturitas 2009; 63(4): 302–314
10. Teschke R. Black cohosh and suspected hepatotoxicity: inconsistencies, confounding variables, and prospective use of a diagnostic causality algorithm. A critical review. Menopause 2010; 17(2): 426–440
11. Teschke R, Frenzel C, Schulze J, Eickhoff A. Spontaneous reports of primarily suspected herbal hepatotoxicity by *Pelargonium sidoides*: was causality adequately ascertained? Regul Toxicol Pharmacol 2012; 63(1): 1–9
12. Teschke R, Schmidt-Taenzer W, Wolff A. USP suspected herbal hepatotoxicity: quality of causality assessment is more important than quantity of counted cases, not vice versa. Pharmocoepidemiol Drug Saf 2012; 21(3): 336–338
13. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA. Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. Nature 2003; 425(6954): 191–196
14. Hubbard BP, Gomes AP, Dai H, Li J, Case AW, Considine T, Riera TV, Lee JE, e SY, Lamming DW, Pentelute BL, Schuman ER, Stevens LA, Ling AJ, Armour SM, Michan S, Zhao H, Jiang Y, Sweitzer SM, Blum CA, Disch JS, Ng PY, Howitz KT, Rolo AP, Hamuro Y, Moss J, Perni RB, Ellis JL, Vlasuk GP, Sinclair DA. Evidence for a common mechanism of SIRT1 regulation by allosteric activators. Science 2013; 339(6124): 1216–1219
15. Morselli E, Maiuri MC, Markaki M, Megalou E, Pasparaki A, Palikaras K, Criollo A, Galluzzi L, Malik SA, Vitale I, Michaud M, Madeo F, Tavernarakis N, Kroemer G. Caloric restriction and resveratrol promote longevity through the Sirtuin-1-dependent induction of autophagy. Cell Death Dis 2010; 1(1): e10
16. Wang JB, Xiao XH, Du XX, Zou ZS, Song HB, Guo XX. Identification and early diagnosis for traditional Chinese medicine-induced liver injury based on translational toxicology. Zhongguo Zhong Yao Za Zhi 2014; 39(1): 5–9 (in Chinese)
17. Wang J, Zou Z, Zhao Y, Qin L, Li Q, Ma Z, Du X, Xiao X. Metabonomic biomarkers for drug-induced liver injury in rats and humans: better than alanine aminotransferase? Hepatology 2013; 58 (4): A379–A380
18. Boocock DJ, Faust GE, Patel KR, Schinas AM, Brown VA, Ducharme MP, Booth TD, Crowell JA, Perloff M, Gescher AJ, Steward WP, Brenner DE. Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent. Cancer Epidemiol Biomarkers Prev 2007; 16(6): 1246–1252
19. Xiaojing W, Liang Z, Chen HB, Zhao Z, Li P. Identification of Daqingye and Banlangen including crude drugs and decoction dregs from three plant species by normal light and fluorescence microscopy. Microsc Res Tech 2013; 76(8): 774–782
20. Chen S, Xu J, Liu C, Zhu Y, Nelson DR, Zhou S, Li C, Wang L, Guo X, Sun Y, Luo H, Li Y, Song J, Henrissat B, Levasseur A, Qian J, Li J, Luo X, Shi L, He L, Xiang L, Xu X, Niu Y, Li Q, Han MV, Yan H, Zhang J, Chen H, Lv A, Wang Z, Liu M, Schwartz DC, Sun C. Genome sequence of the model medicinal mushroom *Ganoderma lucidum*. Nat Commun 2012; 3: 913
21. Qiu X, Zhang J, Huang Z, Zhu D, Xu W. Profiling of phenolic constituents in *Polygonum multiflorum* Thunb. by combination of ultra-high-pressure liquid chromatography with linear ion trap-Orbitrap mass spectrometry. J Chromatogr A 2013; 1292: 121–131
22. The State Pharmacopoeia Commission of PR China. First Div.,

Appendix XID. Pharmacopoeia of the People's Republic of China. Beijing: Chemical Industry Press, 2010: Appendix 67

23. Chen X, Bian Z, Hou H, Yang F, Liu S, Tang G, Hu Q. Development and validation of a method for the determination of 159 pesticide residues in tobacco by gas chromatography-tandem mass spectrometry. J Agric Food Chem 2013; 61(24): 5746–5757
24. Dalton HR, Fellows HJ, Stableforth W, Joseph M, Thurairajah PH, Warshow U, Hazeldine S, Remnarace R, Ijaz S, Hussaini SH, Bendall RP. The role of hepatitis E virus testing in drug-induced liver injury. Aliment Pharmacol Ther 2007; 26(10): 1429–1435
25. Davern TJ, Chalasani N, Fontana RJ, Hayashi PH, Protiva P, Kleiner DE. Acute hepatitis E infection accounts for some cases of suspected drug-induced liver injury. Gastroenterology, 2011, 141: 1665–1672 e1–9
26. Zachou K, Muratori P, Koukoulis GK, Granito A, Gatselis N, Fabbri A, Dalekos GN, Muratori L. Review article: autoimmune hepatitis — current management and challenges. Aliment Pharmacol Ther 2013; 38(8): 887–913
27. Wang JB, Pu SB, Sun Y, Li ZF, Niu M, Yan XZ, Zhao YL, Wang LF, Qin XM, Ma ZJ, Zhang, YM, Li BS, Luo SQ, Gong M, Sun YQ, Zou ZS, Xiao XH. Metabolomic profiling of autoimmune hepatitis: the diagnostic utility of nuclear magnetic resonance spectroscopy. J Proteome Res 2014; 13(8): 3792–3801
28. Coghlan ML, Haile J, Houston J, Murray DC, White NE, Moolhuijzen P, Bellgard MI, Bunce M. Deep sequencing of plant and animal DNA contained within traditional Chinese medicines reveals legality issues and health safety concerns. PLoS Genet 2012; 8(4): e1002657
29. Gökmen MR, Cosyns JP, Arlt VM, Stiborová M, Phillips DH, Schmeiser HH, Simmonds MS, Cook HT, Vanherweghem JL, Nortier JL, Lord GM. The epidemiology, diagnosis, and management of aristolochic acid nephropathy: a narrative review. Ann Intern Med 2013; 158(6): 469–477
30. Ma J, Zheng L, Deng T, Li CL, He YS, Li HJ, Li P. Stilbene glucoside inhibits the glucuronidation of emodin in rats through the down-regulation of UDP-glucuronosyltransferases 1A8: application to a drug-drug interaction study in Radix Polygoni Multiflori. J Ethnopharmacol 2013; 147(2): 335–340
31. Wu X, Chen X, Huang Q, Fang D, Li G, Zhang G. Toxicity of raw and processed roots of *Polygonum multiflorum*. Fitoterapia 2012; 83 (3): 469–475
32. Murias M, Luczak MW, Niepsuj A, Krajka-Kuzniak V, Zielinska-Przyjemska M, Jagodzinski PP, Jäger W, Szekeres T, Jodynis-Liebert J. Cytotoxic activity of 3,3′,4,4′,5,5′-hexahydroxystilbene against breast cancer cells is mediated by induction of p53 and downregulation of mitochondrial superoxide dismutase. Toxicol In Vitro 2008; 22(5): 1361–1370
33. Ünsalan O, Kuş N, Jarmelo S, Fausto R. *Trans*- and *cis*-stilbene isolated in cryogenic argon and xenon matrices. Spectrochim Acta A Mol Biomol Spectrosc 2015: 136(A): 81–94
34. Lin CM, Singh SB, Chu PS, Dempcy RO, Schmidt JM, Pettit GR, Hamel E. Interactions of tubulin with potent natural and synthetic analogs of the antimitotic agent combretastatin: a structure-activity study. Mol Pharmacol 1988; 34(2): 200–208
35. Siles R, Ackley JF, Hadimani MB, Hall JJ, Mugabe BE, Guddneppanavar R, Monk KA, Chapuis JC, Pettit GR, Chaplin DJ, Edvardsen K, Trawick ML, Garner CM, Pinney KG. Combretastatin dinitrogen-substituted stilbene analogues as tubulin-binding and vascular-disrupting agents. J Nat Prod 2008; 71(3): 313–320
36. Sun J, Huang X, Wu H, Huang F. HPLC/IT-MS analysis of glycosides in radix *Polygoni multiflori*. Nat Prod Res Dev 2009; 21: 806–812
37. Navarro VJ, Lucena MI. Hepatotoxicity induced by herbal and dietary supplements. Semin Liver Dis 2014; 34(2): 172–193
38. Verma S, Thuluvath PJ. Complementary and alternative medicine in hepatology: review of the evidence of efficacy. Clin Gastroenterol Hepatol 2007; 5(4): 408–416
39. Wu X, Chen X, Huang Q, Fang D, Li G, Zhang G. Toxicity of raw and processed roots of *Polygonum multiflorum*. Fitoterapia 2012; 83 (3): 469–475
40. la Porte C, Voduc N, Zhang G, Seguin I, Tardiff D, Singhal N, Cameron DW. Steady-state pharmacokinetics and tolerability of trans-resveratrol 2000 mg twice daily with food, quercetin and alcohol (ethanol) in healthy human subjects. Clin Pharmacokinet 2010; 49(7): 449–454
41. Crowell JA, Korytko PJ, Morrissey RL, Booth TD, Levine BS. Resveratrol-associated renal toxicity. Toxicol Sci 2004; 82(2): 614–619
42. Popat R, Plesner T, Davies F, Cook G, Cook M, Elliott P, Jacobson E, Gumbleton T, Oakervee H, Cavenagh J. A phase 2 study of SRT501 (resveratrol) with bortezomib for patients with relapsed and or refractory multiple myeloma. Br J Haematol 2013; 160(5): 714–717