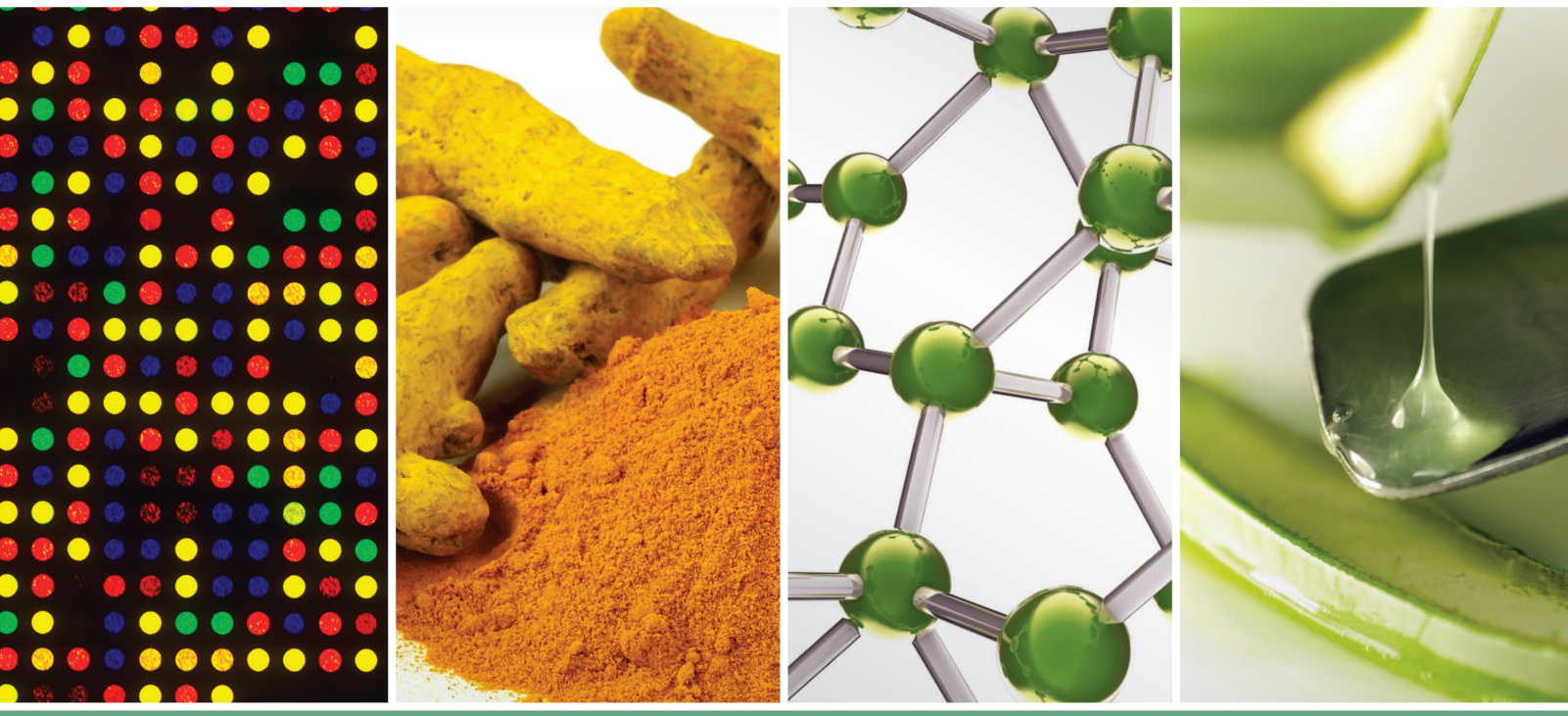
eCAM Evidence-Based Complementary and Alternative Medicine

The Safety of Herbal Medicine: From Prejudice to Evidence

Guest Editors: Junhua Zhang, Igho J. Onakpoya, Paul Posadzki, and Mohamed Eddouks



The Safety of Herbal Medicine: From Prejudice to Evidence

Evidence-Based Complementary and Alternative Medicine

The Safety of Herbal Medicine: From Prejudice to Evidence

Guest Editors: Junhua Zhang, Igho J. Onakpoya, Paul Posadzki, and Mohamed Eddouks

Editorial Board

Contents

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 316706, 3 pages
http://dx.doi.org/10.1155/2015/316706

Editorial

The Safety of Herbal Medicine: From Prejudice to Evidence

Junhua Zhang,[1] Igho J. Onakpoya,[2] Paul Posadzki,[3] and Mohamed Eddouks[4]

[1]*Tianjin University of Traditional Chinese Medicine, Tianjin 300193, China*
[2]*Department of Primary Care Health Sciences, University of Oxford, Oxford OX2 6GG, UK*
[3]*Lee Kong Chian School of Medicine, Singapore 138543*
[4]*Moulay Ismail University, 52000 Errachidia, Morocco*

Correspondence should be addressed to Junhua Zhang; zjhtcm@gmail.com

Received 28 December 2014; Accepted 28 December 2014

About 100 years ago, natural herbs were the main remedy for treating human diseases. It has been estimated that 25% of modern medicines are made from plants first used traditionally [1], such as aspirin, artemisinin, ephedrine, and paclitaxel. However, there is limited scientific evidence to establish the safety and efficacy of most herbal products [2]. With the wide application of chemical drugs, herbal medicine and other traditional therapies have shown sharp contraction. As a country with rich herbal resource, China is not an exception. In recent decades, spectrum of disease has shifted and the complex chronic diseases have become the main part. The effect of Western medicine treatment is not satisfactory and problems of the adverse drug reaction are also very prominent. The complementary and alternative treatment, especially the herbal medicine, has gained more attention and has also become popular.

About 80% of people worldwide rely on herbal medicines for some aspects of their primary health care [1]. In 2008, the global market for herbal remedies was about USD 83 billion, and now it is about 100 billion (see http://www.nutraceutical-sworld.com/). In China, the industry output value of Chinese patent medicines reached about USD 80 billion in 2013.

(1) Safety Issues of Herbal Medicine. Along with the significant increase of worldwide consumption, the safety of herbal medicine has been highlighted. At present, there are misunderstanding and prejudice toward the safety of herbal medicine. So, objective understanding, neutral and fair interpretation, and publicity are warranted.

(a) Herbal Medicine Is Drug, Not Food. Advocates will advertise that herbal medicine originated from nature and belongs to green therapy and has no toxin or adverse effect and people can take it in the long term and so forth. These sayings are slogans of the advocates which have misled people with less medical knowledge. On one hand, it will lead to many severe adverse events by misusing herbal medicine; on the other hand, it will cause people's panic and anxiety due to some adverse events reports. We should clearly recognize that herbal medicinal products are widely considered to be of lower risk compared with synthetic drugs; they are not completely free from the possibility of toxicity or adverse effects. Exaggerated propaganda and giving up using for adverse event are prejudice against herbal medicine. Therefore, to ensure the safety use of herbal medicinal products, herbal medicine should be managed as drug.

(b) The Relative Property of Herbal Medicine Safety. As the Chinese saying goes, "all medicines have their own side effects"; that is, medicine is a double-blade sword: it can cure disease or maintain health, while it may also cause damage to human body. All effective drugs may produce adverse drug reactions; herbal medicines are no exception. Herbal medicine should be adopted by appropriate dosage and course of treatment and for adapted syndrome, rather than unrestricted abusing. Overdosage and course of treatment are bound to safety problems. The toxicity of *Radix Bupleuri Chinensis* in Japan has attracted worldwide attentions. A research on the quantity-toxin relation indicates that the toxic dose of *Radix Bupleuri Chinensis* (192 g/60 kg) is much greater

than clinical common dose (9 g/60 kg). However, high-dose and long-term use may also cause adverse event [3].

(c) The Complexity of Safety of Herbal Medicine. There are a number of causes of adverse events to herbal medicines, which can be divided into "direct" and "indirect" reasons.

Intrinsic Toxicity. Direct reason is the intrinsic toxicity of some herb at normal therapeutic dosage or in overdose. Adverse reactions associated with Ephedra, *Aristolochia*, and *Aconitum* have shown that herbs can produce toxicity in humans.

External Toxicity. Adverse effects associated with herbal medicines may result from contamination of products with toxic metals, adulteration, misidentification or substitution of herbal ingredients, or improperly processed or prepared products [4]. For example, *Caulis Akebiae* replaced by *Caulis Aristolochiae Manshuriensis* and *Stephania tetrandra* replaced by *Aristolochia fangchi* have led to the serious problem of "aristolochic acid nephropathy."

Wrong Indication. Inappropriate use of herbal medicines can cause negative or dangerous effects. For instance, the herb "Ma Huang" (Ephedra) is traditionally used in China to treat respiratory congestion, while it was marketed as dietary supplements formulated for weight reduction in US. Overdosage use led to at least a dozen deaths, heart attacks, and strokes [5, 6].

Herb-Drug Interaction. All herbal medicines are complex mixtures of more than one active ingredient. Multitude of active ingredients will increase the possibilities of interactions between herbal medicines and conventional drugs. Moreover, users of medicinal herbs are usually suffering from chronic conditions for which they are likely to take prescribed drugs concomitantly. This, in turn, further increases the potential of herb-drug interaction [7]. A retrospective cross-sectional study found that the prevalence of concomitant herbal medicinal products and antipsychotic treatment was 36.4% (34.2%–38.6%). Herbal medicine regimens containing *Radix Bupleuri, Fructus Gardenia, Fructus Schisandrae Chinensis, Radix Rehmanniae, Akebia Caulis, and Semen Plantaginis* in concomitant use with quetiapine, clozapine, and olanzapine were associated with nearly 60% of the risk of adverse outcomes [8].

(d) Weak Basic Research in Safety of Herbal Medicine. *Sheng Nong's Herbal,* the first classic of Chinese materia medica, recorded 365 herbs, which were divided into three levels (high grade, moderate, and inferior) according to the toxin size of each herb. In the Chinese Pharmacopoeia 2010, there are 83 types of Chinese materia medica officially recorded and defined as toxic and they were classified into three categories: high toxicity, medium toxicity, and low toxicity. Efficacy and toxicity of the majority of them are mostly based on traditional knowledge and clinical experience. The toxicity classification is lack of scientific standard and objective experimental data. There is no adequate data about toxic herbs, toxic target organs, safe dose range, safety window of effective dose, and minimum toxic dose. Thus, to specify the toxic and adverse effect of each herbal medicine is a vital base to ensure the safe use of herbal medicine.

Processing of herbal slices is an important step to decrease toxicity. For example, heating processing can make the bitter almond enzyme lose activity and then decrease the toxicity of almond which contains cyanophoric glycoside. Diester-diterpenoid *Aconitum* Alkaloids are the strongest toxicity constituents of aconite and monkshood. By heating processing, diester-diterpenoid *Aconitum* Alkaloids can be decomposed into low toxicity monoester-diterpenoid *Alkaloids* and aconine which are of low toxicity and almost not toxic. If preparation was neglected, risk of aconite will increase. Compatibility, which aims to decrease toxicity and improve treating effect for prescription, is the key theory of Chinese herbal medicine. For instance, combining Radix aconiti praeparata with licorice, *Aconitum* Alkaloids can decrease obviously [9]. Ginger can antagonize the toxicity of *Rhizoma Pinelliae* [10]. Therefore, the scientific connotation of compatibility and preparation of herbal medicine should be investigated deeply.

(2) Content of the Special Issue. This special issue wished to recruit high level research articles, including systematic reviews of herbal medicine safety, case observation and monitoring of adverse events, herbal toxic composition, relations of amount and toxicity, the metabolic process, herb-drug interactions, and methods for risk control. However, we have only received 23 articles for this issue and 12 were rejected due to the content being not closely related with our topic issue or low quality. Finally, 11 articles have been published after being reviewed and revised by the editorial committee and reviewers. Although there are a limited number of papers, the contents are extensive, covering the safety evaluation method, herb-drug interaction, safety evaluation of skin-applied herbal medicine, and the influence of herbal ingredients to cytochrome P450 system, which have certain representations for the related work in this field.

(3) Perspective. Based on the current situation, worldwide research on herbal medicine safety is still not broad or deep enough. For next step, more attention should be paid to researches on the toxicity and the herb-drug interaction of commonly used herb medicines, which are the most necessary and urgent work.

For clinical safety monitoring, spontaneous reporting system or active pharmacovigilance is effective in identifying therapeutically relevant safety issues. Even in countries where herbal medicinal products are regularly assessed before market authorisation, pharmacovigilance is a critical activity to promote the safe use of herbal medicines throughout their life cycle.

A regulatory framework for herbal medicines can provide greater assurance to consumers. However, the regulation and specification of herbal medicines vary significantly different countries. Herbal medicines were managed as food supplement, functional food, health products, or drugs, which caused differential standards and chaotic market. In order

to ensure the quality and safety of herbal medicines, the World Health Organization should propose global unified planning, which includes global management standards and quality standards, radical source of herbs, seed and seedling breeding, planting, harvesting and storage, rational proceeding, manufacture, and quality standards. Moreover, safety guarantee system comprised rational clinical practice and risk monitoring should be established to improve the safety of herbal medicine and to play more important role in maintaining human health.

Junhua Zhang
Igho J. Onakpoya
Paul Posadzki
Mohamed Eddouks

References

[1] http://www.who.int/mediacentre/factsheets/2003/fs134/en/.

[2] S. Bent, "Herbal medicine in the United States: review of efficacy, safety, and regulation: grand rounds at University of California, San Francisco Medical Center," *Journal of General Internal Medicine*, vol. 23, no. 6, pp. 854–859, 2008.

[3] L. Lv, W. Huang, X. Yu, H. Ren, and R. Sun, "Comparative research of different Bupleurum chinense composition to influence of hepatotoxicity of rats and oxidative damage mechanism," *China Journal of Chinese Material Medica*, vol. 34, no. 18, pp. 2364–2368, 2009.

[4] J. Zhang, B. Wider, H. Shang, X. Li, and E. Ernst, "Quality of herbal medicines: challenges and solutions," *Complementary Therapies in Medicine*, vol. 20, no. 1-2, pp. 100–106, 2012.

[5] M. K. Lee, B. W. H. Cheng, C. T. Che, and D. P. H. Hsieh, "Cytotoxicity assessment of Ma-Huang (Ephedra) under different conditions of preparation," *Toxicological Sciences*, vol. 56, no. 2, pp. 424–430, 2000.

[6] C. A. Haller and N. L. Benowitz, "Adverse cardiovascular and central nervous system events associated with dietary supplements containing ephedra alkaloids," *The New England Journal of Medicine*, vol. 343, no. 25, pp. 1833–1838, 2000.

[7] E. Ernst, "Herb-drug interactions: potentially important but woefully under-researched," *European Journal of Clinical Pharmacology*, vol. 56, no. 8, pp. 523–524, 2000.

[8] Z. J. Zhang, Q. R. Tan, Y. Tong et al., "An epidemiological study of concomitant use of Chinese medicine and antipsychotics in schizophrenic patients: implication for herb-drug interaction," *PLoS ONE*, vol. 6, no. 2, Article ID e17239, 2011.

[9] J. Chen, B. Tan, W. Wu et al., "Study on the compatibility of monkshood and licorice in Sini decoction," *Chinese Journal of Experimental Traditional Medical Formulae*, vol. 7, no. 3, pp. 16–17, 2001.

[10] H. Wu, W. Shu, L. Qiu, and D. Ye, "The experiment research of ginger detoxification to rhizoma pinelliae," *Journal of Chinese Medicinal Materials*, vol. 21, no. 3, pp. 137–140, 1998.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 258598, 15 pages
http://dx.doi.org/10.1155/2015/258598

Review Article

Efficacy and Safety of Pomegranate Medicinal Products for Cancer

Christian Vlachojannis,[1] Benno F. Zimmermann,[2,3] and Sigrun Chrubasik-Hausmann[1]

[1]*Institute of Forensic Medicine, University of Freiburg, Albertstr. 9, 79104 Freiburg, Germany*
[2]*Institute of Nutrition and Food Sciences, Food Technology and Food Biotechnology, University of Bonn, Römerstraße 164, 53117 Bonn, Germany*
[3]*Institut Prof. Dr. Georg Kurz GmbH, Eupener Straße 161, 50933 Köln, Germany*

Correspondence should be addressed to Sigrun Chrubasik-Hausmann; sigrun.chrubasik@klinikum.uni-freiburg.de

Received 25 February 2014; Revised 8 July 2014; Accepted 2 September 2014

Academic Editor: Junhua Zhang

Preclinical *in vitro* and *in vivo* studies demonstrate potent effects of pomegranate preparations in cancer cell lines and animal models with chemically induced cancers. We have carried out one systematic review of the effectiveness of pomegranate products in the treatment of cancer and another on their safety. The PubMed search provided 162 references for pomegranate and cancer and 122 references for pomegranate and safety/toxicity. We identified 4 clinical studies investigating 3 pomegranate products, of which one was inappropriate because of the low polyphenol content. The evidence of clinical effectiveness was poor because the quality of the studies was poor. Although there is no concern over safety with the doses used in the clinical studies, pomegranate preparations may be harmful by inducing synthetic drug metabolism through activation of liver enzymes. We have analysed various pomegranate products for their content of anthocyanins, punicalagin, and ellagic acid in order to compare them with the benchmark doses from published data. If the amount of coactive constituents is not declared, patients risk not benefiting from the putative pomegranate effects. Moreover, pomegranate end products are affected by many determinants. Their declaration should be incorporated into the regulatory guidance and controlled before pomegranate products enter the market.

1. Introduction

Pomegranate products are amongst most promising antitumorigenic dietary supplements. The polyphenol fraction of pomegranate exerts antiproliferative and proapoptotic effects in a number of cancer cell lines [1]. Various mediators of carcinogenesis are inhibited by the pomegranate active principle *in vitro*, for example, vascular endothelial growth factor [2], insulin-like growth factors [3], cytokine-stimulated NF-κB [4], and others [5, 6]. Fermentation is a new technology that enriches coactive compounds [7]. Fermentation of pomegranate juice with *Lactobacillus plantarum* increased the concentration of ellagic acid and enhanced the antimicrobial activity of the juice. Both fresh and fermented juices inhibited the growth of K562 tumor cells [7]. Polyphenols from fermented pomegranate juice showed about twice the antiproliferative effect shown by polyphenols from fresh pomegranate juice. They also inhibited the activity of aromatase and 17-β-hydroxysteroid dehydrogenase type 1 by 60–80% and inhibited chemically induced formation of cancerous lesions in a murine mammary gland organ culture by about 50% [8] However, a specific purified polyphenol compound and pomegranate seed oil were more effective than fermented juice in this *in vitro* test [9]. The isolated ellagitannins, punicalagin, and ellagic acid also had a high antiproliferative activity against various cancer cell lines [10, 11].

The aromatase enzyme, which converts androgen to estrogen, plays a key role in breast cancer. Tamoxifen is the usual antiestrogen therapy for hormone-receptor-positive breast cancer in premenopausal women, though it carries a risk of development of resistance [12]. Pomegranate fruit

extracts enhanced the action of tamoxifen in both tamoxifen-sensitive and tamoxifenresistant breast cancer cells, through the inhibition of cell viability by inducing the cell-death machinery [13].

The polyphenols also inhibited the expression of genes for key androgen-synthesizing enzymes and androgen receptors, suggesting that the pomegranate polyphenols (mainly the oligomeric punicalagin and the monomeric ellagic acid) affect androgen-independent prostate cancer cells and the subset of human prostate cancer cells where the androgen receptor is upregulated [4, 14]. In investigations of the anti-invasive effects of ellagic acid in androgen-independent human and rat prostate cancer cell lines *in vitro*, ellagic acid significantly inhibited the motility and invasion of cells examined in migration and invasion assays. The secretion of matrix metalloproteinases from androgen-independent human and rat prostate cancer cell lines and the proteolytic activity of collagenase/gelatinase were significantly reduced, indicating that the anti-invasive potential of prostate cancer cells is mediated via protease activity [15].

Although all pomegranate-derived materials contribute to a greater or lesser extent to the anticancer effect of pomegranate, the polyphenol fraction and supercritical CO_2-extracted seed oil were more potent than cold-pressed pomegranate seed oil in inhibiting growth of prostate cancer xenografts in athymic mice [16]. Pomegranate juice was more effective than compounds isolated from the juice [17].

In immunodeficient mice, pomegranate juice and extract inhibited tumor-associated angiogenesis and slowed the growth of prostate cancer [18]. Oral infusion of pomegranate fruit extract resulted not only in a dose-dependent inhibition of tumor growth, but also in a decrease of prostate-specific antigen (PSA) levels in mice implanted with androgen-responsive cancer cells [19]. Tumor reductions were also seen in mice with induced lung, colon, and skin cancers [1].

Of the polyphenols, the chemopreventive pomegranate ellagitannins (e.g., punicalagin and punicalin) are metabolized during absorption. One of the metabolites, ellagic acid, is further metabolized by the colonic microflora to urolithin A. Both ellagic acid and urolithin A contribute to the mechanism of anticancer action, but urolithin was less effective in inhibiting cancer cell proliferation [20–22]. There was no difference in metabolite production between pomegranate juice and extracts thereof [23], though inactive ellagitannin-derived metabolites are also produced by the colonic microflora [24]. This may account for individual differences in the response to pomegranate consumption.

The aim of this study was to summarize data on the clinical effectiveness and safety of pomegranate preparations for the treatment of cancer and to analyse various pomegranate preparations for their content of coactive compounds in order to find out the dose required for an anticancer effect in patients suffering from prostate cancer.

2. Methods

2.1. Systematic Reviews on Effectiveness and Safety. On June 20, 2014 we searched PubMed using the terms: "pomegranate cancer" and "pomegranate prostate" and, on June 29, "pomegranate safety" and "pomegranate toxicity," the reference lists of articles were searched by hand for other publications. No methodological filter was applied and the search was not limited by language. The full manuscript was retrieved for each record that had a chance of meeting the review criteria (clinical trial, safety investigation). Two authors (Christian Vlachojannis and Sigrun Chrubasik-Hausmann) extracted the data independently and evaluated the quality of the studies and the strength of the evidence of clinical effectiveness using the same criteria as in previous reviews [25–35]. Briefly, the assessment of quality was based on "yes" or "no" answers to the following questions: was or were

(i) patients included on the basis of specified eligibility criteria;

(ii) randomization appropriate;

(iii) treatment allocation concealed;

(iv) baseline values of the groups similar

(v) outcome measures and control interventions explicitly described;

(vi) cointerventions comparable;

(vii) outcome measures relevant;

(viii) adverse events fully described;

(ix) attrition of patients from the study (the "drop-outs") fully described;

(x) sample size based on a priori power calculation;

(xi) analysis by intention-to-treat in the event of attrition of patients during the study;

(xii) point estimates and measures of variability presented for the primary outcome measure;

(xiii) studies undertaken over an appropriate time-course to demonstrate the putative effect.

For observational studies, some of the questions are not applicable but the inability to supply a "yes" answer itself marks an "absence of quality" in systematic reviews of this sort. Potential disagreements were discussed and resolved by referring to the original protocol.

Adding up the "yes" answers applicable to each study gave it a total score (TS) out of a maximum of 13. Evidence of effectiveness was defined as (i) "strong": pooling of data from at least 2 confirmatory studies demonstrating a clinically relevant effect;

(ii) "moderate": consistent findings from one confirmatory study with a clinically relevant effect, multiple exploratory studies of high internal validity (TS 10 and higher), or both;

(iii) poor: multiple exploratory studies of low internal validity or one single study of high internal validity.

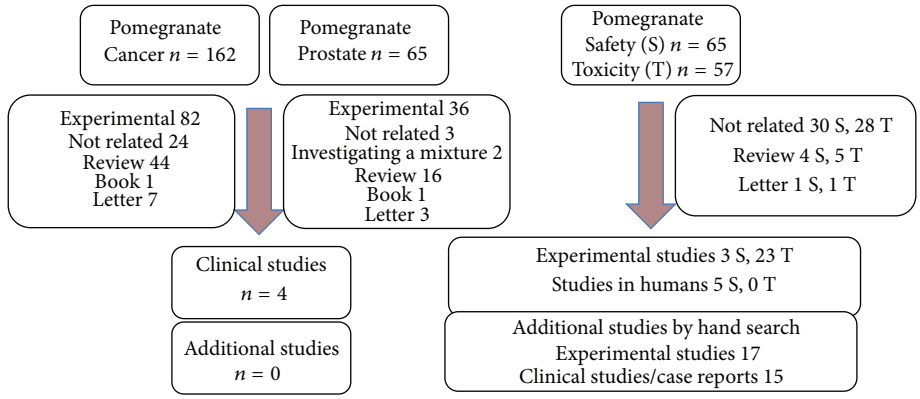


Figure 1: Search profile.

2.2. Analyses of Various Pomegranate Products. The pomegranate preparations we investigated included

(A) three commercially available pure (100%) juices:

(i) 5174-13, expiry date June 2, 2015,
(ii) L3074, expiry date Sept 15, 2014,
(iii) POM Wonderful (expiry date April 21, 2014);

(B) two juice concentrates:

(iv) POM Wonderful expiry date August 1, 2014
(v) F4, a commercially available fermented pomegranate concentrate supplemented with 10% elderberry concentrate (details not stated; photometric assessment on May 30, 2013);

(C) five extracts:

(vi) POMx capsules (1000 mg capsules, expiry date June 17, 2015),
(vii) ultra Granatapfel forte capsules (500 mg, expiry date Sept., 2015),
(viii) extract 20651 (not commercially available, batch 19829, native drug extract ratio 5.3–8 : 1, solvent ethanol),
(ix) GranaProstan capsules (500 mg freeze-dried powder from fermented pomegranate juice (84%),
(x) pomegranate extract (16%, drug extract ratio and solvent not stated, photometric assessment in February 2013, expiry date Feb 18, 2015).

Punicalagins A and B and ellagic acid were analysed by RP-UHPLC-UV using authentic reference compounds. Anthocyanidins were analysed by RP-UHPLC-Vis using cyanidin-3-O-glycoside as reference. Total polyphenols were determined by the Folin-Ciocalteu photometric method using gallic acid as reference. Details of the methods are presented at http://www.uniklinik-freiburg.de/rechtsmedizin/forschung/phytomedizin.html.

3. Results

3.1. Systematic Reviews on Effectiveness and Safety. We identified 162 references for "pomegranate cancer" and 65 references for "pomegranate prostate," both included 4 clinical studies investigating pomegranate products in prostate cancer patients (Figure 1, see webpage: PubMed searches). The quality of the studies is listed in Table 1. According to the criteria set out above in the methods, the evidence of effectiveness of pomegranate products for the treatment of prostate cancer is poor.

We identified 42 references for "pomegranate safety" and 57 for "pomegranate toxicity," respectively. A total of 26 experimental and 5 clinical studies were included in the part on safety together with 17 experimental studies and 15 clinical studies from hand searches (Figure 1, see webpage: PubMed searches).

3.2. Analyses of Various Pomegranate Products. Table 2 summarizes the total polyphenol content by photometric assessment (Folin-Ciocalteu method) as declared by the manufacturers, along with our own Folin-Ciocalteu data. The HPLC chromatograms are placed on the above-mentioned webpage (see Results). The table also summarizes coactive compounds as assessed by HPLC and their sum in mg/L or mg/kg and the daily dose of polyphenols in the doses of product recommended by the manufacturers. It can be seen that the sum of our HPLC measurements of anthocyanins, punicalagins, and ellagic acid is substantially less than the photometrically measured total polyphenols, though there is a correlation of sorts.

The lower part of the table shows the content on coactive compounds in commercially available pomegranate preparations, as taken from the references stated and the calculated sum of polyphenols in the recommended daily doses.

Table 3 lists the individual anthocyanidins measured by HPLC, which also allows a distinction to be made between pomegranate and elderberry anthocyanidins in the juice concentrate F4.

Table 1: Quality criteria considered in the 4 trials investigating pomegranate products.

	Clin cancer Res 2006; 12: 4018–26 Pantuck et al. [36]	Prostate cancer prostatic Dis 2013; 16: 50–5 Paller et al. [37]	J cancer 2013; 4: 597–605 Stenner-Liewen et al. [40]	Cancer prev Res (Phila) 2013; 6: 1120–7 Freedland et al. [38]
	$N = 46$	$N = 92$	$N = 97$	$N = 69$
SM	Juice POM wonderful	extract POMx	Pomegranate blend	Extract POMx
Dose	240 mL/day	1000 mg versus 2000 mg/day	500 mL/day	2 × 1000 mg/day
pa	570 mg/day	Not stated	700 mg/day*	1200 mg/day
cai	Not stated*	400 mg versus 800 mg/day	40 mg/day	Not stated*
	Open, uncontrolled	Low dose (45), high dose (47)	Placebo (48), control (49)	Placebo (36), control (33)
D	13 months	Up to 18 months	4 weeks	4 weeks
R	Lengthening of PSA doubling time	No difference between groups lengthening of PSA doubling time	No difference between groups	No difference between groups
A	Prostate cancer	Prostate cancer	Prostate cancer	Prostate cancer requiring Radical prostatectomy
B	Not applicable	Not stated	Yes	Yes
C	Not applicable	Not stated	Yes	Yes
E	Not applicable	Not stated	Yes	Yes
F	Not applicable	Yes	Yes	Yes
G	Not applicable	Yes	Yes	Yes
H	Yes	Yes	Yes	Yes
I	Yes	Yes	Yes	Not stated
J	Yes	Yes	Yes	Yes (none)
K	Yes	Yes	Yes	Yes
L	No	Yes	No	No
N	No	No	No	No
O	Yes	Yes	No	No
TS	6	9	10	9
	*according to Paller the same as in extract POMx	*from other source see Hong et al., 2008 [14]	*see Chrubasik-Hausmann et al. 2014a [41]	*no details given on request

SM study medication, pa photometrically assessed, cai coactive ingredients/day (HPLC), D duration of treatment, R result.
Quality criteria A: eligibility criteria specified, B: randomization appropriate, C: treatment allocation concealed, E: similarity at baseline, F: outcome measures and control interventions explicitly described, G: cointerventions comparable, H: outcome measures relevant, I: adverse events and J drop-outs fully described, K: sample size based on a priori power calculation, L: intention-to-treat analysis, N: point estimates and measures of variability presented for the primary outcome measure, and O: appropriate timing giving a total score (TS) of 13.

4. Discussion

4.1. Evidence of Effectiveness of Pomegranate Products. Pomegranate preparations have so far been investigated only in patients with prostate cancer. In an uncontrolled study, patients with rising PSA after surgery or radiation for prostate cancer were treated with 240 mL of fermented pomegranate juice per day, containing total polyphenols equivalent to 570 mg of gallic acid [36]. The content of coactive compounds as assessed by HPLC was not stated but was said in another study [37] to be similar to that in extract POMx, which contained 370 mg punicalagin and 30 mg ellagic acid in the daily dosage [14]. Mean PSA doubling time increased with treatment from a mean of 15 months at baseline to 54 months after treatment [36]. The remaining observational study included 104 men with rising PSA but without metastases. Daily doses of either 1000 or 3000 mg of a polyphenol extract of pomegranate were given (POMx, 37% punicalin (POM Wonderful, LLC; Los Angeles, California, http://cms.herbalgram.org/herbclip/474/051321-474.html. Patients were stratified according to their baseline PSA doubling time and Gleason score. The primary endpoint was the increase in PSA doubling time after 6 months. The average PSA doubling time did indeed increase from 12 months to almost 19 months, irrespective of dose. This may or may not indicate a ceiling effect. The data are not conclusive because of the lack of a placebo and the unreliability of the endpoint [37]. The coactive compound urolithin A was detected more often in benign and malignant prostate tissue in patients who had received POMx during the 4 weeks before surgery. An inverse correlation was expected between intraprostatic urolithin A and the oxidative stress tissue marker 8-hydroxy-2′-deoxyguanosine content. The study was powered to detect a 35% reduction in that marker. However, POMx was

Table 2: Content of coactive ingredients in various pomegranate preparations (density of liquid products 1.3).

Preparation	Total polyphenols		Total anthocyanins	Punicalagin A + B	Ellagic acid	***Sum of A, P, and EA	
	Declared	Measured					Daily Dose
Mother juice 5174-13	n.i.	2654 mg/L*	34.47 mg/L	271 mg/L	81.5 mg/L	387 mg/L	39 mg/100 mL
Mother juice L3074	3840 mg/L**	2188 mg/L*	9.45 mg/L	948 mg/L	47.4 mg/L	1005 mg/L	101 mg/100 mL
POM wonderful juice	n.i.	2670 mg/L*	60.7 mg/L	310 mg/L	134 mg/L	505 mg/L	121/240 mL
POM Wonderful Concentrate	n.i.	18900 mg/L*	1.1 mg/L	1400 mg/kg	146 mg/kg	1547 mg/L	77 mg/50 mL
F4 concentrate	71515 mg/L*	73944 mg/L*	179.4 mg/L	29900 mg/L	1378 mg/L	31457 mg/L	315 mg/10 mL
POMx-capsules	n.i.	613000 mg/kg*	n.d.	103000 mg/kg	28700 mg/kg	131700 mg/kg	132 mg/1000 mg
Ultra Granatapfel forte capsules	n.i.	843000 mg/kg*	17.3 mg/kg	45900 mg/kg	13900 mg/kg	59800 mg/kg	30 mg/500 mg
Extract 20651	59000 mg/kg**	189900 mg/kg*	241 mg/kg	38400 mg/kg	1610 mg/kg	40251 mg/kg	40 mg/1000mg
GranaProstan capsules	460000 mg/kg*	394000 mg/kg*	112 mg/kg	74000 mg/kg	69900 mg/kg	144012 mg/kg	142 mg/1000 mg
	Reference						
POM Wonderful juice (mg/L)	McCutcheon et al., 2008 [102]		n.i.	1740[&]	140[&]	1860[&]	94 mg/240 mL
POM Wonderful Concentrate (mg/L)	http://www.google.ca/patents/US7727563[&]		384[§]	1561[§]	121[§]	2066[§]	103 mg/50 mL
Extract 1 (mg/1000 g)	Madrigal-Carballo et al., 2009 [123]		n.i.	177000[§]	33000[§]	n.i.	201 mg/1000 mg
Pomella (mg/kg)	Patel et al., 2008 [48]		none[§]	300000[§]	21500[§]	321500	193 mg/600 mg
POMx wonderful (mg/kg)	Hong et al., 2008 [14]		n.i.	370000[§]	30000[§]	n.i.	400 mg/1000 mg

*total polyphenols calculated as gallic acid equivalents (Folin-Ciocalteau, photometric assessment).
**total polyphenols calculated as pyrogallol (PhEur 2.8.14, photometric assessment).
***sum of anthocyanins (A), punicalagin (P), and ellagic acid (EA) assessed by HPLC; DD in the suggested daily dose.
n.d.: not detectable.
n.i. no information.
[§]according to the manufacturers, [&]according to the POM wonderful monography.

Table 3: Content of individual anthocyanins expressed as cyanidin-3-glucoside equivalents (mg/L at 500 nm) in the pomgranate preparations investigated.

	Delphinidin-3,5-diglucoside	Cyanidin-3,5-diglucoside	Delphinidin-3-glucoside + pelargonidin-3,5-glucoside + cyanidin-rutinoside	Cynanidin-3-glucoside	Delphinidin-pentoside	Pelargonidin-3-glucoside	Cyanidin-pentoside
Mother juice 5174-13 (mg/L)	5.63	15.0	4.07	8.79	n.d.	0.77	0.21
Mother juice L3074 (mg/L)	2.14	5.11	0.79	1.32	n.d.	0.11	n.d.
POM wonderful							
Juice (mg/L)	7.3	13.2	7.0	17.2	1.7	1.3	n.d.
Concentrate (mg/L)	n.d.	n.d.	n.d.	1.1	n.d.	n.d.	n.d.
F4 concentrate (mg/L)	n.d.	20.3*	n.d.	59.9**	n.d.	57.4	n.d.
POMx-capsules (mg/kg)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ultra Granatapfel forte							
Capsules (mg/kg)	12.3	n.d.	4.9	n.d.	n.d.	n.d.	n.d.
Extract 20651 (mg/kg)	20.3	63.4	36.6	88.5	18.0	14.2	n.d.
GranaProstan capsules (mg/kg)	n.d.	n.d.	32.0	13.4	29.0	n.d.	0.9
POM wonderful concentrate[&]	n.i.	n.i.	n.i.	1.1	n.i.	n.i.	n.i.

*Sum of cyanidin-3,5-diglucoside and cyanidin-3-sambubioside-5-glucoside of elderberry.
**Sum cyanidin-3-glucoside and cyanidin-3-sambusoside of elderberry.
n.i. no information.
[&]adopted from http://www.google.ca/patents/US7727563.

associated only with 16% lowermarker content, which was not statistically significant in this short-term clinical trial [38]. It may well be that the 4-week treatment duration was too short. The results are eagerly awaited of two on-going and two as yet unpublished investigations of pomegranate in prostate cancer patients with a juice, a proprietary extract (2 studies) and a liquid extract [39]. Stenner-Liewen et al. [40] carried out a phase IIb, double-blinded, randomized placebo-controlled trial in patients with histologically confirmed prostate cancer in patients with a PSA $\geq$ 5 ng/mL; this used an amount of pomegranate active principle per day (20 mg in 500 mL), which was only 5% of that investigated in the other studies [41]. Unsurprisingly, the study concluded that daily pomegranate intake has no impact on PSA levels in patients with advanced prostate cancer.

In our critique of the Cochrane reviews on herbal medicines [42], we called for rigorous declaration of coactive ingredients in study medications to avoid misleading interpretations of data. For example, Stenner-Liewen and coworkers [40] relied on photometric assessments of the coactive principle in their study medication, failing to take into account the fact that photometric assessments overestimate the true polyphenol content by detecting all polyphenolic or antioxidative compounds regardless of their clinical activity [41]. The photometric assessment of the total mixed polyphenols in 500 mL of the proprietary pomegranate blend was 1147 mg of gallic acid equivalents. Subtracting the various polyphenols from other components of the blend (white tea and chokeberry—agave concentrate does not contain polyphenols) amounted to 445 mg/500 mL; the remaining value of around 700 mg of pomegranate polyphenols does not reflect the dose of coactive compounds (e.g., punicalagin and ellagic acid), our HPLC analysis resulted in a total of 20 mg. Bench-mark doses of coactive ingredients are given in the study by Paller and coworkers [37], a total of 400 mg per day as assessed by HPLC. Thus the conclusion of the Stenner-Liewen group that daily pomegranate intake has no impact on PSA levels in patients with advanced prostate cancer is wrong since it was based on an inadequate amount of coactive ingredients in their pomegranate mixture. Likewise, similar confusion exists for cranberry products in which the photometric assessments do not reflect the true content of coactive ingredients [43]. HPLC assessments provided bench-mark doses for the prevention of urinary tract infections [44].

Recently, a significant decrease in PSA levels during treatment with pomegranate extract Pomella (225 mg/kg, Table 2) has been demonstrated in a mouse model of prostate cancer. The production of testosterone, DHT, DHEA, androstenedione, androsterone, and pregnenolone was inhibited in prostate cancer cell lines and serum steroids reduced after 20 weeks of treatment (0.17 g/L in drinking water) [45]. In metastatic castration-resistant PCa cells, POMx exhibited potent *in vitro* cytotoxicity and in athymic nude mice, the extract retarded C4-2 tumor growth in skeleton and significantly enhanced the efficacy of docetaxel [46]. These studies and the experiments mentioned in the Introduction of our manuscript suggest that the clinical effectiveness of pomegranate products in the treatment of prostate and other cancers deserves further evaluation.

5. Safety Aspects

5.1. Based on Experiments. A diet containing 6% punicalagin given to rats for 37 days caused no obvious toxicity [47]. The oral LD_{50} of a pomegranate extract standardized to 30% punicalagins, 5% ellagic acid, and 0.3% gallic acid (photometric assessment 70% polyphenols, trade name POMELLA) was found to be greater than 5 g/kg body weight in rats and mice. The respective *intraperitoneal* LD_{50}s in rats and mice were determined as 217 and 187 mg/kg body weight. In a subchronic study in rats, a diet containing up to 600 mg/kg body weight/day of this extract was given over 90 days with or without a 28-day recovery phase. Compared with the control group, giving the extract did not result in any clinically relevant treatment-related organ changes. The "no observed-adverse-effect level" was defined as 600 mg/kg body weight/day, the highest extract dose tested [48].

Pomegranate fruit extract exerted an embryoprotective effect against adriamycin-induced oxidative stress in 12-day old chick embryos. After 24 and 48 h of incubation, 70 μg/egg of adriamycin on its own produced a significant dose versus time-dependent reduction in body weight and volume of amniotic fluid and a dose-related increase in gross embryological deformities and significant changes in the levels of biochemical markers in amniotic fluid. These changes were significantly reduced by preadministration of pomegranate fruit extract at a dose of 200 μg/egg [49]. Lead acetate administration inhibited spermatogenesis in rats by reducing the length of the stages related to spermiation and onset of mitosis. The epididymal sperm number and daily sperm production were reduced. Giving ethanolic pomegranate extract along with the lead acetate resulted in longer spermiation stages than with the lead acetate only. The deleterious effects on epididymal sperm number and daily sperm production were reduced. Thus, pomegranate may prevent lead acetate-induced spermatogenic disruption in rats possibly owing to antioxidant effects [50]. Pomegranate also reduced the RNA-damaging effect of doxorubicin, H_2O_2, and spermine. Its inhibitory activity could be related to its ability to form complexes with doxorubicin and H_2O_2 or its interaction with the intracellular formation of reactive substances that mediated their toxicity [51]. In adult Wistar rats, pomegranate juice augmented the antioxidant defence mechanism against carbon tetrachloride-induced reproductive toxicity [52]. In other tests, pomegranate extract was found to be protective against methotrexate-induced oxidative bone marrow damage [53], reduced methotrexate-induced neurotoxicity [54], and reversed methotrexate-induced oxidative stress and apoptosis in hepatocytes by modulating Nrf2-NF-κB pathways in male Swiss albino rats. Preparations of pomegranate may, thus, help to reduce some adverse effects of methotrexate. Further tests demonstrated that pomegranate methanolic peel extract inhibited aluminum-induced hepatorenal toxicity [55], mercuric chloride-induced oxidant toxicity [56] and gentamicin-induced nephrotoxicity [57]. Pomegranate seed oil in doses up to 0.64 mg/kg, one hour before 100 mg/kg of the nephrotoxic agent diazinon had a nephroprotective effect [58]. This has been confirmed with hexachlorobutadiene as the nephrotoxic agent [59]. The "no observable adverse effect

level" (NOAEL) of pomegranate seed oil was 50.000 ppm PSO (=4.3 g PSO/kg body weight/day) [60].

In hepatitis induced in rats by D-galactosamine/lipopolysaccharide, a 2-week pretreatment with pomegranate juice 20 mL/kg body weight per day protected against hepatic damage by suppressing oxidative stress. Histopathology showed that the pomegranate juice restored the hepatic architecture to normal [61]. Histopathological studies of the liver of rats fed pomegranate fruit extract and carbon tetrachloride also indicated a hepatoprotective effect. Likewise, pomegranate juice protected against carbon tetrachloride-induced hepatotoxicity [62] and nephrotoxicity [63] and protected against ethylene glycol-induced crystal deposition in renal tubules [64] and the development of azoxymethane-induced aberrant crypt foci [65]. Oral pomegranate extract had a protective effect against cisplatin ototoxicity in rats. Cisplatin ototoxicity was assessed by analysing "distortion product otoacoustic emissions" 3 days before and after the cisplatin injections. Histological changes in the cochleas were observed by light microscopy [66]. This was confirmed in an experimental study with aminoglycoside as the ototoxic agent [67] A whole fruit extract of pomegranate was cardioprotective against doxorubicin-induced toxicity [68].

In the chick embryo model, doses of whole fruit extract (DER 3 : 1, solvent ethanol 50%) of less than 0.1 mg per embryo were not toxic. The LD_{50} of the extract, determined after intraperitoneal administration in mice, was 731 mg/kg (confidence limits 565–945 mg/kg). At the doses of 0.4 and 1.2 mg/kg of extract, repeated intranasal administration to Wistar rats produced no toxic effects in terms of food intake, weight gain, behavioural or biochemical measurables, nor was it associated with histopathological changes [69]. Aqueous and lipophilic pomegranate peel extracts have demonstrated a dose-dependent antimutagenic activity in *Salmonella typhimurium* strains [70]; this was probably attributable to the content on ellagitannins [71]. No toxic effects were observed in mice treated with aqueous pomegranate fruit extracts [72]. A study in Swiss mice treated with ethanolic extracts of pomegranate leaf or fruit confirmed the absence of mutagenic effects and the dose-dependent protective effects against cyclophosphamide-induced oxidative DNA damage [73]. However, a later study was carried out on the genotoxicity of whole pomegranate fruit extract (solvent 50% ethanol) using different *in vitro* and *in vivo* assays to detect DNA damage at different expression levels: it indicated that this extract can induce genetic damage at different expression levels: recombinogenic, mutagenic, and clastogenic [74]. Thus, the use of this extract may well carry a genetic risk and an analysis of the balance of risk and benefit is probably crucial. Whereas pomegranate bark [75] and root [76] contain toxic alkaloids, the presence of alkaloids in peel was considered equivocal [77]. Studies of cytotoxicity affecting the Caco-2 cell line and human peripheral blood mononuclear cells (PBMC) could provide preliminary information about toxicity on intestinal cancer cells and normal cells. The effective dose of pomegranate peel extract for stimulating proliferation in Caco-2 cells was 4.7 μg/mL and for PBMCs 44.4 μg/mL [78]. One should therefore be cautious about using peel extract in humans as a natural dietary antioxidant or a therapy (http://archive.lib.cmu.ac.th/full/T/2008/pha0808st_ch4.pdf). However, one should also note that the toxic effects of pomegranate fruit extract occurred at higher doses than the doses used either those in animal experiments or in Cuban folk medicine [69].

5.2. Based on Data from Humans. In the clinical study investigating a pomegranate extract in doses of 1000 and 3000 mg, diarrhea occurred more often in the high dose group [37]. Heber et al. [79] carried out two clinical pilot studies on the safety of a pomegranate ellagitannin-enriched polyphenol extract. Sixty-four overweight individuals took one, two, or three 710 mg capsules per day of pomegranate extract for 28 days, each capsule containing 435 mg of gallic acid equivalents (GAEs). In none of the subjects were there any serious adverse events on complete blood count, blood chemistry, and urinalysis. In another 22 overweight subjects, levels of thiobarbituric acid reactive substances (TBARS) were significantly less after receiving 1000 mg pomegranate extract (610 mg of GAEs) versus baseline measurements. Diabetic indicators were not worsened in diabetic patients taking pomegranate juice; serum lipid peroxidases were reduced by 56% and TBSARs by 24% whereas serum SH groups increased by 12% and paraoxonase activity by 24% [80]. In other clinical studies, consumption of pomegranate juice or extracts were also well tolerated [36, 38, 40, 81–85]. No toxic effects were seen in a one-year pilot study of the proprietary pomegranate extract POMx in 10 patients with carotid artery stenosis (5 of whom continued taking the extract another 2 years) [86]. Interestingly, the improvement in clinical signs took place during the first 12 months of the study but was maintained over the following 2 years. Pomegranate fruit and peel extracts have so far been used safely from a toxicological perspective [87].

Allergies to pomegranate may occur but are very rare [88–92]. One case report described exercise-induced anaphylaxis triggered by the ingestion of pomegranate, the allergy being confirmed by immunoblotting and absence of lipid transfer protein cross-reactivity, although exercise-induced anaphylaxis is generally independent of the kind of food ingested before exercise [93]. Mannitol which is also contained in pomegranate has been identified as causing IgE-mediated hypersensitivity [94].

5.3. Risk of Interactions. If pomegranate preparations are taken over longer periods, putative interactions with other medications need to be considered. This is because the pomegranate active principle interacts with hepatic cytochrome P450 [95, 96]. The *in vitro* 1′-hydroxylase activity of midazolam, catalysed by human CYP3A, was inhibited less by a commercial pomegranate juice than by the juices from grapefruit, black mulberry, and wild grape [97]. Pomegranate juice did not impair the clearance of oral or intravenous midazolam in volunteers, [98]. However, rhabdomyolysis has been associated with pomegranate juice consumption in a patient taking synthetic rosuvastatin, though the latter is not known to be metabolized by hepatic P450 3A4 [99]. More

studies are needed to determine whether these and other interactions such as the interaction between pomegranate-containing products and the immunosuppressive agent tacrolimus [100] are clinically significant [101].

5.4. Analyses of Various Pomegranate Products. The review of the literature indicates that the active principle of pomegranate may well have a potent anticancer potential, but the clinical evidence of effectiveness is still poor because of the poor quality of the available clinical studies. (The results of four further studies are awaited.) Hong and coworkers described the POMx extract as containing monomeric and oligomeric ellagitannins (punicalagin 37–40% and 3.4% free ellagic acid) but no anthocyanins as determined by high performance-liquid chromatography. Thus, a dose of 1000 mg of extract contained 400 mg of both ellagitannins. Paller et al. [37] stated that each POMx capsule contained 1000 mg of polyphenol extract, comparable to about 8 oz (about 240 mL) of pomegranate juice. According to the "POM wonderful pomegranate juice monograph" of the American Botanical Council [102], the juice contained 1.74 mg/mL punicalagin and 0.14 mg/mL ellagic acid, a dose of 94 mg all together in 240 mL. According to the voice message from the company (see webpage: voice message) one POMx pill contained 370 mg punicalagin. This dose has also been mentioned in the review by Kroeger et al. [39]. But according to our measurements, POMx capsules contained only 132 mg of punicalagin and ellagic acid (combined). Since 3000 mg have not been more effective than 1000 mg, the optimum dose until a ceiling effect occurs may be in between these doses (1000 and 3000 mg) or the ceiling effect may even occur at a dose *less than* 1000 mg. This needs to be clarified in a careful dose-finding study.

A competing company has developed their extract POMELLA based on work at the University of California, Los Angeles. The extract (drug : extract ratio: 50 : 1, solvent not stated) is standardized by HPLC on 30% of punicalagins in addition to smaller amounts of other marker compounds that exist at concentrations less than 5% (ellagic acid, gallic acid, and gallagic acid). Batch (Lot number LPR1EP1212L09) contained 300 mg/1000 mg punicalagin and 20 mg/ellagic acid/1000 mg (see http://pomextract.com/Pomella-Story_fc7cfcf6fd873a1634.html). A daily dose of 1000 mg of this extract contains at least 320 mg of total polyphenols and is presently being tested in a clinical study [39]. The photometrically assessed polyphenol content varies between 60 and 70% ([48]; see webpage POMELLA)

For colorimetric quantification of polyphenolic antioxidants in general, the Folin-Ciocalteu assay is used with gallic acid as reference [103]. Theoretically, however, any polyphenol could be used as reference compound (e.g., pyrogallol (Table 1)). Martin et al. [104] proposed replacing gallic acid by a purified pomegranate pomace extract in the Folin-Ciocalteu assay. This purified extract contained at least 5.6% nonpolyphenols (identified as sugars, moisture, ash, and nitrogen (Kjeldhal assay)) compared with 9.1% in the raw POMx extract (trademark) [104]. The polyphenolic composition of this purified extract has not been quantified in terms of pure reference compounds. Data are not presented as absolute values but as rough estimates of polyphenol contents expressed as percentages of total polyphenols. When POMx extract was analyzed by Folin-Ciocalteu using the purified extract as reference, the result (unsurprisingly) was 92.6%. This percentage does not necessarily reflect 92.6% of polyphenols, since the absolute polyphenolic content of the purified pomegranate pomace extract has not been analysed. Thus, though the purified pomegranate pomace extract may well be appropriate for quality control of the POMx extraction process, it does not allow quantification of polyphenols in pomegranate products as suggested by Martin et al. [104]. The 15.7% of punicalagin expressed as a percentage of the 92.6% total polyphenols suggests a putative absolute value of 14.5% of punicalagin in POMx. It remains questionable why POMx has been characterized as extract standardized on 37–40% punicalagin assessed by HPLC [14]. The Folin-Ciocalteu assay has never claimed to reflect the absolute polyphenol content of a sample. It has been designed as an index for comparing similar samples [105] by using gallic acid as reference compound. The Folin-Ciocalteu values were not declared on the POMx capsules we bought.

Pomegranate preparations can contain up to 48 phenolic compounds, and the complexity of their polyphenolic profiles necessitates the use of hyphenated techniques for a thorough evaluation of their composition [106–108]. For reasons of expense, only punicalagin, ellagic acid, and anthocyanins are measured in routine laboratories despite the presence of larger concentrations of other ellagitannins in processed pomegranate preparations, as shown by Fischer et al. 2011 [107, 108]. Although, if all polyphenols in pure pomegranate preparations were included in the HPLC analysis, resulting estimates of total polyphenol content correlated well with photometric estimates, the correlations were very poor if only punicalagin, ellagic acid, and anthocyanins were used. Our results substantiate this (Table 2).

Standardization of products solely on photometric assessments can be misleading and the content of punicalagin, ellagic acid, and anthocyanins as assessed by routine HPLC should be declared on product labels in addition to the photometric estimates. Both indicators should replace descriptions such as the one that came with the extract "Ultra Granatapfel forte," claiming that "the punicalagin dose in one capsule is equivalent to 840 mL mother juice" (see webpage: Ultra Granatapfel forte Capsules). Such information is unhelpful because one 500 mg capsule contained only 20 mg polyphenols as assessed by HPLC. Depending on what is taken as the benchmark daily dose for prostate cancer—130 or 400 mg ellagitannins—, many capsules of this product may need to be taken daily, which would be inconvenient as well as expensive.

The dose of oral pomegranate fruit extract chosen in mice to inhibit tumorigenesis was based on the assumption that a typical healthy 70 kg individual may be persuaded to drink 500 mL of pomegranate juice extracted from two fruits [109], containing a putative polyphenol dose of around 350 mg per day (Table 2). Of the products investigated, this dose is contained in 1000 mg of POMx extract if we can rely on the study by Hong et al. [14] or 3 POMx capsules as currently available (Table 2), in 350 mL (3.5 cups) of pure

juice L3074, in 12 mL of the concentrated fermented juice F4 supplemented with elderberry concentrate, in 2-3 of the proprietary capsules GranaProstan, or in 16 of the proprietary capsules Ultra Granatapfel forte (an inappropriate dose). Though these doses are large, they can be used safely in patients [110].

Tables 2 and 3 show that the quantity and the spectrum of phenolic compounds vary greatly in different products, depending partly on the ripeness of the fruits [111]. Fresh pomegranates contained between 11 and 1543 mg anthocyanins/L depending on the colour of the variety, white, rose, dark red, and purple [112]. Fresh juices contained 904 to 2067 mg/L of total phenols as assessed by Folin-Ciocalteu [112]. This is in accordance with the study by Gómez-Caravaca and coworkers [113] who found that the total phenolic content ranged from 581 to 2551 mg/L in the pomegranate juices they investigated. Table 2 shows that only one of our pure juices was within this range. Our anthocyanin: polyphenol ratios were lower than those reported by Gómez-Caravaca and coworkers [113], which varied between 20 to 82%; this may well indicate anthocyanin degradation in the samples we analysed.

Reductions or losses of phenolic compounds have been reported in commercial juices, and these have been attributed to commercial processing procedures [114]. Although mother juices (100% pure juices) should contain more polyphenols than blended juices, only 3 of 6 pure juices were rich in ellagitannins and antioxidant capacity. Only one of the 6 pure juices that were rich in ellagitannin was also rich in anthocyanins. Some of the other pure juices had even a lower antioxidant capacity than blended juices. In some juices the antioxidant capacity was attributable to vitamin C rather than to phenolic compounds [106]. Vitamin C may preserve coactive compounds [115].

Factors affecting the stability of anthocyanins in juices include pH, the presence of enzymes and copigments such as metallic ions and sugars and, such processing features as the intensity and duration of heating, the storage temperature, and time and the presence of oxygen and/or light. Short-term thermal treatments (65 and 90°C for 30 or 5 s) decreased the percentage of polymeric anthocyanins and increased the amount of monomeric anthocyanins and thus the bioavailability of coactive compounds [116]. Pasteurization had no influence on the total polyphenols and antioxidant capacity of juices. However, the storage temperature was the main factor affecting all coactive compounds, the total monomeric and individual anthocyanins, the total phenolic compounds, and therefore also the antioxidant activity [116]. Fast degradation of anthocyanins was observed in juices stored at 25°C, while refrigerated storage at 5°C resulted in much slower degradation. Cyanidin-3-O-glucoside was less stable than delphinidin- and cyanidin-3,5-diglucosides. There was a linear relationship between total monomeric anthocyanins and antioxidative capacity [116]. Consistently, liquid pomegranate peel extracts had acceptable thermal stability after sterilization and storage at low temperature [117]. Longer thermal treatment of juices (heating at 90°C for 5 h) resulted in total anthocyanin losses ranging from 76% to 87% of the initial anthocyanin levels. The anthocyanin stability was independent of the total phenolic content and of low and high molecular weight pomegranate matrix components (such as organic acids and sugars) [118]. Exposure to light during storage also affects loss of coactive compound [107, 117]. Good correlation of the anthocyanins with red colour was observed for all samples at elevated temperatures (70–90°C), but the visual appearance did not adequately reflect the quality and storage stability of pomegranate juices [118].

Ellagitannins seem to be the major antioxidants in pomegranate juices [106]. Commercial juices from whole pomegranates contained about 1500–1900 mg/L punicalagin while only traces of this compound were detected in self-made freshly squeezed juice from pomegranate arils. The ellagitannins in extracts from whole pomegranate are therefore derived from the peel [119]. Punicalagin concentrations ranged from 1100 to 2000 mg/kg dry matter of mesocarp and peel and from 4 to 565 mg/L in aril juices [119]. The punicalagin contents in the two pure juices analysed here are within or above this range (Table 2). For the whole pomegranate fruit extracts the punicalagin content of 95% relative to the total polyphenols and the low anthocyanin content of around 1% reflect the extraction from whole pomegranates (Table 2). The anthocyanins in the F4 preparation are derived from the additional elderberry as revealed by the individual elderberry anthocyanin components (Table 3).

Homogenates prepared from the whole fruit showed about a 20-fold higher antioxidant activity than did aril homogenates, which correlated significantly with the content of the four major hydrolyzable tannins (predominantly punicalagin) [120]. Likewise, when extracted with an ethanol-acetone extraction solvent, pomegranate peel showed greater antioxidant capacity than did pulp. This was consistent with the higher contents of total phenolics, flavonoids, and proanthocyanidins in the peel extract [121]. No correlation between antioxidant activity and level of anthocyanins was found [120]. When polyphenols purified from peel containing juice products were compared with those from peel-free juice, the radical scavenging effect was lower with the latter [122]. Juicing with peel made the juice bitter and astringent [122].

When 19 pomegranate food supplements were compared, only a limited number of pomegranate supplements were believably authentic. Product labels were inconsistent with polyphenol composition and antioxidant content. Thirteen samples contained disproportionately large amounts of ellagic acid and low or no detectable pomegranate tannins. Only six products had a tannin profile (punicalagin, punicalin, ellagitannins, and gallotannins) consistent with pomegranate. Natural pomegranate extract was the most representative of pomegranate fruit polyphenols with 99% total pomegranate polyphenol and the highest antioxidant capacity across all measures (Extract 1, lower part of Table 2. There were strong correlations between total polyphenols and antioxidant capacity in products that had polyphenol compositions consistent with a pomegranate source but not with products that contained large amounts of ellagic acid and little or no detectable pomegranate tannins. Thus, regulation of the market is required to assure consumers of the quality of pomegranate supplements [123, 124]. The content of saccharose and D-sorbit should be negligible, the

glucose to fructose ratio should be below 1, the quantity of titratable acid (calculated as citric acid, pH 8.1) should vary between 1.9, and 45 g/L and malic acid should never be present. A comparative analysis of pomegranate juices and concentrates verified that many commercial products are mixed with sugar, colouring ingredients, and other fruit juices [112, 125]: these may or may not influence therapeutic effectiveness.

Processing conditions of the fruits, that is, coextraction of arils and peel and pressure, markedly affected the profiles and contents of phenolics in the pomegranate juices [108], underlining the necessity to optimise these features for obtaining products with well-defined and reproducible functional properties [119]. Heating plus refrigeration may help to reduce anthocyanin degradation in pasteurized pomegranate juice, avoiding a dramatic impact on its colour and preserving the beneficial effects [116]. Future research should identify the optimum coactive compound composition of a pomegranate preparation for the treatment of prostate cancer [120] with the primary outcome of 5-year survival. Declaration of the content of coactive constituents helps to identify quality products. However, direct evidence for bioequivalence between products can only come from well-planned clinical studies. Because of the complexity of the coactive pomegranate compounds, similar bioavailabilities of coactive compounds cannot provide indirect evidence for bioequivalence unless the clinical effectiveness for the leading polyphenol mixture has been confidently established [126]. This is because bioequivalence requires not only pharmaceutical similarity of components, but also their pharmacological and therapeutic equivalence.

Ellagic acid and its metabolites are found in human plasma after ingestion of pomegranate. Its antioxidant capacity was retained *ex vivo* [127]. An average ellagic acid serum concentration of 0.14 μM/L was attained after consumption of a proprietary pomegranate extract and was associated with a putative anticancer effect [36]. Although similar ellagic acid serum concentrations were attained after taking extract or juice [23], another study showed lower concentrations of 0.06 μM/L after drinking 180 mL of a pomegranate juice [128]. As long as we do not know which polyphenol (or polyphenols) is (or are) responsible for the putative anticancer effect, it is unwise to base dosing of pomegranate products on ellagic acid, because serum ellagic acid or its metabolites are the metabolites of various oligomeric polyphenols. Pharmacodynamic or *ex vivo/in vitro* tests are not surrogates for bioequivalence unless the results can be shown to correlate with therapeutic effectiveness [126].

In summary, there is evidence that pomegranate has a putative anticancerogenic effect in prostate cancer and can safely be used in high doses. But commercial pomegranate products vary greatly in their content of coactive ingredients. For reasons of transparency, consumers should know not only the photometric quantification of the polyphenols in the daily recommended dosage, but also the content of HPLC-analysed polyphenols. Only then can they choose a dose that has a chance of being effective in the treatment of cancer. The preparation of pomegranate end-products is affected by many determinants. Their declaration should be incorporated into the regulatory guidance and controlled before pomegranate products are allowed on the market.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] V. M. Adhami, N. Khan, and H. Mukhtar, "Cancer chemoprevention by pomegranate: laboratory and clinical evidence," *Nutrition and Cancer*, vol. 61, no. 6, pp. 811–815, 2009.

[2] M. Toi, H. Bando, C. Ramachandran et al., "Preliminary studies on the anti-angiogenic potential of pomegranate fractions in vitro and in vivo," *Angiogenesis*, vol. 6, no. 2, pp. 121–128, 2003.

[3] S. Koyama, L. J. Cobb, H. H. Mehta et al., "Pomegranate extract induces apoptosis in human prostate cancer cells by modulation of the IGF-IGFBP axis," *Growth Hormone and IGF Research*, vol. 20, no. 1, pp. 55–62, 2010.

[4] M. B. Rettig, D. Heber, J. An et al., "Pomegranate extract inhibits androgen-independent prostate cancer growth through a nuclear factor-κB-dependent mechanism," *Molecular Cancer Therapeutics*, vol. 7, no. 9, pp. 2662–2671, 2008.

[5] A. Malik, F. Afaq, S. Sarfaraz, V. M. Adhami, D. N. Syed, and H. Mukhtar, "Pomegranate fruit juice for chemoprevention and chemotherapy of prostate cancer," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 41, pp. 14813–14818, 2005.

[6] A. Bishayee, R. J. Thoppil, A. S. Darvesh, V. Ohanyan, J. G. Meszaros, and D. Bhatia, "Pomegranate phytoconstituents blunt the inflammatory cascade in a chemically induced rodent model of hepatocellular carcinogenesis," *Journal of Nutritional Biochemistry*, vol. 24, no. 1, pp. 178–187, 2013.

[7] P. Filannino, L. Azzi, I. Cavoski et al., "Exploitation of the health-promoting and sensory properties of organic pomegranate (*Punica granatum* L.) juice through lactic acid fermentation," *International Journal of Food Microbiology*, vol. 163, no. 2-3, pp. 184–192, 2013.

[8] N. D. Kim, R. Mehta, W. Yu et al., "Chemopreventive and adjuvant therapeutic potential of pomegranate (*Punica granatum*) for human breast cancer," *Breast Cancer Research and Treatment*, vol. 71, no. 3, pp. 203–217, 2002.

[9] R. Mehta and E. P. Lansky, "Breast cancer chemopreventive properties of pomegranate (*Punica granatum*) fruit extracts in a mouse mammary organ culture," *European Journal of Cancer Prevention*, vol. 13, no. 4, pp. 345–348, 2004.

[10] F. Aqil, R. Munagala, M. V. Vadhanam et al., "Anti-proliferative activity and protection against oxidative DNA damage by punicalagin isolated from pomegranate husk," *Food Research International*, vol. 49, no. 1, pp. 345–353, 2012.

[11] M. Larrosa, F. A. Tomás-Barberán, and J. C. Espín, "The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway," *Journal of Nutritional Biochemistry*, vol. 17, no. 9, pp. 611–625, 2006.

[12] C. Palmieri, D. K. Patten, A. Januszewski, G. Zucchini, and S. J. Howell, "Breast cancer: current and future endocrine therapies," *Molecular and Cellular Endocrinology*, vol. 382, no. 1, pp. 695–723, 2014.

[13] S. Banerjee, S. Kambhampati, I. Haque, and S. K. Banerjee, "Pomegranate sensitizes Tamoxifen action in ER-α positive breast cancer cells," *Journal of Cell Communication and Signaling*, vol. 5, no. 4, pp. 317–324, 2011.

[14] M. Y. Hong, N. P. Seeram, and D. Heber, "Pomegranate polyphenols down-regulate expression of androgen-synthesizing genes in human prostate cancer cells overexpressing the androgen receptor," *Journal of Nutritional Biochemistry*, vol. 19, no. 12, pp. 848–855, 2008.

[15] P. Pitchakarn, T. Chewonarin, K. Ogawa et al., "Ellagic acid inhibits migration and invasion by prostate cancer cell lines," *Asian Pacific Journal of Cancer Prevention*, vol. 14, no. 5, pp. 2859–2863, 2013.

[16] M. Albrecht, W. Jiang, J. Kumi-Diaka et al., "Pomegranate extracts potently suppress proliferation, xenograft growth, and invasion of human prostate cancer cells," *Journal of Medicinal Food*, vol. 7, no. 3, pp. 274–283, 2004.

[17] N. P. Seeram, L. S. Adams, S. M. Henning et al., "In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice," *Journal of Nutritional Biochemistry*, vol. 16, no. 6, pp. 360–367, 2005.

[18] M. R. Sartippour, N. P. Seeram, J. Y. Rao et al., "Ellagitannin-rich pomegranate extract inhibits angiogenesis in prostate cancer in vitro and in vivo," *International Journal of Oncology*, vol. 32, no. 2, pp. 475–480, 2008.

[19] A. Malik and H. Mukhtar, "Prostate cancer prevention through pomegranate fruit," *Cell Cycle*, vol. 5, no. 4, pp. 371–373, 2006.

[20] N. P. Seeram, W. J. Aronson, Y. Zhang et al., "Pomegranate ellagitannin-derived metabolites inhibit prostate cancer growth and localize to the mouse prostate gland," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 19, pp. 7732–7737, 2007.

[21] L. S. Adams, Y. Zhang, N. P. Seeram, D. Heber, and S. Chen, "Pomegranate ellagitannin-derived compounds exhibit antiproferative and antiaromatase activity in breast cancer cells in vitro," *Cancer Prevention Research*, vol. 3, no. 1, pp. 108–113, 2010.

[22] R. Vicinanza, Y. Zhang, S. M. Henning, and D. Heber, "Pomegranate juice metabolites, ellagic acid and urolithin a, synergistically inhibit androgen-independent prostate cancer cell growth via distinct effects on cell cycle control and apoptosis," *Evidence-based Complementary and Alternative Medicine*, vol. 2013, Article ID 247504, 12 pages, 2013.

[23] N. P. Seeram, Y. Zhang, R. McKeever et al., "Pomegranate juice and extracts provide similar levels of plasma and urinary ellagitannin metabolites in human subjects," *Journal of Medicinal Food*, vol. 11, no. 2, pp. 390–394, 2008.

[24] B. Cerdá, J. C. Espín, S. Parra, P. Martínez, and F. A. Tomás-Barberán, "The potent in vitro antioxidant ellagitannins from pomegranate juice are metabolised into bioavailable but poor antioxidant hydroxy-6H-dibenzopyran-6-one derivatives by the colonic microflora of healthy humans," *European Journal of Nutrition*, vol. 43, no. 4, pp. 205–220, 2004.

[25] R. Agosti, R. K. Duke, J. E. Chrubasik, and S. Chrubasik, "Effectiveness of *Petasites hybridus* preparations in the prophylaxis of migraine: a systematic review," *Phytomedicine*, vol. 13, no. 9-10, pp. 743–746, 2006.

[26] S. Chrubasik, C. Conradt, and B. D. Roufogalis, "Effectiveness of *Harpagophytum* extracts and clinical efficacy," *Phytotherapy Research*, vol. 18, no. 2, pp. 187–189, 2004.

[27] J. J. Gagnier, S. Chrubasik, and E. Manheimer, "Harpgophytum procumbens for osteoarthritis and low back pain: a systematic review," *BMC Complementary and Alternative Medicine*, vol. 4, article 13, 2004.

[28] C. Chrubasik, R. K. Duke, and S. Chrubasik, "The evidence for clinical efficacy of rose hip and seed: a systematic review," *Phytotherapy Research*, vol. 20, no. 1, pp. 1–3, 2006.

[29] J. E. Chrubasik, B. D. Roufogalis, H. Wagner, and S. A. Chrubasik, "A comprehensive review on nettle effect and efficacy profiles, part I: herba urticae," *Phytomedicine*, vol. 14, no. 6, pp. 423–435, 2007.

[30] J. E. Chrubasik, B. D. Roufogalis, H. Wagner, and S. Chrubasik, "A comprehensive review on the stinging nettle effect and efficacy profiles. Part II: urticae radix," *Phytomedicine*, vol. 14, no. 7-8, pp. 568–579, 2007.

[31] C. Chrubasik, B. D. Roufogalis, U. Müller-Ladner, and S. Chrubasik, "A systematic review on the *Rosa canina* effect and efficacy profiles," *Phytotherapy Research*, vol. 22, no. 6, pp. 725–733, 2008.

[32] C. Chrubasik, G. Li, and S. Chrubasik, "The clinical effectiveness of chokeberry: a systematic review," *Phytotherapy Research*, vol. 24, no. 8, pp. 1107–1114, 2010.

[33] J. E. Vlachojannis, M. Cameron, and S. Chrubasik, "A systematic review on the effectiveness of willow bark for musculoskeletal pain," *Phytotherapy Research*, vol. 23, no. 7, pp. 897–900, 2009.

[34] J. E. Vlachojannis, M. Cameron, and S. Chrubasik, "A systematic review on the Sambuci fructus effect and efficacy profiles," *Phytotherapy Research*, vol. 24, no. 1, pp. 1–8, 2010.

[35] J. E. Vlachojannis, M. Cameron, and S. Chrubasik, "Medicinal use of potato-derived products: a systematic review," *Phytotherapy Research*, vol. 24, no. 2, pp. 159–162, 2010.

[36] A. J. Pantuck, J. T. Leppert, N. Zomorodian et al., "Phase II study of pomegranate juice for men with rising prostate-specific antigen following surgery or radiation for prostate cancer," *Clinical Cancer Research*, vol. 12, no. 13, pp. 4018–4026, 2006.

[37] C. J. Paller, X. Ye, P. J. Wozniak et al., "A randomized phase II study of pomegranate extract for men with rising PSA following initial therapy for localized prostate cancer," *Prostate Cancer and Prostatic Diseases*, vol. 16, no. 1, pp. 50–55, 2013.

[38] S. J. Freedland, M. Carducci, N. Kroeger et al., "A double-blind, randomized, neoadjuvant study of the tissue effects of POMx pills in men with prostate cancer before radical prostatectomy," *Cancer Prevention Research*, vol. 6, no. 10, pp. 1120–1127, 2013.

[39] N. Kroeger, A. S. Belldegrun, and A. J. Pantuck, "Pomegranate extracts in the management of men's urologic health: Scientific rationale and preclinical and clinical data," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 701434, 9 pages, 2013.

[40] F. Stenner-Liewen, H. Liewen, R. Cathomas et al., "Daily pomegranate intake has no impact on PSA levels in patients with advanced prostate cancer—results of a phase IIb randomized controlled trial," *Journal of Cancer*, vol. 4, no. 7, pp. 597–605, 2013.

[41] S. Chrubasik-Hausmann, C. Vlachojannis, and B. Zimmermann, "Pomegranate juice and prostate cancer: importance of the characterisation of the active principle," *Phytotherapy Research*, 2014.

[42] E. Davidson, J. Vlachojannis, M. Cameron, and S. Chrubasik, "Best available evidence in cochrane reviews on herbal medicine?" *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 163412, 7 pages, 2013.

[43] S. Chrubasik-Hausmann, C. Vlachojannis, and B. F. Zimmermann, "Proanthocyanin content in cranberry CE medicinal products," *Phytotherapy Research*, 2014.

[44] E. Davidson, B. F. Zimmermann, E. Jungfer, and S. Chrubasik-Hausmann, "Prevention of urinary tract infections with *Vaccinium* products," *Phytotherapy Research*, vol. 28, no. 3, pp. 465–470, 2014.

[45] D. S. Ming, S. Pham, S. Deb et al., "Pomegranate extracts impact the androgen biosynthesis pathways in prostate cancer models *in vitro* and *in vivo*," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 143, pp. 19–28, 2014.

[46] Y. Wang, S. Zhang, S. Iqbal et al., "Pomegranate extract inhibits the bone metastatic growth of human prostate cancer cells and enhances the in vivo efficacy of docetaxel chemotherapy," *Prostate*, vol. 74, no. 5, pp. 497–508, 2014.

[47] B. Cerdá, J. J. Cerón, F. A. Tomás-Barberán, and J. C. Espín, "Repeated oral administration of high doses of the pomegranate ellagitannin punicalagin to rats for 37 days is not toxic," *Journal of Agricultural and Food Chemistry*, vol. 51, no. 11, pp. 3493–3501, 2003.

[48] C. Patel, P. Dadhaniya, L. Hingorani, and M. G. Soni, "Safety assessment of pomegranate fruit extract: acute and subchronic toxicity studies," *Food and Chemical Toxicology*, vol. 46, no. 8, pp. 2728–2735, 2008.

[49] R. K. Kishore, D. Sudhakar, and P. R. Parthasarathy, "Embryo protective effect of pomegranate (*Punica granatum* L.) fruit extract in adriamycin-induced oxidative stress," *Indian Journal of Biochemistry and Biophysics*, vol. 46, no. 1, pp. 106–111, 2009.

[50] K. P. Leiva, J. Rubio, F. Peralta, and G. F. Gonzales, "Effect of Punica granatum (pomegranate) on sperm production in male rats treated with lead acetate," *Toxicology Mechanisms and Methods*, vol. 21, no. 6, pp. 495–502, 2011.

[51] C. Fimognari, P. Sestili, M. Lenzi, A. Bucchini, G. Cantelli-Forti, and P. Hrelia, "RNA as a new target for toxic and protective agents," *Mutation Research—Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 648, no. 1-2, pp. 15–22, 2008.

[52] E. M. Al-Olayan, M. F. El-Khadragy, D. M. Metwally, and A. E. Abdel Moneim, "Protective effects of pomegranate (*Punica granatum*) juice on testes against carbon tetrachloride intoxication in rats," *BMC Complementary and Alternative Medicine*, vol. 14, article 164, 2014.

[53] V. Şen, M. Bozkurt, S. Söker et al., "The effects of pomegranate and carvacrol on methotrexate-induced bone marrow toxicity in rats," *Clinical and Investigative Medicine*, vol. 37, no. 2, pp. E93–E101, 2014.

[54] F. Çelik, C. Gocmez, M. Bozkurt et al., "Neuroprotective effects of carvacrol and pomegranate against methotrexate-induced toxicity in rats," *European Review for Medical and Pharmacological Sciences*, vol. 17, no. 22, pp. 2988–2993, 2013.

[55] A. E. Abdel Moneim, M. S. Othman, S. M. Mohmoud, and K. M. El-Deib, "Pomegranate peel attenuates aluminum-induced hepatorenal toxicity," *Toxicology Mechanisms and Methods*, vol. 23, no. 8, pp. 624–633, 2013.

[56] D. Kumar, S. Singh, A. K. Singh, and S. I. Rizvi, "Pomegranate (*Punica granatum*) peel extract provides protection against mercuric chloride-induced oxidative stress in Wistar strain rats," *Pharmaceutical Biology*, vol. 51, no. 4, pp. 441–446, 2013.

[57] M. Cekmen, A. Otunctemur, E. Ozbek et al., "Pomegranate extract attenuates gentamicin-induced nephrotoxicity in rats by reducing oxidative stress," *Renal Failure*, vol. 35, no. 2, pp. 268–274, 2013.

[58] M. T. Boroushaki, D. Arshadi, H. Jalili-Rasti, E. Asadpour, and A. Hosseini, "Protective effect of pomegranate seed oil against acute toxicity of diazinon in rat kidney," *Iranian Journal of Pharmaceutical Research*, vol. 12, no. 4, pp. 821–827, 2013.

[59] M. T. Bouroshaki, H. R. Sadeghnia, M. Banihasan, and S. Yavari, "Protective effect of pomegranate seed oil on hexachlorobutadiene-induced nephrotoxicity in rat kidneys," *Renal Failure*, vol. 32, no. 5, pp. 612–617, 2010.

[60] I. A. T. M. Meerts, C. M. Verspeek-Rip, C. A. F. Buskens et al., "Toxicological evaluation of pomegranate seed oil," *Food and Chemical Toxicology*, vol. 47, no. 6, pp. 1085–1092, 2009.

[61] A. Faria, R. Monteiro, N. Mateus, I. Azevedo, and C. Calhau, "Effect of pomegranate (*Punica granatum*) juice intake on hepatic oxidative stress," *European Journal of Nutrition*, vol. 46, no. 5, pp. 271–278, 2007.

[62] M. Pirinççioğlu, G. Kizil, M. Kizil, G. Özdemir, Z. Kanay, and M. A. Ketani, "Protective effect of Öküzgözü (*Vitis vinifera* L. cv.) grape juice against carbon tetrachloride induced oxidative stress in rats," *Food and Function*, vol. 3, no. 6, pp. 668–673, 2012.

[63] A. E. Abdel Moneim and M. F. El-Khadragy, "The potential effects of pomegranate (Punica granatum) juice on carbon tetrachloride-induced nephrotoxicity in rats," *Journal of Physiology and Biochemistry*, vol. 69, no. 3, pp. 359–370, 2013.

[64] V. Tugcu, E. Kemahli, E. Ozbek et al., "Protective effect of a potent antioxidant, pomegranate juice, in the kidney of rats with nephrolithiasis induced by ethylene glycol," *Journal of Endourology*, vol. 22, no. 12, pp. 2723–2731, 2008.

[65] J. Boateng, M. Verghese, L. Shackelford et al., "Selected fruits reduce azoxymethane (AOM)-induced aberrant crypt foci (ACF) in Fisher 344 male rats," *Food and Chemical Toxicology*, vol. 45, no. 5, pp. 725–732, 2007.

[66] Z. M. Yazici, A. Meric, A. Midi, Y. V. ArInc, V. Kahya, and G. HafIz, "Reduction of cisplatin ototoxicity in rats by oral administration of pomegranate extract," *European Archives of Oto-Rhino-Laryngology*, vol. 269, no. 1, pp. 45–52, 2012.

[67] V. Kahya, B. Ozucer, R. Dogan et al., "Pomegranate extract: a potential protector against aminoglycoside ototoxicity," *Journal of Laryngology and Otology*, vol. 128, no. 1, pp. 43–48, 2014.

[68] M. Hassanpour Fard, A. E. Ghule, S. L. Bodhankar, and M. Dikshit, "Cardioprotective effect of whole fruit extract of pomegranate on doxorubicin-induced toxicity in rat," *Pharmaceutical Biology*, vol. 49, no. 4, pp. 377–382, 2011.

[69] A. Vidal, A. Fallarero, B. R. Peña et al., "Studies on the toxicity of *Punica granatum* L. (Punicaceae) whole fruit extracts," *Journal of Ethnopharmacology*, vol. 89, no. 2-3, pp. 295–300, 2003.

[70] P. S. Negi, G. K. Jayaprakasha, and B. S. Jena, "Antioxidant and antimutagenic activities of pomegranate peel extracts," *Food Chemistry*, vol. 80, no. 3, pp. 393–397, 2003.

[71] M. Zahin, F. Aqil, and I. Ahmad, "Broad spectrum antimutagenic activity of antioxidant active fraction of *Punica granatum* L. peel extracts," *Mutation Research—Genetic Toxicology and Environmental Mutagenesis*, vol. 703, no. 2, pp. 99–107, 2010.

[72] A. de Amorim, H. R. Borba, and J. L. Armada, "Test of mutagenesis in mice treated with aqueous extracts from *Punica granatum* L. (Pomegranate)," *Revista Brasileira de Farmacia*, vol. 76, no. 4, pp. 110–111, 1995.

[73] M. C. Valadares, E. R. T. Pereira, P. L. Benfica, and J. R. Paula, "Assessment of mutagenic and antimutagenic effects of *Punica granatum* in mice," *Brazilian Journal of Pharmaceutical Sciences*, vol. 46, no. 1, pp. 121–127, 2010.

[74] A. Sánchez-Lamar, G. Fonseca, J. L. Fuentes et al., "Assessment of the genotoxic risk of *Punica granatum* L. (Punicaceae) whole fruit extracts," *Journal of Ethnopharmacology*, vol. 115, no. 3, pp. 416–422, 2008.

[75] S. M. Tripathi and D. K. Singh, "Molluscicidal activity of *Punica granatum* bark and *Canna indica* root," *Brazilian Journal of Medical and Biological Research*, vol. 33, no. 11, pp. 1351–1355, 2000.

[76] L. Ferrara, O. Schettino, P. Forgione, V. Rullo, and S. Di Gennaro, "Identification of the root of *Punica granatum* in galenic preparations using TLC," *Bollettino della Societa Italiana di Biologia Sperimentale*, vol. 65, no. 5, pp. 385–390, 1989.

[77] E. P. Lansky and R. A. Newman, "*Punica granatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer," *Journal of Ethnopharmacology*, vol. 109, no. 2, pp. 177–206, 2007.

[78] S. Okonogi, C. Duangrat, S. Anuchpreeda, S. Tachakittirungrod, and S. Chowwanapoonpohn, "Comparison of antioxidant capacities and cytotoxicities of certain fruit peels," *Food Chemistry*, vol. 103, no. 3, pp. 839–846, 2007.

[79] D. Heber, N. P. Seeram, H. Wyatt et al., "Safety and antioxidant activity of a pomegranate ellagitannin-enriched polyphenol dietary supplement in overweight individuals with increased waist size," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 24, pp. 10050–10054, 2007.

[80] M. Rosenblat, T. Hayek, and M. Aviram, "Anti-oxidative effects of pomegranate juice (PJ) consumption by diabetic patients on serum and on macrophages," *Atherosclerosis*, vol. 187, no. 2, pp. 363–371, 2006.

[81] M. Aviram and L. Dornfeld, "Pomegranate juice consumption inhibits serum angiotensin converting enzyme activity and reduces systolic blood pressure," *Atherosclerosis*, vol. 158, no. 1, pp. 195–198, 2001.

[82] M. D. Sumner, M. Elliott-Eller, G. Weidner et al., "Effects of pomegranate juice consumption on myocardial perfusion in patients with coronary heart disease," *The American Journal of Cardiology*, vol. 96, no. 6, pp. 810–814, 2005.

[83] B. Cerdá, C. Soto, M. D. Albaladejo et al., "Pomegranate juice supplementation in chronic obstructive pulmonary disease: a 5-week randomized, double-blind, placebo-controlled trial," *European Journal of Clinical Nutrition*, vol. 60, no. 2, pp. 245–253, 2006.

[84] C. P. Forest, H. Padma-Nathan, and H. R. Liker, "Efficacy and safety of pomegranate juice on improvement of erectile dysfunction in male patients with mild to moderate erectile dysfunction: a randomized, placebo-controlled, double-blind, crossover study," *International Journal of Impotence Research*, vol. 19, no. 6, pp. 564–567, 2007.

[85] A. Basu, E. D. Newman, A. L. Bryant, T. J. Lyons, and N. M. Betts, "Pomegranate polyphenols lower lipid peroxidation in adults with type 2 diabetes but have no effects in healthy volunteers: a pilot study," *Journal of Nutrition and Metabolism*, vol. 2013, Article ID 708381, 7 pages, 2013.

[86] M. Aviram, M. Rosenblat, D. Gaitini et al., "Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation," *Clinical Nutrition*, vol. 27, no. 4, p. 671, 2008.

[87] T. Ismail, P. Sestili, and S. Akhtar, "Pomegranate peel and fruit extracts: a review of potential anti-inflammatory and anti-infective effects," *Journal of Ethnopharmacology*, vol. 143, no. 2, pp. 397–405, 2012.

[88] T. Yamamoto, K. Asakura, H. Shirasaki, and T. Himi, "Questionnaire about the intake of and hypersensitivity to fruits, vegetables and nuts including birch pollen related foods," *Journal of Otolaryngology of Japan*, vol. 116, no. 7, pp. 779–788, 2013.

[89] B. Buyuktiryaki, B. Bartolomé, U. M. Sahiner et al., "Pomegranate allergy and pathogenesis-related protein 4," *Annals of Allergy, Asthma and Immunology*, vol. 111, no. 3, pp. 231–232, 2013.

[90] E. Damiani, A. M. Aloia, M. G. Priore, S. Nardulli, and A. Ferrannini, "Pomegranate (*Punica granatum*) allergy: clinical and immunological findings," *Annals of Allergy, Asthma and Immunology*, vol. 103, no. 2, pp. 178–180, 2009.

[91] P. Gaig, B. Bartolomé, R. Lleonart, P. García-Ortega, R. Palacios, and C. Richart, "Allergy to pomegranate (*Punica granatum*)," *Allergy*, vol. 54, no. 3, pp. 287–288, 1999.

[92] J. M. Igea, J. Cuesta, M. Cuevas et al., "Adverse reaction to pomegranate ingestion," *Allergy*, vol. 46, no. 6, pp. 472–474, 1991.

[93] S. Gangemi, G. Mistrello, D. Roncarolo, S. Amato, and P. L. Minciullo, "Pomegranate-dependent exercise-induced anaphylaxis," *Journal of Investigational Allergology and Clinical Immunology*, vol. 18, no. 6, pp. 491–492, 2008.

[94] V. L. Hegde and Y. P. Venkatesh, "Anaphylaxis to excipient mannitol: evidence for an immunoglobulin E-mediated mechanism," *Clinical and Experimental Allergy*, vol. 34, no. 10, pp. 1602–1609, 2004.

[95] M. Nagata, M. Hidaka, H. Sekiya et al., "Effects of pomegranate juice on human cytochrome P450 2C9 and tolbutamide pharmacokinetics in rats," *Drug Metabolism and Disposition*, vol. 35, no. 2, pp. 302–305, 2007.

[96] M. Hidaka, M. Okumura, K.-I. Fujita et al., "Effects of pomegranate juice on human cytochrome P450 3A (CYP3A) and carbamazepine pharmacokinetics in rats," *Drug Metabolism and Disposition*, vol. 33, no. 5, pp. 644–648, 2005.

[97] H. Kim, Y. J. Yoon, J. H. Shon, I. J. Cha, J. G. Shin, and K. H. Liu, "Inhibitory effects of fruit juices on CYP3A activity," *Drug Metabolism and Disposition*, vol. 34, no. 4, pp. 521–523, 2006.

[98] D. Farkas, L. E. Oleson, Y. Zhao et al., "Pomegranate juice does not impair clearance of oral or intravenous midazolam, a probe for cytochrome P450-3A activity: comparison with grapefruit juice," *Journal of Clinical Pharmacology*, vol. 47, no. 3, pp. 286–294, 2007.

[99] A. V. Sorokin, B. Duncan, R. Panetta, and P. D. Thompson, "Rhabdomyolysis associated with pomegranate juice consumption," *The American Journal of Cardiology*, vol. 98, no. 5, pp. 705–706, 2006.

[100] T. Khuu, A. Hickey, and M. C. Deng, "Pomegranate-containing products and tacrolimus: a potential interaction," *Journal of Heart and Lung Transplantation*, vol. 32, no. 2, pp. 272–274, 2013.

[101] D. Farkas and D. J. Greenblatt, "Influence of fruit juices on drug disposition: discrepancies between in vitro and clinical studies," *Expert Opinion on Drug Metabolism and Toxicology*, vol. 4, no. 4, pp. 381–393, 2008.

[102] A. McCutcheon, J. Udani, and D. J. Brown, *Therapeutic Monograph and Clinical Overview of POM Wonderful Pomegranate Juice*, American Botanical Council, 2008.

[103] E. A. Ainsworth and K. M. Gillespie, "Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent," *Nature Protocols*, vol. 2, no. 4, pp. 875–877, 2007.

[104] K. R. Martin, C. G. Krueger, G. Rodriquez, M. Dreher, and J. D. Reed, "Development of a novel pomegranate standard and new method for the quantitative measurement of pomegranate polyphenols," *Journal of the Science of Food and Agriculture*, vol. 89, no. 1, pp. 157–162, 2009.

[105] V. L. Singleton, R. Orthofer, and R. M. Lamuela-Raventós, "Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent," *Methods in Enzymology*, vol. 299, pp. 152–178, 1998.

[106] G. Borges, W. Mullen, and A. Crozier, "Comparison of the polyphenolic composition and antioxidant activity of European commercial fruit juices," *Food and Function*, vol. 1, no. 1, pp. 73–83, 2010.

[107] U. A. Fischer, R. Carle, and D. R. Kammerer, "Identification and quantification of phenolic compounds from pomegranate (*Punica granatum* L.) peel, mesocarp, aril and differently produced juices by HPLC-DAD-ESI/MSn," *Food Chemistry*, vol. 127, no. 2, pp. 807–821, 2011.

[108] U. A. Fischer, J. S. Dettmann, R. Carle, and D. R. Kammerer, "Impact of processing and storage on the phenolic profiles and contents of pomegranate (*Punica granatum* L.) juices," *European Food Research and Technology*, vol. 233, no. 5, pp. 797–816, 2011.

[109] N. Khan, F. Afaq, M. H. Kweon, K. Kim, and H. Mukhtar, "Oral consumption of pomegranate fruit extract inhibits growth and progression of primary lung tumors in mice," *Cancer Research*, vol. 67, no. 7, pp. 3475–3482, 2007.

[110] M. Viladomiu, R. Hontecillas, P. Lu, and J. Bassaganya-Riera, "Preventive and prophylactic mechanisms of action of pomegranate bioactive constituents," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 789764, 18 pages, 2013.

[111] A. Galindo, Á. Calín-Sánchez, J. Collado-González et al., "Phytochemical and quality attributes of pomegranate fruits for juice consumption as affected by ripening stage and deficit irrigation," *Journal of the Science of Food and Agriculture*, vol. 94, no. 11, pp. 2259–2265, 2014.

[112] M. Fischer-Zorn and V. Ara, "Granatapfelsaft—Chemische Zusammensetzung und mögliche Verfälschungen," *Flüssiges Obst*, vol. 8, pp. 386–393, 2007.

[113] A. M. Gómez-Caravaca, V. Verardo, M. Toselli, A. Segura-Carretero, A. Fernández-Gutiérrez, and M. F. Caboni, "Determination of the major phenolic compounds in pomegranate juices by HPLC-DAD-ESI-MS," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 22, pp. 5328–5337, 2013.

[114] G. A. Spanos, R. E. Wrolstad, and D. A. Heatherbell, "Influence of processing and storage on the phenolic composition of apple juice," *Journal of Agricultural and Food Chemistry*, vol. 38, no. 7, pp. 1572–1579, 1990.

[115] B. W. Bolling, Y.-Y. Chen, and C.-Y. O. Chen, "Contributions of phenolics and added vitamin C to the antioxidant capacity of pomegranate and grape juices: synergism and antagonism among constituents," *International Journal of Food Science and Technology*, vol. 48, no. 12, pp. 2650–2658, 2013.

[116] S. Vegara, P. Mena, N. Martí, D. Saura, and M. Valero, "Approaches to understanding the contribution of anthocyanins to the antioxidant capacity of pasteurized pomegranate juices," *Food Chemistry*, vol. 141, no. 3, pp. 1630–1636, 2013.

[117] W. Qu, P. Li, J. Hong et al., "Thermal stability of liquid antioxidative extracts from pomegranate peel," *Journal of the Science of Food and Agriculture*, vol. 94, no. 5, pp. 1005–1012, 2013.

[118] U. A. Fischer, R. Carle, and D. R. Kammerer, "Thermal stability of anthocyanins and colourless phenolics in pomegranate (*Punica granatum* L.) juices and model solutions," *Food Chemistry*, vol. 138, no. 2-3, pp. 1800–1809, 2013.

[119] M. I. Gil, F. A. Tomas-Barberan, B. Hess-Pierce, D. M. Holcroft, and A. A. Kader, "Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing," *Journal of Agricultural and Food Chemistry*, vol. 48, no. 10, pp. 4581–4589, 2000.

[120] R. Tzulker, I. Glazer, I. Bar-Ilan, D. Holland, M. Aviram, and R. Amir, "Antioxidant activity, polyphenol content, and related compounds in different fruit juices and homogenates prepared from 29 different pomegranate accessions," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 23, pp. 9559–9570, 2007.

[121] Y. Li, C. Guo, J. Yang, J. Wei, J. Xu, and S. Cheng, "Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract," *Food Chemistry*, vol. 96, no. 2, pp. 254–260, 2006.

[122] H. Wasila, X. Li, L. Liu, I. Ahmad, and S. Ahmad, "Peel effects on phenolic composition, antioxidant activity, and making of pomegranate juice and wine," *Journal of Food Science*, vol. 78, no. 8, pp. C1166–C1172, 2013.

[123] S. Madrigal-Carballo, G. Rodriguez, C. G. Krueger, M. Dreher, and J. D. Reed, "Pomegranate (*Punica granatum*) supplements: authenticity, antioxidant and polyphenol composition," *Journal of Functional Foods*, vol. 1, no. 3, pp. 324–329, 2009.

[124] G. Borges and A. Crozier, "HPLC-PDA-MS fingerprinting to assess the authenticity of pomegranate beverages," *Food Chemistry*, vol. 135, no. 3, pp. 1863–1867, 2012.

[125] F. Tezcan, S. Uzaşçi, G. Uyar, N. Öztekin, and F. Bedia Erim, "Determination of amino acids in pomegranate juices and fingerprint for adulteration with apple juices," *Food Chemistry*, vol. 141, no. 2, pp. 1187–1191, 2013.

[126] S. Chrubasik and B. Roufogalis, "Bioequivalence of herbal medicines," *The New Zealand Journal of Pharmaceutical*, vol. 53, pp. 39–44, 2003.

[127] S. U. Mertens-Talcott, P. Jilma-Stohlawetz, J. Rios, L. Hingorani, and H. Derendorf, "Absorption, metabolism, and antioxidant effects of pomegranate (*Punica granatum* L.) polyphenols after ingestion of a standardized extract in healthy human volunteers," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 23, pp. 8956–8961, 2006.

[128] N. P. Seeram, S. M. Henning, Y. Zhang, M. Suchard, Z. Li, and D. Heber, "Pomegranate juice ellagitannin metabolites are present in human plasma and some persist in urine for up to 48 hours," *Journal of Nutrition*, vol. 136, no. 10, pp. 2481–2485, 2006.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 379586, 13 pages
http://dx.doi.org/10.1155/2015/379586

Research Article

Safety Evaluation of Chinese Medicine Injections with a Cell Imaging-Based Multiparametric Assay Revealed a Critical Involvement of Mitochondrial Function in Hepatotoxicity

Meng Wang,[1,2] Chen-Xiang Liu,[1,2] Ran-Ran Dong,[1,2] Shuang He,[1,2] Ting-Ting Liu,[1,2] Tie-Chan Zhao,[1,2] Zhi-Long Wang,[1,2] Xi-Ya Shen,[1,2] Bo-Li Zhang,[1] Xiu-Mei Gao,[1] and Yan Zhu[1,2,3]

[1] *Tianjin State Key Laboratory of Modern Chinese Medicine, Tianjin University of Traditional Chinese Medicine, 312 Anshanxi Road, Nankai District, Tianjin 300193, China*
[2] *Research and Development Center of TCM, Tianjin International Joint Academy of Biotechnology & Medicine, 220 Dongting Road, TEDA, Tianjin 300457, China*
[3] *Molecular Cardiology Research Institute, Tufts Medical Center and Tufts University School of Medicine, 750 Washington Street, Boston, MA 02111, USA*

Correspondence should be addressed to Yan Zhu; yanzhu.harvard@gmail.com

Received 19 June 2014; Accepted 25 September 2014

Academic Editor: Junhua Zhang

The safety of herbal medicine products has been a widespread concern due to their complex chemical nature and lack of proper evaluation methods. We have adapted a sensitive and reproducible multiparametric cell-based high-content analysis assay to evaluate the hepatic-safety of four Chinese medicine injections and validated it with classical animal-based toxicity assays. Our results suggested that the reported hepatotoxicity by one of the drugs, Fufangkushen injection, could be attributed at least in part to the interference of mitochondrial function in human HepG2 cells by some of its constituents. This method should be useful for both preclinical screen in a drug discovery program and postclinical evaluation of herbal medicine preparations.

1. Introduction

Traditional Chinese medicine (TCM) injection is an innovative and quick-acting dosage form of herbal medicine products. It plays a significant role in clinical treatment of acute, severe syndromes in China. However, the safety of TCM injection has been a long concern of the public, especially after consecutive reports of adverse drug reaction (ADR) recently. The ADR induced by TCM injection accounts for 77.2% of all the ADR induced by TCM in national ADR case report database [1]. Hepatotoxicity is a major cause for the termination of drug development programs and frequently results in regulatory actions including denied approval and black box warnings [2]. Drug-induced hepatotoxicity accounts for one-half of cases of acute liver failure in America and Great Britain [3]. Long term and acute animal toxicity test on liver injury was demanded officially in preclinical research. However, classical animal study is inefficient, for only approximately half of new drugs with hepatotoxicity can be found in animal test [4]. A more precise, rapid, and high-throughput method to evaluate the hepatotoxicity of drugs, especially for TCM injection, was needed.

The major mechanistic classifications of hepatotoxicity include inhibition of mitochondrial function, disruption of intracellular calcium homeostasis, activation of apoptosis, oxidative stress, inhibition of specific enzymes or transporters, and formation of reactive metabolites that cause direct toxicity or immunogenicity [5]. HCA is considered as an important predictive tool for application of the above mechanistic understanding for the assessment of hepatotoxicity. It is a recent advance in the automation of quantitative

epifluorescence microscopy and image analysis and in the application of microfluorescent, multiprobe technology. It enables kinetic monitoring *in vitro* of cells in real time for multiple cellular biomarkers that are critically involved in the pathogenesis of toxicity [6]. A HCA assay was established to investigate hepatotoxicity of 243 drugs to HepG2 cells. When the data were adjusted to take account of the reported maximum human plasma concentrations of the drugs, a specificity of 98% and a sensitivity of 93% for detection of compounds that cause hepatotoxicity were observed.

TCM injection commonly is a compound preparation without completely clear therapeutic material basis that makes it difficult to evaluate the toxicity effect, especially on its mechanism. The characteristics of visualization, intuition, and multiparameter of HCA are suitable for toxicity assessment of TCM preparations. Four TCM injections, Danhong injection (DHI), Xiangdan injection (XDI), Mailuoning injection (MLNI), and Fufangkushen injection (FFKSI), were selected for the HCA assay. They are all widely used in clinic practice in China with a total sales amount of more than 4 billion RMB in 2013. The hepatotoxicity ADR reports of four injections are varying [7]. XDI and MLNI were reported in ADR information bulletin by SFDA [8, 9]. It was also observed that DHI and FFKSI could increase ALT, AST, and ALP in individual clinical cases [10–14].

The study aimed to develop and validate a practical, reproducible, *in vitro* multiparametric cell-based HCA assay to assess hepatotoxicity of TCM injections and to suggest their mechanisms of action. The assay was applied to HepG2 human cell line [15–17] cultured in 96-well plates. Fluorescent dyes with optical compatibility were adopted to evaluate multiple parameters concerning drug-induced liver injury, including cell number (CN), nuclear area (NA), mitochondrial mass (MS), mitochondrial membrane potential (MMP), and plasma membrane permeability (PMP). Drugs with known hepatotoxic mechanism, P-fluoromethoxyphenylhydrazone (FCCP), acetaminophen, and doxorubicin hydrochloride, were used as positive controls. FCCP, a potent uncoupler of mitochondrial oxidative phosphorylation [18], also reduces MMP activity and induces apoptosis [19]. Acetaminophen is converted by cytochrome P450 enzymes to a reactive metabolite, NAPQI, which was efficiently detoxified by conjugation with GSH. However, after toxic doses, GSH was depleted by the conjugation reaction and the metabolite covalently bounded to protein. Since the loss of GSH, peroxidation reactions occurred by Fenton-type mechanisms which can lead to activation of proteases and endonucleases and DNA strand breaks. The effect of the excess NAPQI on isolated mitochondria and inhibition of mitochondrial respiration are considered to cause damage on hepatocyte [20]. Doxorubicin causes an imbalance between free oxygen radicals (ROS) and antioxidants. The disturbance in oxidant-antioxidant systems results in tissue injury that is demonstrated with lipid peroxidation and protein oxidation in tissue. It is demonstrated that inflammatory processes, free radicals, oxidative stress, and lipid peroxidation are frequently associated with liver damage induced by toxic agents such as doxorubicin [21].

To validate the assay, a 28-day subacute animal study was carried out. Animal mortality, state of survival, body and liver weights, and antioxidant activity in both plasma and liver homogenates were determined and compared with the results of HCA. Hepatotoxicity of the four TCM injections was assessed and the mechanism of action was suggested by the method established.

2. Materials and Methods

2.1. Drugs and Reagents. FCCP (Sigma, St. Louis, MO, USA, batch number 122M4004V), acetaminophen (Sigma, St. Louis, MO, USA, batch number 061M0042V), and doxorubicin hydrochloride (Meilun Biotech Co., Ltd., Dalian, China, batch number 20120205) were used as positive control to validate the assay. The median concentrations (IC 50) of FCCP and acetaminophen were 5.2 μM and 1.3 mM [22], respectively. The lowest concentration of doxorubicin hydrochloride was 0.1 μM [4]. For dose-response relationship, FCCP at 0.3–30 μM, acetaminophen at 0.3–10 mM, and doxorubicin hydrochloride at 0.1–10 μM were selected. Four TCM injections, DHI (Buchang Pharmaceutical Co., Ltd., Shandong, China, batch number 12081024077), XDI (Bicon Pharmaceutical Co., Ltd., Jiangsu, China, batch number 120812), MLNI (Jinling Pharmaceutical Co., Ltd., Jiangsu, China, batch number 20121208), and FFKSI (Zhendong Pharmaceutical Co., Ltd., Shanxi, China, batch number 20130228), were purchased from the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine. The TCM injections were serially diluted up to 1000-fold by DMEM/high glucose supplemented with 10% FBS. HepG2 cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The Mitotracker Deep Red FM fluorescent probe was obtained from Invitrogen (CA, USA), while other fluorescent probes were obtained from Sigma-Aldrich (MO, USA).

2.2. Cell Culture and Drug Treatment. HepG2 cells were subcultured less than fifteen generations after acquisition from the culture collection. Cells were cultured in DMEM/high glucose with 4500 mg·L^{-1} glucose and 4 mM·L^{-1}-glutamine (Thermo Fisher Scientific, UT, USA) and supplemented with 10% FBS (Gibco, NY, USA) and 100 IU·mL^{-1} penicillin-streptomycin (Gibco, NY, USA) at 37°C in a 5% CO_2 atmosphere. Cells were subcultured following trypsinization with a 0.25% trypsin-EDTA solution (Gibco, NY, USA), plated onto a Collagen I (Shengyou, Hangzhou, China) coated 96-well microplate (Corning, MA, USA) at a density of 8×10^3 per well, and cultured at 37°C with 5% CO_2. Only the inner 60 wells of 96-well microplate were used due to evaporation-related edge effects in the outside wells.

2.3. HCA Imaging and Image Analysis. In the assay, cell parameters associated with CN, NA (Hoechst 33342), MS (Mitotracker Deep Red FM), MMP (Rhodamine 123), and PMP (PI iodide) were measured. After 24-hour incubation, 50 μL of a fluorophore mixture containing 3 μmol·L^{-1} Hoechst 33342, 0.9 μmol·L^{-1} Mitotracker Deep Red FM, and

9 μmol·L^{-1} PI iodide in DMEM/high glucose was added to each well. Cells were incubated 30 minutes in dark. After removing the medium, 100 μL 1.2 μg·mL^{-1} Rhodamine 123 was added in each well. The cells were cultured for additional half hour prevented from light, and then they were washed once with warm DMEM before image scan. The assay plate was imaged and analyzed using the Operetta HCA system (Perkin Elmer, MA, USA) at 25°C with a relative humidity of 45% using a 20x objective. The fluorescent images of six fields per well in Hoechst 33342, Mitotracker Deep Red FM, PI iodide, and Rhodamine 123 channels were acquired by confocal scan, respectively. CN, NA, MS, MMP, and PMP were measured and calculated by the mean values of six images by adopting Graph Pad Grism (Graph Pad Software, Inc. CA, USA) software.

2.4. Subacute Toxicity Test. Animal care and operation procedures were in strict accordance with the China Laboratory Animal Use Regulations, and the animals were performed on in accordance with the institutional ethical guideline, and the experiment was approved by the institutional Animal Care and Use Committee of Tianjin International Joint Academy of Biotechnology and Medicine. Sprague-Dawley rats (male, 200 ± 20 g) were purchased from the Laboratory Animal Center of Academy of Military Medical Sciences and housed in stainless steel wire bottom cages with a control environment (25 ± 1°C, 50–60% humidity, 12 h fluorescent lighting per day) for 10 days of acclimatization. All rats were fed with standard food and water *ad libitum*.

Rats were randomly divided into nine groups of six animals each. 1.2 times of clinical dose was set as low-dose group. Four low-dose groups were administrated 4.4 mL·kg^{-1}, 2.2 mL·kg^{-1}, 1.3 mL·kg^{-1}, and 2.2 mL·kg^{-1} of DHI, XDI, FFKSI, and MLNI, respectively while the high-dose groups were given 6 times of clinical dose of each injection. Control group received 22 mL·kg^{-1} physiological saline. All agents were administered daily for 28 days via tail vein injection. Body weights were recorded on the first day of the experiment and weekly thereafter. The dosage was corrected according to the weight change. Physical condition of animals was observed daily including changes in skin and fur, eyes and mucous membranes, and manure and behavior patterns. The rat sacrificed during the experiment was necropsied immediately; all macroscopic abnormalities of heart, liver, spleen, lung, kidney, and other organs were recorded. Since the intense adverse reactions of rats in FFKSI high-dose group, the rats were necropsied, and all organs and tissues were routinely processed on the 14th day.

2.5. Serum Biochemical Assays. After 28 days of drug treatment, rats were fasted overnight and blood samples were obtained from the abdominal aorta following anesthesia with 10% chloral hydrate (5 mL·kg^{-1}) and plated at 4°C to clot for five hours. Serum was separated by centrifugation at 3000 r for 15 min. Biochemical parameters of serum enzyme activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride (TG), and alkaline phosphatase (ALP) were measured by commercial ALT reagent kit (Biosino, Beijing, China), AST reagent kit (Biosino, Beijing, China), GPO-PAP TG kit (Biosino, Beijing, China), and ALP kit (Biosino, Beijing, China) using a Hitachi 7020 Clinical Chemistry Analyzer (HITACHI, Tokyo, Japan).

2.6. Antioxidative Effects. The livers of the rats were isolated immediately and washed in ice-cold physiological saline. 0.2 g liver tissue of each rat was weighted precisely and homogenized with physiological saline in a Teflon homogenizer and centrifuged at 3000 r for 10 min to get 10% liver homogenates (w/v). The activity of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and malondialdehyde (MDA) was determined by using commercially SOD WST-kit (Jiancheng, Nanjing, China), CAT assay kit (Jiancheng, Nanjing, China), GSH assay kit (Jiancheng, Nanjing, China), and MDA assay kit (Jiancheng, Nanjing, China).

2.7. Statistical Analysis. IC 50 of six image fields per well was calculated by Graph Pad Grism software. All data was expressed as mean ± SEM. Statistical analysis was performed using ANOVA with LSD test by SPSS 17.0. Value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Method Validation with Positive Control Drugs. The sensitivity of the multiparametric HCA assay was first validated by three known hepatotoxic compounds. Representative images of Hoescht 33342, Mitotracker Deep Red FM, PI iodide, and Rhodamine 123 channels captured by the HCA established typical cytotoxic effects caused by FCCP (3 μM), acetaminophen (3 mM), and doxorubicin hydrochloride (3 μM). As shown in Figure 1, an increased intensity of Mitotracker Deep Red FM indicated MS increase caused by FCCP and doxorubicin hydrochloride (Figures 1(g) and 1(h)); a decreased intensity of Rhodamine 123 indicated the MMP decrease caused by all three positive drugs (Figures 1(j), 1(k), and 1(l)). The cell nucleuses shrunk were founded by FCCP, Figure 1(d). As shown in Figure 2, FCCP at 3 μM, 10 μM, and 30 μM ($P < 0.01$), acetaminophen at 1 mM, 3 mM, and 10 mM ($P < 0.01$), and doxorubicin hydrochloride at 0.1 μM to 10 μM ($P < 0.01$) dramatically decreased the CN by 35.8%–70.4% compared with blank group. FCCP at 30 μM ($P < 0.05$), acetaminophen at 100 μM ($P < 0.01$), and doxorubicin hydrochloride at 3 μM and 10 μM ($P < 0.01$) significantly increased the PMP by 17.5-, 61.4-, 19.0-, and 44.9-fold, respectively. Acetaminophen significantly increased the NA by 25.7% at 3 mM ($P < 0.01$), while it increased the MMP by 26.7% at 100 μM ($P < 0.01$). In addition, for FCCP, acetaminophen, and doxorubicin hydrochloride, the IC 50 of CN was 5.18 μM, 1.84 mM, and 1.67 μM, respectively. For NA, the IC 50 was 0.60 μM, 111.63 mM, and 0.17 μM, respectively. For MS, the IC 50 was 7.02 μM, 9.78 mM, and 1.94 μM, respectively. For MMP, the IC 50 was 0.77 μM, 113.18 mM, and 0.63 μM, respectively. For FCCP, acetaminophen and doxorubicin hydrochloride, the IC 50 of PMP was 25.69 μM, 0.01 mM, and 10.73 μM.

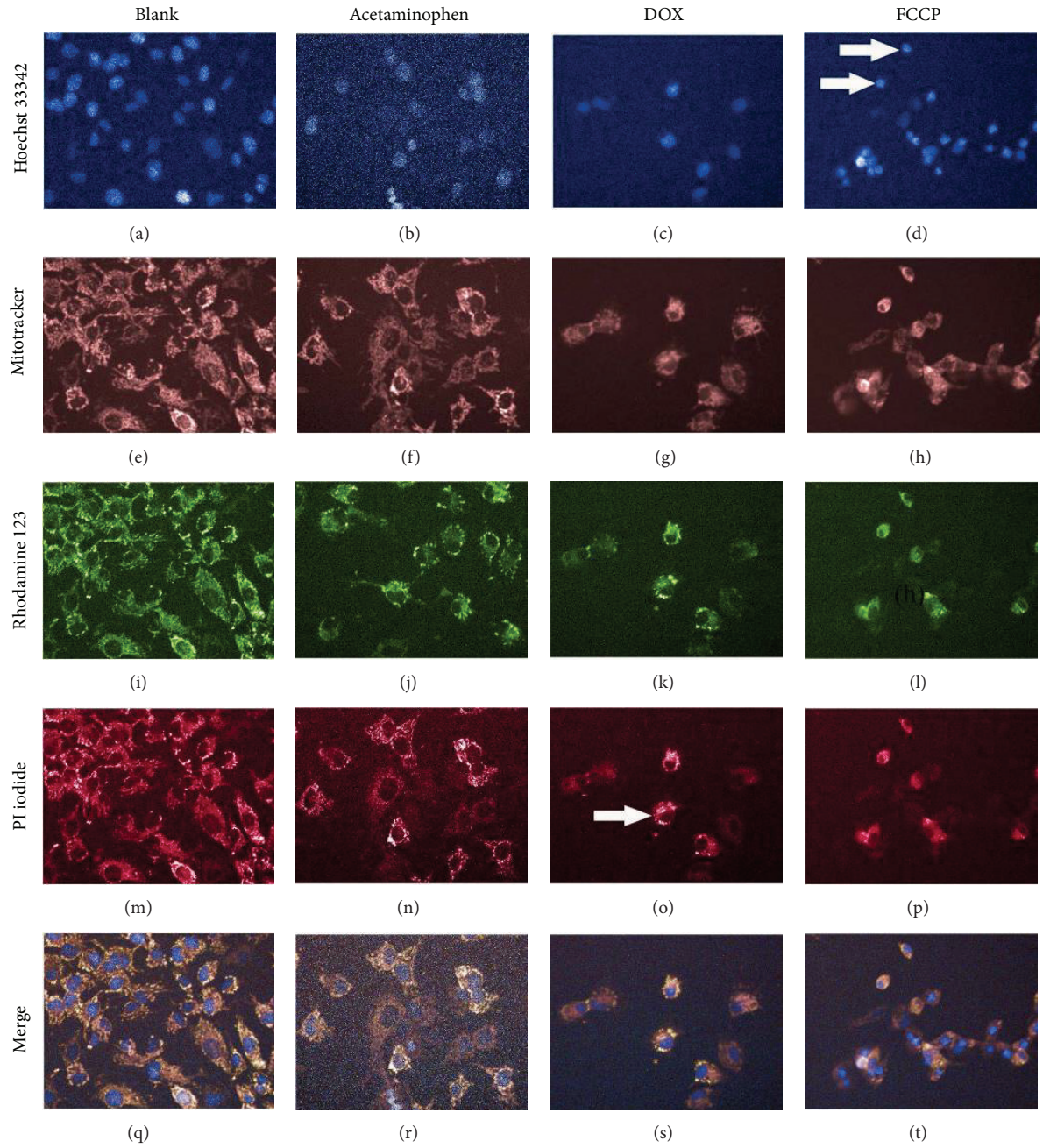


Figure 1: Representative images of HCA for positive drugs induced hepatotoxicity on Hoescht 33342, Mitotracker Deep Red FM, PI iodide, and Rhodamine 123 channels. HepG2 cells treated with blank (a, e, i, m, and q), 3 μM FCCP (d, h, l, p, and t), 3 mM acetaminophen (b, f, j, n, and r), and 3 μM doxorubicin hydrochloride (DOX) (c, g, k, o, and s) are shown. The increased intensity of Mitotracker Deep Red FM indicated an increased mitochondrial mass (MS) (g, h). The decreased intensity of Rhodamine 123 indicated a decreased mitochondrial membrane potential (MMP) (j, k, and l). The MMP decrease was indicated by white arrow (g, k, and o). The intensity of PI was increased when plasma membrane permeability (PMP) increased. The PMP increase was indicated by white arrow (o). The cell nucleuses stained by Hoechst 33342 shrunk and CN decreased by FCCP, indicated by white arrow (d).

3.2. Effect of TCM Injections in HCA Hepatotoxicity Assay. Five fixed concentrations (1000-, 800-, 500-, 300-, 100-fold dilution) of four injections, DHI, XDI, FFKSI, and MLNI were selected. As shown in Figure 3, the representative images were captured by the HCA method established for typical cytotoxic effects on hepatocyte induced by FFKSI 100-fold dilution, XDI 100-fold dilution, MLNI 100-fold dilution, and DHI 100-fold dilution. It is noteworthy that 1000-, 800-, 500-, 300- ($P < 0.05$), and 100-fold dilution ($P < 0.01$) of FFKSI significantly decreased the MMP by 11.1%–22.8% as compared to blank group (Figure 4(d)). XDI and FFKSI at 100-fold dilution caused a significant decrease of CN by

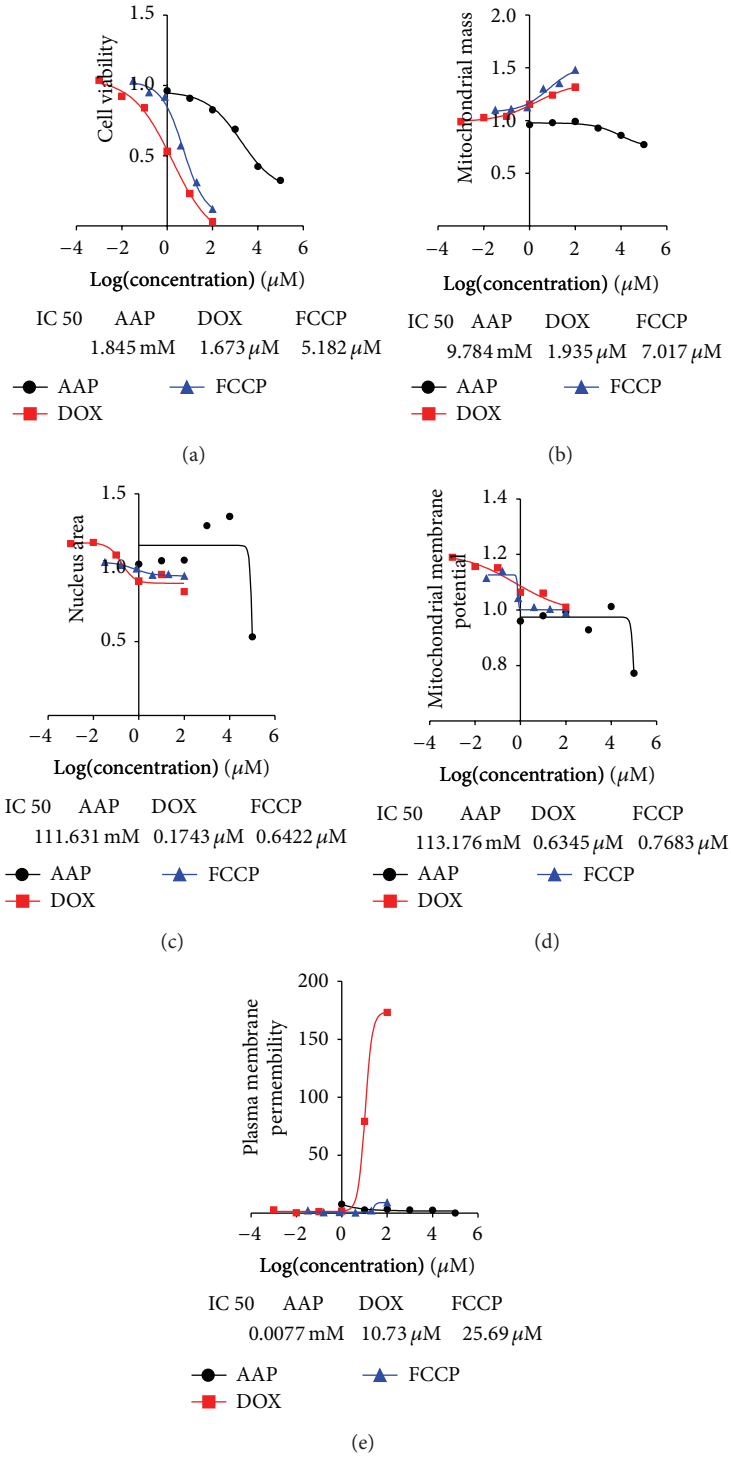


Figure 2: Acetaminophen (AAP), doxorubicin hydrochloride (DOX), and FCCP-generated dose-response curves deduced from cell number (a), mitochondrial mass (b), nuclear area (c), mitochondrial membrane potential (d), and plasma membrane permeability (e). Exposed to acetaminophen (1 μM, 10 μM, 100 μM, 1 mM, 10 mM, and 100 mM), exposed to doxorubicin hydrochloride (1 nM, 10 nM, 100 nM, 1 μM, 10 μM, and 100 μM), and exposed to FCCP (32 nM, 160 nM, 800 nM, 4 μM, 20 μM, and 100 μM), HepG2 cells are studied. Data is expressed as mean ± SEM, $n = 3$.

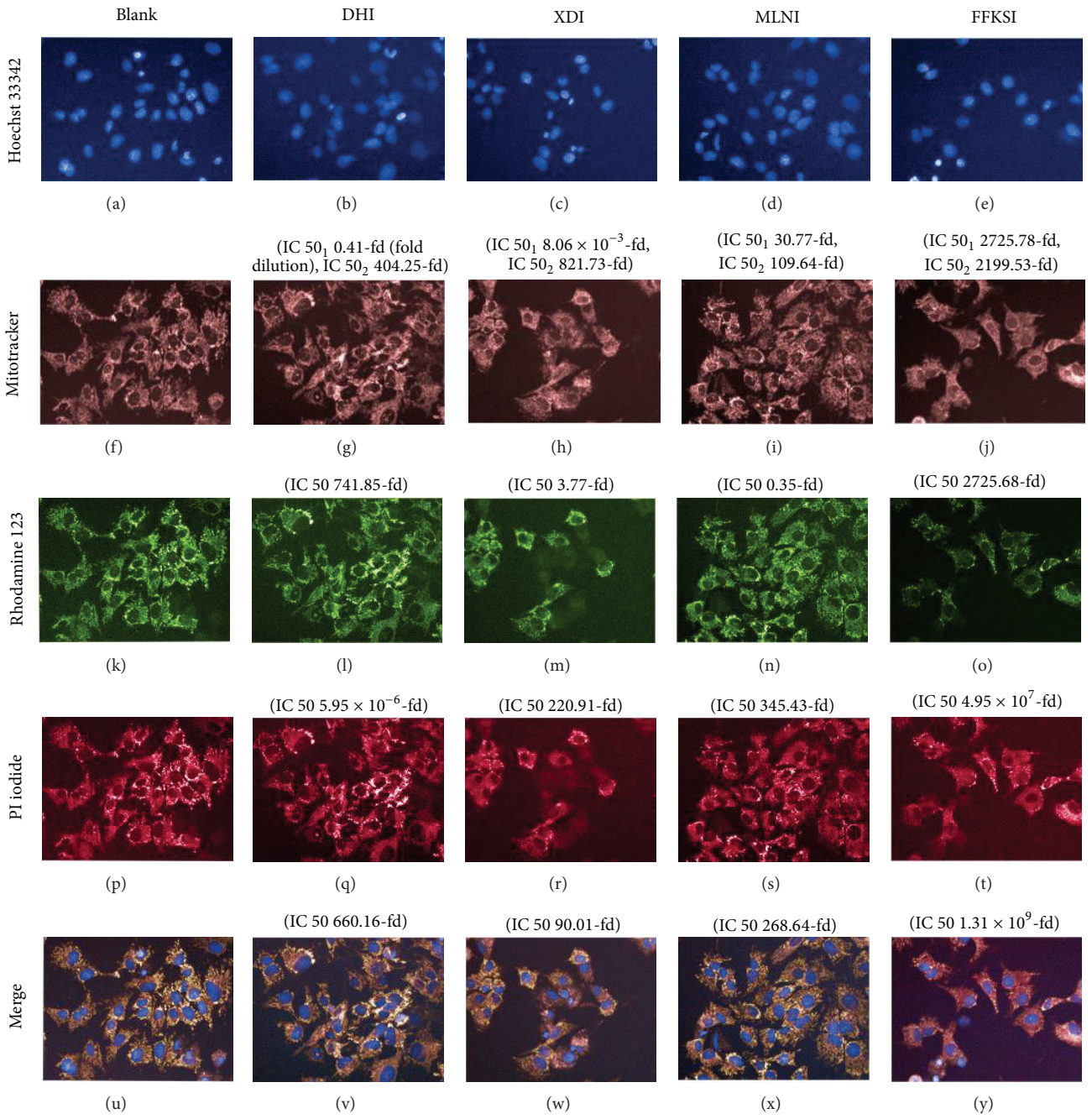


FIGURE 3: Representative images of the HCA analysis of HepG2 cells for the evaluation of nuclei number and morphology stained by 4 dyes in control (a, f, k, p, and u), Danhong injection (DHI) 100-fold dilution (b, g, l, q, and v), Xiangdan injection (XDI) 100-fold dilution (c, h, m, r, and w), Mailuoning injection (MLNI) 100-fold dilution (d, i, n, s, anad x), and Fufangkushen injection (FFKSI) 100-fold dilution (e, j, o, t, and y) groups. The cell number (CN) decreased by XDI 100-fold dilution and FFKSI 100-fold dilution (c, e). The intensity of Rhodamine 123 (green) was lower in FFKSI-treated group than control group indicating that the MMP decreased (o). Note: IC 50 of cell parameters associated with cell number (CN) was IC 50$_1$ and IC 50 of cell parameters associated with nuclear area (NA) was IC 50$_2$.

50.8% and 48.4% ($P < 0.05$), respectively (Figure 4(a)). XDI at 100-fold dilution also showed a significant increase of MS (3237.24 ± 78.38) by 34.8% compared with blank group (2265.40 ± 109.58) ($P < 0.01$) (Figure 4(b)). As shown in Figure 4, DHI and MLNI at all concentrations did not have significant changes in any of the parameters studied. The result of HCA assay suggested that the high concentration of FFKSI and XDI may cause mitochondrial damage of HepG2 cells. In addition, for DHI, XDI, MLNI, and FFKSI, the IC 50 of CN was 0.41-fold, 8.6 × 10^{-3}-fold, 30.77-fold, and 2725.78-fold; the IC 50 of MS was 741.85-fold, 3.77-fold, 0.35-fold, and 2725.68-fold; the IC 50 of NA was 404.25-fold,

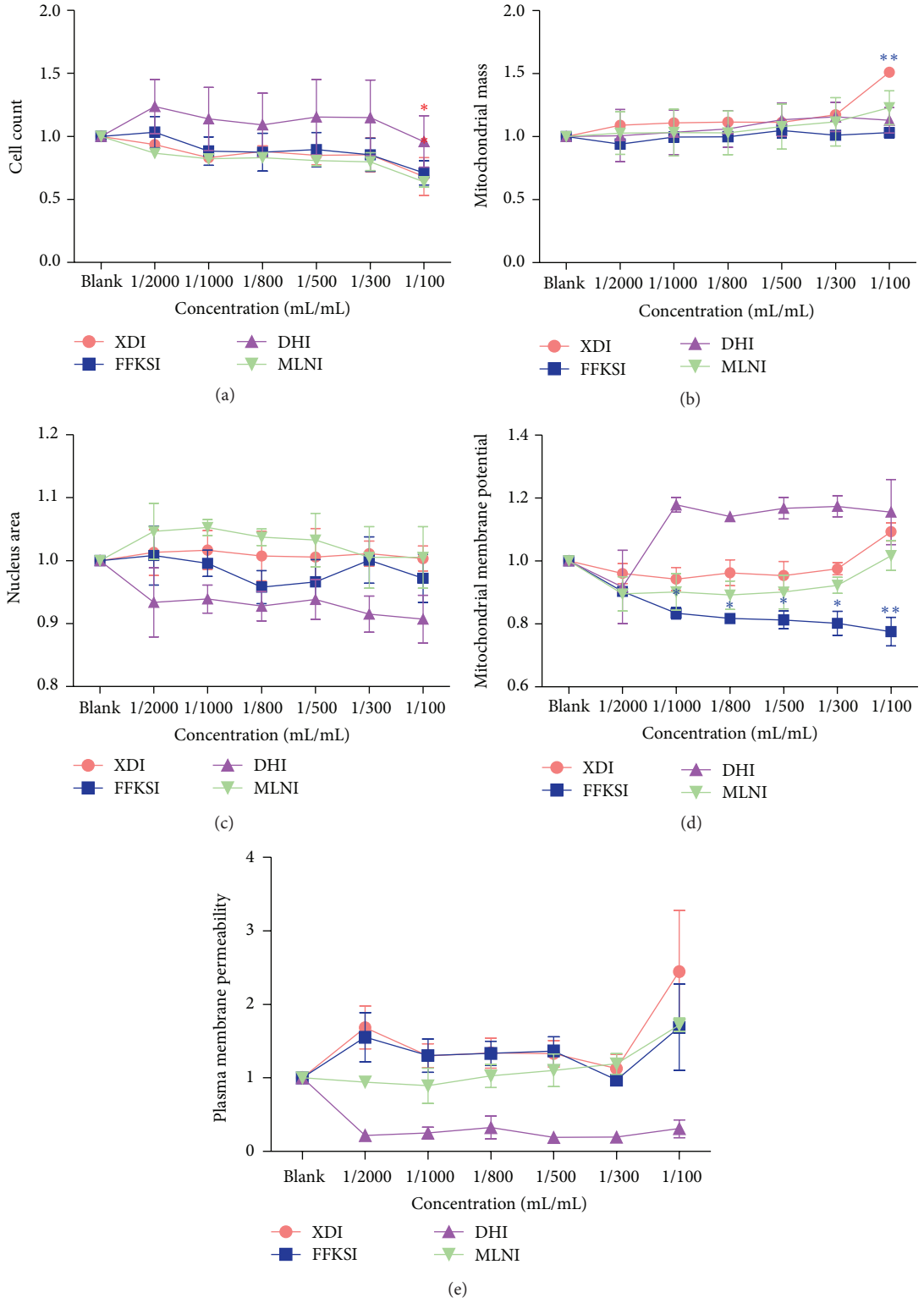


Figure 4: Effects of Danhong injection (DHI), Xiangdan injection (XDI), Mailuoning injection (MLNI), and Fufangkushen injection (FFKSI) on cell number (a), nuclear area (b), mitochondrial mass (c), mitochondrial membrane potential (d), and plasma membrane permeability (e). HepG2 cells were treated with DHI, XDI, MLNI, and FFKSI. FFKSI (100-fold dilution) and XDI (100-fold dilution) caused deviations from the control group with a significant decrease of the cell number (a). A significant increase of MS was induced by XDI (100-fold dilution) (b). All FFKSI group (100-, 300-, 500-, 800-, and 1000-fold dilution) induced a decrease of MMP (d). Data is expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 3$.

Table 1: Phenotypic changes in subacute toxicity test.

Group	Death/total animals	Abnormalities
Control	0/6	Normal
DHI high-dose	0/6	Normal
DHI low-dose	0/6	Normal
XDI high-dose	1/6	1/6 hind limb paralyzed
XDI low-dose	0/6	Normal
MLNI high-dose	0/6	Normal
MLNI low-dose	0/6	Normal
FFKSI high-dose	2/6	6/6 twitched after injection
FFKSI low-dose	1/6	1/6 hind limb paralyzed

821.73-fold, 109.64-fold, and 2199.53-fold; the IC 50 of MMP was 5.95×10^{-6}-fold, 220.91-fold, 345.43-fold, and 4.95×10^{7}-fold; the IC 50 of PMP was 660.16-fold, 90.01-fold, 268.64-fold, and 1.31×10^{9}-fold.

3.3. Effect of TCM Injections in Subacute Toxicity Test. To confirm the HCA results, a subacute animal toxicity test was carried out. Of all 54 animals subjected to the subacute toxicity test, four were sacrificed during the experiment, two from FFKSI high-dose group, one from FFKSI low-dose group, and one from XDI high-dose group (Table 1). For the animals in the high-dose FFKSI group, twitching was observed in six rats immediately after drug injection and sustained nearly 3 minutes. Since intense adverse reactions occurred in the group with high-dose FFKSI, the rats were necropsied and all organs and tissues were routinely processed on the 14th day. Body weight of rats in FFKSI low-dose group increased slowly. On the 7th day after injection, the rats were irritable and the fur became pale brown. Lower limbs of one rat were paralyzed after drug administration on the 23th day and the rat died 2 days later. The body weight of rats in XDI high-dose group increased slowly and the fur was lackluster. One rat lower limbs were paralyzed on the 17th day after injection and the rat died 3 days later.

The effects of four injections on the growth of rats are shown in Figure 5. The body weight decreased significantly in both FFKSI high-dose and low-dose group ($P < 0.01$) as well as in XDI low-dose group ($P < 0.05$) by 16.9%, 26.8%, and 16.0%, respectively (Figure 5(a)). DHI and MLNI did not change body weight after 28 days of injection. XDI in high-dose ($P < 0.05$) as well as MLNI in both low-dose and high-dose groups ($P < 0.01$) decreased the ratio of liver weight to body weight significantly compared to those in control group (Figure 5(b)), while DHI and FFKSI did not change it.

3.4. Effect of 4 TCM Injections by Serum Biochemistry Analysis. After 28 days of administration of four injections, serum activities of AST, ALT, TG, and ALP enzymes in all groups did not change significantly compared to control group. No influence on liver function caused by 4 TCM injections was observed from the serum biochemical study.

3.5. Effect of TCM Injections on Hepatic Antioxidant Enzyme Activities and Lipid Peroxidation. To further assess the antioxidant effect of the four injections on liver function *in vivo*, SOD, MDA, CAT, and GSH were measured. As shown in Figure 6, the SOD, MDA, CAT, and GSH activities of 4 TCM injections did not show significant change except for the high-dose group of FFKSI. The CAT activity of FFKSI high-dose group (41.61 ± 3.11, $P < 0.01$) decreased 40.4% compared to the blank; meanwhile the MDA activity (1.57 ± 0.18, $P < 0.01$) increased significantly by 70.0%. It indicated that the high dose of FFKSI increased reactive oxygen species (ROS) and decreased the antioxidant capacity of liver tissue that was in accordance with the decrease of MMP found in HCA assay established. The SOD value of XDI high-dose group was lower than all other groups; the MDA level was also higher than other groups except for FFKSI high-dose group without significant difference. It indicated that long-term use of XDI at a high concentration may also cause certain oxidative damage on liver (Figure 7).

4. Discussion

With increased application of TCM injections in clinical practice, safety problems have been increasing. The four TCM injections investigated in this study were widely used in clinical practice in China with large patient population. In 2013, the sales amount of the four injections totaled to more than 4 billion RMB. Different degrees of hepatotoxicity ADR reports of the four TCM injections were collected from clinical centers and SFDA reports. XDI and MLNI were reported in ADR information bulletin [8, 9]. FFKSI increased ALT, AST, and ALP [7]; XDI increased ALT, chronic hepatitis B, and liver cirrhosis in clinical treatment [10, 11]. MLNI increased ALT and AST at 68–229 U/L and 30–157 U/L after 2 weeks of use [12, 13]. The incidence of ADR by DHI is relatively low (5.15‰) [14] and its hepatotoxicity adverse reaction has rarely been recorded. Therefore, a rapid and accurate method is needed to postmarketing reevaluation of hepatotoxicity of TCM injection.

Cell-based assays have been increasingly employed in predicting drug-induced toxicities [23], which is an efficient means to reduce the dependence on animals. Multiparameter HCA assays have so far been applied to measure genotoxicity, hepatotoxicity, drug-induced phospholipidosis, and developmental neurotoxicity. In terms of hepatotoxicity, O'Brien et al. [5] applied HCA and tested 243 known hepatotoxicity drugs using HepG2 human hepatocytes with a specificity of 98% and a sensitivity of 93%. Based on O'Brien's work, a live cell multiparametric HCA cytotoxicity assay was developed by Abraham et al. [24]. Seventeen compounds with various hepatotoxicity mechanisms were evaluated. The capability of HCA assay on sensitively detection with different mechanism was approved. Garside et al. [25] developed a HCA hepatotoxicity assay on HepG2 human hepatocytes and 144 compounds were assessed. The specificity and sensitivity were 58% and 75%, respectively. However, HCA-based hepatotoxicity assay has not been attempted for evaluating either drug combinations or TCM or herbal medicine that

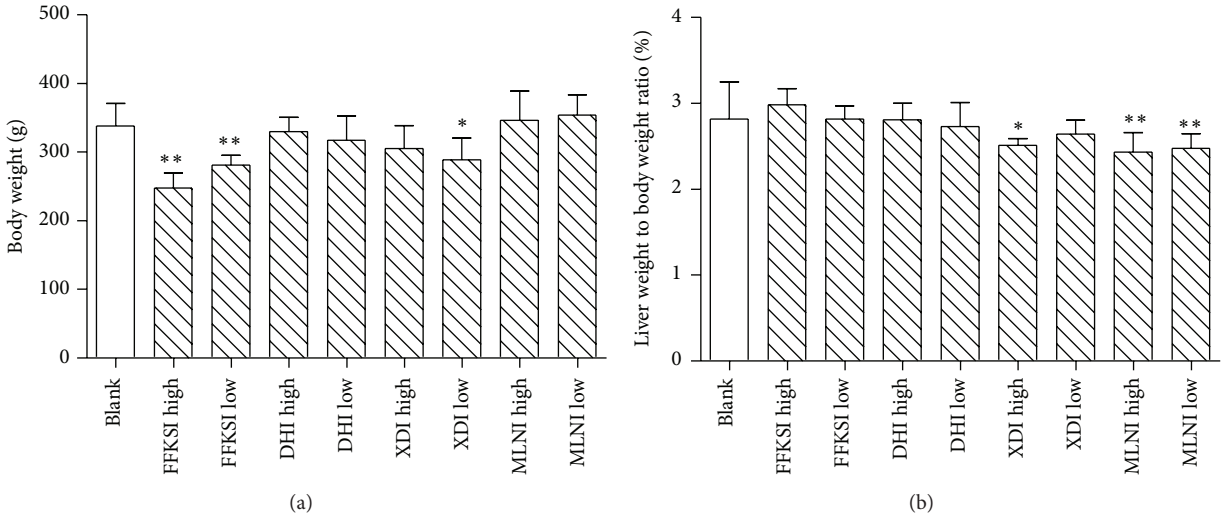


Figure 5: The body weight (a) and the ratio of liver weight to body weight (b) of rats after 28-day injection of Danhong injection, Xiangdan injection, Mailuoning injection, and Fufangkushen injection. Data are given as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$.

have complex chemical constituents and potential multiple toxicity determinants. Due to its complex chemical nature, safety evaluation of TCM preparations has been a challenging task, especially on providing an organ-specific toxicity profile and a mechanistic insight. The ability to determine multiple cell parameters associated with cell health by the application of microfluorescent, multiprobe technology simultaneously makes HCA a suitable tool for safety assessment of TCM preparations with complex chemicals. Compared to conventional assays, the higher sensitivity of HCA assay can provide clear image of cells and quantify multiple cellular biomarkers to evaluate the effect of compound and analysis the pathogenesis of toxicity. Long term and acute toxicity test on various animal species is necessary for new drug approval. However, unpredictable ADR still cannot be avoided according to the postmarket surveillance. The HCA assay established on human cells is highly predictive for drug-induced liver injury. It can be employed in drug discovery and postmarket reevaluation to optimize compound series on their safety profile and provide a risk assessment tool towards candidate selection with higher output and sensitivity.

The median concentrations of FCCP and acetaminophen referred to IC 50 from the assay protocol of Operetta HCA system were 5.2 μM and 1.3 mM. The lowest concentration of doxorubicin hydrochloride was set as 0.1 μM according to previous research [4]. In order to confirm dose-response relationship, FCCP (0.32–100 μM), acetaminophen (0.001–100 mM), and doxorubicin hydrochloride (0.001–100 μM) were selected. Since there is no sufficient evidence of relationship between the amount used in *in vitro* cell experiment and clinical dose of TCM injections, the formulation was chosen to calculate the highest concentration of injections in the HCA assay. Consider the following:

$$\text{Highest concentration of TCM injection} = \left[\frac{(60 \times D)/5000}{50\%}\right], \quad (1)$$

where 60 is average weight of adult, D is the clinic dosage of TCM injections ($\text{mL}\cdot\text{kg}^{-1}\text{d}^{-1}$), 5000 is average blood volume (mL) of adult, and 50% is the volume ratio of blood cells in plasma. The clinic dosage of DHI is 0.67 $\text{mL}\cdot\text{kg}^{-1}\text{d}^{-1}$; for instance, 100-fold diluted DHI was calculated and set as the highest concentration [26].

In the subacute animal test, according to "Guide on New Drug research of Chinese Medicine" [27], 1.2 times of the clinical dose was set as low-dose group with administration amount of 4.4 $\text{mL}\cdot\text{kg}^{-1}$, 2.2 $\text{mL}\cdot\text{kg}^{-1}$, 1.3 $\text{mL}\cdot\text{kg}^{-1}$, and 2.2 $\text{mL}\cdot\text{kg}^{-1}$ of DHI, XDI, FFKSI, and MLNI, respectively. Meanwhile 6 times of clinical dose was the high-dose group.

Given the need for a proliferating cell model for predictive cytotoxicity studies, the effectiveness of the choice of HepG2 human cells as a cell line to drug safety evaluation is supported by other studies. Schoonen et al. found them slightly more predictive than HeLa, ECC-1, and CHO-k1cells [15, 16]. Others have made similar findings (Bugelski et al. 2000) [17]. A cell line with metabolic competence (HepaRG cells) and morphology more representative of the *in vivo* state (L-02 cells) would likely further enhance the predictability of cytotoxicity assays in our following study.

In this study, five parameters (CN, MS, MMP, NA, and PMP) were simultaneously assessed to evaluate cell proliferation, mitochondrial function, and cellular structure integrity. Cell permeable dye Hoechst 33342 was used to locate the cell and measure NM and CN. CN is one of the most sensitive cell health indicators directly affected by drug toxicity, while NA change indicates the damage of nucleus, alive cell impermeable DNA binding dye, PI iodide, measures PMP via quantification of nuclear brightness.

Mitochondria is one of the most important target sites 14 of hepatotoxicity drugs. According to the three-step model of drug induced liver injury, the decrease of MMP is the most important step in the process [28]. When the mitochondrial function was impaired, the MMP would decrease and

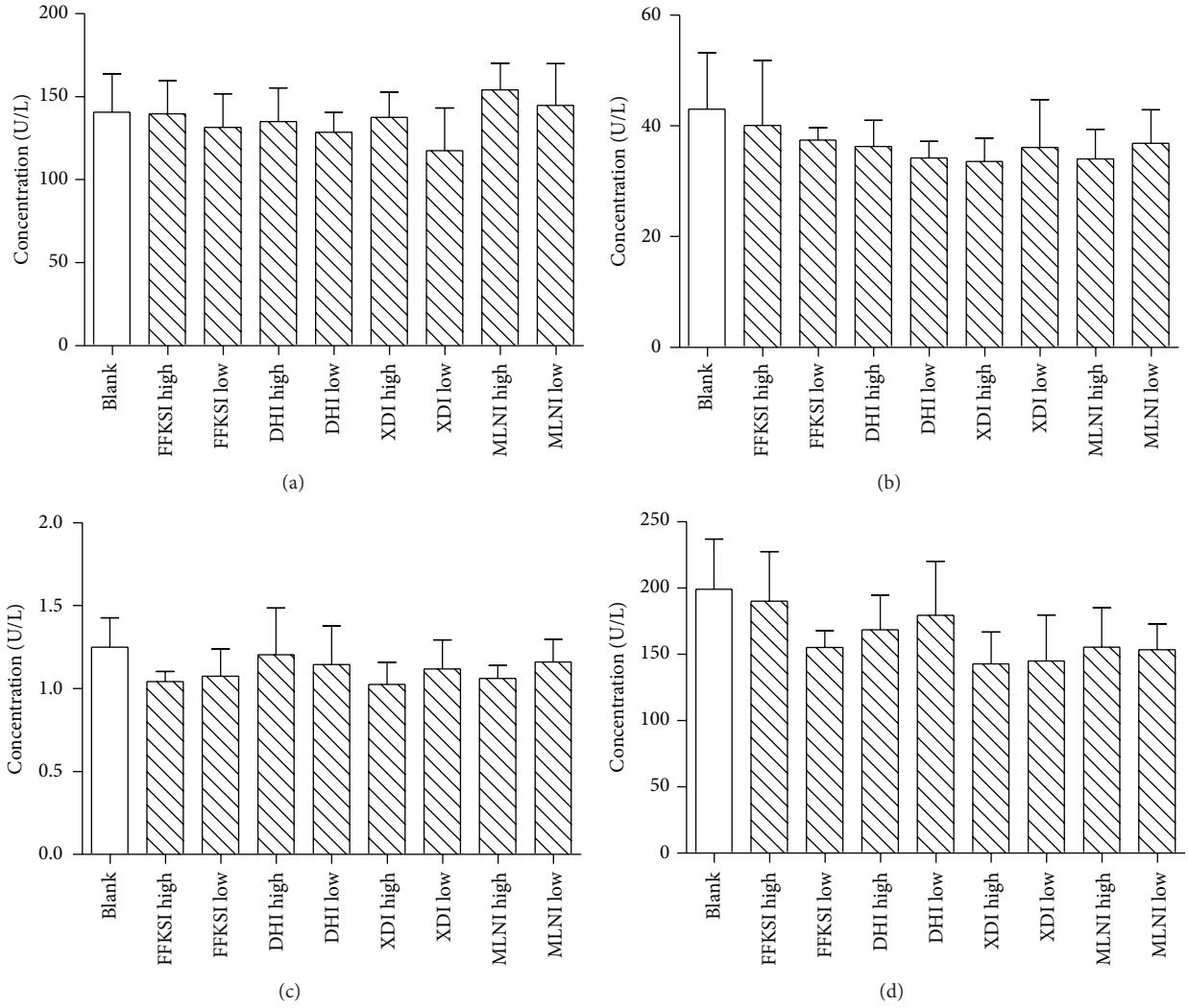


Figure 6: Serum AST (a), ALT (b), TG (c), and ALP (d) profile of Danhong injection, Xiangdan injection, Mailuoning injection, and Fufangkushen injection after 4-week treatment to rats. Data are given as mean ± SEM for each point of at least four separate rats for each point, $^{*}P < 0.05$, $^{**}P < 0.01$.

cytochrome C and other apoptosis inducing factors would be released from mitochondria into cytosol to trigger subsequent activation of procaspase-9 and downstream apoptotic effectors to cause apoptosis [6, 29]. In our assay, Mitotracker Deep Red FM and Rhodamine 123 were chosen to measure mitochondrial function. Rhodamine 123 accumulates in mitochondria depending on MMP whereas Mitotracker Deep Red FM stains mitochondria without the influence from MMP. The combined application enhanced the accuracy in deeply damaged mitochondria. Since mitochondria is the main target in the process of liver injury and insensitivity of intracellular calcium, mitochondrial mass was adopted as a substitution of Ca2+ membrane permeability in the HCA assay established by O'Brien et al. [5]. It is appropriate for hepatotoxicity evaluation of the natural product with complex chemical constituents.

In the subacute animal test, 4 rats were sacrificed because of faulty operation on injection rate and infusing air, two from FFKSI high-dose group, one from FFKSI low-dose group, and one from XDI high-dose group. The rats were fiercely struggled when injecting FFKSI; twitching was observed immediately after injection and sustained nearly 3 minutes on six rats and 2 rats died in the process of injecting in high-dose group. No more rats died when injection speed was turned down. In the FFKSI low-dose group, the body weight of rats increased slowly, and on the 7th day after injection, the rats were irritable and the fur became pale brown. One rat lower limbs were paralyzed after administrated on the 23th day after injection and the rat died 2 days later.

Serum concentrations of AST, ALT, ALP, and TG are important indicators of hepatic health [30, 31]. The activity of AST, ALT, and TG enzymes in all groups of the four injections showed no significant difference compared with control after 28 days of injection. It indicated that the 4 TCM injections would not induce severe histopathological hepatic lesions in rats.

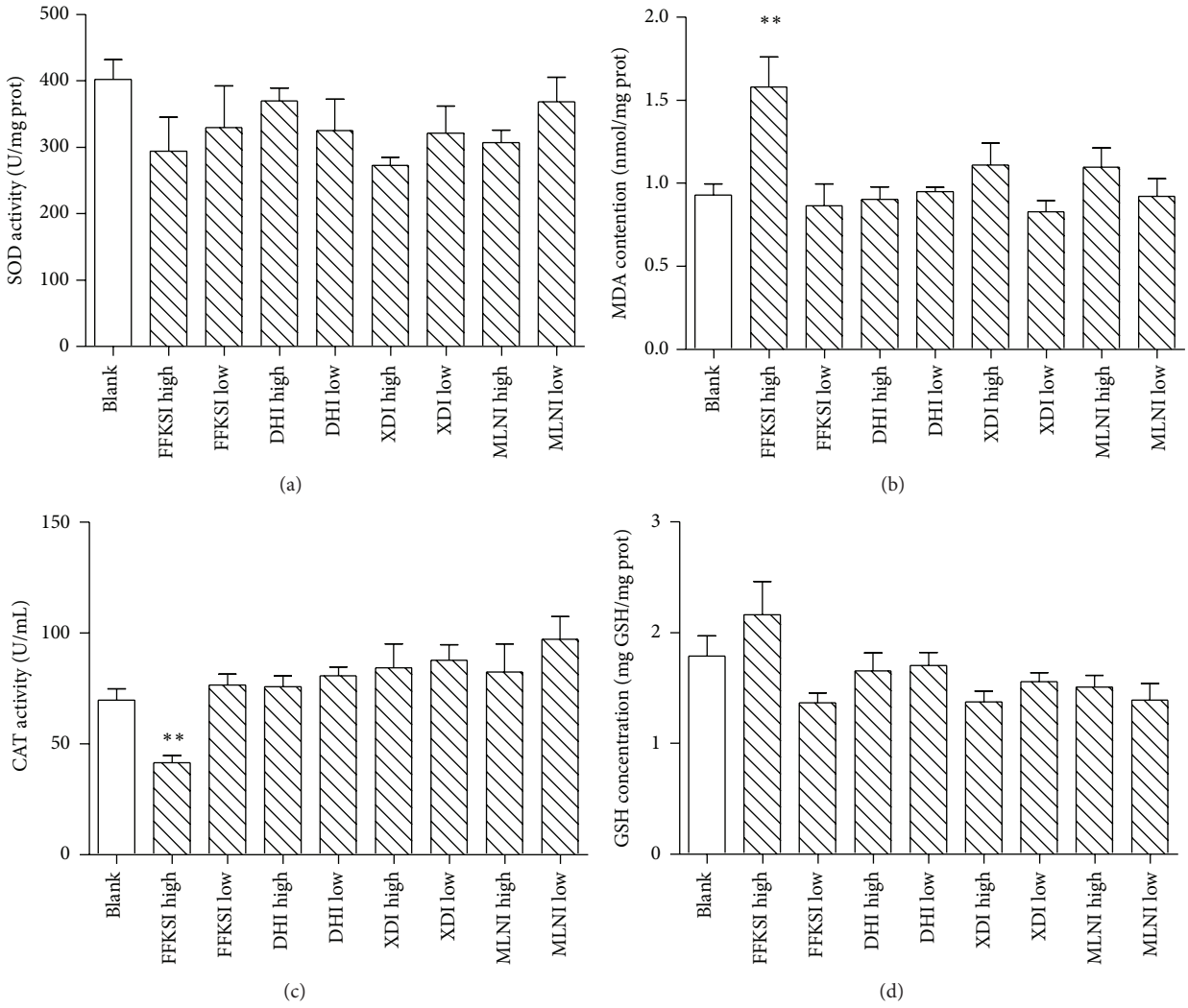


Figure 7: The effect of Danhong injection, Xiangdan injection, Mailuoning injection, and Fufangkushen injection on SOD (a), MDA (b), CAT (c), and GSH (d) in liver homogenate. In FFKSI high-dose group, the CAT activity decreased extremely significantly (b) and MDA concentration increased significantly (c). Data are given as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$.

Excessive free radicals would consume intracellular SOD, CAT, and GSH and cause hepatocyte damage in liver cells [32]. MDA is an end product of lipid peroxidation. The concentration of MDA is widely used as a marker of free radical mediated lipid peroxidation injury of liver tissue [33]. According to biochemical experiment of FFKSI high-dose group, cell antioxidant activity weakened along with the increased MDA content and decrease of CAT activity in liver homogenate, indicating that the high-dose FFKSI weakened the antioxidant capacity of liver cells. A concentration dependent decrease of CN and MMP was found from 1000- to 100-fold dilution of FFKSI by the HCA assay established, indicating that FFKSI damaged the cells by inducing mitochondrial dysfunction. The hepatotoxicity caused by FFKSI was probably relative with the damage of liver cells by reducing the antioxidant ability, promoting the formation of ROS and damaging the mitochondrial membrane structure.

The 100-fold dilution of XDI caused significant decrease of CN and significant increase of MS from the HCA result. In subacute toxicity test, one rat in XDI high-dose group was sacrificed during the experiment. The SOD value of XDI was lower than other groups while the MDA level was higher than all other groups except for FFKSI high-dose group, though there was no significant difference among those groups. In addition, the body weight in XDI low-dose group and the ratio of liver weight to body weight in XDI high-dose group are significantly lower than blank. It indicated that long-term use of XDI at a high concentration may also cause certain oxidative damage on liver by affecting the function of mitochondria. High correlation was found between the results of HCA hepatotoxicity assay and the subacute animal test.

In summary, we present an *in vitro* cell-based high-content assay using PerkinElmer HCA system, which is

highly predictive for human drug-induced liver injury. The hepatotoxicity of four TCM injections was tested. The result is in accordance with subacute animal study. Compared with conventional hepatotoxicity assays, the assay is sensitive and accurate with higher throughput, lower cost, and less animals needed that is suitable for TCM preparations with complex chemicals.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This study was supported by funding from the Innovation Team of Tianjin Higher Education (TD2-5031), State Key Development Program for Basic Research of China (Grant no. 2012CB723504), and the National Natural Science Foundation of China (Grant nos. 81202850, 81274128).

References

[1] L. Zhang, X.-H. Yang, L.-Y. Cao, and S. Ji, "Current status and development of monitoring on adverse reaction of traditional Chinese medicine in China," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 25, no. 7, pp. 581–584, 2005.

[2] M. Chen, H. Bisgin, L. Tong et al., "Toward predictive models for drug-induced liver injury in humans: are we there yet?" *Biomarkers in Medicine*, vol. 8, no. 2, pp. 201–213, 2014.

[3] J. A. Hinson, D. W. Roberts, and L. P. James, "Mechanisms of acetaminophen-induced liver necrosis," *Handbook of Experimental Pharmacology*, vol. 196, pp. 369–405, 2010.

[4] H. Olson, G. Betton, D. Robinson et al., "Concordance of the toxicity of pharmaceuticals in humans and in animals," *Regulatory Toxicology and Pharmacology*, vol. 32, no. 1, pp. 56–67, 2000.

[5] P. J. O'Brien, W. Irwin, D. Diaz et al., "High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening," *Archives of Toxicology*, vol. 80, no. 9, pp. 580–604, 2006.

[6] J. A. Kim, E. Han, C. J. Eun, Y. K. Tak, and J. M. Song, "Real-time concurrent monitoring of apoptosis, cytosolic calcium, and mitochondria permeability transition for hypermulticolor high-content screening of drug-induced mitochondrial dysfunction-mediated hepatotoxicity," *Toxicology Letters*, vol. 214, no. 2, pp. 175–181, 2012.

[7] R. H. Chen, C. Zhang, X. H. Li et al., "Fufangkushen injection caused liver injury," *Adverse Drug Reactions Journal*, vol. 15, no. 5, pp. 296–297, 2013.

[8] China Food and Drug Administration, "Vigilance Xiangdan injection serious adverse reactions," Adverse Drug Reaction Information Bulletin, no. 45, 2012.

[9] China Food and Drug Administration, *Vigilance Yanxiping Injection and Mailuoning Injection Serious Adverse Reactions*, Adverse Drug Reaction Information Bulletin no. 48, 2012.

[10] L. M. Zhong and S. L. Zhao, "Analysis for 178 cases of adverse reaction report about Xiangdan injection," *Chinese Journal of Pharmacovigilance*, vol. 3, no. 6, pp. 353–355, 2006.

[11] Y. Wu and C. F. Qian, "Analysis of 178 adverse reaction reports of Xiangdan injections," *Chinese Journal of Pharmacoepidemiol*, vol. 22, no. 1, pp. 12–14, 2013.

[12] W. Hu, "Analysis of 113 serious adverse reaction reports of Mailuoning injections," *Zhejiang Journal of Integrated Traditional Chinese and Western Medicine*, vol. 28, no. 2, pp. 116–117, 2008.

[13] G. N. Xu, P. H. Shen, J. Lin et al., "Analysis of 97 cases of adverse reactions of literature about mailuoning injection," *China Journal of Hospital Pharmacy*, vol. 17, no. 9, pp. 594–595, 2007.

[14] J. F. Tang and X. L. Lee, "Literature research of the adverse reaction of Danhong injection," *China Pharmacy*, vol. 22, no. 3, pp. 261–263, 2011.

[15] W. G. E. J. Schoonen, W. M. A. Westerink, J. A. D. M. de Roos, and E. Débiton, "Cytotoxic effects of 100 reference compounds on Hep G2 and HeLa cells and of 60 compounds on ECC-1 and CHO cells. I Mechanistic assays on ROS, glutathione depletion and calcein uptake," *Toxicology in Vitro*, vol. 19, no. 4, pp. 505–516, 2005.

[16] W. G. E. J. Schoonen, J. A. D. M. de Roos, W. M. A. Westerink, and E. Débiton, "Cytotoxic effects of 110 reference compounds on HepG2 cells and for 60 compounds on HeLa, ECC-1 and CHO cells: II mechanistic assays on NAD(P)H, ATP and DNA contents," *Toxicology in Vitro*, vol. 19, no. 4, pp. 491–503, 2005.

[17] P. J. Bugelski, U. Atif, S. Molton, I. Toeg, P. G. Lord, and D. G. Morgan, "A strategy for primary high throughput cytotoxicity screening in pharmaceutical toxicology," *Pharmaceutical Research*, vol. 17, no. 10, pp. 1265–1272, 2000.

[18] P. Niethammer, H. Y. Kueh, and T. J. Mitchison, "Spatial patterning of metabolism by mitochondria, oxygen, and energy sinks in a model cytoplasm," *Current Biology*, vol. 18, no. 8, pp. 586–591, 2008.

[19] S. Kuruvilla, C. W. Qualls Jr., R. D. Tyler et al., "Effects of minimally toxic levels of carbonyl cyanide P-(trifluoromethoxy) phenylhydrazone (FCCP), elucidated through differential gene expression with biochemical and morphological correlations," *Toxicological Sciences*, vol. 73, no. 2, pp. 348–361, 2003.

[20] L. P. James, P. R. Mayeux, and J. A. Hinson, "Acetaminophen-induced hepatotoxicity," *Drug Metabolism and Disposition*, vol. 31, no. 12, pp. 1499–1506, 2003.

[21] M. Yagmurca, O. Bas, H. Mollaoglu et al., "Protective effects of erdosteine on doxorubicin-induced hepatotoxicity in rats," *Archives of Medical Research*, vol. 38, no. 4, pp. 380–385, 2007.

[22] "Multiparametric Applications with Assay Protocol," Operetta Application Guide.

[23] H. L. Martin, M. Adams, J. Higgins et al., "High-content, high-throughput screening for the identification of cytotoxic compounds based on cell morphology and cell proliferation markers," *PLoS ONE*, vol. 9, no. 2, Article ID e88338, 2014.

[24] V. C. Abraham, D. L. Towne, J. F. Waring, U. Warrior, and D. J. Burns, "Application of a high-content multiparameter cytotoxicity assay to prioritize compounds based on toxicity potential in humans," *Journal of Biomolecular Screening*, vol. 13, no. 6, pp. 527–537, 2008.

[25] H. Garside, K. F. Marcoe, J. Chesnut-Speelman et al., "Evaluation of the use of imaging parameters for the detection of compound-induced hepatotoxicity in 384-well cultures of HepG2 cells and cryopreserved primary human hepatocytes," *Toxicology in Vitro*, vol. 28, no. 2, pp. 171–181, 2014.

[26] X. Niu, L. Gu, C. Zhang, and F. Wang, "Influences of Chinese herbal remedies on transcriptional activity of NF-κB and TNF-α-mRNA level in cells infected by influenza virus," *Journal of*

Beijing University of Traditional Chinese Medieine, vol. 32, no. 12, pp. 822–829, 2009.

[27] "Health bureau of the People's Republic of China drug administration policy," Guide of New Drug and Chinese Medicine.

[28] S. Russmann, G. A. Kullak-Ublick, and I. Grattagliano, "Current concepts of mechanisms in drug-induced hepatotoxicity," *Current Medicinal Chemistry*, vol. 16, no. 23, pp. 3041–3053, 2009.

[29] S. W. Perry, J. P. Norman, J. Barbieri, E. B. Brown, and H. A. Gelbard, "Mitochondrial membrane potential probes and the proton gradient: a practical usage guide," *BioTechniques*, vol. 50, no. 2, pp. 98–115, 2011.

[30] A. Ogutcu, Z. Suludere, and Y. Kalender, "Dichlorvos-induced hepatotoxicity in rats and the protective effects of vitamins C and E," *Environmental Toxicology and Pharmacology*, vol. 26, no. 3, pp. 355–361, 2008.

[31] M. Xue, Z.-Z. Jiang, T. Wu et al., "Anti-inflammatory effects and hepatotoxicity of Tripterygium-loaded solid lipid nanoparticles on adjuvant-induced arthritis in rats," *Phytomedicine*, vol. 19, no. 11, pp. 998–1006, 2012.

[32] Y.-H. Yeh, Y.-L. Hsieh, Y.-T. Lee, and C.-C. Hu, "Protective effects of *Geloina eros* extract against carbon tetrachloride-induced hepatotoxicity in rats," *Food Research International*, vol. 48, no. 2, pp. 551–558, 2012.

[33] J. Quan, X. Yin, and H. Xu, "Boschniakia rossica prevents the carbon tetrachloride-induced hepatotoxicity in rat," *Experimental and Toxicologic Pathology*, vol. 63, no. 1-2, pp. 53–59, 2011.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 815358, 9 pages
http://dx.doi.org/10.1155/2015/815358

Review Article

Beneficial Effects and Safety of Corticosteroids Combined with Traditional Chinese Medicine for Pemphigus: A Systematic Review

Tingting Zhou, Peiru Zhou, Hong Hua, and Xiaosong Liu

Department of Oral Medicine, Peking University School and Hospital of Stomatology, Beijing 100081, China

Correspondence should be addressed to Hong Hua; honghua68@hotmail.com and Xiaosong Liu; songxiaoliu@yahoo.com.cn

Received 19 June 2014; Revised 4 November 2014; Accepted 4 November 2014

Academic Editor: Paul Posadzki

Objective. To evaluate the beneficial effects and safety of corticosteroids combined with traditional Chinese medicine (TCM) for pemphigus. *Methods*. Seven electronic databases were searched to identify any potential randomized controlled trials (RCTs) or clinical controlled trials (CCTs) that compared corticosteroids with and without TCM for the treatment of pemphigus, published in any language. Remission of the mucocutaneous lesions, therapeutic duration, dosage of corticosteroids, and specific antibody titers were employed as the main outcome measures. The methodological quality of the included studies was assessed using the Cochrane Handbook for Systematic Review of Interventions and Rev Man 5.1.0 software. *Results*. Four RCTs with a total of 199 patients were included in the present review. Management with corticosteroids combined with TCM seemed to benefit pemphigus patients in terms of healing of lesions, prevention of complications and relapse, and reduced interferon-gamma (IFN-γ) level. The trials were not of high methodological quality. No study mentioned allocation concealment and blinding. Only one trial reported adverse events, and it indicated that the safety of corticosteroids combined with TCM was uncertain. *Conclusion*. There is some, albeit weak, evidence to show that combined treatment with corticosteroids with TCM could be of benefit for some patients with pemphigus. The efficacy and safety of this combined treatment should be evaluated further in better designed, fully powered, and confirmatory RCTs.

1. Introduction

Pemphigus is a rare group of autoimmune bullous diseases characterized by widespread blistering and erosions on the skin and mucous membranes. The incidence of pemphigus has been estimated to be 1 to 16 new cases per million people per year [1]. Antibodies against desmoglein-1 and/or desmoglein-3 are the main cause of these diseases [2].

Pemphigus is a chronic and potentially life-threatening condition [3]. With the introduction of corticosteroids in the 1950s, the mortality rate of pemphigus patients decreased from 75% to 30% [4]. To date, systemic treatment with corticosteroids is recommended clinically as the first-line remedy. However, high doses of and prolonged exposure to systemic corticosteroids, together with the use of adjuvant immunosuppressive and anti-inflammatory agents, cause severe adverse effects in PV patients [5], such as Cushing syndrome, necrosis of the femoral head, and digestive bleeding. To minimize the side effects of corticosteroids, traditional Chinese medicine (TCM) has been used as an adjuvant in China since the 1980s [6]. Many clinical studies have shown that it can effectively improve patients' conditions and therapeutic efficacy and reduce corticosteroid dosage, complications, and the risk of recurrence [7–10]. However, no evidence-based studies using corticosteroids combined with TCM for treatment of pemphigus have appeared in the literature. In this analysis, randomized controlled trials (RCTs) and clinical controlled trials (CCTs) were collected to evaluate the beneficial effects and safety of corticosteroids combined with TCM for pemphigus.

2. Materials and Methods

2.1. Database and Search Strategies. We selected all clinical trials that compared corticosteroid treatment with combined

treatment of corticosteroids and TCM for treatment of pemphigus in the Chinese National Knowledge Infrastructure (CNKI), the Chinese Biomedical Literature Database (CBM), the Chinese Scientific Journal Database (VIP), WANFANG, PubMed, EMBASE, SCI, Current Controlled Trials, and the Cochrane Central Register of Controlled Trials in the Cochrane Library (March 2014). No restrictions were placed on language. The following search terms were used individually or combined: "Chinese patent medicine," "Chinese patent drugs," "traditional Chinese medicine," "Chinese herbology," "Chinese medicine," "Chinese material medical," "Chinese herbs," "Chinese herbal medicine," "herbal medicine," "Chin Tradit Pat Med," corticoid, corticosteroids, "rat ATH," glucocorticoid, "cortical hormone," pemphigus, "controlled clinical trial," "clinical trial," and "randomized controlled trials."

2.2. Inclusion and Exclusion Criteria and Process

Inclusion Criteria. RCTs and CCTs that compared corticosteroids with and without TCM for pemphigus were collected. Based on the "Diagnosis and classification of pemphigus and bullous pemphigoid" [11], patients fulfilling the diagnostic criteria for pemphigus were enrolled, without restrictions on age, gender, or race. Outcome measures included clinical outcome (e.g., remission of the lesions, dosage of the corticosteroid, and complications) and laboratory outcome (e.g., titers of Dsg1 and 3 and IIF).

Exclusion Criteria. Exclusion criteria were as follows: duplicate publications reporting the same groups of participants; case reports, reviews, workshop summaries, and studies about clinical observations; studies that included pemphigoid or other autoimmune bullous diseases; research reports without relevant information on patients and interventions; studies that included any adjuvant (i.e., azathioprine, methotrexate, or steroid sparing agents) before starting TCM.

2.3. Data Extraction and Quality Assessment. Two authors (Tingting Zhou and Peiru Zhou) were responsible for searching the literature, selecting studies, and extracting data independently. Various data were extracted including the title of the study, author, year of publication, article source, study size, sample size, diagnostic criteria, methodological details, therapeutic duration, and clinical standards, as well as interventional details of controls, outcomes, and adverse effects for each study. Disagreements were resolved by discussion, and consensus was achieved through a third party (Hong Hua, Xiaosong Liu). The quality of the enrolled trials was assessed according to the Cochrane Handbook for Systematic Review of Interventions, Version 5.1.0, and Rev Man 5.1.0 software [12]. The assessment criteria used were random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), selective reporting (reporting bias), and other bias.

2.4. Data Synthesis. A descriptive analysis of the data was conducted in this systematic review because of statistical heterogeneity and the limited number of patients enrolled.

3. Results

3.1. Description of the Included Studies. A total of 450 abstracts (431 in Chinese and 19 in English) were obtained from 9 databases and were evaluated on the basis of the inclusion criteria of the present analysis. Figure 1 shows details regarding the full flow of information, presented according to the PRISMA format [13]. A total of 193 studies were excluded for duplication. Among the 257 remaining abstracts, 106 abstracts were not relevant, and 42 described bullous disorders other than pemphigus. Therefore, 10 full-text articles were reviewed. Further examination showed that only four trials met the inclusion criteria; they were included in the present analysis [6, 14–16].

3.2. Characteristics of Studies. Table 1 shows the characteristics of the four clinical trials included in this review. A total of 199 pemphigus patients were studied in these trials. Of these patients, 103 were prescribed a combination of oral corticosteroids and TCM, while 96 patients were treated with corticosteroids only. The sample size in each trial ranged from 20 to 32.

3.3. Risk of Bias and Quality Assessment of Studies. The quality of most trials was poor according to the Cochrane quality assessment criteria (as shown in Table 2). Figure 2 shows the risk of bias. No information about allocation concealment, blindness, or withdrawals and dropouts was recorded in any trial. Only one trial reported the randomized allocation of participants, follow-up, and adverse reactions.

3.4. Effects of the Interventions. To summarize the data, a descriptive analysis was conducted due to the limitations of both statistical heterogeneity and the number of articles analyzed.

3.5. Main Outcome Measure. The remission of mucocutaneous lesions, therapeutic duration, dosage of corticosteroids, complications, and relapse were evaluated as main outcome measures.

3.5.1. Remission of Lesions. Three out of four trials reported the remission of lesions. In Li's study, the improvement of blisters was significant (t-test, $P < 0.05$) in the group with TCM, while no significant difference was demonstrated between groups in fever or causalgia [14]. The response of the pemphigus patients in the active condition was documented by Peng and Jie [15]. After 2 months of treatment, only 6.25% (2/32) and 24.14% (7/29) of the patients remained unchanged in the groups with and without TCM, respectively. The ratio of improvement in the group with TCM was significantly higher than that in the group without TCM ($P < 0.05$). In the third trial [13], 4.5% (1/22) and 35% (7/20) of the patients

Table 1: Characteristics of the studies included in this review.

(a)

Study	Authors	Year	Article source	Type of study	Sample size (treatment/control)	Diagnosis standard
[14]	Li and Liang	2009	CJCM2009 Vol. (1)	RCT	29/27	Clinical manifestation histopathology and DIF
[15]	Chen and Xu	2007	Journal of New Chinese Medicine, January 2007 VoI. 39 No.1	RCT	32/29	Clinical manifestation histopathology and DIF
[6]	Luo et al.	2003	Journal of Clinical Dermatology, 2003; 32(1): 38–41.	RCT	20/20	Clinical manifestation histopathology and DIF
[16]	Wang et al.	2009	Journal of China Traditional Chinese Medicine Information, April 2009, Vo1. 1 No. 2	RCT	22/20	Clinical manifestation histopathology and immunopathology

(b)

Study	Intervention	Control
	Treatment	
[14]	*Systemic therapy*: (Radix rehmanniae, cornu bubali, coptidis rhizoma, *Gardenia*, radix scutellariae, red peony root, phillyrin, cortex moutan, folium phyllostachytis, herba artemisiae scopariae, *Fritillaria cirrhosa*, caulis akebiae, acorus tatarinowii, rhizoma belamcandae, mechanism, agastache rugosus, cardamom) combined 20 mg dexamethasone iv, regularly reduced after seven days, 5 mg for maintenance and stopped when lesion was controlled. *Topical therapy:* (gallnut, fructus mume, pepper, chaulmoogra, cochinchina momordicae seed, calamine, *Hibiscus syriacus*, *Sophora flavescens*, cortex dictamni, senecio scandens) decoction for external application 3-4 times for one day.	*Systemic therapy*: 12 mg prednisone, once a day, appropriate antibiotics and protecting stomach medicine added according to the symptoms. *Topical therapy*: Gentian violet, nitrofurazone solution, permanganate armour, rivanol gauze for external application.
[15]	(White atractylodes rhizome, raw Gordon euryale seed, cortex phellodendri, *Paeonia suffruticosa*, raw hovenia dulcis, raw semen coicis, SevenlobedYam Rhizome, herba artemisiae scopariae, honeysuckle, cortex sclerotii poriae) combined prednisone.	Oral administration of prednisone.
[6]	Pemphigus I (Copitidis rihizoma, radix scutellariae, cortex phellodendri, *Gardenia*, honeysuckle, glabrous greenbrier rhizome, radix rehmanniae, coix seed, rhizoma atractylodis) combined glucocorticoids.	Glucocorticoids only
[16]	Tonifying kidney herb (Liuweidihuang pill) combined small doses of corticosteroids for maintenance treatment.	Only small doses of corticosteroids for maintenance treatment

(c)

Study	Treatment course	Outcome measure				
		Clinical standards	Laboratory standards	Complications	Follow-up	Adverse event
[14]	More than six months	① The regression of the blister, fever, and the causalgiar ② the treatment time, ③ relapse	No	Gastric ulcer, fungal infection, and osteoporosis	Not mentioned	Not mentioned
[15]	Two months	① The regression of the blister, ② the dosage of corticosteroids	No	No	Not mentioned	Not mentioned

(c) Continued.

Study	Treatment course	Outcome measure		Complications	Follow-up	Adverse event
		Clinical standards	Laboratory standards			
[6]	Not mentioned	(1) The time used when the lesion was controlled, (2) the highest dosage of corticosteroids used when the lesion was controlled	The titer of the antibodies and the level of IL-10, IFN-γ, and slL-2R	No	Not mentioned	One patient with subtotal gastrectomy experienced diarrhea.
[16]	14 days as one course of treatment, just one course every month	The lesion control rate (patients in stable condition and regressed)	No	Osteoporosis, hypertension, diabetes, and peptic ulcer	Two years	Not mentioned

Table 2: Quality assessment of included randomized controlled trials.

Study	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other bias	Summary
[14]	Uncertain	Uncertain	Low bias	Low bias	Low bias	Uncertain	Uncertain	Unclear risk of bias
[15]	Uncertain	Uncertain	Low bias	Low bias	Low bias	Uncertain	Uncertain	Unclear risk of bias
[6]	Low bias	Uncertain	Low bias	Low bias	Low bias	Uncertain	Uncertain	Unclear risk of bias
[16]	Uncertain	Uncertain	Low bias	Low bias	Low bias	Uncertain	Uncertain	Unclear risk of bias

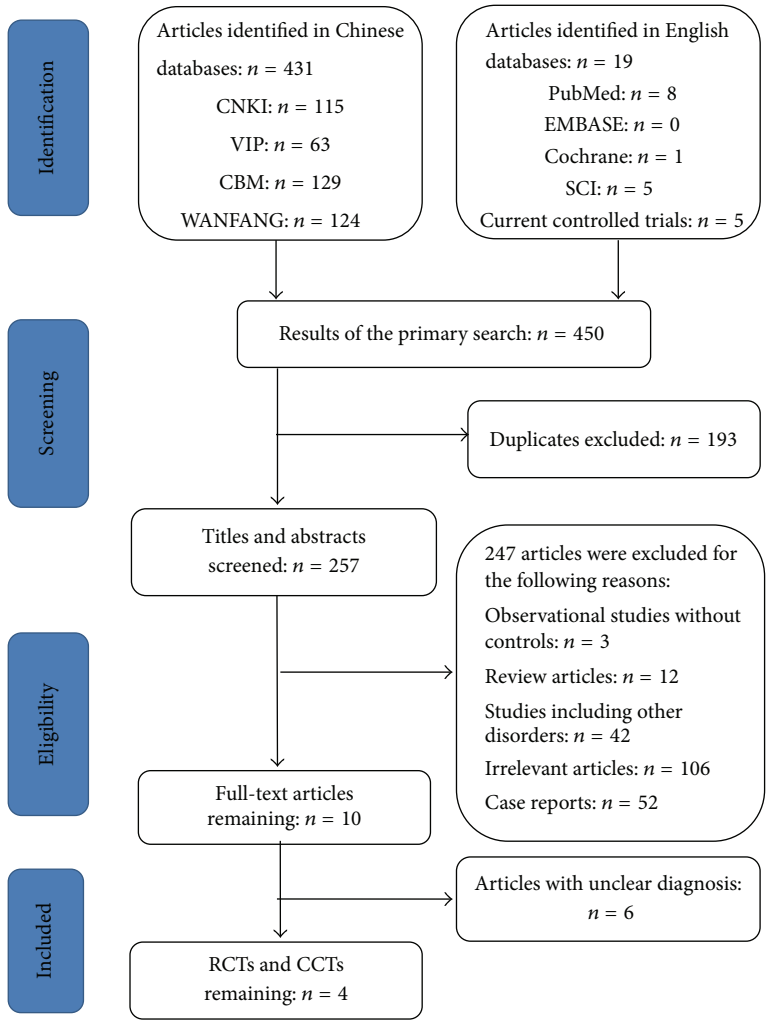


Figure 1: Study selection process.

relapsed during the follow-up period (2 years) in the group with and without TCM respectively (χ^2 test, $P < 0.05$).

3.5.2. Time of Treatment. Two trials described the therapeutic duration. The size of lesions was measured at different time points of treatment in one trial [14]. In this trial, therapeutic effects were classified into three grades: cured: all previous rashes faded without any new eruption; improved: more than 30% of rashes faded and new rashes appeared occasionally; and not improved: less than 30% of rashes faded and new rashes appeared all the time [17, 18]. Significant differences in the percentage of cured patients at various time points of treatment (2 months, 4 months, and 6 months) were observed between groups (t-test, $P < 0.01$). The other trial [6] just mentioned that there was a significant difference between groups in healing time of the lesions ($P < 0.05$), but no detailed times were presented.

3.5.3. Dosage of Corticosteroids. Dosage of corticosteroids was reported in only two trials. In Chen's study [15], no significant difference between the groups was found in the percentage of patients taking less than 30 mg of prednisone per day at the end of 2 months' treatment, although the ratio was higher in the TCM group than in the non-TCM group. However, the difference was significant ($P < 0.01$) when the dosage was more than 30 mg per day. The other trial [6] only mentioned that there was a significant difference ($P < 0.05$) in corticosteroid dosage when the lesions could be controlled well, but no exact figure was given.

3.5.4. Complications. Two trials reported complications. In one study, during treatment, gastric ulcer occurred in two patients, and fungal infection and osteoporosis developed in one and three patients, respectively, in the TCM group. In the group without TCM, 5 patients suffered from gastric ulcer, 7 patients suffered from fungal infection, and 10 patients suffered from osteoporosis. A t-test demonstrated a highly significant difference between groups ($P < 0.01$) [14]. In the other study, osteoporosis and hypertension were observed in one patient receiving TCM, while in the group receiving corticosteroids alone, two patients with osteoporosis,

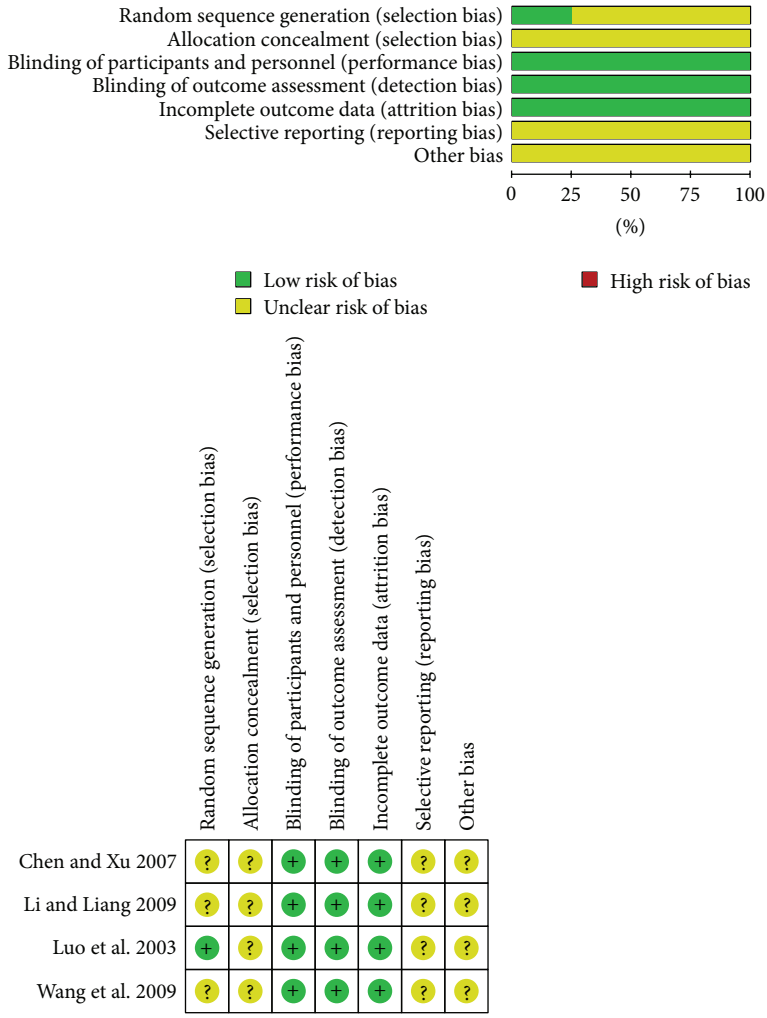


Figure 2: The Cochrane Collaboration's tool for assessing risk of bias.

three patients with hypertension, two patients with diabetes mellitus, and one patient with digestive tract ulcer were observed (chi-square test, $P < 0.05$). The chi-square test demonstrated a significant difference between the two groups ($P < 0.05$) [16].

3.5.5. Relapse Rate. Only one trial reported the relapse rate [14]. Two patients receiving TCM and six patients receiving corticosteroid alone experienced relapse. A t-test demonstrated a highly significant difference between the two groups ($P < 0.01$).

3.6. Secondary Outcome Measure. Antibody titer in circulation was set as a secondary outcome measure. One [6] of the four studies compared the titer of specific antibodies for pemphigus between groups, as well as the levels of interleukin-10 (IL-10), interferon-gamma (IFN-γ), and soluble interleukin-2 receptor (slL-2R). Prior to treatment, no differences were detected between groups in circulating levels of these markers. However, the levels of IFN-γ decreased remarkably in both groups after treatment. The reduction in the group with TCM was much more remarkable than that in the group without TCM ($P < 0.05$). In addition, the level of IL-10 increased in the combined TCM group more than that in the corticosteroid group, but there were no significant differences ($P > 0.05$).

3.7. Adverse Events. One case of diarrhea was observed in the combined group by Luo et al. [6]. No other adverse events were reported.

3.8. Subgroup Analysis and Publication Bias. The number of trials in the present review was too small to conduct analyses of subgroups and publication bias.

4. Discussion

Pemphigus is an acquired autoimmune blistering disease, in which the immune system becomes dysregulated and produces antibodies against normal mucocutaneous components. The use of systemic corticosteroids has dramatically reduced mortality from this disease, but treatment outcome

is still associated with profound corticosteroid-related morbidities. To minimize the side effects of these medicines, TCM has been used in China since the 1980s. Yu et al. [7] reported that a combination of corticosteroids and TCM can effectively improve patients' conditions and therapeutic efficacy and reduce corticosteroid dosage and complications. Wu et al. [10] found that this therapeutic strategy could reduce the toxicity of corticosteroids and promote drug absorption. Based on traditional Chinese medicine, pemphigus is attributed to damp, heat, and toxin, which result in the holistic Yin-yang imbalance and dysfunction in the visceral organs of the patient and consequently cause various clinical manifestations [19]. Unlike Western medicine, the core idea of traditional Chinese herbal medicines is to correct the holistic condition, such as with *Coptidis Rhizoma*, *Radix Scutellariae*, and *Cortex Phellodendri*, which are most commonly used for heat-clearing and detoxicating. Modern clinical trials have demonstrated that some herbal medicines have immunosuppressive and anti-inflammatory properties [20].

In the present analysis, four clinical trials consisting of 199 patients were evaluated. Compared to corticosteroids alone, management with corticosteroids combined with TCM seemed to benefit pemphigus patients in terms of healing of lesions, prevention of complications and relapse, and reducing levels of IFN-γ. However, conclusions could not be drawn because of several limitations in the present analysis. First, only a few clinical trials were available, and they were small in size and poor in quality of study design. Second, publication bias resulting from the difficulty in publishing negative results may have occurred. Third, a meta-analysis could not be performed because of the small number of available studies and statistical heterogeneity. Nevertheless, the alternative strategy of using corticosteroids combined with TCM for pemphigus may be considered one option to prevent complications, especially when patients are experiencing side effects from corticosteroid treatment.

However, to date, no well-designed or high-quality RCT on the safety and efficacy of combining steroid and TCM treatment for pemphigus has been reported. This analysis suggests that high-quality RCTs are essential to demonstrate the beneficial effects of Chinese herbs for patients with pemphigus. To achieve a strong clinical trial, clinical practitioners should follow the international standards of CONSORT in assessing TCM [21]. Furthermore, this analysis suggests that high-quality RCTs are essential to TCM clinical investigation. In order to achieve an ideal clinical trial, clinical practitioners should follow the international standards of CONSORT for TCM [19]. To make sure that the rationale of the study design is the first step. The study design model, such as the TCM syndrome-oriental model and the integrated syndrome and disease-oriental model, could have effect on the inclusion and exclusion criteria, the treatment protocols, and the outcomes. Secondly, the information of the TCM which included the TCM formula, dosage, treatment course, control intervention, outcome measurement method, and index should be defined clearly. Thirdly, the primary and secondary outcomes and selecting the rationale ones which could improve the reliability of the assessment also should be defined clearly. In addition, statisticians should participate throughout the research process and be responsible for calculating sample size, monitoring the performance of the research, and analyzing data.

5. Conclusion

There is some evidence on the use of corticosteroids combined with TCM in promoting healing of lesions, reducing circulating specific antibody levels, and decreasing adverse events and relapse. However, considering the quality of experimental designs and methodologies in existing studies, the evidence remains weak. More rigorous RCTs with high-quality study designs are needed to assess whether corticosteroids combined with TCM are an effective and safe treatment of pemphigus.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Hong Hua selected the topic, designed the study, and modified the paper. Xiaosong Liu modified the paper. Tingting Zhou and Peiru Zhou searched the literature and wrote the paper. Tingting Zhou and Peiru Zhou equally contributed to this paper.

Acknowledgment

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see http://www.textcheck.com/certificate/DlTMZT. This work was supported by the National Key Clinic Program (2013) and Beijing Natural Science Foundation (7142168).

References

[1] C. Chams-Davatchi, M. Valikhani, M. Daneshpazhooh et al., "Pemphigus: analysis of 1209 cases," *International Journal of Dermatology*, vol. 44, no. 6, pp. 470–476, 2005.

[2] L. K. Martin, V. Werth, E. Villanueva, J. Segall, and D. F. Murrell, "Interventions for pemphigus vulgaris and pemphigus foliaceus," *The Cochrane Database of Systematic Reviews*, no. 1, Article ID CD006263, 2009.

[3] L. K. Martin, V. P. Werth, E. V. Villaneuva, and D. F. Murrell, "A systematic review of randomized controlled trials for pemphigus vulgaris and pemphigus foliaceus," *Journal of the American Academy of Dermatology*, vol. 64, no. 5, pp. 903–908, 2011.

[4] J. C. Bystryn, "Adjuvant therapy of pemphigus," *Archives of Dermatology*, vol. 120, no. 7, pp. 941–951, 1984.

[5] F. R. Rosenberg, S. Sanders, and C. T. Nelson, "Pemphigus: a 20 year review of 107 patients treated with corticosteroids," *Archives of Dermatology*, vol. 112, no. 7, pp. 962–970, 1976.

[6] X. Q. Luo, L. C. Zhu, L. D. Tao et al., "The clinical and laboratory research for Chinese traditional medicine in the treatment of

pemphigus," *Journal of Clinical Dermatology*, vol. 32, no. 1, pp. 38–41, 2003.

[7] L. P. Yu, H. Xun, and D. F. Tan, "The clinical research of the combination of corticosteroids and tripterygium glycoside in treatment of pemphigus," *Modern Journal of Integrated Traditional Chinese and Western Medicine*, vol. 18, no. 3, p. 268, 2009.

[8] Z. Qu, "The clinical research of the combination of tripterygium glycoside and prednisone in treatment of pemphigus vulgaris," *China Foreign Medical Treatment*, vol. 17, p. 93, 2009.

[9] L. Wang, L. H. Liao, Y. J. Huang, and J. Q. Deng, "The clinical research for Chinese traditional medicine in the treatment of pemphigus vulgaris," *Journal of Sichuan of Traditional Chinese Medicine*, vol. 24, no. 9, pp. 77–78, 2006.

[10] M. Q. Wu, Q. Y. Han, and Z. H. Pu, "A review on treating pemphigus in the integrative medicine," *Clinical Journal of Chinese Medicine*, vol. 4, no. 15, pp. 120–122, 2012.

[11] R. Kershenovich, E. Hodak, and D. Mimouni, "Diagnosis and classification of pemphigus and bullous pemphigoid," *Autoimmunity Reviews*, vol. 13, no. 4-5, pp. 477–481, 2014.

[12] J. P. T. Higgins and S. Green, *Cochrane Handbook for Systematic Reviews of Interventions, Version 5.1.0*, The Cochrane Collaboration, 2011, http://handbook.cochrane.org/.

[13] D. Moher, A. Liberati, J. Tetzlaff, and D. G. Altman, "Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement," *International Journal of Surgery*, vol. 8, no. 5, pp. 336–341, 2010.

[14] Y. X. Li and P. Liang, "The clinical research of the combination of traditional Chinese and western medicine in treatment of 29 pemphigus vulgaris," *Clinical Journal of Chinese Medicine*, vol. 1, pp. 21–23, 2009.

[15] C. P. Chen and L. J. Xu, "The clinical research of the combination of traditional Chinese and western medicine in treatment of 32 pemphigus vulgaris," *Journal of New Chinese Medicine*, vol. 39, no. 1, pp. 75–76, 2007.

[16] L. M. Wang, J. J. Li, J. T. Yuan, and M. H. Zhou, "The clinical research of tonifying kidney herb combined small doses of corticosteroids in maintenance treatment of pemphigus," *Journal of China Traditional Chinese Medicine Information*, vol. 1, no. 2, p. 65, 2009.

[17] State Administration of Traditional Chinese Medicine, *Standards for Diagnosis and Curative Effect of Chinese Medical Symptom*, Nanjing University Publishing House, 1st edition, 1994.

[18] Hunan Provincial Department of Health, *Standards for Diagnosis and Curative of Common Diseases*, Hubei Medical University Publishing House, 1st edition, 1999.

[19] Y. B. Liu, "The general situation about the combination of traditional Chinese and western medicine in treatment of pemphigus," *Modern Journal of Integrated Traditional Chinese and Western Medicine*, vol. 14, no. 5, pp. 698–700, 2005.

[20] X. X. Huang, "The mechanism and clinical application of traditional Chinese immunosuppressant," *LiShiZhen Medicine and Materia Medica Research*, vol. 16, no. 12, p. 1296, 2005.

[21] Z. Bian, B. Liu, D. Moher et al., "Consolidated standards of reporting trials (CONSORT) for traditional Chinese medicine: current situation and future development," *Frontiers of Medicine in China*, vol. 5, no. 2, pp. 171–177, 2011.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 868731, 8 pages
http://dx.doi.org/10.1155/2015/868731

Research Article

Can Herbal Medicine Cause Hematoma Enlargement of Hypertensive Intracerebral Hemorrhage within 24 hrs Time Window? A Retrospective Study of 256 Cases from a Single Center in China

Yafa Xu,[1,2] Jianwen Guo,[1] Xian Liu,[1] Juehui Li,[1] Jing Wang,[1] and Lingbo Hou[1]

[1]*No. 1 Neurology Department, Guangdong Province Hospital of TCM, The Second Teaching Hospital of Guangzhou University of Traditional Chinese Medicine, 111 Da'de Road, Yuexiu District, Guangzhou, Guangdong Province 510120, China*
[2]*Neurology Department, Qingyuan Traditional Chinese Medicine Hospital, Qingyuan, Guangdong Province 511500, China*

Correspondence should be addressed to Jianwen Guo; jianwen_guo@me.com

Received 27 May 2014; Accepted 14 October 2014

Academic Editor: Paul Posadzki

A retrospective review was performed of consecutive patients presenting with HICH within 24 hours of ictus presenting between March 2008 and March 2013 who were diagnosed as having HICH by CT scan. Of the 256 patients who matched study inclusion standard, 43 patients hematoma was enlarged (16.8%). The number of the patients who did not take PBC or RBC herbal medicine, took the PBC herbal medicine, and took RBS herbal medicine was 19 (44.2%), 2 (4.7%), and 22 (51.2%) in hematoma enlargement group and 78 (36.6%), 26 (12.2%), and 109 (51.2%) in nonhematoma enlargement group, individually. There was no significant difference between two groups ($P = 0.671$). PBC and RBS herbal medicine did not increase the incidence of hematoma expansion of ICH within 24 hours after onset of symptom.

1. Introduction

Hypertensive intracerebral hemorrhage (HICH) is the most devastating form of stroke. Approximately 40% of patients with intracerebral hemorrhage die within 30 days, and the majority of survivors are left with severe disability [1, 2]. Hematoma growth occurs in up to two-third of ICH patients within 24 hours after the onset of symptoms [3]. Furthermore, hemorrhage expansion is an independent determinant of death and disability [4, 5]. Several reasons may be related to the hematoma enlargement in the early stage of HICH, including high blood pressure, "spot" sign of CT scan, sex, age, time window, and anticoagulation drugs [6]. Herbal medicine of promoting blood circulation (PBC) and removing blood stasis (RBS) are widely used in Chinese hospitals to treat HICH; however, whether this herbal medicine can cause hematoma enlargement is undefined until now [7].

In order to evaluate the safety of PBC and RBS herbal drugs, we designed a retrospective study on the hematoma enlargement in HICH patients of 256 cases treated with PBC and RBS herbal medicine within 24-hour time window from the symptom onset in Guangdong Province Hospital of Traditional Chinese Medicine.

2. Material and Methods

2.1. Materials. A retrospective review was performed of consecutive patients presenting with ICH within 24 hours of ictus presenting between March 2008 and March 2013 who were diagnosed as having hypertensive intracerebral hemorrhage by CT scan. The inclusion criteria also included the following: (1) the patient had hypertensive history; (2) patients got follow-up 24-hour unenhanced CT scan; (3) the patients administrated the herbal medicine within 24 hours from symptom onset of HICH. The exclusion criteria were (1) the time window over 24 hrs from onset to the first CT scan; (2) other reasons causing ICH, such as cerebral tumor, aneurysm, arteriovenous malformation, trauma, anticoagulation drugs,

and hematological disorder; (3) lack of second CT scan; (4) lack of other important data of the study. Finally, two hundred and fifty-six cases were reviewed.

2.2. Methods. We searched the patients data from the electronic medical record system platform developed by IBM, inc. The searching strategy was "diagnosis=intracerebral hemorrhage" OR "The International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) [8]" =I61, Intracerebral haemorrhage (Excl.: sequelae of intracerebral haemorrhage) (I69.1) OR I61.0 Intracerebral haemorrhage in hemisphere, subcortical (Deep intracerebral haemorrhage) OR I61.1 Intracerebral haemorrhage in hemisphere, cortical (Cerebral lobe haemorrhage, Superficial intracerebral haemorrhage) OR I61.2 (Intracerebral haemorrhage in hemisphere, unspecified) OR I61.3 (Intracerebral haemorrhage in brain stem) OR I61.4 (Intracerebral haemorrhage in cerebellum) OR I61.5 (Intracerebral haemorrhage, intraventricular) OR I61.6 (Intracerebral haemorrhage, multiple localized) OR I61.8 (Other intracerebral haemorrhage) OR I61.9 (Intracerebral haemorrhage, unspecified), "Admission time=March 2008 to March 2013", "SEX=BOTH", "AGE=ALL".

The patients' raw data were recorded in the well-designed case report forms (CRFs) by two researchers, which contained human demography, medical history, personal history, clinical feather, CT scan, laboratory examination, and herbal medicine treatment. The hematoma volume was measured by ABC/2 Coniglobus formula [9, 10]. Hemorrhage growth was operationally defined as an increase in the volume of intracerebral hemorrhage of >33% as measured by image analysis on the 24-hour CT compared with the baseline CT scan [11].

We defined the herbal medicine as PBC or RBS under the criteria of Chinese Pharmacopoeia of 2010 version. The combined herbal drugs, such as relieving heat and calming liver Yang, decreasing wind and dispersing phlegm, and loosing the bowels, were also under the criteria of Chinese Pharmacopoeia of 2010 version.

Two neuroradiologists analysis on the CT scan data at the work station independently. We divided the patients into hematoma enlargement group and nonhematoma enlargement group. Thus, all the data were analyzed in the statistic software.

2.3. Statistical Analysis. Statistical Product and Service Solutions (SPSS Inc.) 19.0 version was used in our study. Firstly, Univariate analysis was used. χ^2 and nonpaired t-tests were used to compare patients with and without hemorrhage growth as to the following variables: age, sex, race, current smoking, prior stroke, diabetes, history of hypertension, blood pressure, location of hemorrhage, volume of ICH on baseline CT, time to first CT scan, baseline platelet count, and baseline prothrombin and partial thromboplastin times. The Wilcoxon rank sum test was used to compare the initial GCS score in patients with and without hemorrhage growth. The Wilcoxon rank sum test was also used to compare patients with and without hemorrhage growth as to the change in the GCS score, hematoma enlargement from baseline to 24 hours. Logistic regression was used to investigate possible multiple risk factors and PBC herbal drugs or RBS herbal drugs for growth in hemorrhage volume from baseline to 24 hours. We also analyzed the possible risk factors and PBC and RBS herbal drugs of 3-month outcome followup (mRS 0-1 as independent outcome, mRS 2–6 as dependent outcome) by logistic regression. All statistical tests were two-tailed, and $P \leq 0.05$ was considered significant. Data are presented as mean ± SD.

3. Results

3.1. Patients. Between March 2008 and March 2013, ICH was diagnosed in 901 patients at our hospital. Of these 901 patients, 31 were diagnosed as having bleeding infarction, 43 were diagnosed as having arteriovenous malformation (AVM), 19 were diagnosed as having intracerebral aneurysm, 9 were diagnosed as having cerebral tumor, and 8 were diagnosed as having cerebral trauma. So 791 who were cause by hypertension.

Of the 791 ICH patients, 357 who were admitted after 24 hours of onset were excluded, including 95 patients whose duration was from 24 hrs to 2 ws, 96 patients from 2 ws to 6 ms, and 166 patients more than 6 ms. 70 failed to undergo the second CT because of surgery or death. 108 received emergency surgery within 24 hours after first CT scan. Thus, 256 patients, all of whom underwent the first CT within 24 hours of onset and the second CT within 24 hours after first CT scan, were reviewed in this retrospective study.

We also reviewed the patients three months later after entering hospital through telephone or outpatient department visiting. The modified Rankin scale (mRS) was recorded in the followup (see Figure 1).

3.2. Hematoma Growth and the Age. Of the 256 patients who matched study inclusion standard, 43 patients hematoma were enlarged (16.8%) within 24 hrs from onset. The mean age was 66 ± 24.00 yrs in hematoma enlargement group and 65.00 ± 22.00 yrs in nonhematoma group; they included 173 male patients and 83 female patients.

3.3. PBC and RBS Herbal Medicine Use in Two Groups. The number of the patients who did not take the PBC and RBS herbal medicine was 19 (44.2%) in hematoma enlargement group and 78 (36.6%) in nonhematoma enlargement group. The number of the patients who took the PBC and RBS herbal medicine was 24 (55.8%) in hematoma enlargement group and 135 (63.4%) in nonhematoma enlargement group ($P = 0.390$). The number of the patients who took the PBC herbal medicine was 2 (4.7%) in hematoma enlargement group and 26 (12.2%) in nonhematoma enlargement group. The number of the patients who took the RBS herbal medicine were 22 (51.2%) in hematoma enlargement group and 109 (51.2%) in nonhematoma enlargement group. There was no significant difference between two groups ($P = 0.671$). Thus, PBC and RBS herbal medicine could not cause hematoma enlargement of HICH within 24 hrs time window (Figure 2).

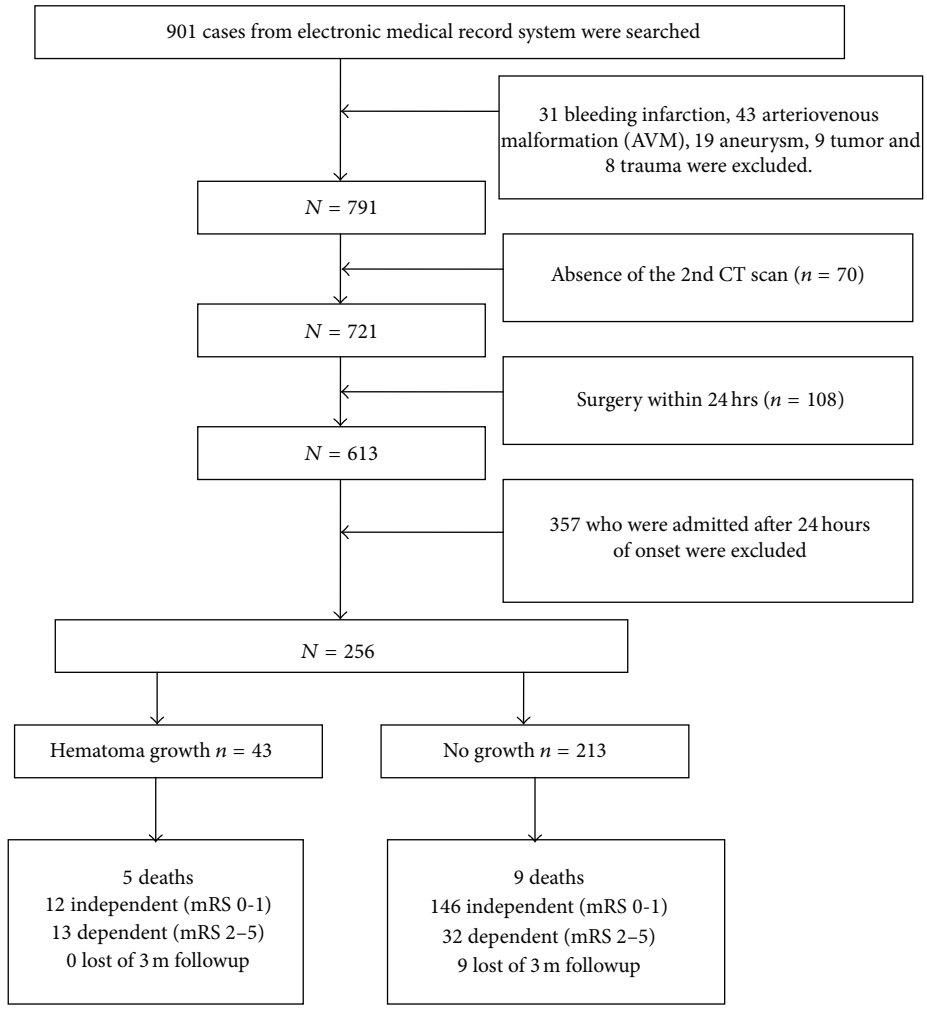


Figure 1: Patients recruited chart.

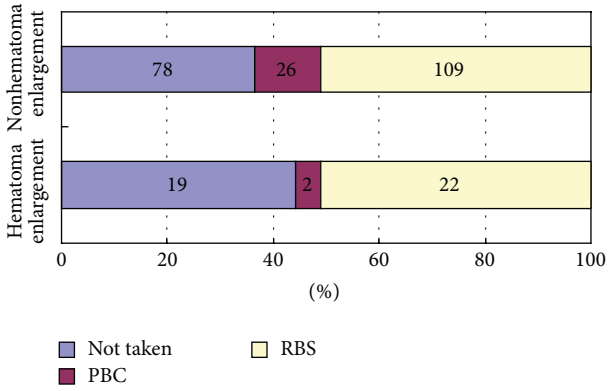


Figure 2: Comparison of PBC and RBS herbal medicine use between two groups.

3.4. Univariate Analysis on the Hematoma Enlargement. We found that patients' sex, baseline GCS, baseline NIHSS, duration from onset to the first CT scan, and aspartate aminotransferase (AST) had significant difference between two groups ($P < 0.05$) (Table 1).

3.5. Multivariate Analysis on the Hematoma Enlargement. Patients' sex, baseline Glasgow coma scale, baseline NIHSS, duration from onset to the first CT scan, aspartate aminotransferase (AST), and PBC and RBS herbal medicine use were an independent variable in the multivariate logistic regression analysis and hematoma growth an outcome variable (dependent variable) (Table 2).

There were two independent factors that can cause hematoma growth. The first one was patient's sex ($P = 0.019$). The second one was duration from onset to the first CT scan, 0-1 hr ($P = 0.046$), 1-2 hrs ($P = 0.041$). PBC herbal medicine use ($P = 0.197$) or RBS herbal medicine use ($P = 0.946$) was not independent risk fact. On the other hand, the utilization rate of PBC and RBS herbal medicine was higher in the nonhematoma growth group (63.4%) than in the hematoma growth group (55.8%). The coefficient of regression β of RBS herbal medicine use was −1.166, OR = 0.312. The coefficient of regression β of PBC herbal medicine use was −0.026, OR = 0.975.

Table 1: The univariate analysis on the hematoma enlargement (%).

Factor	Hematoma enlargement (%) (n = 43)	Nonhematoma enlargement (%) (n = 213)	Value	P
Age (yrs)	66.00 ± 24.00	65.00 ± 22.00	−0.578$^{\triangle}$	0.563
Male	35 (81.4)	138 (64.8)	4.503$^{\diamond}$	0.034*
Hypertension history	27 (62.80)	139 (65.30)	0.096$^{\diamond}$	0.757
DM history	4 (9.30)	25 (11.70)	0.038$^{\bullet}$	0.845
ICH history	4 (9.30)	13 (6.10)	0.591$^{\diamond}$	0.442
Alcohol intake				
Yes	11 (25.6)	44 (20.7)		
Stopped	1 (2.3)	10 (4.7)	0.903$^{\diamond}$	0.637
No	31 (72.1)	159 (74.6)		
Smoking				
Yes	14 (32.60)	47 (22.10)		
Stopped	3 (7.00)	35 (16.40)	3.814$^{\diamond}$	0.149
No	26 (60.50)	131 (61.50)		
Duration from onset to the first CT scan (hrs)				
0~1	6 (14.0)	10 (4.7)		
>1~2	9 (20.90)	20 (9.40)		
>2~4	13 (30.20)	59 (27.70)	9.952$^{\#}$	0.002*
>4~6	3 (7.00)	26 (12.20)		
>6~24	12 (27.90)	98 (46.00)		
Baseline systolic blood pressure (mmHg)	176.42 ± 31.09	167.82 ± 25.84	−1.920$^{\blacktriangle}$	0.056
Baseline GCS				
3~7	4 (9.30)	4 (1.90)		
8~13	17 (39.5)	45 (21.1)	12.995$^{\#}$	0.000*
14~15	22 (51.2)	164 (77.0)		
Baseline NIHSS	10 ± 6	5 ± 7	3.791$^{\triangle}$	0.000*
Hematoma location				
Basal ganglion	19 (44.2)	116 (54.4)		
Thalamus	4 (9.3)	37 (17.4)		
Lobar	16 (37.2)	46 (21.6)	6.667$^{\diamond}$	0.155
Cerebellar	1 (2.3)	6 (2.8)		
Brain stem	3 (7.0)	8 (3.8)		
Intraventricular hemorrhage	5 (11.6)	28 (13.1)	0.073$^{\diamond}$	0.786
Hematoma volume (mL)				
≤15	28 (65.1)	166 (77.9)		
>15~30	9 (20.9)	33 (15.5)	3.290$^{\#}$	0.070
>30	6 (14.0)	14 (6.5)		
Irregular hematoma	34 (79.1)	139 (65.3)	3.115$^{\diamond}$	0.078
PLT (10^9/L)	203.00 ± 82.00	209.00 ± 63.50	−0.768$^{\triangle}$	0.443
PT (s)	11.50 ± 1.80	12.00 ± 1.80	−0.270$^{\triangle}$	0.787
APTT (s)	30.00 ± 11.20	30.00 ± 9.25	−0.026$^{\triangle}$	0.979
FIB (g/L)	3.07 ± 0.63	3.09 ± 0.71	−0.466$^{\triangle}$	0.641
ALT (IU/L)	28.00 ± 17.00	21.00 ± 17.00	1.525$^{\triangle}$	0.127
AST (IU/L)	29.00 ± 11.00	24.00 ± 14.00	1.969$^{\triangle}$	0.049*
Urea (mmol/L)	5.00 ± 1.44	5.00 ± 1.72	−0.285$^{\triangle}$	0.775
Crea (mmol/L)	80.00 ± 28.00	80.00 ± 23.50	0.388$^{\triangle}$	0.698

Table 1: Continued.

Factor	Hematoma enlargement (%) (n = 43)	Nonhematoma enlargement (%) (n = 213)	Value	P
PBC and RBS herbal				
Not used	19 (44.2)	78 (36.6)		
PBC	2 (4.7)	26 (12.2)	0.181#	0.149
RBS	22 (51.2)	109 (51.2)		
PBC and RBS	24 (55.8)	135 (63.4)	0.870	0.390
Leech	22 (51.2)	109 (51.2)	0.00	1.00
Leonurus	24 (58.1)	124 (58.2)	0.085	0.866
Rhizoma	22 (51.2)	109 (51.2)	0.00	1.00

Note: *$P < 0.05$, ▲t-test, △Mann-Whitney U test, ●continuous correction chi-square test, ◇Pearson chi-square test, and #Kruskal-Wallis test.

Table 2: Multivariate regression analysis on the independent risk factors of hematoma enlargement in 256 patients.

Independent variable	Coefficient of regression	OR	95% CI		P value
			Lower	Upper	
Male patient	1.066	2.903	1.189	7.086	0.019*
Baseline NIHSS	0.089	1.094	0.993	1.204	0.089
Baseline GCS (14~15)					
Baseline GCS (8~13)	0.970	1.346	0.525	3.451	0.536
Baseline GCS (3~7)	1.054	2.869	0.478	17.238	0.249
Duration (>6~24 h)					
Duration (>4~6 h)	0.106	1.112	0.273	4.524	0.883
Duration (>2~4 h)	0.698	2.009	0.815	4.954	0.130
Duration (>1~2 h)	1.126	3.082	1.046	9.083	0.041*
Duration (0~1 h)	1.324	3.759	1.025	13.789	0.046*
AST	0.008	1.008	0.997	1.018	0.145
Not used					
RBS	−1.166	0.312	0.053	1.835	0.197
PBC	−0.026	0.975	0.461	2.058	0.946

Note: * means $P < 0.05$.

3.6. Comparison of Herbal Drugs Combined with PBS and RBC. HICH patients were given herbal drugs formula including several mixed herbal drugs besides PBS and RBS drugs, such as relieving heat and calming liver Yang, decreasing wind and dispersing phlegm, and loosing bowls. We analyse the effect as in Table 3.

The results showed that there was no significant difference between two groups combined with the above three types of herbal drugs (all $P > 0.05$).

3.7. Multivariate Analysis on the 3-Month Outcome Followup (mRS). Patients' sex, baseline Glasgow coma scale, baseline NIHSS, duration from onset to the first CT scan, aspartate aminotransferase (AST), PBC and RBS herbal medicine use, and hematoma growth were an independent variable in the multivariate logistic regression analysis and mRS a dependent variable. We defined mRS 0-1 as independent outcome and mRS 2–6 as dependent outcome.

The results showed that baseline NIHSS and hematoma growth were the independent risk factors of outcome of three-month followup (see Table 4).

There were two independent factors that affect the 3-month outcome. The first one was baseline NIHSS (P = 0.000). The second one was hematoma growth (P = 0.003). PBC and RBS herbal medicine use was not independent risk factor (P = 0.651).

4. Discussion

The safety of the herbal medicine administration became more and more critical since aristolochic acids were reported to cause renal injury in 1993 [12, 13].

It is long history that PBC and RBS herbal medicine were used in China to treat HICH. A multicenter, prospective clinical trial showed PBC and RBS herbal medicine can reduce the death and also improve the neurological function [14]. Meta-analysis showed that PBC and RBS herbal medicine seems

Table 3: Herbal drugs combined with PBS and RBC between two groups (n, %).

Combined herbal drugs	PBS and RBC used in hematoma enlargement (n, %) ($n = 24$)	PBS and RBC used in nonhematoma enlargement (n, %) ($n = 135$)	Value	P
Relieving heat and calming liver Yang	21 (87.5)	116 (85.9%)	0.042	0.837
Decreasing wind and dispersing phlegm	14 (58.3%)	104 (77.0%)	3.725	0.054
Loosing bowls	19 (79.2%)	101 (74.8%)	0.208	0.648

Table 4: Multivariate regression analysis on the independent risk factors of 3-month outcome in 247 patients.

Independent variable	Coefficient of regression	OR	95% CI		P value
			Lower	Upper	
Sex	0.293	1.341	0.630	2.852	0.447
Baseline NIHSS	−0.397	0.672	0.598	0.755	0.000
Baseline GCS	0.211	1.234	0.565	2.697	0.598
Duration from onset	0.022	1.023	0.776	1.347	0.874
AST	−0.006	0.994	0.980	1.009	0.439
PBC and RBS used	0.164	1.178	0.580	2.392	0.651
Hematoma growth	−1.482	0.227	0.085	0.609	0.003

effective to treat HICH [15]. PBC and RBS were also adopted by textbook and guideline in treating HICH [16].

However, safety data about hematoma enlargement were also reported. Bin and Jian declared that danshen injection and mailuoning injection (one of the PBC and RBS herbal medicine) could induce the uncontrolled bleeding [17]. Leech prevents not only fibrinogen clotting but also other thrombin-catalyzed hemostatic reactions such as the activation of clotting factors V, VIII, and XIII and the thrombin-induced platelet activation [18]. Other researchers advocated that PBC and RBC herbal medicine should be used with few side effects in the clinical application because they added to some other stopping bleeding herbal medicine to make the prescription balance [19]. So it is necessary that we perform this study on the safety of treating HICH with PBC and RBS herbal medicine.

In this study, forty-three (16.8%) of the 256 patients demonstrated enlargement of the hematoma after the first CT scan. The growth rate was near the rate 14.3% (60/419) of Fujii et al.'s report [20]. In this study, 159 patients were administrated PBC and RBS herbal medicine prescription within 24-hour time window, including 24 patients in hematoma enlargement group and 135 patients in no hematoma group, which were not significantly different ($P > 0.05$).

Hematoma enlargement in HICH has significant associations with the duration of time since onset of neurological symptoms, the shape and volume of the bleeding, the initials deep coma degree, the presence of liver dysfunction, and male patients [20, 21]. In this study, the univariate analysis showed that duration, baseline GCS and NIHSS, the liver dysfunction (AST), and male patients were the risk factor of hematoma expansion, while the shape and size of the hematoma were not significant because the sample was too small.

There were only two risk factors that induced hematoma growth according to the multivariate analysis, male patients and duration of onset since ICH symptoms. Hematoma enlargement was the independent risk factor of outcome of three-month followup; the result was similar as Seiji Kazui's study [22]. PBC and RBS herbal drug was not the independent risk factor of hematoma growth of the outcome of three-month followup.

Some herbal drugs have strong PBC and RBS function; for example, *leech* caused rebleeding [21]. Some others have two-way adjustment pharmacological effect; for example, *Radix notoginseng* can not only PBC but also stopping bleeding. In our opinion, the prescription of Chinese medicine to learn is through reasonable compatibility other than a single drug, to eliminate this rebleeding risk.

PBC and RBS herbal medicine was administrated within the time window that was uncertain. A majority (83%) of patients with hematoma enlargement underwent the initial CT scan within 6 hours of onset; Enlargement after 24 hours of onset seems extremely rare [22]. So some neurologists showed their opinion that PBC and RBS herbal medicine should be used after 24 hrs of onset in order to prevent rebleeding risk [23]. Others supported that PBC and RBS herbal medicine should be administrated as sooner as possible [24]. Guo and his colleagues' study did not show deterioration of condition of the AICH patients who were treated with herbal compound within 6 hrs time window from onset [25].

PBC and RBS herbal medicine should be combined with other drugs correctly in the Traditional Chinese Medicine Formula in order to avoid the rebleeding risk, as recorded in the Chinese Pharmacopoeia of 2010 version [26]. In this study, the treatment of acute cerebral hemorrhage frequently

used herbal medicine by turns as follows: *Leonurus japonicus* Houtt. (148 cases), *Rhizoma Polygoni Cuspidati* (131 cases), leech (131 cases), *Radix Achyranthis Bidentatae* (83 cases), *Ligusticum chuanxiong* Hort. (21 cases), *Radix Salviae Miltiorrhizae* (18 cases), *Radix et Rhizoma Rhei Palmati* (6 cases), *Cortex moutan* (15 cases), peach seed (13 cases), *Radix Curcumae Wenyujin* (12 cases), *Radix notoginseng* (6 cases), *Carthamus tinctorius* L. (5 cases), *Caulis Spatholobi* (4 cases), and *Rhizoma corydalis* (2 cases). They were used in balance between two groups ($P > 0.05$).

In this retrospective study, 159 patients (24 in hematoma growth group and 135 in no hematoma growth group) were given PBC and RBS herbal medicine prescription, which was also combined with other herbal drugs (showed as Table 4). PBC and RBS drugs reasonable compatibility with other herbs perhaps reduced the risk of hematoma expansion caused by single PBC and RBS herb. This result undoubtedly is instructive for further clinical application.

This is the first retrospective study of the hematoma growth on the early HICH treated with traditional Chinese medicine since now. Though the results showed it seems safe, the retrospective study has many limitations. Firstly, there are too many herbals medication to control the quality of the clinical study. Secondly, a lot of patients were excluded because of important data absent, for example, their second CT scan data. Thirdly, some scales, including NIHSS and GCS, had recall bias from raw medical records.

In order to make up for these limitations, we have designed a prospective, 13 hospitals, randomized, placebo control clinical trial (clinicaltrials.gov: NCT01918722) to confirm if PBC and RBS herbal medicine induces the incidence of hematoma enlargement of AICH patient within the 6 hrs time window from onset. 62 cases have been recruited since February 2014 and all 300 patients will be completed in December 2015.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

The authors thank Professor Zehuai Wen because he directed the statistics of the data. He is the director of Key Unit of Methodology in Clinical Research of Guangdong Province Hospital of Traditional Chinese Medicine. The authors also thank China National Clinical Study Base Project of Traditional Chinese Medicine, sponsored by State Administration of Traditional Chinese Medicine of the People's Republic of China (Grant no. JDZX2012074).

References

[1] A. I. Qureshi, A. D. Mendelow, and D. F. Hanley, "Intracerebral haemorrhage," *The Lancet*, vol. 373, no. 9675, pp. 1632–1644, 2009.

[2] C. J. van Asch, M. J. Luitse, G. J. Rinkel, I. van der Tweel, A. Algra, and C. J. Klijn, "Incidence, case fatality, and functional outcome of intracerebral haemorrhage over time, according to age, sex, and ethnic origin: a systematic review and meta-analysis," *The Lancet Neurology*, vol. 9, no. 2, pp. 167–176, 2010.

[3] J. K. Lim, H. S. Hwang, B. M. Cho et al., "Multivariate analysis of risk factors of hematoma expansion in spontaneous intracerebral hemorrhage," *Surgical Neurology*, vol. 69, no. 1, pp. 40–45, 2008.

[4] S. M. Davis, J. Broderick, M. Hennerici et al., "Hematoma growth is a determinant of mortality and poor outcome after intracerebral hemorrhage," *Neurology*, vol. 66, no. 8, pp. 1175–1181, 2006.

[5] G.-Q. Wang, S.-Q. Li, Y.-H. Huang et al., "Can minimally invasive puncture and drainage for hypertensive spontaneous Basal Ganglia intracerebral hemorrhage improve patient outcome: a prospective non-randomized comparative study," *Military Medical Research*, vol. 1, article 10, 2014.

[6] J. C. Hemphill III, D. C. Bonovich, L. Besmertis, G. T. Manley, and S. C. Johnston, "The ICH score: a simple, reliable grading scale for intracerebral hemorrhage," *Stroke*, vol. 32, no. 4, pp. 891–896, 2001.

[7] G. Jian-wen and L. Ming-jie, "Meta-analysis of acute intracerebral hemorrhage treated with traditional Chinese medicine or/and composition of promoting blood circulation and removing blood stasis," *Journal of China-Japan Friendship Hospital*, vol. 15, no. 5, pp. 283–286, 2001.

[8] ICD-10 Version, 2010, http://apps.who.int/classifications/icd10/browse/2010/en.

[9] R. U. Kothari, T. Brott, J. P. Broderick et al., "The ABCs of measuring intracerebral hemorrhage volumes," *Stroke*, vol. 27, no. 8, pp. 1304–1305, 1996.

[10] J. M. Gebel, C. A. Sila, M. A. Sloan et al., "Comparison of the ABC/2 estimation technique to computer-assisted volumetric analysis of intraparenchymal and subdural hematomas complicating the GUSTO-1 trial," *Stroke*, vol. 29, no. 9, pp. 1799–1801, 1998.

[11] T. Brott, J. Broderick, R. Kothari et al., "Early hemorrhage growth in patients with intracerebral hemorrhage," *Stroke*, vol. 28, no. 1, pp. 1–5, 1997.

[12] J.-L. Vanherweghem, M. Depierreux, C. Tielemans et al., "Rapidly progressive interstitial renal fibrosis in young women: association with slimming regimen including Chinese herbs," *The Lancet*, vol. 341, no. 8842, pp. 387–391, 1993.

[13] M. Jadoul, J. F. de Plaen, J. P. Cosyns, and C. van Ypersele de Strihou, "Adverse effects from traditional Chinese medicine," *The Lancet*, vol. 341, no. 8849, pp. 892–893, 1993.

[14] P.-X. Huang, Y. Huang, and M. Lu, "Treatment of acute intracerebral hemorrhage with a comprehensive protocol of integrated Chinese and Western medicine," *Zhongguo Zhong Xi Yi Jie He Za Zhi*, vol. 26, no. 7, pp. 590–593, 2006.

[15] J. Guo and M. Liu, "Meta-analysis of acute intracerebral hemorrhage treated with traditional Chinese medicine or/and composition of promoting blood circulation and removing blood stasis," *Journal of China-Japan Friendship Hospital*, vol. 15, no. 5, pp. 283–286, 2001.

[16] W. Mian-hua and W. Xin-yue, *Traditional Chinese Internal Medicine*, China Press of Traditional Chinese Medicine, 3rd edition, 2000.

[17] L. Bin and L. Jian, "Clinical observation of early use promoting blood circulation and removing blood stasis herbal injection to treat acute intracerebral hemorrhage," *Shandong Journal of Traditional Chinese Medicine*, vol. 19, no. 8, pp. 461–462, 2000.

[18] F. Markwardt, "The development of hirudin as an antithrombotic drug," *Thrombosis Research*, vol. 74, no. 1, pp. 1–23, 1994, Review.

[19] J. W. Guo, Y. F. Cai, and B. X. Du, "Thinking and challenge induced by the hypothesis of breaking stagnant and eliminating blood stasis in Treating acute cerebral hemorrhage by rF VII a," *Zhongguo Zhong Xi Yi Jie He Za Zhi*, vol. 25, no. 11, pp. 1027–1029, 2005 (Chinese).

[20] Y. Fujii, R. Tanaka, S. Takeuchi, T. Koike, T. Minakawa, and O. Sasaki, "Hematoma enlargement in spontaneous intracerebral hemorrhage," *Journal of Neurosurgery*, vol. 80, no. 1, pp. 51–57, 1994.

[21] L. Liu, J.-A. Duan, Y. Tang et al., "Taoren-Honghua herb pair and its main components promoting blood circulation through influencing on hemorheology, plasma coagulation and platelet aggregation," *Journal of Ethnopharmacology*, vol. 139, no. 2, pp. 381–387, 2012.

[22] S. Kazui, H. Naritomi, H. Yamamoto, T. Sawada, and T. Yamaguchi, "Enlargement of spontaneous intracerebral hemorrhage: incidence and time course," *Stroke*, vol. 27, no. 10, pp. 1783–1787, 1996.

[23] Y. Wanzhng, Z. Zhilan, Z. Ming et al., "Once again on bleeding after cerebral hemorrhage and the time window for therapy of "promoting blood circulation and removing blood stasis"," *Chinese Journal of Integrative Medicine on Cardio-/Cerebrovascular Disease*, vol. 11, pp. 662–665, 2004.

[24] W. Li, "Discussion of early use promoting blood circulation and removing blood stasis herbal medicine," *Chinese Journal of Basic Medicine in Traditional Chinese Medicine*, vol. 5, pp. 58–59, 2003.

[25] J.-W. Guo, Y.-C. He, and S.-H. Chen, "Clinical study on the therapeutic time window of acute intracerebral hemorrhage treated with herbal composition of Zhongfengxingnao taken orally," *Chinese Journal of Cerebrovascular Diseases*, vol. 2, no. 6, pp. 255–259, 2005.

[26] Chinese Pharmacopoeia Commission, *The Pharmacopoeia of the People's Republic of China*, vol. 1, Chemical Industry Press, Beijing, China, 2010.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2014, Article ID 691574, 8 pages
http://dx.doi.org/10.1155/2014/691574

Research Article

Acute and Long-Term Toxicity of Mango Leaves Extract in Mice and Rats

Yi Zhang,[1] Jian Li,[2] Zhizhen Wu,[1] Erwei Liu,[3] Pingping Shi,[1] Lifeng Han,[1] Lingling Guo,[2] Xiumei Gao,[3] and Tao Wang[1]

[1] *Tianjin State Key Laboratory of Modern Chinese Medicine, 312 Anshanxi Road, Nankai District, Tianjin 300193, China*
[2] *Institute of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine, 312 Anshanxi Road, Nankai District, Tianjin 300193, China*
[3] *Key Laboratory of Pharmacology of Traditional Chinese Medical Formulae, Tianjin University of Traditional Chinese Medicine, Ministry of Education, 312 Anshanxi Road, Nankai District, Tianjin 300193, China*

Correspondence should be addressed to Tao Wang; wangt@263.net

Received 14 June 2014; Accepted 15 August 2014; Published 27 August 2014

Academic Editor: Junhua Zhang

The acute toxicity of mango leaves extract (MLE) at the maximal dose (18.4 g/kg) was studied in ICR mice and no abnormalities were detected during the experiment. The long-term studies at various doses of MLE (100 mg/kg, 300 mg/kg, and 900 mg/kg) in SD rats for 3 consecutive months revealed that, compared with the control group, rats in MLE treated groups showed slight body weight increase and higher fat weight; the serum TG and CHOL levels and the epididymis weight of male rats were a little higher; the serum K^+ level of female rats was on the low side but the weights of liver, kidney, and adrenal gland were on the high side. In addition to this, no other obvious abnormalities were detected.

1. Introduction

Mango tree (*Mangifera indica* L.), a tropical plant belonging to Anacardiaceae, has been distributed worldwide as the most cultivated fruits in the tropics. Mango leaves were used for diabetes and asthma treatment in traditional Chinese medicine (TCM). Mango leaves contain phenolic constituents such as caffeic acid [1], polyphenols such as mangiferin and gallic acid [2], flavonoids [3], volatile compounds [4], and so forth. Pharmacology studies showed that the extract of mango leaves possesses many effects like antioxidant, antimicrobial, antihelminthic, antidiabetic, antiallergic, and so forth [5]. In previous study, we reported benzophenone C-glycosides with triglyceride accumulation inhibitory effects in adipocyte [6, 7]. We also reported that ethanol extract of mango leaves dose-dependently decreased serum glucose and triglyceride in KK-A^y mice, and mechanism on glucose and lipid homeostasis is mediated, at least in part, through PI3K/AKT and AMPK signaling pathway [8]. Although mango tree leaves were used for a long period in TCM clinic, there are few reports on the safety evaluations. In this study, we carried out the acute toxicity and long-term toxicity of mango leaves extract (MLE), aiming at providing reference basis for other safety evaluation studies and selecting clinical dosage.

2. Materials and Methods

2.1. Materials

2.1.1. Plant Material. In the present study, mango leaves were collected from Zhejiang Province, China, and identified by Dr. Tianxiang Li at Tianjin University of TCM as *Mangifera indica* L. Voucher specimen was deposited at the Academy of Traditional Chinese Medicine of Tianjin University of TCM

2.1.2. Animals. Forty ICR mice, half male and half female, weighted 18–21 g, and 48 SD rats, composed of male and female in half, weighted 117–160 g, were used in the study and feed in room of SPF grade laboratory. These animals were all

Table 1: The effect on body weight of MLE in ICR mice ($\overline{X} \pm$ SD, g).

Sex	Groups	Preceding the first dose	Preceding the second dose	After administration			
				Day 1	Day 3	Day 7	Day 14
♂	Control	20.84 ± 0.42	20.06 ± 0.40	21.72 ± 0.78	24.70 ± 0.81	28.06 ± 0.92	30.91 ± 1.02
	Drug	20.83 ± 0.71	20.31 ± 0.69	20.98 ± 1.28	24.13 ± 1.37	27.21 ± 1.52	29.94 ± 1.27
♀	Control	19.27 ± 0.64	18.59 ± 0.59	19.42 ± 1.27	21.04 ± 1.36	22.99 ± 0.88	25.33 ± 1.05
	Drug	19.19 ± 0.73	18.81 ± 0.84	18.99 ± 0.97	20.99 ± 0.86	23.42 ± 1.31	24.42 ± 1.28

Ten mice in each group; no difference was considered to be significant between the two groups.

Table 2: The effect on body weight of MLE in SD rats ($\overline{X} \pm$ SD, g).

Sex	Time	n	Control group	n	Low dose group	n	Medium dose group	n	High dose group
♂	0 d	18	149.1 ± 8.2	18	148.2 ± 8.6	18	147.6 ± 8.3	18	147.9 ± 7.9
	3 d	18	174.8 ± 10.9	18	172.3 ± 10.1	18	172.2 ± 10.1	18	169.6 ± 11.8
	7 d	18	206.4 ± 14.1	18	200.0 ± 14.5	18	201.0 ± 13.6	18	202.9 ± 15.9
	10 d	18	231.2 ± 17.0	18	227.7 ± 16.2	18	226.1 ± 15.3	18	227.5 ± 18.6
	14 d	18	261.4 ± 20.2	18	260.6 ± 19.9	18	257.8 ± 18.7	18	259.8 ± 22.1
	17 d	18	285.9 ± 22.8	18	284.4 ± 23.3	18	283.8 ± 21.2	18	285.3 ± 22.0
	21 d	18	311.3 ± 24.1	18	312.2 ± 28.4	18	313.2 ± 23.6	18	316.0 ± 23.9
	24 d	18	325.9 ± 26.6	18	331.4 ± 30.7	18	332.2 ± 25.0	18	336.4 ± 24.9
	28 d	18	347.6 ± 31.4	18	353.6 ± 35.7	18	350.0 ± 25.9	18	359.4 ± 27.5
	31 d	18	363.4 ± 34.9	18	370.1 ± 38.1	18	368.6 ± 28.1	18	377.1 ± 29.5
	35 d	18	379.2 ± 38.2	18	388.1 ± 40.6	18	387.1 ± 31.6	18	396.2 ± 30.3
	42 d	18	409.9 ± 44.1	18	418.0 ± 45.7	18	416.6 ± 34.7	18	425.0 ± 34.1
	49 d	18	435.1 ± 46.5	18	443.2 ± 47.5	18	439.4 ± 35.4	18	448.4 ± 35.2
	56 d	18	455.7 ± 51.1	18	465.7 ± 49.8	18	461.1 ± 38.3	18	471.5 ± 37.4
	63 d	18	482.6 ± 51.2	18	493.8 ± 51.8	18	487.8 ± 40.8	18	498.1 ± 39.6
	70 d	18	500.2 ± 51.8	18	508.7 ± 54.0	18	497.3 ± 44.6	18	516.4 ± 43.2
	77 d	18	516.8 ± 53.5	18	525.1 ± 57.8	18	517.6 ± 45.6	18	531.8 ± 44.9
	84 d	18	535.9 ± 56.1	18	538.2 ± 60.7	18	532.8 ± 47.5	18	546.9 ± 47.4
♀	0 d	18	132.4 ± 6.7	18	134.1 ± 7.3	18	133.0 ± 7.2	18	131.2 ± 7.4
	3 d	18	146.1 ± 7.7	18	149.4 ± 7.9	18	148.2 ± 9.0	18	147.8 ± 9.9
	7 d	18	159.3 ± 8.2	18	163.4 ± 8.7	18	165.1 ± 12.5	18	163.9 ± 13.1
	10 d	18	168.2 ± 9.3	18	176.4 ± 9.0*	18	176.3 ± 12.6*	18	174.8 ± 14.6
	14 d	18	181.5 ± 11.3	18	186.3 ± 9.6	18	186.9 ± 15.2	18	188.1 ± 15.3
	17 d	18	194.3 ± 11.4	18	197.0 ± 11.9	18	199.2 ± 17.6	18	202.6 ± 18.7
	21 d	18	204.3 ± 13.4	18	207.8 ± 12.0	18	211.1 ± 20.9	18	216.8 ± 21.3*
	24 d	18	212.6 ± 16.3	18	215.1 ± 13.0	18	222.1 ± 19.8	18	224.0 ± 24.7
	28 d	18	221.4 ± 18.4	18	223.1 ± 14.8	18	232.0 ± 21.9	18	233.8 ± 25.1
	31 d	18	229.7 ± 16.9	18	232.3 ± 13.0	18	239.9 ± 23.7	18	243.2 ± 24.7
	35 d	18	237.2 ± 17.5	18	241.8 ± 14.3	18	248.7 ± 25.3	18	250.4 ± 23.6
	42 d	18	250.4 ± 22.2	18	255.6 ± 15.0	18	262.2 ± 27.5	18	265.7 ± 24.5
	49 d	18	258.9 ± 18.2	18	263.6 ± 17.3	18	272.9 ± 29.3	18	274.6 ± 24.1*
	56 d	18	265.7 ± 18.3	18	267.8 ± 17.5	18	280.1 ± 26.5	18	276.8 ± 26.8
	63 d	18	279.2 ± 20.2	18	284.2 ± 16.8	18	292.8 ± 29.6	18	290.1 ± 26.8
	70 d	18	284.6 ± 21.1	18	290.4 ± 14.5	18	296.5 ± 30.3	18	295.1 ± 29.1
	77 d	18	290.0 ± 19.8	18	294.4 ± 14.4	18	302.6 ± 33.4	18	303.3 ± 26.8
	84 d	18	295.4 ± 19.1	18	300.6 ± 14.3	18	306.1 ± 29.6	18	308.4 ± 28.8

*Means $P < 0.05$; n means the number of animals.

Table 3: The effect on food consumption of MLE in SD rats ($\overline{X} \pm$ SD, g).

Sex	Time	Cage number	n	Control group	n	Low dose group	n	Medium dose group	n	High dose group
	0 d	3	18	22.7 ± 0.9	18	23.1 ± 0.3	18	23.4 ± 0.8	18	22.4 ± 2.5
	7 d	3	18	25.5 ± 2.4	18	26.2 ± 1.1	18	26.5 ± 0.7	18	25.6 ± 0.8
	14 d	3	18	28.1 ± 1.4	18	28.4 ± 0.2	18	27.9 ± 1.3	18	27.9 ± 1.5
	21 d	3	18	28.4 ± 2.2	18	31.2 ± 1.5	18	28.8 ± 2.9	18	30.6 ± 1.0
	28 d	3	18	28.8 ± 2.9	18	30.6 ± 1.2	18	30.7 ± 0.4	18	31.9 ± 0.2
	35 d	3	18	29.7 ± 2.3	18	30.9 ± 2.1	18	31.6 ± 0.4	18	30.7 ± 1.2
♂	42 d	3	18	30.1 ± 2.3	18	29.6 ± 1.3	18	30.4 ± 0.7	18	29.6 ± 1.4
	49 d	5	18	32.4 ± 3.7	18	31.5 ± 1.1	18	30.6 ± 1.5	18	30.6 ± 3.0
	56 d	5	18	32.5 ± 2.0	18	33.4 ± 1.1	18	30.7 ± 0.7	18	31.3 ± 2.2
	63 d	5	18	32.7 ± 1.8	18	33.2 ± 2.3	18	32.6 ± 1.3	18	34.6 ± 2.2
	70 d	5	18	33.7 ± 3.4	18	33.6 ± 2.1	18	33.7 ± 1.8	18	33.1 ± 2.4
	77 d	5	18	32.3 ± 2.6	18	32.6 ± 4.0	18	32.4 ± 2.3	18	33.7 ± 2.4
	84 d	5	18	30.7 ± 3.1	18	31.1 ± 1.2	18	29.8 ± 1.2	18	30.7 ± 2.1
	0 d	3	18	17.6 ± 0.8	18	18.1 ± 0.5	18	18.8 ± 1.5	18	18.6 ± 0.9
	7 d	3	18	18.9 ± 1.2	18	17.3 ± 0.6	18	19.7 ± 1.5	18	17.9 ± 0.8
	14 d	3	18	20.0 ± 1.8	18	18.9 ± 0.5	18	21.4 ± 1.4	18	20.8 ± 1.1
	21 d	3	18	21.9 ± 2.4	18	22.1 ± 1.1	18	22.4 ± 3.2	18	21.4 ± 2.3
	28 d	3	18	19.4 ± 3.6	18	22.1 ± 1.2	18	22.5 ± 1.5	18	22.7 ± 1.2
	35 d	3	18	21.3 ± 0.4	18	21.1 ± 1.2	18	22.6 ± 1.7	18	23.1 ± 2.0
♀	42 d	3	18	22.2 ± 1.2	18	22.8 ± 1.9	18	23.4 ± 1.7	18	24.3 ± 1.0
	49 d	3	18	22.5 ± 2.4	18	24.6 ± 1.4	18	23.2 ± 3.0	18	22.1 ± 0.8
	56 d	3	18	21.7 ± 1.0	18	22.1 ± 1.1	18	22.8 ± 2.1	18	21.6 ± 0.4
	63 d	3	18	22.0 ± 1.3	18	22.3 ± 1.0	18	22.2 ± 2.2	18	20.8 ± 1.3
	70 d	3	18	23.9 ± 1.8	18	23.6 ± 0.9	18	23.8 ± 2.5	18	22.7 ± 1.3
	77 d	3	18	21.8 ± 2.0	18	23.8 ± 1.4	18	22.4 ± 2.8	18	21.1 ± 1.4
	84 d	3	18	20.3 ± 1.1	18	21.9 ± 0.6	18	21.2 ± 1.3	18	21.5 ± 1.9

n means the number of animals.

provided by Vital River Laboratory Animal Technology Co., Ltd.

2.1.3. Instruments. PL203 electronic balance and ML203 electronic balance were purchased from Changzhou Mettler Toledo Instrument Co., Ltd. ADVIA2120 hematology analyzer was made by Germany Siemens Electrical Apparatus Ltd. ACL9000 coagulometer was made by American Beckman Coulter Inc. 7080 automatic biochemical analyzer was made by Japanese Hitachi Ltd.

2.2. Methods

2.2.1. Preparation of MLE. Dried mango leaves, collected from Hainan province of China, was extracted by 70% ethanol (1 g in 10 mL) under reflux for 2 h, and the residue was extracted under the same condition. The 70% ethanol solutions were combined together and further subjected to a D101 macroporous absorption resin column eluted with water, 15% ethanol. 15% ethanol fraction was concentrated and reextracted by 50% ethanol (50 degrees for 2 h). The 50% ethanol extract was dried under vacuum to obtain mango leaves extract, which contains 62% mangiferin (HPLC method). The final extract based on the above process was effective and has the functions of mango leaves.

2.2.2. Acute Toxicity of MLE in ICR Mice. Forty ICR mice of mixed sexes were randomly divided into two groups: MLE treated group and control group. Mice in MLE treated group were given MLE at the maximal dose of 18.4 g/kg by intragastric administration, in a volume of 0.1 mL per 10 g, twice a day with a 4 h interval, while the control group received an equal volume of deionized water. After oral administration, the various responses of mice including toxic reactions and mortality were observed and recorded every day for successive 14 days. At the end of the experiment, animals were executed for gross anatomy check. Evaluating and recording whether there were any obvious changes in major organs by macroscopic observation.

2.2.3. Long-Term Toxicity of MLE in SD Rats

(1) Group Setup and Administration. After seven days of stabilization, 48 SD rats of mixed sexes that have moderate body weight and body weight gain speed were selected for the study. Depending on the weight, they were randomly divided into four groups, including one blank group for control and three administration groups of different doses (100 mg/kg, 300 mg/kg, and 900 mg/kg) which are equivalent to 17.2, 51.7, and 155.2 times of clinical daily dose (5.8 mg/kg), respectively. Rats in MLE treated group were given MLE by

Table 4: The effects on hematology and coagulation function of MLE in SD rats ($\overline{X} \pm$ SD, g).

Sex	Parameters	Control group ($n = 6$)	Low dose group ($n = 6$)	Medium dose group ($n = 6$)	High dose group ($n = 6$)
♂	WBC ($\times 10^9$/L)	10.08 ± 1.29	12.47 ± 3.03	11.66 ± 2.32	11.58 ± 3.36
	Neut (%)	12.06 ± 1.38	15.48 ± 4.58	12.33 ± 2.89	12.30 ± 5.21
	Lymph (%)	83.32 ± 1.64	79.10 ± 4.80	81.78 ± 3.14	81.85 ± 5.50
	Mono (%)	2.58 ± 0.64	3.02 ± 0.66	2.95 ± 0.71	3.00 ± 0.87
	Eos (%)	1.24 ± 0.33	1.10 ± 0.18	1.33 ± 0.56	1.65 ± 0.32
	RBC ($\times 10^{12}$/L)	9.24 ± 0.36	9.52 ± 0.59	9.57 ± 0.36	9.79 ± 0.34*
	HGB (g/L)	167.40 ± 6.80	166.17 ± 9.33	162.33 ± 4.27	167.50 ± 3.15
	HCT (%)	48.32 ± 1.57	48.40 ± 3.48	48.00 ± 1.08	49.87 ± 1.64
	MCV (fL)	52.28 ± 0.55	50.82 ± 0.77**	50.20 ± 1.44*	50.98 ± 2.29
	MCH (pg)	18.16 ± 1.10	17.50 ± 0.67	16.98 ± 0.57*	17.13 ± 0.72
	MCHC (g/L)	347.20 ± 20.66	344.50 ± 13.40	338.33 ± 3.33	336.67 ± 5.99
	PLT ($\times 10^9$/L)	1145.00 ± 121.6	1208.00 ± 158.73	1216.17 ± 98.95	1217.00 ± 104.08
	Retic (‰)	2.96 ± 0.42	2.80 ± 0.43	2.96 ± 0.36	2.56 ± 0.40
	PT (s)	16.04 ± 1.13	16.48 ± 2.71	15.68 ± 0.93	16.15 ± 1.22
♀	WBC ($\times 10^9$/L)	5.10 ± 1.44	5.97 ± 0.89	5.77 ± 1.01	5.45 ± 1.41
	Neut (%)	15.92 ± 2.58	15.62 ± 3.79	17.77 ± 2.32	16.38 ± 5.62
	Lymph (%)	78.27 ± 2.64	78.38 ± 4.06	76.48 ± 2.70	78.08 ± 7.14
	Mono (%)	2.57 ± 0.46	3.02 ± 0.58	2.95 ± 1.11	2.73 ± 1.18
	Eos (%)	2.00 ± 0.40	1.60 ± 0.67	1.73 ± 0.29	1.97 ± 0.58
	RBC ($\times 10^{12}$/L)	8.63 ± 0.23	8.81 ± 0.56	8.55 ± 0.26	8.64 ± 0.48
	HGB (g/L)	159.17 ± 2.93	158.50 ± 7.34	157.50 ± 2.88	159.17 ± 6.55
	HCT (%)	46.53 ± 1.29	46.52 ± 2.61	46.28 ± 1.16	46.45 ± 2.14
	MCV (fL)	53.92 ± 1.66	52.83 ± 1.26	54.18 ± 1.70	53.78 ± 0.95
	MCH (pg)	18.43 ± 0.46	18.05 ± 0.63	18.43 ± 0.37	18.47 ± 0.32
	MCHC (g/L)	342.17 ± 5.78	341.33 ± 5.79	340.50 ± 6.06	343.50 ± 2.88
	PLT ($\times 10^9$/L)	1134.67 ± 63.64	1268.33 ± 182.79	1211.17 ± 121.14	1197.83 ± 73.19
	Retic (‰)	2.86 ± 0.71	2.45 ± 0.63	2.79 ± 0.37	2.57 ± 0.57
	PT (s)	16.52 ± 0.82	16.00 ± 1.20	16.27 ± 0.63	16.57 ± 1.29

*Means $P < 0.05$; **means $P < 0.01$; n means the number of animals.

intragastric administration at the corresponding dose, in a volume of 0.1 mL per 10 g, for 3 consecutive months (6 times a week), while the control group received an equal volume of deionized water. During the study, all rats were allowed access to food and water ad libitum.

(2) Observational Indices. After oral administration, observe the general symptom, such as appearance, behavior, glandular secretion, breathing, and so on. The body weight and food consumption of each animal were recorded weekly and the differences among groups were compared.

After 90 days of treatment, all the 48 SD rats were sacrificed, and blood samples were collected from the abdominal aorta for hematology and coagulation tests. The white blood corpuscles (WBC) count, red blood corpuscles (RBC) count, hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), and prothrombin time (PT) were carried out.

Serum was separated by spinning the blood and was used for biochemical studies. ALT, AST, ALP, BUN, CREA, TP, ALB, GLU, CHOL, Na^+, K^+, and Cl^- contents in serum and many other blood biochemical parameters were determined using the automatic biochemistry analyzer.

Organs (liver, heart, spleen, lung, kidney, brain, thymus, etc.) were collected from each sacrificed rat and weighed. The relative organ weights (organ/body weight ratio and organ/brain weight ratio) were calculated and compared with the value of the control.

2.2.4. Statistical Analysis. The intragroup difference of measurement data was detected with the t-test. The data obtained were subjected to SPSS NPar Tests Mann-Whitney Test. Values were expressed as the mean ± standard error and were considered statistically significant at $P < 0.05$.

3. Results and Discussion

3.1. Acute Toxicity

3.1.1. General Observation. During the course of the study, all the mice were healthy without any abnormal responses, and no distinct lesions were revealed anatomically.

3.1.2. Body Weight. After the second oral administration of MLE, the body weight of the mice in both of the two groups decreased slightly than before the first dose, and the control

Table 5: The effects on blood biochemical and electrolyte indicators of MLE in SD rats ($\overline{X} \pm$ SD, g).

Sex	Parameters	Control group ($n = 6$)	Low-dose group ($n = 6$)	Medium-dose group ($n = 6$)	High-dose group ($n = 6$)
♂	ALT (U/L)	33.85 ± 5.16	40.90 ± 12.38	34.50 ± 4.71	34.82 ± 3.32
	AST (U/L)	143.57 ± 32.64	164.42 ± 49.16	144.92 ± 15.15	152.03 ± 48.87
	ALP (U/L)	83.73 ± 11.32	91.22 ± 30.39	83.65 ± 18.66	82.45 ± 17.09
	BUN (mmol/L)	6.22 ± 0.64	6.35 ± 0.73	6.72 ± 0.60	6.12 ± 0.57
	CREA (μmol/L)	73.67 ± 7.55	70.35 ± 3.17	76.50 ± 3.31	72.05 ± 5.29
	TP (g/L)	57.53 ± 4.49	59.52 ± 1.09	59.22 ± 1.26	59.87 ± 3.34
	ALB (g/L)	22.90 ± 1.28	23.43 ± 0.74	23.67 ± 0.74	24.25 ± 0.90
	GLU (mmol/L)	10.14 ± 1.07	8.93 ± 1.13	10.35 ± 1.88	9.25 ± 0.93
	CHOl (mmol/L)	1.36 ± 0.26	1.78 ± 0.38	1.50 ± 0.39	1.74 ± 0.23*
	TBIL (μmol/L)	2.34 ± 0.16	2.44 ± 0.28	2.39 ± 0.24	2.35 ± 0.15
	TG (mmol/L)	0.41 ± 0.16	0.48 ± 0.17	0.56 ± 0.28	0.64 ± 0.18*
	CK (U/L)	775.18 ± 354.51	812.22 ± 295.98	817.42 ± 143.23	795.83 ± 221.83
	Na^+ (mmol/L)	139.62 ± 2.32	140.77 ± 2.27	139.65 ± 1.74	139.88 ± 0.37
	K^+ (mmol/L)	4.52 ± 0.45	4.73 ± 0.19	4.58 ± 0.25	4.65 ± 0.30
	Cl^- (mmol/L)	104.00 ± 1.37	104.12 ± 1.40	102.90 ± 1.88	103.30 ± 1.11
♀	ALT (U/L)	26.35 ± 3.31	29.80 ± 4.60	25.98 ± 3.80	24.93 ± 4.54
	AST (U/L)	137.12 ± 18.95	128.62 ± 21.23	129.70 ± 23.13	114.12 ± 14.57*
	ALP (U/L)	42.28 ± 9.34	48.72 ± 13.80	43.47 ± 6.99	47.52 ± 10.20
	BUN (mmol/L)	7.67 ± 0.64	7.84 ± 0.74	7.56 ± 0.99	6.87 ± 1.31
	CREA (μmol/L)	74.63 ± 4.22	76.52 ± 4.24	79.57 ± 4.44	73.15 ± 4.37
	TP (g/L)	65.97 ± 3.99	67.60 ± 3.47	67.98 ± 6.41	65.13 ± 3.23
	ALB (g/L)	29.37 ± 2.71	30.25 ± 1.94	30.57 ± 2.82	28.45 ± 2.26
	GLU (mmol/L)	7.96 ± 1.08	8.92 ± 1.00	8.93 ± 1.41	8.76 ± 1.00
	CHOl (mmol/L)	1.72 ± 0.19	1.79 ± 0.50	1.79 ± 0.43	1.63 ± 0.20
	TBIL (μmol/L)	2.69 ± 0.20	2.68 ± 0.15	2.76 ± 0.43	2.42 ± 0.21*
	TG (mmol/L)	0.32 ± 0.06	0.34 ± 0.06	0.30 ± 0.06	0.31 ± 0.07
	CK (U/L)	607.28 ± 213.08	568.75 ± 167.69	670.88 ± 153.16	612.25 ± 136.05
	Na^+ (mmol/L)	139.87 ± 1.30	140.55 ± 1.07	140.87 ± 1.24	139.68 ± 0.92
	K^+ (mmol/L)	4.35 ± 0.31	4.18 ± 0.34	3.98 ± 0.23*	4.04 ± 0.32
	Cl^- (mmol/L)	104.92 ± 2.49	104.95 ± 0.36	104.82 ± 1.21	103.82 ± 0.60

*Means $P < 0.05$; n means the number of animals.

group decreased more, but there was no significant difference ($P > 0.05$). It is speculated that the body weight loss may result from the fasting between the two doses and the high drug concentration (the maximum dispensing concentration) that induced satiety may affect the short-time body weight change.

Throughout the recovery period, the animal weight, both of the two groups, showed a general increase, but in several mice it decreased a day after the treatment (one in control group and six in MLE treated group); and after fourteen-day treatment, five mice in MLE treated group showed a slight weight loss (less than 1 g). Speculated by the whole growth trend, we guess that the body weight change after one-day treatment, which was recovered three days later, may be associated with the drug's effect and the change after fourteen days was likely to be coursed by physiological fluctuations, but it produced no significant difference ($P > 0.05$) compared with control group (Table 1). As a result, the effect of MLE on mice body weight was not obvious.

3.1.3. Gross Anatomy and Histopathological Examination. After a fourteen-day recovery, all the mice were executed for gross anatomy check. Because there were no gross lesions on major organs, no histopathological examination was conducted.

3.2. Long-Term Toxicity

3.2.1. General Observation. After three consecutive months of oral administration, all the animals showed no marked abnormalities during the study.

3.2.2. Body Weight and Food Consumption. The body weight of rats (presented in Table 2) in each group showed a steady increase trend, while MLE treated groups had a higher body-mass index than control group. However, there were no significant differences ($P > 0.05$), except in the female rats in low and medium dose groups at day 10 and in high dose group at days 21 and 49 ($P < 0.05$).

Table 6: The effects on organ weights and the relative organ weights of MLE in male SD rats ($\overline{X} \pm$ SD, g).

Parameters	Control group ($n = 6$)	Low dose group ($n = 6$)	Medium dose group ($n = 6$)	High dose group ($n = 6$)
Organ weight (g)				
Heart	1.720 ± 0.311	1.715 ± 0.312	1.669 ± 0.127	1.640 ± 0.189
Liver	12.370 ± 1.468	12.673 ± 2.428	12.803 ± 2.070	13.091 ± 2.106
Spleen	0.783 ± 0.099	0.847 ± 0.121	0.788 ± 0.053	0.816 ± 0.124
Lung	1.623 ± 0.070	1.666 ± 0.215	1.711 ± 0.163	1.701 ± 0.147
Kidney	3.229 ± 0.418	3.040 ± 0.300	3.090 ± 0.318	3.207 ± 0.450
Brain	2.024 ± 0.057	2.138 ± 0.113	2.120 ± 0.123	2.058 ± 0.074
Adrenal gland	0.063 ± 0.010	0.062 ± 0.013	0.070 ± 0.008	0.070 ± 0.006
Thymus	0.397 ± 0.106	0.317 ± 0.064	0.396 ± 0.087	0.347 ± 0.038
Testis	3.348 ± 0.302	3.587 ± 0.293	3.529 ± 0.367	3.571 ± 0.320
Epididymis	1.557 ± 0.096	1.700 ± 0.121*	1.647 ± 0.208	1.787 ± 0.135**
Fat	14.949 ± 3.320	17.607 ± 5.071	18.648 ± 8.385	17.647 ± 4.952
Organ/body weight ratio (mg/g)				
Weight	514.2 ± 41.1	514.8 ± 55.9	520.0 ± 51.1	525.7 ± 59.0
Heart	3.335 ± 0.440	3.356 ± 0.696	3.217 ± 0.121	3.125 ± 0.218
Liver	24.012 ± 1.433	24.430 ± 2.333	24.504 ± 1.484	24.823 ± 1.562
Spleen	1.519 ± 0.097	1.640 ± 0.101	1.521 ± 0.115	1.552 ± 0.158
Lung	3.166 ± 0.174	3.233 ± 0.199	3.312 ± 0.406	3.249 ± 0.244
Kidney	6.269 ± 0.502	5.924 ± 0.453	5.974 ± 0.718	6.095 ± 0.424
Brain	3.953 ± 0.257	4.201 ± 0.587	4.114 ± 0.504	3.950 ± 0.406
Adrenal gland	0.124 ± 0.021	0.121 ± 0.022	0.135 ± 0.016	0.133 ± 0.017
Thymus	0.765 ± 0.164	0.622 ± 0.163	0.763 ± 0.176	0.664 ± 0.086
Testis	6.571 ± 1.005	7.010 ± 0.657	6.840 ± 0.972	6.848 ± 0.845
Epididymis	3.048 ± 0.349	3.317 ± 0.210	3.188 ± 0.472	3.426 ± 0.365
Fat	28.878 ± 4.757	33.652 ± 6.862	34.940 ± 11.830	33.614 ± 8.958
Organ/brain weight ratio (g/g)				
Brain	2.024 ± 0.057	2.138 ± 0.113	2.120 ± 0.123	2.058 ± 0.074
Heart	0.848 ± 0.140	0.810 ± 0.184	0.791 ± 0.090	0.797 ± 0.084
Liver	6.100 ± 0.585	5.955 ± 1.240	6.083 ± 1.263	6.365 ± 1.017
Spleen	0.386 ± 0.040	0.398 ± 0.065	0.373 ± 0.033	0.396 ± 0.057
Lung	0.802 ± 0.031	0.781 ± 0.112	0.810 ± 0.097	0.826 ± 0.062
Kidney	1.592 ± 0.172	1.428 ± 0.179	1.458 ± 0.123	1.556 ± 0.187
Adrenal gland	0.031 ± 0.005	0.029 ± 0.007	0.033 ± 0.004	0.034 ± 0.003
Thymus	0.196 ± 0.050	0.147 ± 0.024	0.188 ± 0.046	0.168 ± 0.017
Testis	1.656 ± 0.166	1.679 ± 0.123	1.669 ± 0.189	1.734 ± 0.119
Epididymis	0.770 ± 0.057	0.798 ± 0.080	0.781 ± 0.125	0.869 ± 0.063*
Fat	7.356 ± 1.451	8.266 ± 2.462	8.949 ± 4.533	8.578 ± 2.372

*Means $P < 0.05$; **means $P < 0.01$; n means the number of animals.

The food consumption of rats (presented in Table 3) in each group showed some fluctuation, but there were no significant differences among those groups ($P > 0.05$).

As a result, after a long-term administration of MLE, the effects on the body weight and food consumption of rats were not obvious.

3.2.3. Hematology and Coagulation Function. After a 3-month of oral administration of MLE, some hematology indices and coagulation function of rats were determined as were shown in Table 4.

Compared with control group, the mean corpuscular volume (MCV) of male rats in low and medium dose groups and the mean corpuscular hemoglobin (MCH) of male rats in medium dose group were significantly decreased ($P < 0.05$ or $P < 0.01$). The RBC, HGB, and HCT in serum, however, were normal and the change extents of MCV and MCH were in a narrow range and no abnormalities were found in high dose group but the RBC was significantly elevated ($P < 0.05$).

Prothrombin times of rats in each group were approximately the same ($P > 0.05$) and were in normal range.

As a result, 3-month consecutive oral administration of MLE caused no obvious influences to the hematology and coagulation function of rats.

3.2.4. Biochemical Parameters. Serum levels of TG and CHOL of the male rats in high dose group were significantly higher ($P < 0.05$) than that of control group. Among the female rats,

Table 7: The effects on organ weights and the relative organ weights of MLE in female SD rats ($\overline{X} \pm$ SD, g).

Parameters	Control group ($n = 6$)	Low dose group ($n = 6$)	Medium dose group ($n = 6$)	High dose group ($n = 6$)
Organ weight (g)				
Heart	1.074 ± 0.162	1.059 ± 0.054	1.058 ± 0.100	1.092 ± 0.097
Liver	6.629 ± 0.449	7.320 ± 1.076	7.342 ± 0.747	7.215 ± 1.015
Spleen	0.484 ± 0.030	0.514 ± 0.058	0.470 ± 0.049	0.496 ± 0.073
Lung	1.230 ± 0.098	1.223 ± 0.084	1.256 ± 0.064	1.280 ± 0.116
kidney	1.751 ± 0.152	1.832 ± 0.169	1.875 ± 0.134	2.014 ± 0.227*
Brain	1.911 ± 0.054	1.905 ± 0.113	1.869 ± 0.084	1.905 ± 0.082
Adrenal gland	0.064 ± 0.013	0.082 ± 0.017	0.070 ± 0.012	0.084 ± 0.013*
Thymus	0.266 ± 0.052	0.282 ± 0.074	0.308 ± 0.065	0.264 ± 0.096
Uterus	0.726 ± 0.156	0.733 ± 0.107	0.756 ± 0.107	0.641 ± 0.184
Ovary	0.186 ± 0.025	0.194 ± 0.030	0.174 ± 0.036	0.195 ± 0.028
Fat	8.844 ± 2.096	9.452 ± 2.210	10.023 ± 4.202	8.612 ± 4.182
Organ/body weight ratio (mg/g)				
Weight	283.2 ± 13.8	286.5 ± 14.2	289.0 ± 26.4	291.8 ± 35.2
Heart	3.791 ± 0.516	3.705 ± 0.283	3.666 ± 0.244	3.770 ± 0.440
Liver	23.404 ± 0.952	25.490 ± 2.803	25.445 ± 1.930*	24.696 ± 1.327
Spleen	1.709 ± 0.079	1.793 ± 0.171	1.630 ± 0.130	1.702 ± 0.146
Lung	4.341 ± 0.244	4.267 ± 0.190	4.364 ± 0.286	4.405 ± 0.269
kidney	6.190 ± 0.561	6.388 ± 0.388	6.503 ± 0.327	6.919 ± 0.411*
Brain	6.757 ± 0.252	6.667 ± 0.568	6.509 ± 0.622	6.608 ± 0.872
Adrenal gland	0.226 ± 0.043	0.286 ± 0.050	0.242 ± 0.035	0.294 ± 0.068
Thymus	0.937 ± 0.163	0.986 ± 0.254	1.064 ± 0.197	0.900 ± 0.299
Uterus	2.567 ± 0.557	2.563 ± 0.379	2.636 ± 0.438	2.190 ± 0.518
Ovary	0.659 ± 0.102	0.682 ± 0.137	0.608 ± 0.153	0.674 ± 0.095
Fat	31.288 ± 7.420	32.888 ± 6.624	33.915 ± 11.504	28.450 ± 11.684
Organ/brain weight ratio (g/g)				
Brain	1.911 ± 0.054	1.905 ± 0.113	1.869 ± 0.084	1.905 ± 0.082
Heart	0.562 ± 0.081	0.559 ± 0.063	0.567 ± 0.060	0.573 ± 0.047
Liver	3.468 ± 0.195	3.857 ± 0.622	3.926 ± 0.318*	3.790 ± 0.526
Spleen	0.253 ± 0.011	0.270 ± 0.029	0.252 ± 0.026	0.261 ± 0.042
Lung	0.643 ± 0.050	0.643 ± 0.050	0.672 ± 0.023	0.673 ± 0.063
kidney	0.916 ± 0.075	0.966 ± 0.119	1.005 ± 0.084	1.057 ± 0.103*
Brain	0.033 ± 0.006	0.043 ± 0.010	0.037 ± 0.006	0.044 ± 0.005**
Adrenal gland	0.139 ± 0.024	0.149 ± 0.041	0.165 ± 0.033	0.139 ± 0.053
Thymus	0.380 ± 0.082	0.385 ± 0.049	0.406 ± 0.062	0.338 ± 0.104
Uterus	0.098 ± 0.014	0.102 ± 0.018	0.094 ± 0.022	0.103 ± 0.016
Ovary	4.645 ± 1.166	4.979 ± 1.183	5.390 ± 2.372	4.525 ± 2.194

*Means $P < 0.05$; **means $P < 0.01$; n means the number of animals.

the AST and TBIL levels in high dose group were significantly lower ($P < 0.05$) but within a narrow range than the controls, which means no toxicological significance. Moreover, a lower serum K^+ level was detected in female rats in high dose group ($P < 0.05$); in addition, no other biochemical parameters were found abnormal (Table 5).

3.2.5. Relative Organ Weight. The effects of MLE on the relative organ weights of male rats are presented in Table 6. Compared with control group, epididymis weight in medium and high dose groups were significantly higher ($P < 0.05$ or $P < 0.01$) and epididymis/brain weight ratio in high dose group was significantly different ($P < 0.05$), while the organ/body weight ratio revealed no marked differences ($P > 0.05$). The fat weight and the relative fat weight were higher but produced no remarkable differences than the controls ($P > 0.05$) and there were no obvious changes with the increasing of the dosage of MLE. Besides, all the other organ weights were normal.

The effects of MLE on the relative organ weights of female rats are presented in Table 7. Compared with control group, the liver/body weight ratio and the liver/brain weight ratio in

medium dose group were remarkably different ($P < 0.05$), and in the high dose group, the kidney weight, the relative kidney weights, the adrenal gland weight, and the ratio of adrenal gland to brain weight were also significantly different ($P < 0.05$ or $P < 0.01$). While in the low and medium groups, the fat weight and the relative fat weights were higher but produced no remarkable differences than the controls ($P > 0.05$) and there were no obvious changes with the increasing of the dosage of MLE. All the other organ weights were normal.

4. Conclusions

The acute toxicity study showed that MLE was safe at the maximum dose (18.4 g/kg) on ICR mice; animals behaved normally during the experiment and no gross lesions on major organs were examined.

Throughout the 3 consecutive months of oral administration of MLE at different doses (100 mg/kg, 300 mg/kg, and 900 mg/kg), rats in each group were normal in body weight and food consumption and various tests showed that higher serum TG and CHOL levels were found in male rats; lower serum K^+ level was detected in female rats; the epididymis weight of male rats and the liver, kidney, and adrenal gland weights of female rats were found higher. In addition to this, all animals were normal in hematology, coagulation function, biochemical criteria, gross anatomy, and relative organs weight.

From the foregoing, in male rats, as the body and fat weight increased, the TG and CHOL levels increased; more attentions should be paid to body fat examination. Although the liver and kidney weights of rats were changed, no blood biochemistry changes were hence brought about.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This research was supported by Important Drug Develop of MOST, China, (2012ZX09103201-031) and Program for Tianjin Innovative Research Team in University (TD12-5033).

References

[1] U. P. Singh, D. P. Singh, M. Singh et al., "Characterization of phenolic compounds in some Indian mango cultivars," *International Journal of Food Sciences and Nutrition*, vol. 55, no. 2, pp. 163–169, 2004.

[2] J. C. Barreto, M. T. S. Trevisan, W. E. Hull et al., "Characterization and quantitation of polyphenolic compounds in bark, kernel, leaves, and peel of mango (*Mangifera indica* L.)," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 14, pp. 5599–5610, 2008.

[3] Q. Kanwal, I. Hussain, H. Latif Siddiqui, and A. Javaid, "Antifungal activity of flavonoids isolated from mango (*Mangifera indica* L.) leaves," *Natural Product Research*, vol. 24, no. 20, pp. 1907–1914, 2010.

[4] S. S. Gebara, W. De Oliveira Ferreira, N. Ré-Poppi, E. Simionatto, and E. Carasek, "Volatile compounds of leaves and fruits of *Mangifera indica* var. coquinho (Anacardiaceae) obtained using solid phase microextraction and hydrodistillation," *Food Chemistry*, vol. 127, no. 2, pp. 689–693, 2011.

[5] A. Hannan, S. Asghar, T. Naeem et al., "Antibacterial effect of mango (*Mangifera indica* Linn.) leaf extract against antibiotic sensitive and multi-drug resistant Salmonella typhi," *Pakistan Journal of Pharmaceutical Sciences*, vol. 26, no. 4, pp. 715–719, 2013.

[6] Y. Zhang, L. Han, D. Ge et al., "Isolation, structural elucidation, MS profiling, and evaluation of triglyceride accumulation inhibitory effects of benzophenone C-glucosides from leaves of *Mangifera indica* L.," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 8, pp. 1884–1895, 2013.

[7] Y. Zhang, Q. Qian, D. Ge et al., "Identification of benzophenone C-glucosides from mango tree leaves and their inhibitory effect on triglyceride accumulation in 3T3-L1 adipocytes," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 21, pp. 11526–11533, 2011.

[8] Y. Zhang, X. Liu, L. Han, X. Gao, E. Liu, and T. Wang, "Regulation of lipid and glucose homeostasis by mango tree leaf extract is mediated by AMPK and PI3K/AKT signaling pathways," *Food Chemistry*, vol. 141, no. 3, pp. 2896–2905, 2013.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2014, Article ID 979414, 7 pages
http://dx.doi.org/10.1155/2014/979414

Research Article

Pharmacokinetics and Tissue Distribution Study of Chlorogenic Acid from Lonicerae Japonicae Flos Following Oral Administrations in Rats

Yulu Zhou,[1] Ting Zhou,[1,2] Qi Pei,[1] Shikun Liu,[1] and Hong Yuan[1]

[1] *Department of Pharmacy, The Third Xiangya Hospital, Central South University, Changsha, Hunan 410013, China*
[2] *School of Pharmaceutical Sciences, Central South University, Changsha, Hunan 410013, China*

Correspondence should be addressed to Hong Yuan; yuanhong01@vip.sina.com

Received 31 March 2014; Accepted 20 June 2014; Published 21 July 2014

Academic Editor: Mohamed Eddouks

Chlorogenic acid (ChA) is proposed as the major bioactive compounds of Lonicerae Japonicae Flos (LJF). Forty-two Wistar rats were randomly divided into seven groups to investigate the pharmacokinetics and tissue distribution of ChA, via oral administration of LJF extract, using ibuprofen as internal standard, employing a high performance liquid chromatography in conjunction with tandem mass spectrometry. Analytes were extracted from plasma samples and tissue homogenate by liquid–liquid extraction with acetonitrile, separated on a C_{18} column by linear gradient elution, and detected by electrospray ionization mass spectrometry in negative selected multiple reaction monitoring mode. Our results successfully demonstrate that the method has satisfactory selectivity, linearity, extraction recovery, matrix effect, precision, accuracy, and stability. Using noncompartment model to study pharmacokinetics, profile revealed that ChA was rapidly absorbed and eliminated. Tissue study indicated that the highest level was observed in liver, followed by kidney, lung, heart, and spleen. In conclusion, this method was suitable for the study on pharmacokinetics and tissue distribution of ChA after oral administration.

1. Introduction

Lonicerae Japonicae Flos (LJF) (*Lonicera japonica* Thunb), as a traditional Chinese medicine, was used widely in diseases such as wind-heat common cold and hot blood poison dysentery for its main property of clearing heat and detoxication [1]. The accumulated evidence has demonstrated that LJF has dozens of chemical components such as chlorogenic acid (ChA) (Figure 1), adinoside A, and stryspinoside [2, 3]. As the main active ingredient of LJF, ChA not only is the most abundant phenolic acid in LJF but also is usually used to control the quality of the LJF by its exact content [4]. ChA can prevent oxidation and microbial infection, protect cardiovascular system and liver, decrease blood pressure, and attenuate inflammation and pain [5, 6]. Furthermore, ChA can also inhibit the replication and viability of *Enterovirus* 71 *in vitro* [7] and show effective activity of antibacterial and antibiofilm [8, 9]. Additionally, ChA can reduce liver inflammation and fibrosis through inhibition of toll-like receptor 4 signaling pathway [10], attenuate ventricular remodeling after myocardial infarction [11], relieve acute and inflammatory pain [12], and prevent from lens opacity and cytotoxicity in human lens epithelial cells [13]. Concerning the multiple biological activity of ChA, investigating the pharmacokinetic profile and tissue distribution of ChA is instant requirement for its clinic application.

In this study, we investigated firstly the pharmacokinetic and tissue distribution of ChA extract from LJF *in vivo* by using high performance liquid chromatography in conjunction with tandem mass spectrometry (HPLC-MS/MS). A rapid and sensitive HPLC-MS/MS method was developed and validated to describe the pharmacokinetics and tissue distribution of ChA after oral administration of LJF extract in rats.

2. Experiment

2.1. Materials and Reagents. LJF was bought in Changsha. The reference standards of ChA and ibuprofen were supplied

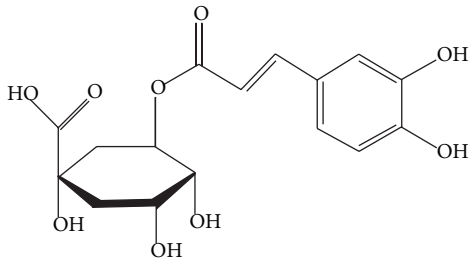


Figure 1: Chemical structures of chlorogenic acid.

by China National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile (ACN) and methanol (MEOH), HPLC grade, were acquired from Merck (Darmstadt, Germany). Formic acid was of HPLC grade and purchased from ROE SCIENTIFIC INC. (Beijing, China).

2.2. Animals, Drug Administration, and Sampling. Wistar rats weighting 180–220 g, half male and half female, were purchased from Changsha Tianqing Biotechnology Limited Company (Changsha, China). The rats were housed for 1 week, room temperature (22 ± 2°C), relative humidity (45–60%), and 12 h dark/light cycle controlled facility with free access to food and tap water. In this study, 42 rats were randomly assigned to seven groups, and rats fasted with free access to water for 12 h before being dosed. LJF extract was dissolved with 20% PEG400 and administered orally at a dose of 400 mg/kg.

Blood samples and tissue samples were collected at 10, 30, 60, 120, 180, and 240 min after dose. Blood samples were put into heparinized microcentrifuge tubes, followed by centrifuging at approximately 11000 r/min for 5 min. The resulting plasma layers were separated and stored in microcentrifuge tubes at −80°C until the analysis. Tissue samples were weighed rapidly and put into normal saline solution to remove the blood by blotting on filter paper and were minced and homogenized with physiological saline solution (1 : 2, w/v) thoroughly in ice-bath. These tissue homogenates were stored at −80°C until the analysis.

2.3. Preparation of Calibration Standard and Quality Control Samples. The stock solution of ChA was prepared by accurately weighing 10.00 mg of ChA reference substance into 10 mL volumetric flasks and dissolving in MEOH to give a final concentration of 1.000 g/L, so did ibuprofen. The standard solutions of ChA with concentrations of 78.20, 156.30, 312.50, 625.00, 1250.00, 2500.00, 5000.00, and 10000.00 μg/L were prepared by further dilution of the stock solution with MEOH. The working solutions of ibuprofen (5 mg/L) were obtained by dilution of the stock solution with MEOH. All the solutions were stored at 4°C and brought to room temperature before use.

To prepare the standard calibration samples, 20 μL of ChA standard solutions was added to 200 μL of controlled blank plasma. The mixture was vortex-mixed thoroughly to get the final standard concentrations of 7.820, 15.630, 31.250, 62.500, 125.000, 250.000, 500.000, and 1000.000 μg/L. The quality control (QC) samples with the concentration of 15.630, 125.000, and 800.000 μg/L were prepared by fortifying 20 μL of ChA standard solution to 200 μL of controlled blank plasma. These samples were stored at −20°C.

2.4. Sample Preparation. The preparation of LJF extract was operated as follows: 200 g of LJF with 2000 mL 85% ETOH was refluxed three times each for 2 hours. Subsequently, the solution was concentrated under reduced pressure and dried with microwave, yielding an extract with the content of 16.7% (ChA) detected by HPLC-MC/MC, which was much higher than the content of 2.51% (ChA) in LJF.

Twenty microliters of internal standard liquid (5 mg/L ibuprofen) which was added, during analysis, to 200 μL of biosamples of tissue homogenate, plasma, standard calibration samples, or QC samples followed by addition of 20 μL of internal standard liquid (5 mg/L ibuprofen), was added; then 400 μL of acetonitrile was added to precipitate protein. The biological samples were swirled for 1 min and were centrifuged at 11000 r/min for 10 min at 4°C. The obtained supernatant was filtered via a 0.22 μm filter membrane, and 5 μL of supernatant was injected into the HPLC-MS for analysis.

2.5. Instrumentation and HPLC-MS/MS Conditions. The HPLC-MS/MS system consists of an UFLC-20A high performance liquid chromatograph (Shimadzu Corporation, Japan), including an autosampler and temperature controlled column compartment, and an API 4000 mass spectrometer/mass spectrometer (AB SCIEX, America) with an electrospray ionization source (ESI). The signal acquisition, peak integration, and concentration determination were performed using the Analyst 1.5.1 software, supplied by AB SCIEX (Boston, America), too.

Chromatographic separation was performed on guard column C_{18} column (3 μm, 4 mm × 2.0 mm, Phenomenex, Torrance, CA, USA) and Luna C_{18} column (3 μm, 50 mm × 2.0 mm, Phenomenex). The autosampler temperature was maintained at 25°C and the column at 4°C. A gradient of 0.01% formic acid in methanol (solvent A) and 0.01% formic acid in water (solvent B) was used as follows: 95% B at 0.00 to 0.50 mins, 20% B at 0.50 to 2.00 mins, and 95% B at 2.00 to 4.50 mins. The flow rate was 0.4 mL/min.

The mass spectrometer equipped with an ESI (in the positive mode) source was performed in negative ion MRM mode, set with the capillary voltage of 4500 V; the pressure of ion source gas (N_2) 1 is 4.5×10^5 Pa and 2 is 5.5×10^5 Pa; the air curtain gas (N_2) is 2.0×10^5 Pa. The monitor ions of chlorogenic acid are from m/z 353.0 to m/z 191.1, declustering potential (DP) of 56 V, collision energy (CE) of 21 eV, while ibuprofen is from m/z 204.9 to m/z 161.0, DP of 53 V, CE of 11 eV.

2.6. Method Validation

2.6.1. Selectivity. In order to investigate potential interference from endogenous compounds that could coelute with the

analyte and the internal standard, 200 μL of blank rat plasma from six different sources with or without standard solutions of ChA and ibuprofen and plasma samples was tested after the administration.

2.6.2. Linearity and Lower Limit of Quantification (LLOQ). Linearity of calibration curve was determined by plotting the peak area ratio (y) of ChA to internal standard versus the concentration (x) of ChA. Series calibration standards of plasma and different tissues were prepared as descried above for analysis. Results were fitted to linear regression analysis using $1/x$ as the weighting factor.

2.6.3. Extraction Recovery and Matrix Effect. To calculate recovery, QC samples were analyzed at low (15.63 μg/L), medium (125.00 μg/L), and high (800.00 μg/L) concentration in quintuplicate after the preparation method described above. The peak area of ChA (A1) and ibuprofen (A2) in plasma samples was noted. Besides, 20 μL of internal standards as well as 600 μL of mobile phases was added to each concentration of 200 μL of QC samples followed by swirling for 30 sec. And 5 μL of mix solution was taken for injection. The peak area of ChA (A1′) and ibuprofen (A2′) in blood samples was noted.

The recovery was calculated by using the following formula: ChA in standard plasma samples (%) = A1/A1′; ibuprofen in standard plasma samples (%) = A1/A2′.To calculate matrix effect (ME), 20 μL of internal standards was added to each concentration of 200 μL of QC samples; also, 600 μL of blank plasma or mobile phases, swirled for 30 sec, and 5 μL of mix solution were taken for injection. The peak area of ChA (A3, A4) and ibuprofen (A3′, A4′) in plasma samples was recorded. ME can be determined by using the following formula: ChA in standard plasma samples (%) = A3/A3′; ibuprofen in standard plasma samples (%) = A4/A4′.

2.6.4. Accuracy and Precision. In order to determine the intraday accuracy and precision, five replications of all low, medium, and high concentration QC samples were performed on the same day and calculated each concentration of samples according to the calibration curve. The interday accuracy and precision were assessed by analyzing three batches on different days. The criteria for data acceptability are as follows: accuracy was determined by the ratio of calculated concentration and nominal concentration, precision was evaluated by relative standard derivative (RSD), and both accuracy and precision were within 15%.

2.6.5. Stability. Freeze-thaw stability was determined by assessing the QC samples after three freeze and thaw cycles at room temperature and at −80°C. The short-term stability was evaluated by keeping the QC samples at room temperature for 3 h and 6 h. The postpreparative stability was conducted by reanalyzing the QC samples after 6 h and 12 h in the autosampler at 4°C. The long-term stability of ChA was determined by placing the QC samples at −80°C for 30 days. For all storage conditions, replications of all low, medium, and high concentration QC samples were analyzed after the operation and the experimental results were obtained through chromatographic area and compared with the nominal values.

Table 1: Accuracy and precision of ChA in plasma samples.

Added concentration (μg/L)	Mean measured concentration (μg/L)	Accuracy (%)	RSD (%)
Intraday (n = 5)			
15.63	15.70	100.4	6.4
125.00	127.60	102.1	7.1
800.00	839.00	104.9	7.6
Interday (n = 15)			
15.63	15.71	100.5	6.7
125.00	123.90	99.1	6.4
800.00	834.00	104.3	7.9

3. Results

3.1. Method Validation

3.1.1. Selectivity. No interfering peaks were observed at the retention time of ChA (2.1 min) and internal standard (ibuprofen, 2.8 min) in all conditions (Figure 2).

3.1.2. Linearity and Lower Limit of Quantification (LLOQ). The back-calculated concentrations (mean ± SD) of ChA from the representative calibration standards by HPLC-MS/MS determination were within the acceptance limits. The correlation coefficient of calibration curve is larger than 0.99, suggesting a good linearity within the range from 7.820 μg/L to 1000.000 μg/L. The LLOQ of ChA is 7.820 μg/L in plasma sample and tissues.

3.1.3. Extraction Recovery and Matrix Effect. Extraction recovery and matrix effect were computed. The mean extraction recovery at three concentrations of ChA was 94.7% and of ibuprofen was 93.8%. The mean matrix effect at three concentrations of ChA was 96.7% and of ibuprofen was 94.9%.

3.1.4. Accuracy and Precision. Accuracy and precision data for intra- and interday plasma samples were within the scope of the standard (Table 1). The intra- and interday mean accuracy were within 5.0%; the intra- and interday precision (RSD%) values were less than 8%.

3.1.5. Stability. The stability data of ChA under four conditions are listed in Table 2. The ChA in plasma has been stable for 6 h at room temperature, for 12 h in autosampler, after 3 freeze-thaw cycles, and for 30 days stored at −80°C.

3.2. Pharmacokinetic Analysis. The pharmacokinetic analysis was processed with WinNonlin 6.1 software simulating data

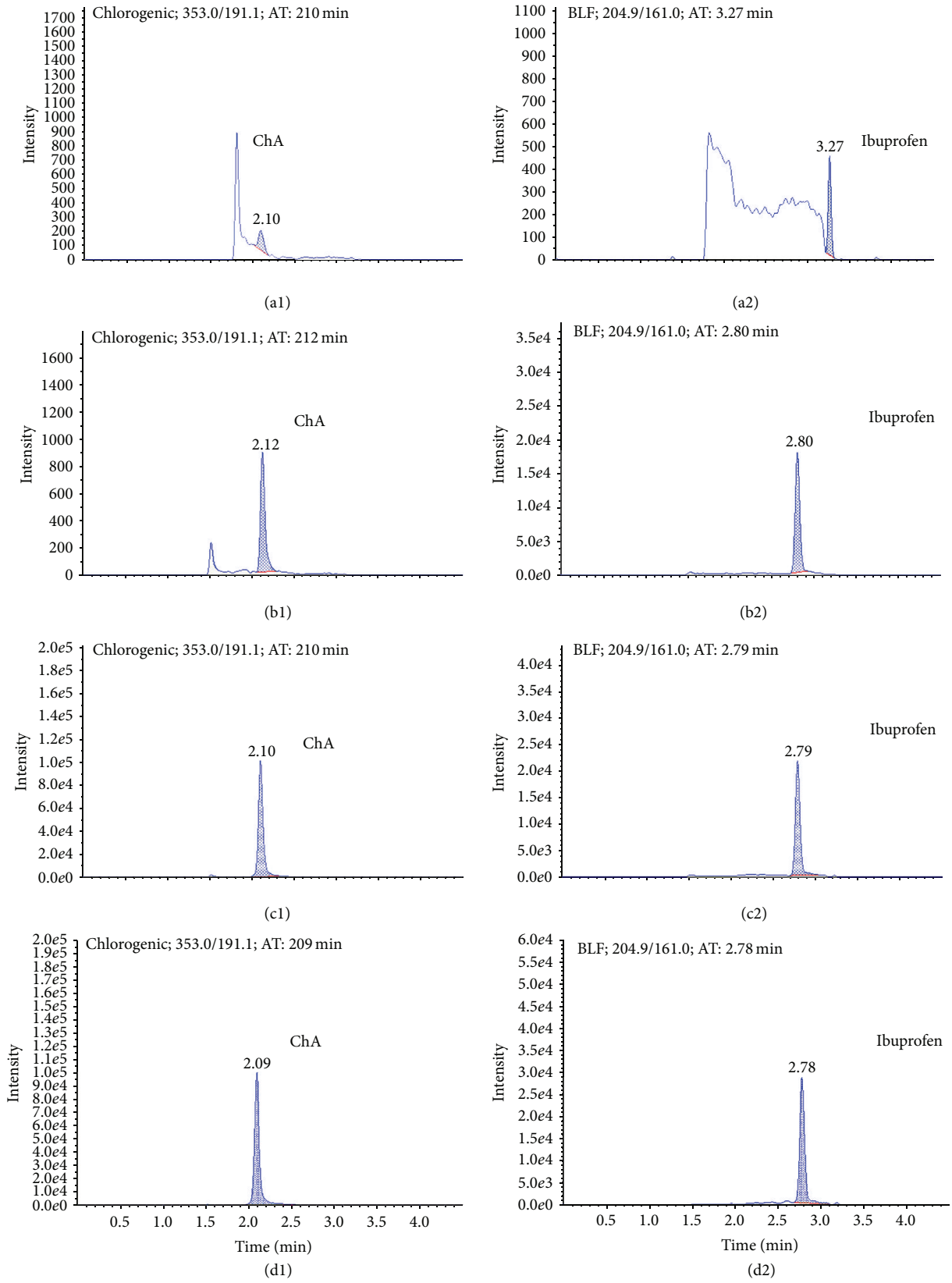


Figure 2: Representative mass spectrum of ChA and ibuprofen. (a1) Blank plasma sample of ChA. (a2) Blank plasma sample of ibuprofen. (b1) Standard calibration plasma sample with ChA at LLOQ level (7.820 μg/L). (b2) Standard calibration plasma sample with ibuprofen at LLOQ level. (c1) Plasma sample after administration of LJF extract of ChA. (c2) Plasma sample after administration of LJF extract of ibuprofen. (d1) Liver sample of ChA. (d2) Liver sample of ibuprofen.

Table 2: The stability of plasma samples.

Stability type	Time and n	Added concentration (μg/L)	Mean measured concentration (μg/L)	Accuracy (%)	RSD (%)
Short-term stability	3 h (n = 3)	15.63	14.30	91.5	7.0
		125.00	131.4	105.1	5.7
		800.00	825.5	103.2	3.0
	6 h (n = 3)	15.63	14.22	91.0	6.8
		125.00	133.6	106.9	6.2
		800.00	806.5	100.8	5.4
Postpreparative stability	6 h (n = 5)	15.63	14.30	91.5	7.0
		125.00	131.4	105.1	5.7
		800.00	825.5	103.2	3.0
	12 h (n = 5)	15.63	15.252	97.6	9.5
		125.00	130.3	104.2	6.4
		800.00	842	105.3	7.1
Freeze-thaw stability	After 3 freeze-thaw cycles (n = 3)	15.63	13.74	87.9	7.7
		125.00	127.3	101.9	6.4
		800.00	771.3	96.4	4.6
Long-term stability	After 30 days (n = 3)	15.63	13.66	87.4	5.2
		125.00	122.1	97.7	3.1
		800.00	804.9	100.6	4.0

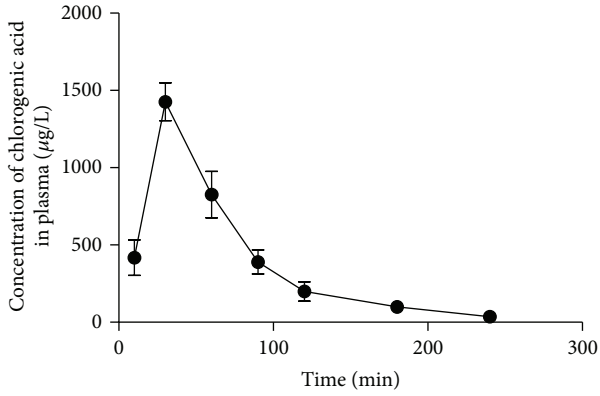


Figure 3: Plasma concentration-time profiles of ChA after oral administration. Each point represents the mean ± SD of 6 rats.

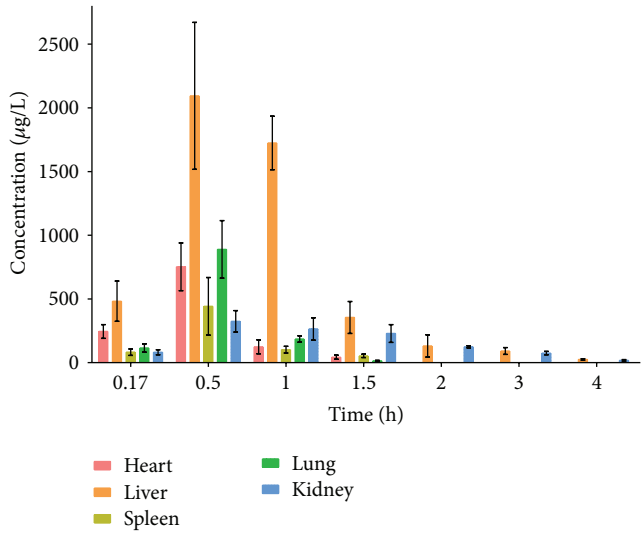


Figure 4: Tissue distribution of ChA in rats after oral administration. Each point represents the mean ± SD of 6 rats.

with noncompartmental model. Mean plasma concentration-time profile and the corresponding pharmacokinetic parameters of ChA after oral administration are shown in Figure 3 and Table 3. In this study, we found that ChA was absorbed and eliminated rapidly in rats, with low oral bioavailability. The half-lives of ChA in the plasma were about 0.8 h; the t_{max} was 0.58 ± 0.13 h and C_{max} was 1490 ± 0.16 ug/L.

3.3. Tissue Distribution Analysis. The concentration of ChA was determined in several organs, which indicated that ChA rapidly increased and then decreased accompanied by a wide distribution. Tissue distribution showed that the highest level was in the liver, followed by kidney, lung, heart, and spleen as shown in Figure 4. The concentrations in organs revealed that ChA was metabolized quickly and it almost cannot be detected in tissues after 4 h.

4. Discussion

Considering the low solubility of ChA, 20% PEG400 was selected as solubilization to improve the dissolving capacity of ChA. And, according to our preliminary experiments,

Table 3: Pharmacokinetic parameters of ChA in rat plasma after oral administration ($n = 6$).

Parameters	Value	Parameters	Value
C_{max} (μg/L)	1490.00 ± 160.00	$t_{1/2}$ (h)	0.80 ± 0.54
t_{max} (h)	0.58 ± 0.13	$AUC_{0\to t}$(μ∗gh/L)	1700.00 ± 320.00
V/F (L)	266.85 ± 144.89	$AUC_{0\to\infty}$(μ∗gh/L)	1730.00 ± 330.00
CL/F (L/h)	238.53 ± 49.76	$MRT_{0\to t}$ (h)	1.07 ± 0.09

ChA is mainly distributed to the most abundant blood-supply tissues, such as liver and kidney, which implied that the distribution of ChA might depend on the blood flow and perfusion rate of the organ. Then, we explored the biotransformation of primary ChA in liver and kidney. ChA decreased more rapidly in liver than that in kidney, which showed that liver played a more important role as compared to kidney. Furthermore, it can be inferred that ChA might target liver and induce a protective effect. Farrell et al. have studied the absorption and metabolism of ChA in cultured gastric epithelial monolayers [14], but cell experiment has its intrinsic limits. Pharmacokinetics of ChA extracted from Shuang-Huang-Lian [15], Yin-Huang granules [16], Aidi lyophilizer [17], or Daqingye [18] was also studied, while little in LJF. Other methods, such as HPLC [17, 19], LC-MS/MS [15], RP-HPLC [16], or HPLCDAD [4], were used to detect ChA.

By using the animal experiment, we can explore multiple pharmacological effects of LJF extract (ChA) as possible. And HPLC-MS/MS applied in our study exhibited higher sensitivity than LC-MS/MS. As we know, *in vivo* study of pharmacokinetic and tissue distribution of LJF extract (ChA) is significantly meaningful. Pharmacokinetic study can contribute to better understand the efficacy and toxicity of ChA, furthermore, tissue distribution study is crucial to discover the main target sites and account for disposition [20]. So, we first found a HPLC-MS/MS method, which is simple, sensitive, and with a highly detective C_{max} and short t_{max}.

Although we established this method and found some important meaningful knowledge, there are still some respects that need to ameliorate. For the pharmacokinetic, further experimental data from animals even human *in vivo* study is needed to be conducted. For the tissue distribution, we determined the concentration of ChA in each organ; the method which is used for quantification of ChA in tissues needs to be verified, just like the method that is applied in quantification of ChA in rat plasma.

5. Conclusion

In conclusion, for the first time, a rapid, simple, and sensitive HPLC-MS/MS method was validated for the quantification of ChA in rat plasma and tissues. The specificity, linearity, extraction recovery, accuracy, and stability of the method were successfully established.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

The authors wish to thank Professor Zengrong, Hunan University of Chinese Medicine, for his help in identifying LJF and detecting its content.

References

[1] S. Li and C. Chang, "Biological effects of chlorogenic acid and body health," *Wei Sheng Yan Jiu*, vol. 34, no. 6, pp. 762–764, 2005.

[2] Y. Chen, Y. Zhao, S. Wu, M. Wang, X. Feng, and J. Liang, "Water-soluble chemical constituents in flower buds of Lonicera macranthoides," *Zhong Yao Cai*, vol. 35, no. 2, pp. 231–234, 2012.

[3] F. Wang, Y. Jiang, X. Wang et al., "Chemical constituents from flower buds of *Lonicera japonica*," *Zhongguo Zhongyao Zazhi*, vol. 38, no. 9, pp. 1378–1385, 2013.

[4] C. Kuixia, Z. Yingtao, Y. Xiuwei et al., "Simultaneous quantification of ten phenolic acids in Lonicerae Japonicae Flos by HPLC-DAD," *Journal of Chinese Pharmaceutical Sciences*, vol. 22, no. 6, pp. 521–526, 2013.

[5] Y. Zhao, J. Wang, O. Ballevre, H. Luo, and W. Zhang, "Antihypertensive effects and mechanisms of chlorogenic acids," *Hypertension Research*, vol. 35, no. 4, pp. 370–374, 2012.

[6] X. Shang, H. Pan, M. Li, X. Miao, and H. Ding, "Lonicera japonica Thunb.: ethnopharmacology, phytochemistry and pharmacology of an important traditional Chinese medicine," *Journal of Ethnopharmacology*, vol. 138, no. 1, pp. 1–21, 2011.

[7] X. Li, Y. Liu, X. Hou et al., "Chlorogenic acid inhibits the replication and viability of enterovirus 71 *in vitro*," *PLoS ONE*, vol. 8, no. 9, Article ID e76007, 2013.

[8] A. Karunanidhi, R. Thomas, A. Van Belkum, and V. Neela, "In vitro antibacterial and antibiofilm activities of chlorogenic acid against clinical isolates of stenotrophomonas maltophilia including the trimethoprim/sulfamethoxazole resistant strain," *BioMed Research International*, vol. 2013, Article ID 392058, 7 pages, 2013.

[9] A. Luis, F. Silva, S. Sousa, A. P. Duarte, and F. Domingues, "Antistaphylococcal and biofilm inhibitory activities of gallic, caffeic, and chlorogenic acids," *Biofouling*, vol. 30, no. 1, pp. 69–79, 2013.

[10] H. Shi, L. Dong, J. Jiang et al., "Chlorogenic acid reduces liver inflammation and fibrosis through inhibition of toll-like receptor 4 signaling pathway," *Toxicology*, vol. 303, pp. 107–114, 2013.

[11] Y. Kanno, R. Watanabe, H. Zempo, M. Ogawa, J. Suzuki, and M. Isobe, "Chlorogenic acid attenuates ventricular remodeling after myocardial infarction in mice," *International Heart Journal*, vol. 54, no. 3, pp. 176–180, 2013.

[12] K. Hara, Y. Haranishi, K. Kataoka et al., "Chlorogenic acid administered intrathecally alleviates mechanical and cold

hyperalgesia in a rat neuropathic pain model," *European Journal of Pharmacology*, vol. 723, pp. 459–464, 2014.

[13] Y. S. Kim, N. H. Kim, Y. M. Lee, and J. S. Kim, "Preventive effect of chlorogenic acid on lens opacity and cytotoxicity in human lens epithelial cells," *Biological & Pharmaceutical Bulletin*, vol. 34, no. 6, pp. 925–928, 2011.

[14] T. L. Farrell, T. P. Dew, L. Poquet, P. Hanson, and G. Williamson, "Absorption and metabolism of chlorogenic acids in cultured gastric epithelial monolayers," *Drug Metabolism and Disposition*, vol. 39, no. 12, pp. 2338–2346, 2011.

[15] J. X. Ye, W. Wei, L. H. Quan, C. Y. Liu, Q. Chang, and Y. H. Liao, "An LC-MS/MS method for the simultaneous determination of chlorogenic acid, forsythiaside A and baicalin in rat plasma and its application to pharmacokinetic study of Shuang-huang-lian in rats," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 52, no. 4, pp. 625–630, 2010.

[16] X. Li, J. Yu, J. Luo et al., "Determination and pharmacokinetic study of chlorogenic acid in rat dosed with Yin-Huang granules by RP-HPLC," *Biomedical Chromatography*, vol. 20, no. 2, pp. 206–210, 2006.

[17] Q. Li, L. Sun, Y. Jia, Z. Wang, Z. Shen, and K. Bi, "Determination and pharmacokinetic study of syringin and chlorogenic acid in rat plasma after administration of Aidi lyophilizer," *Biomedical Chromatography*, vol. 20, no. 12, pp. 1315–1320, 2006.

[18] B. Yuan, M. Qiao, H. Xu, L. Wang, and F. Li, "Determination of chlorogenic acid in rat plasma by high performance chromatography after peritoneal administration of compound Daqingye injection," *Yakugaku Zasshi*, vol. 126, no. 9, pp. 811–814, 2006.

[19] R. Gao, Y. Lin, G. Liang, B. Yu, and Y. Gao, "Comparative pharmacokinetic study of chlorogenic acid after oral administration of Lonicerae Japonicae Flos and Shuang -Huang- Lian in normal and febrile rats," *Phytotherapy Research*, vol. 28, no. 1, pp. 144–147, 2014.

[20] F. Zhang, X. Gong, B. Xiao, C. Zhang, and Z. Wang, "Pharmacokinetics and tissue distribution of a bioactive sesquiterpenoid from Polygonum jucundum following oral and intravenous administrations to rats," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 83, pp. 135–140, 2013.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2014, Article ID 879396, 8 pages
http://dx.doi.org/10.1155/2014/879396

Review Article

Valerian: No Evidence for Clinically Relevant Interactions

Olaf Kelber,[1,2] Karen Nieber,[1,3] and Karin Kraft[1,4]

[1] *Working Group Efficacy, Safety and Interactions of Kooperation Phytopharmaka GbR, Plittersdorfer Straße 218, 53173 Bonn, Germany*
[2] *Medical Information and Clinical Research, Steigerwald Arzneimittelwerk GmbH, 64295 Darmstadt, Germany*
[3] *Institute of Pharmacy, University of Leipzig, 04103 Leipzig, Germany*
[4] *Center of Internal Medicine, Universitätsmedizin Rostock, 18057 Rostock, Germany*

Correspondence should be addressed to Olaf Kelber; olaf@kelber.org

Received 8 January 2014; Revised 23 March 2014; Accepted 12 May 2014; Published 30 June 2014

Academic Editor: Igho J. Onakpoya

In recent popular publications as well as in widely used information websites directed to cancer patients, valerian is claimed to have a potential of adverse interactions with anticancer drugs. This questions its use as a safe replacement for, for example, benzodiazepines. A review on the interaction potential of preparations from valerian root (*Valeriana officinalis* L. root) was therefore conducted. A data base search and search in a clinical drug interaction data base were conducted. Thereafter, a systematic assessment of publications was performed. Seven *in vitro* studies on six CYP 450 isoenzymes, on p-glycoprotein, and on two UGT isoenzymes were identified. However, the methodological assessment of these studies did not support their suitability for the prediction of clinically relevant interactions. In addition, clinical studies on various valerian preparations did not reveal any relevant interaction potential concerning CYP 1A2, 2D6, 2E1, and 3A4. Available animal and human pharmacodynamic studies did not verify any interaction potential. The interaction potential of valerian preparations therefore seems to be low and thereby without clinical relevance. We conclude that there is no specific evidence questioning their safety, also in cancer patients.

1. Introduction

Valerian "can interfere in an unwanted way with the oncological cancer therapy" or valerian "can diminish the efficacy of cancer therapeutics." Statements like these can be found in published features on complementary medicine for cancer patients (Sloan Kettering Center 2012 [1]). In a very popular book on integrative oncology published by Sparreboom and Baker [2], it is stated that it is known for some herbal drugs that they can influence the activity of the cytochrome system and thereby may change the effect of chemotherapy. It is further stated that cancer patients often have difficulties to sleep and are restless, so that they can use herbal drugs such as valerian in order to avoid chemically defined sedatives and hypnotics as they fear becoming addicted. However, they state that warnings that valerian (*Valeriana officinalis*) may stimulate CYP 3A4.

This would reduce the effect of several cytostatic substances. For tamoxifen, the plasma level is reduced by CYP3A4 inductors, while inhibitors of CYP2D6 can lower the level of active metabolites, both leading to reduced efficacy. In case of cyclophosphamide, metabolic activation by CYP2D6 is necessary that is increased by inductors of this isoenzyme. The plasma level of several other cytostatic substances is lowered by CYP3A4 inductors. Examples are the epipodophyllotoxin derivative teniposide, camptothecin, tyrosine kinase inhibitors like imatinib, taxanes like paclitaxel and docetaxel, and vinca alkaloids like vincristine. CYP3A4 inductors also may reduce the activity of alkylating substances like ifosfamide and some antitumor antibiotics [2].

In several websites directed to patients (http://www.cancer.org, http://www.mskcc.org), the use of valerian in cancer is also questioned due to its interaction potential. By the way, Scientific American elected the latter website just a year after foundation as one of the best five US medicinal websites.

More than 70% of all cancer patients are very interested in herbal drugs. However, many of them do not inform their oncologist about their use [3, 4]. As 19 to 75% of all cancer patients suffer from sleep disturbances [5], warnings against the use of valerian preparations have a high impact on these

patients. However, it has to be ascertained that warnings that valerian may cause interactions with other medicines are backed by valid evidence supporting such an assumption and that they are therefore scientifically correct. Therefore, we conducted a review in order to assess the interaction potential of valerian.

2. Efficacy and Safety of Valerian Extracts

The clinical relevance of acute as well as chronic sleep disorders is obvious: epidemiological data show that they affect approximately one-third of the adult population [6, 7]. Treatment is indicated in about 15% of these cases [8, 9]. Insomnia, defined as insufficient quantity or quality of sleep resulting in compromised daytime alertness and activity, is a common condition. It can result in serious adverse consequences, including attention and memory impairment, depression, falls, and perceived reduced quality of life [10].

To the most common treatments of insomnia belong drugs [11], this however bears some problems. Benzodiazepines and imidazopyridines offer only short-term relief, while data on their long-term efficacy are scarce. Both drug classes have significant adverse effects such as serious psychomotor symptoms, behavioral aberrations, memory impairment resulting in injuries, respiratory depression, rebound insomnia, and paradoxical agitation. Especially for benzodiazepines, the potential for abuse is high [10]. Therefore, the National Institutes of Health consensus conference strongly discouraged chronic treatment of insomnia with benzodiazepines already in 2005 [11].

Sedating antihistamines, such as diphenhydramine, the active ingredient in most over-the-counter sleep aids, are associated with cognitive impairment, daytime drowsiness, and anticholinergic effects. There is no evidence-based data available on their efficacy improving insomnia or prolonging sleep. It therefore was recommended that they should be avoided in the elderly [10, 12]. Finally, antidepressants used for treating insomnia, such as trazodone, can produce dangerous and life-threatening adverse events due to their anticholinergic, cardiovascular, and neurologic actions [10, 12].

Herbal substances improving insomnia, such as valerian, hops, or passion flower, are well-known sleep aids. While marketed as food in the US, they are authorized or registered as medicines in Europe and many other regions, being mostly used in self-medication and holding widespread appeal, presumably because of their lower cost and higher range of safety when compared to chemically defined pharmaceuticals [13]. Among these, the roots of valerian (*Valeriana officinalis* L.) are the most familiar ones, especially in Europe. They improve the subjective experience of sleep when taken in the evening over a period of one or two weeks [14]. The constituents of valerian root include, among others, valepotriates (iridoids) and volatile oil, including monoterpenes and sesquiterpenes (valerenic acids). Commercially available extracts are free from valepotriates [15]. Recommended daily doses of valerian root extracts are about 600 mg, usually taken as capsules or tablets.

Several controlled clinical trials with various valerian extracts are available, and also a meta-analysis on eighteen randomized placebo-controlled trails was published. Its qualitative results suggest that valerian would be effective for a subjective improvement of insomnia, although its effectiveness could not be demonstrated with quantitative or objective measurements [16]. In a study conducted in cancer patients, an improvement in the primary variable, a sleep quality index based mainly on objective parameters, could not be demonstrated; however, fatigue and sleep problems were significantly improved [17].

The clinical studies available show an excellent short-term tolerability, and from several decades of clinical use within the frame of pharmacovigilance systems no data questioning its long-term safety have evolved, while prospective data are missing [18]. In contrast to classical sedatives, valerian extracts did not impair the ability to drive or to use machines, neither after single [19–22] nor after repeated doses [23]. Reports of putative adverse reactions are extremely rare and include one case of hepatic symptoms after prolonged treatment and one case of cardiac symptoms after discontinuation of a long-term treatment with very high doses, which were interpreted as a withdrawal reaction [24, 25]. In both case reports, outcome was benign, causality was questionable, and characteristics of the extract preparations were not provided.

While the side effect profile therefore is benign, a potential for adverse drug interactions has been claimed by some reviews [26], while other reviews did not [18, 27–32], so that a reevaluation of the existing evidence is necessary.

3. Material and Methods

For evaluation of the scientific data on the interaction potential of valerian (*Valeriana officinalis* L.), published data (experimental *in vitro* and *in vivo* studies, pharmacovigilance data from its long standing use as an authorized medicinal drug or supplement) were assessed and evaluated for identifying data on possible drug-herb interactions (Table 1). A search with the search terms (valerian or valeriana) was conducted in the databases MEDLINE and TOXLIT, via DIMDI (Cologne, Germany). All records, for which a relevance to the subject of herb-drug interactions of *Valeriana officinalis* L. could not be clearly excluded by the article title, were screened based on the abstracts. In all cases, where a relevance to the subject could thereby not be clearly ruled out, full-text articles were assessed and, as far as relevant, included into the review. Additionally MedIQ [33], a leading online database for the assessment of potential interactions in pharmacotherapy, was searched. Thereafter cross-referencing was conducted in order to identify and close potential gaps. Studies were assessed for completeness and validity of data on material and methods, on the potential clinical relevance of the results and on potential bias in the presentation of results and conclusions.

4. Results

Eligible and included were all scientific publications on interactions of *Valeriana officinalis* L. root and preparations

Table 1: Potential pharmacokinetic interactions of valerian according to the published literature. The conclusions by the respective authors, the ratings given by MedIQ (http://www.mediq.ch, a Swiss independent interaction data base, which is structured according to mechanisms of interaction and includes herbal drugs), and the conclusions regarding the potential of clinically relevant interactions based on a critical analysis of the published studies are reported.

Metabolic pathway	*In vitro* studies	*In vivo* studies	Interaction potential (according to medIQ database [33])	Indication for a clinically relevant interaction
CYP 1A2	No effect [34]	No effect [35]		None
CYP 2D6	Induction [36]	No effect [35, 37]	Weak inhibitor	None
CYP 2E1		No effect [35]		None
CYP 3A4	Induction [36, 38]	No effect [34, 35, 37]	Weak inductor	None
P-glycoprotein	Weak inhibition? [39, 40]	No data available	Weak inhibitor	None
UGT 1A1	Weak inhibition? [41, 42]	No data available	Weak inhibitor	None
UGT 2B7	Weak inhibition? [41]	No data available	Weak inhibitor	None

CYP: cytochrome P450; UGT: uridine 5′-diphosphate glucuronosyltransferase.

thereof. Among these are altogether 21 original publications. 11 of them present data from *in vitro* studies on pharmacokinetic interactions. One of these publications [34] contains, in addition, data on animal studies. Two publications [35, 37] present clinical studies on pharmacokinetic interactions. 8 studies are available on the subject of pharmacodynamic interactions, thereof two *in vitro* and three animal studies, one clinical study, and in addition two case reports.

4.1. Pharmacokinetic Interactions. Predicting pharmacokinetic herb-drug interactions is difficult because the pharmacological actions of the interacting drugs are often not related. This is also the case with the subject of this review, valerian as a treatment of insomnia, and chemically defined cytostatic therapies. Herb-drug interactions can occur on the levels of absorption, distribution, metabolism, or excretion and can change the amount and duration of the availability of a drug at the site of action. The interactions due to drug metabolism can be, as mentioned above, based on phase 1 metabolism (mainly involving cytochrome P 450 isoenzymes) or, rather rare, on phase 2 metabolism involving, for example, P-glycoproteins (P-gp), which is relevant for outward bound transport processes, for example, in the intestinal wall, or on conjugation, for example, with glucuronic acid [43]. In case that a specific herb-drug interaction is identified, its clinical significance depends on the degree of accumulation and the therapeutic window of the respective drug [44]. Also the dosage, time of administration, galenic properties, and coadministration as well as intrinsic and extrinsic factors may be of importance.

4.2. In Vitro Studies. Budzinski et al. [38] used a fluorometric assay for analysis of the *in vitro* CYP 3A4 inhibitory capability of dilutions of a valerian fluid extract (no further information available) using human CYP 3A4. An IC_{50} of 1.8% the undiluted extract in the reaction mixture was identified. The authors stated that "the *in vitro* interactions, though weak, may have clinical importance." However, the valerian fluid extract tested was not specified, and the inhibitory concentration identified was very high. Additionally, the use of fluorometric methods is highly susceptible to interference by fluorescent herbal components [45]. Therefore, the results do not allow a valid extrapolation towards a clinically relevant effect.

Lefebvre et al. [39] determined the *in vitro* effects of 14 commercially available single entity and combination herbal products containing extracts of valerian root, on CYP 3A4-mediated metabolism and on P-gp transport. The extracts were prepared by extracting 100 mg of the powdered commercial preparations with 1 mL of water and 70% ethanol or acetonitrile and characterized by determination of total valerenic acids by HPLC. In a proportion of 1–5% of total assay volume, most extracts showed an inhibition to different extents. Six extracts had some inhibitory effects on P-gp. The authors concluded that "there is wide variation in commercially available samples of valerian root. The findings from this study suggest that valerian root may have an initial inhibitory effect, when taken with therapeutic products. Further work is warranted to determine whether valerian root can affect other CYP 450 isozymes and how the results of this *in vitro* investigation can be extrapolated to *in vivo* situations." Indeed, several of the tested preparations are insufficiently characterized, as only the valerenic acid content is provided. In addition, only one rather high concentration of each extract was tested and, again, a fluorometric method was used, which is highly susceptible to interference by fluorescent herbal components. These limitations do not allow valid extrapolations with respect to a clinical relevance of the results.

The aim of a further study was to evaluate the *in vitro* effect of commercially available valerian medicinal products on the metabolic activities of the CYP 450 isoenzymes 1A2, 2D6, and 3A4. A valerian extract (drug extraction ratio 6 : 1, extraction solvent ethanol 60%) was incubated with human primary hepatocytes for three times within 48 h. The activities of the CYP isoenzymes were determined by analyzing the metabolites of test substances by HPLC [36, 46]. The herbal extract concentrations used in *in vitro* metabolic

systems were claimed to cover the whole range of herbal concentrations occurring *in vivo*. Dose dependent and statistically significant increases in CYP 2D6 and CYP 3A4 were observed only with two concentrations (0.188 mg/mL and 1.875 mg/mL). The authors postulate an allosteric antagonism but also clearly point to the limitations of their study, as hepatocytes from only one donor were used. Therefore, the well-known polymorphisms in the CYP superfamily were not covered.

In a follow-up study, the same preparation was used, and an IC_{50} for CYP 3A4 inhibition at a concentration of 0.756 mg/mL was observed [40]. Slight but significant effects on bidirectional digoxin transport (involving P-gp) were found only with 1.875 mg/mL. The authors conclude that both CYP 3A4 and P-gp interactions are unlikely to be clinically relevant, as the systemic concentrations are probably much lower; therefore, respective IC_{50} values for P-gp cannot be reached *in vivo*. This conclusion is also applicable to their previous study [36] and to their study on CYP 2C19, which showed a weak induction by valerian root extracts [47].

In a further *in vitro* study conducted on mouse and human liver microsomes [34], the postulated inhibitory action of 2.5–75 μg/mL of a valerian extract (aqueous ethanolic, not further defined) on CYP 1A1, CYP 1A2, CYP 2C, and CYP 3A was not confirmed.

In another study, the effect of valerian, valerian/hops extracts, and valerenic acid on the glucuronide conjugation of various substrates (17α-estradiol, acetaminophen, morphine, and testosterone) was determined using human liver microsomes [41]. Test substance was a valerian capsule (250 mg extract corresponding to 3.48 mg valerenic acid per capsule, further specification not available), which was extracted with 80% methanol (5 mL/capsule). Also, the activities of UGT 1A1 and UGT 2B7 were tested in the presence of 2.5 or 5.0 mL of valerian or valerian/hops extract per 250 mL final incubation volume. Valerenic acid significantly inhibited the glucuronidation by both microsomes and UGTs with the rather high concentration of 1 mg/mL. Due to this fact and the very high glucuronidation capacity of the liver, a clinical relevance of these results cannot be assumed.

Mohamed at al. [42] used an UGT 1A1 assay to test a commercially available valerian preparation (≥0.1% valerenic acid; extraction medium 70% ethanol, recommended daily dose 1000 mg) for inhibition of human estradiol-3-O-glucuronidation (E-3-G) in the same concentrations as Hellum et al. [36]. E-3-G was quantified by HPLC. IC_{50} was 0.562 mg/mL, which would be reached by dissolving one daily dose in a volume of 1.8 L. According to the authors, this volume is in the same order of magnitude as the volume of the intestine, and therefore a potential effect in the intestine could not be excluded [48]. However, even if a transient partial inhibition of UGT 1A1 in the intestine would occur, it would be transient, rather than a long-lasting enzyme induction, and could not to lead to a persistent change of bioavailability of another drug.

4.3. In Vivo Studies. In an *in vivo* study on mice, a valerian extract (aqueous ethanolic, not further described), given in a dose of 0.5% with the diet over 28 days (corresponding to a daily dose of 595 mg/kg in average) did neither affect the CYP content of the liver nor the activities of CYP 1A1, CYP 1A2, CYP 2C9, and CYP 3A4 [34].

In twelve healthy volunteers (6 males and 6 females, age 30.9 ± 7.2 years, nonsmokers from South Carolina), the effect of a valerian root extract on the activity of the drug-metabolizing enzymes CYP 2D6 and CYP 3A4 was tested [37]. Daily before going to bed, participants took two tablets, with 500 mg valerian extract each (extraction solvent ethanol 70%, valerenic acid content 5.51 mg/tablet), for 14 subsequent days. This dose is well within the range of recommended doses for valerian preparations. The probe drugs dextromethorphan (30 mg; CYP 2D6 activity) and alprazolam (2 mg; CYP 3A4 activity) were administered orally at baseline and after treatment with valerian, and dextromethorphan to dextrorphan metabolic ratios and alprazolam pharmacokinetics were determined. The ratio of dextromethorphan to dextrorphan increased slightly but significantly from 0.214 to 0.254. The maximum concentration of alprazolam was moderately increased for about 20% from 25 to 31 ng/mL ($P < 0.05$). This change is therefore within the frame of 80% to 125% rated as equivalent by FDA. Bioavailability of other medicines would therefore not be relevantly diminished and therapeutic efficacy of a therapy not questioned. Changes of other pharmacokinetic parameters were not detected. In conclusion valerian in therapeutic doses is unlikely to produce clinically relevant effects on CYP 2D6 and CYP 3A4 pathways which could diminish the therapeutic efficacy of other drugs.

In another study, twelve healthy volunteers (six men and six women, age mean ± SD = 24 ± 3 years, weight 69.3 ± 14.2 kg KGW) were randomly assigned to receive valerian (DER 4 : 1, no standardization claim) for 28 days, three times daily 125 mg [35]. Before and after the test period, the activities of CYP 3A4/5 (1-hydroxymidazolam/midazolam serum ratio), CYP 1A2 (paraxanthine/caffeine serum ratio), CYP 2E1 (hydroxychlorzoxazone/chlorzoxazone serum ratio), and CYP 2D6 (debrisoquine urinary recovery ratio) were determined. All subjects were nonsmokers and extensive metabolizers of CYP 2D6. No changes at all in phenotypic ratios were observed. The daily dose applied in this study was corresponding to 1.5 g drug, which is in the lower range of doses recommended, for example, in the monograph of the HMPC. Despite that, the fact that there was not even a tendency of an effect in the CYP isoenzymes tested underlines the assumption of the lack of an interaction potential in CYP enzymes.

4.4. Pharmacodynamic Interactions. Pharmacodynamic interactions include the concurrent administration of drugs having the same or opposing pharmacologic actions and also the change of the sensitivity or the responsiveness of the tissues to one drug, induced by another one. Many of these interactions can be predicted from knowledge of the pharmacology of each drug. They were proposed for valerian mainly with drugs influencing vigilance such as codeine, citalopram, and benzodiazepines. A presumable interaction with benzodiazepines, which are positive allosteric modulators on GABA-receptors, is based on *in*

vitro data suggesting GABAergic mechanisms of action of valerian extracts [49, 50]. These studies however are inconclusive and require independent replication.

In rats an *in vivo* study was conducted on interactions between valerian root tincture (ethanol 100%, 1 : 10, not further characterized, daily therapeutic dose 3060 mg for an adult human) and haloperidol with respect to impaired liver or kidney functions. Valerian tincture was applied with the drinking water (1%, corresponding to an extract dose of 200–250 mg/kg bw/d). Haloperidol (38 mg/kg bw) was applied intramuscularly once every 4 weeks over 12 weeks beginning after 15 days of treatment with valerian [51]. While renal effects were lacking, in some of the parameters measured in liver homogenates, slight and statistically significant deviations from control values were observed, suggesting an additive effect of haloperidol and valerian, however of questionable relevance. Authors concluded that in humans a possible toxic additive effect would occur only at supratherapeutic doses. The same working group evaluated the effect of valerian in a rat model of orofacial dyskinesia using the same application procedure as described above. Valerian did not influence orofacial dyskinesia induced by haloperidol. Also oxidative stress parameters were not changed [52].

In another *in vivo* study on mice [53], 25 mg/kg of a valerian root dry extract was combined with 25 mg/kg of a liquorice extract (both prepared with ethanol 70%, not further characterized) or the benzodiazepine alprazolam (0.7 mg/kg). Tests were conducted in an elevated plus maze. Valerian and alprazolam, rather than liquorice, significantly increased time spent in the open arm, pointing to an anxiolytic effect. The effect of alprazolam, combined with liquorice or valerian, was significantly increased compared to each of the single substances. The authors discuss an improved bioavailability, for example, due to an increased gastrointestinal absorption induced by liquorice. The relevance in humans remains unclear.

In 48 healthy volunteers, pharmacodynamic interactions of single doses of valerian (100 mg/d, extract specification lacking) and propranolol (20 mg/d) were evaluated. The results were not presented in detail but indicated that the two drugs act independently from each other. Interactions with respect to heart rate and parameters of psychic strain could not be demonstrated [54].

A possible pharmacodynamic interaction of valerian preparations with other drugs is supported only by two case reports. A 40-year-old male patient had taken lorazepam (2 mg/d) for two month without side effects. Then he additionally took for two days an infusion of valerian root two hours before bedtime and, just before going to bed, an infusion of valerian root and passion flower herb (dose unknown) without side effects. On the third day he took, instead of the infusion, three tablets containing a combination of valerian root extract (300 mg) and passion flower root and herb extracts (350 mg; no further information available) before bedtime. He thereafter suffered from transient mild handshaking and drowsiness [55]. It was suspected that these symptoms were caused by an interaction between the herbal drugs and lorazepam, as they ceased after stopping herbal treatment. It is however questionable to ascribe the adverse event to valerian alone as the herbal preparation also contained a root and herb extract of passion flower, for which monographs are lacking. As the symptoms are also potential adverse effects of lorazepam itself, and no other similar cases have been described since then, and also a coincidence cannot be ruled out.

Another patient (39-year-old female) had taken for two month a daily dose of two tablets of a St. John's wort preparation and one tablet of a valerian preparation and additionally Loperamide [56]. After this time, she was hospitalized with a severe delirium. She claimed that the herbal treatment was a replacement for the opioid meperidine she had been taken before for reducing migraine. The authors proposed an interaction between the herbal drugs and Loperamide, involving MAO-inhibitory properties. Given that none of these herbal medicines has been proven to have MAO-inhibitory properties, while induction of delirium, and a positive drug screening on opioids was reported, the assumption of an involvement of the valerian preparation does not seem to be plausible.

5. Discussion

A good understanding of the mechanisms of drug-drug interactions is essential for assessing and minimizing clinical risks. Indeed many drug interactions are a result of inhibition or induction of CYP enzymes. This is especially true for many antineoplastic substances. However, interactions on P-glycoprotein and conjugation mechanisms should not be neglected. Additionally, pharmacodynamic interactions have to be considered.

Herbal medicinal drugs consist of multiple components. The complex nature of herbal drugs can provide broader information on multiple interaction mechanisms and the results may change due to environmental or manufacturing differences. More information is obtained than that with a single pure natural substance.

During the past decade, several potential mechanisms of interactions of valerian preparations, involving CYP enzymes, P-glycoprotein, and UGTs, have been studied *in vitro* [36, 38–42, 46]. Some of these studies pointed to a possible drug interaction potential by valerian extracts. However, critical assessment of these studies suggests that the clinical relevance of the findings is questionable due to various methodological limitations (Table 1). This is underlined by the available clinical interaction studies on CYP isoenzymes, which do not indicate a relevant drug interaction potential of valerian in healthy volunteers [35, 37].

In this context also the amiability of *in vitro* data to the situation in humans should be addressed. Especially the open questions of *in vivo* bioavailability and of the metabolism of extract components relevant for *in vitro* effects often prevent reliable extrapolations from *in vitro* to *in vivo* data. As *in vitro* and *in vivo* correlations usually are not available, results from *in vitro* interaction studies on herbal medical drugs may not simply be transferred to the *in vivo* situation. For valerian, and similarly also for *Echinacea*, *Ginkgo*, and hawthorn, clinically relevant interactions are lacking in *in vivo* studies, despite *in vitro* studies pointing to drug interactions *in vivo*. Since

for herbal extracts a positive *in vitro and in vivo* correlation of data on drug bioavailability is rare, results from *in vitro* studies should be carefully interpreted [57].

Also the interpretation of metabolic studies in animals of different species should be critically evaluated, as often dosages far above those applied to humans are used and also regarding the enzyme variations in the species.

As for pharmacodynamic interactions of valerian, neither animal studies nor human data provide solid information for a possible risk [51, 52, 54]. Also the case reports are by no means convincing [55, 56]. At present therefore a relevant risk of pharmacodynamic interactions is not proven by valid clinical observations.

Altogether, this review could not identify studies showing a clinically relevant interaction effect of valerian. This confirms pharmacovigilance reviews of herbal medicinal products, which do not mention valerian at all [58–60] or claim valerian as safe [16, 27–31]. Block et al. [5] specifically pointed out that valerian is safe and efficient in patients undergoing cancer therapy. That therapeutic safety regarding interactions is high in valerian and is mentioned in the respective monograph of the HMPC [18] and also in a fact sheet of the Office of Dietary Supplements at the NIH [32]. In a data base for interactions of medicinal drugs (MedIQ), the rating "weak interaction" is given for CYP-isoenzymes, P-gp, and UGT, based on *in vitro* data. However, this rating is apparently the result of a merely formalistic approach and not the result from an analysis of the clinical relevance of these data.

6. Summary and Conclusions

The use of valerian preparations is very common in patients with cancer, who often receive medicines with a narrow therapeutic window. It was claimed as not being safe with regards to interactions in earlier scientific reviews.

A critical analysis of interaction data and experimental setups in papers addressed to CYP 450 isoenzymes and P-gp as targets showed limited *in vitro* interactions. However, the relevance of herb-drug interactions becomes apparent only during clinical use. So the available studies on interactions on CYP 450 enzymes do not reveal clinically relevant interactions. Also the studies and other data on pharmacodynamic interactions do not support the assumption of clinically relevant interactions. Further adverse effects, if any, seem to be very rare and reversible.

In summary, it can be concluded that warnings regarding a specific risk of interactions of valerian are without any recent evidence. Instead, valerian turns out to be an advisable treatment option, as it has a more favorable safety profile than chemically defined hypnotics and as it also improves subjective parameters of sleep quality. Warnings directed to cancer patients, in whom restless nights are responsible for continued disruption of well-being and for further impairment of health, to abstain from its use, should therefore be substantiated and specifically take the comedication into account. Otherwise, they are likely to do more harm than good.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] Sloan Kettering Center, "Integrative Medicine—Valerian," 2012, http://www.mskcc.org/cancer-care/herb/valerian.

[2] A. Sparreboom and S. D. Baker, "CAM-Chemo interactions—what is known?" in *Integrative Oncology*, D. Abrams and A. T. Weil, Eds., vol. 8 of *Weil Integrative Medicine Library*, pp. 171–194, Oxford University Press, New York, NY, USA, 2009.

[3] Deutsche Krebshilfe e.V., 2014, http://www.krebshilfe.de.

[4] M. Donaldson and R. Touger-Decker, "Dietary supplement interactions with medications used commonly in dentistry," *Journal of the American Dental Association*, vol. 144, no. 7, pp. 787–794, 2013.

[5] K. I. Block, C. Gyllenhaal, and M. N. Mead, "Safety and efficacy of herbal sedatives in cancer care," *Integrative Cancer Therapies*, vol. 3, no. 2, pp. 128–148, 2004.

[6] J. L. Hossain and C. M. Shapiro, "The prevalence, cost implications, and management of sleep disorders: an overview," *Sleep and Breathing*, vol. 6, no. 2, pp. 85–102, 2002.

[7] R. C. Kessler, P. A. Berglund, C. Coulouvrat et al., "Insomnia, comorbidity, and risk of injury among insured Americans: results from the America insomnia survey," *Sleep*, vol. 35, no. 6, pp. 825–834, 2012.

[8] M. Ohayon, "Epidemiological study on insomnia in the general population," *Sleep*, vol. 19, supplement 3, pp. S7–S15, 1996.

[9] C. M. Shapiro and W. C. Dement, "Impact and epidemiology of sleep disorders," in *ABC of Sleep Disorders*, C. M. Shapiro, Ed., pp. 1–5, BMJ, London, UK, 1993.

[10] N. S. Kamel and J. K. Gammack, "Insomnia in the elderly: cause, approach, and treatment," *The American Journal of Medicine*, vol. 119, no. 6, pp. 463–469, 2006.

[11] National Institute of Health, "NIH state-of-the-science conference statement on manifestations and management of chronic insomnia in adults," *NIH Consensus and State-of-the-Science Statements*, vol. 22, no. 2, pp. 1–30, 2005.

[12] S. H. Tariq and S. Pulisetty, "Pharmacotherapy for insomnia," *Clinics in Geriatric Medicine*, vol. 24, no. 1, pp. 93–105, 2008.

[13] P. J. Houghton, "The scientific basis for the reputed activity of Valerian," *Journal of Pharmacy and Pharmacology*, vol. 51, no. 5, pp. 505–512, 1999.

[14] S. Hadley and J. J. Petry, "Valerian," *American Family Physician*, vol. 67, no. 8, pp. 1755–1758, 2003.

[15] A. Navarrete, B. Avula, Y. Choi, and I. A. Khan, "Chemical fingerprinting of Valeriana species: simultaneous determination of valerenic acids, flavonoids, and phenylpropanoids using liquid chromatography with ultraviolet detection," *Journal of AOAC International*, vol. 89, no. 1, pp. 8–15, 2006.

[16] M. I. Fernandez-San-Martin, R. Masa-Font, L. Palacios-Soler, P. Sancho-Gomez, C. Calbo-Caldentey, and G. Flores-Mateo, "Effectiveness of Valerian on insomnia: a meta-analysis of randomized placebo-controlled trials," *Sleep Medicine*, vol. 11, no. 6, pp. 505–511, 2010.

[17] D. L. Barton, P. J. Atherton, B. A. Bauer et al., "The use of *Valeriana officinalis* (Valerian) in improving sleep in patients who are undergoing treatment for cancer: a phase III randomized, placebo-controlled, double-blind study (NCCTG Trial,

N01C5)," *Journal of Supportive Oncology*, vol. 9, no. 1, pp. 24–31, 2011.

[18] *Community Herbal Monograph on* Valeriana officinalis *L., Radix*, EMA, 2007.

[19] U. Gerhard, N. Linnenbrink, C. Georghiadou, and V. Hobi, "Vigilanzmindernde effekte zweier pflanzlicher schlafmittel," *Rundschau für Medizin*, vol. 85, no. 15, pp. 473–481, 1996.

[20] J. R. Glass, B. A. Sproule, N. Herrmann, D. Streiner, and U. E. Busto, "Acute pharmacological effects of Temazepam, Dsiphenhydramine, and Valerian in healthy elderly subjects," *Journal of Clinical Psychopharmacology*, vol. 23, no. 3, pp. 260–268, 2003.

[21] S. Gutierrez, M. K. Ang-Lee, D. J. Walker, and J. P. Zacny, "Assessing subjective and psychomotor effects of the herbal medication Valerian in healthy volunteers," *Pharmacology Biochemistry and Behavior*, vol. 78, no. 1, pp. 57–64, 2004.

[22] K. T. Hallam, J. S. Olver, C. McGrath, and T. R. Norman, "Comparative cognitive and psychomotor effects of single doses of *Valeriana officianalis* and triazolam in healthy volunteers," *Human Psychopharmacology*, vol. 18, no. 8, pp. 619–625, 2003.

[23] J. Kuhlmann, W. Berger, H. Podzuweit, and U. Schmidt, "The influence of Valerian treatment on "reaction time, alertness and concentration" in volunteers," *Pharmacopsychiatry*, vol. 32, no. 6, pp. 235–241, 1999.

[24] H. P. Garges, I. Varia, and P. M. Doraiswamy, "Cardiac complications and delirium associated with Valerian root withdrawal," *Journal of the American Medical Association*, vol. 280, no. 18, pp. 1566–1567, 1998.

[25] M. K. Ang-Lee, J. Moss, and C. S. Yuan, "Herbal medicines and perioperative care," *Journal of the American Medical Association*, vol. 286, no. 2, pp. 208–216, 2001.

[26] W. Abebe, "Herbal medication: potential for adverse interactions with analgesic drugs," *Journal of Clinical Pharmacy and Therapeutics*, vol. 27, no. 6, pp. 391–401, 2002.

[27] A. A. Izzo, "Interactions between herbs and conventional drugs: overview of the clinical data," *Medical Principles and Practice*, vol. 21, no. 5, pp. 404–428, 2012.

[28] D. M. Taibi, C. A. Landis, H. Petry, and M. V. Vitiello, "A systematic review of Valerian as a sleep aid: safe but not effective," *Sleep Medicine Reviews*, vol. 11, no. 3, pp. 209–230, 2007.

[29] S. L. Plushner, "Valerian: *Valeriana officinalis*," *American Journal of Health-System Pharmacy*, vol. 57, no. 4, pp. 328–335, 2000.

[30] B. Steinhoff, "Current perspectives on herb-drug interactions in the European regulatory landscape," *Planta Medica*, vol. 78, no. 13, pp. 1416–1420, 2012.

[31] D. Wheatley, "Medicinal plants for insomnia: a review of their pharmacology, efficacy and tolerability," *Journal of Psychopharmacology*, vol. 19, no. 4, pp. 414–421, 2005.

[32] Office of Dietary Supplements at the NIH, "Factsheet Valerian," 2012, http://ods.od.nih.gov/factsheets/Valerian-HealthProfessional.

[33] MedIQ-Datenbank, 2014, http://www.mediq.ch.

[34] K. Yokotani, T. Chiba, Y. Sato, T. Nakanishi, M. Murata, and K. Umegaxj, "Effect of three herbal extracts on cytochrome P450 and possibility of interaction with drugs," *Shokuhin Eiseigaku Zasshi*, vol. 54, no. 1, pp. 56–64, 2013.

[35] B. J. Gurley, S. F. Gardner, M. A. Hubbard et al., "In vivo effects of goldenseal, kava kava, black cohosh, and Valerian on human cytochrome P450 1A2, 2D6, 2E1, and 3A4/5 phenotypes," *Clinical Pharmacology and Therapeutics*, vol. 77, no. 5, pp. 415–426, 2005.

[36] B. H. Hellum, Z. Hu, and O. G. Nilsen, "The induction of CYP1A2, CYP2D6 and CYP3A4 by six trade herbal products in cultured primary human hepatocytes," *Basic and Clinical Pharmacology and Toxicology*, vol. 100, no. 1, pp. 23–30, 2007.

[37] J. L. Donovan, C. L. DeVane, K. D. Chavin et al., "Multiple night-time doses of Valerian (*Valeriana officinalis*) had minimal effects on CYP3A4 activity and no effect on CYP2D6 activity in healthy volunteers," *Drug Metabolism and Disposition*, vol. 32, no. 12, pp. 1333–1336, 2004.

[38] J. W. Budzinski, B. C. Foster, S. Vandenhoek, and J. T. Arnason, "An in vitro evaluation of human cytochrome P450 3A4 inhibition by selected commercial herbal extracts and tinctures," *Phytomedicine*, vol. 7, no. 4, pp. 273–282, 2000.

[39] T. Lefebvre, B. C. Foster, C. E. Drouin et al., "In vitro of commercial Valerian root extracts against human cytochrome P450 3A4," *Journal of Pharmacy and Pharmaceutical Sciences*, vol. 7, no. 2, pp. 265–273, 2004.

[40] B. H. Hellum and O. G. Nilsen, "In vitro inhibition of CYP3A4 metabolism and P-glycoprotein-mediated transport by trade herbal products," *Basic and Clinical Pharmacology and Toxicology*, vol. 102, no. 5, pp. 466–475, 2008.

[41] K. M. Alkharfy and R. F. Frye, "Effect of Valerian, Valerian/hops extracts, and valerenic acid on glucuronidation in vitro," *Xenobiotica*, vol. 37, no. 2, pp. 113–123, 2007.

[42] M. F. Mohamed, T. Tseng, and R. F. Frye, "Inhibitory effects of commonly used herbal extracts on UGT1A1 enzyme activity," *Xenobiotica*, vol. 40, no. 10, pp. 663–669, 2010.

[43] A. Mozayani and L. Raymon, *Handbook of Drug Interactions: A Clinical and Forensic Guide*, Humana Press, New York, NY, USA, 2nd edition, 2012.

[44] K. A. Bachmann and J. D. Lewis, "Predicting inhibitory drug-drug interactions and evaluating drug interaction reports using inhibition constants," *Annals of Pharmacotherapy*, vol. 39, no. 6, pp. 1064–1072, 2005.

[45] J. S. Markowitz and H. J. Zhu, "Limitations of in vitro assessments of the drug interaction potential of botanical supplements," *Planta Medica*, vol. 78, no. 13, pp. 1421–1427, 2012.

[46] B. H. Hellum and O. G. Nilsen, "The in vitro inhibitory potential of trade herbal products on human CYP2D6-mediated metabolism and the influence of ethanol," *Basic and Clinical Pharmacology and Toxicology*, vol. 101, no. 5, pp. 350–358, 2007.

[47] B. H. Hellum, Z. Hu, and O. G. Nilsen, "Tradeherbal products and induction of cyp2c19 and cyp2e1 in cultured human hepatocytes," *Basic and Clinical Pharmacology and Toxicology*, vol. 105, no. 1, pp. 58–63, 2009.

[48] M. F. Mohamed and R. F. Frye, "Effects of herbal supplements on drug glucuronidation. Review of clinical, animal, and in vitro studies," *Planta Medica*, vol. 77, no. 4, pp. 311–321, 2011.

[49] G. Trauner, S. Khom, I. Baburin, B. Benedek, S. Hering, and B. Kopp, "Modulation of GABAA receptors by Valerian extracts is related to the content of valerenic acid," *Planta Medica*, vol. 74, no. 1, pp. 19–24, 2008.

[50] S. Khom, I. Baburin, E. Timin et al., "Valerenic acid potentiates and inhibits GABAA receptors: molecular mechanism and subunit specificity," *Neuropharmacology*, vol. 53, no. 1, pp. 178–187, 2007.

[51] C. L. Dalla Corte, R. Fachinetto, D. Colle et al., "Potentially adverse interactions between haloperidol and Valerian," *Food and Chemical Toxicology*, vol. 46, no. 7, pp. 2369–2375, 2008.

[52] R. Fachinetto, J. G. Villarinho, C. Wagner et al., "*Valeriana officinalis* does not alter the orofacial dyskinesia induced by

haloperidol in rats: role of dopamine transporter," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 31, no. 7, pp. 1478–1486, 2007.

[53] C. Bhatt, N. Kanaki, R. Nayak, and G. Shah, "Synergistic potentiation of anti-anxiety activity of Valerian and alprazolam by liquorice," *Indian Journal of Pharmacology*, vol. 45, no. 2, pp. 202–203, 2013.

[54] R. Kohnen and W.-D. Oswald, "The effects of Valerian, propranolol, and their combination on activation, performance, and mood of healthy volunteers under social stress conditions," *Pharmacopsychiatry*, vol. 21, no. 6, pp. 447–448, 1988.

[55] M. C. Carrasco, J. R. Vallejo, M. Pardo-de-Santayana, D. Peral, M. A. Martin, and J. Altimiras, "Interactions of *Valeriana officinalis* L. and *Passiflora incarnata* L. in a patient treated with lorazepam," *Phytotherapy Research*, vol. 23, no. 12, pp. 1795–1796, 2009.

[56] I. S. Khawaja, R. F. Marotta, and S. Lippmann, "Herbal medicines as a factor in delirium," *Psychiatric Services*, vol. 50, no. 7, pp. 969–970, 1999.

[57] M. Unger, "Pharmakokinetische arzneimittelinteraktionen durch pflanzliche arzneimittel," *Wiener Medizinische Wochenschrift*, vol. 160, no. 21-22, pp. 571–577, 2010.

[58] K. I. Block and C. Gyllenhaal, "Clinical corner: herb-drug interactions in cancer chemotherapy: theoretical concerns regarding drug metabolizing enzymes," *Integrative Cancer Therapies*, vol. 1, no. 1, pp. 83–89, 2002.

[59] A. Paoletti, E. Gallo, S. Benemei et al., "Interactions between natural health products and oral anticoagulants: spontaneous reports in the Italian surveillance system of natural health products," *Evidence-Based Complementary and Alternative Medicine*, vol. 2011, Article ID 612150, 5 pages, 2011.

[60] R. Nowack, C. Balle, F. Birnkammer, W. Koch, R. Sessler, and R. Birck, "Impact of food and herbal medication on calcineurin inhibitor dose in renal transplant patients: a cross-sectional study," *Journal of Medicinal Food*, vol. 14, no. 7-8, pp. 756–760, 2011.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2014, Article ID 150351, 5 pages
http://dx.doi.org/10.1155/2014/150351

Research Article

Inhibition of Human Cytochrome P450 Enzymes by Allergen Removed *Rhus verniciflua* Stoke Standardized Extract and Constituents

Hyunsik Jung and Sanghun Lee

Department of Medical Consilience, Graduate School, Dankook University, 152, Jukjeon-ro, Suji-gu, Yongin-si, Gyeonggi-do 448-701, Republic of Korea

Correspondence should be addressed to Sanghun Lee; integrative@korea.com

Received 22 April 2014; Accepted 9 June 2014; Published 30 June 2014

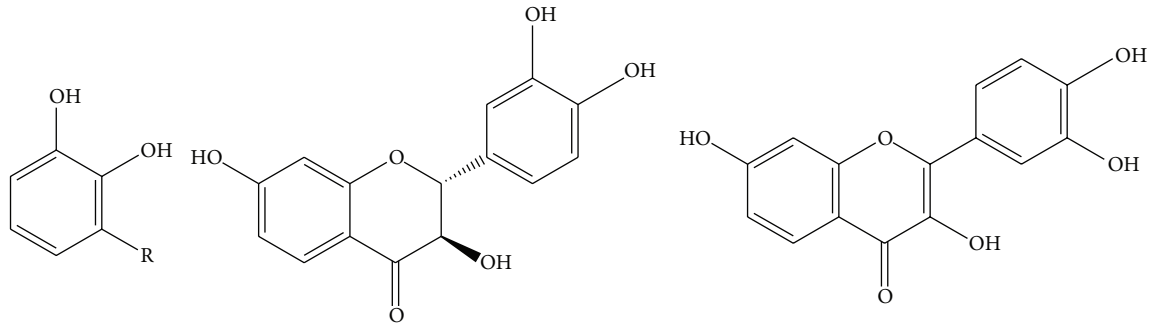
Academic Editor: Mohamed Eddouks

Objective. Potential interactions between herbal extracts and the cytochrome P450 (CYP) system lead to serious adverse events or decreased drug efficacy. *Rhus verniciflua* stoke (RVS) and its constituents have been reported to have various pharmacological properties. We evaluated the inhibitory potential of RVS and its constituents on the major CYP isoforms. *Methods*. The effects of allergen removed RVS (aRVS) standardized extract and major components, fustin and fisetin isolated from aRVS, were evaluated on CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 isoenzyme activity by a luminescent CYP recombinant human enzyme assay. *Results*. The aRVS extract showed relative potent inhibitory effects on the CYP2C9 (IC_{50}, <0.001 μg/mL), CYP2C19 (IC_{50}, 9.68 μg/mL), and CYP1A2 (IC_{50}, 10.0 μg/mL). However, it showed weak inhibition on CYP3A4 and CYP2D6. Fustin showed moderate inhibitory effects on the CYP2C19 (IC_{50}, 64.3 μg/mL) and weak inhibition of the other CYP isoforms similar to aRVS. Fisetin showed potent inhibitory effects on CYP2C9, CYP2C19, and CYP1A2. Fisetin showed moderate inhibition of CYP2D6 and weak inhibition of CYP3A4. *Conclusions*. These results indicate that aRVS, a clinically available herbal medicine, could contribute to herb-drug interactions when orally coadministered with drugs metabolized by CYP2C9, CYP2C19, and CYP1A2.

1. Introduction

Nowadays, the paradigm of chemotherapies in cancer patients has changed with the development of chemotherapy such as capecitabine and targeted agents including tyrosine kinase inhibitors (TKI) and mammalian target of rapamycin (mTOR) inhibitors [1, 2]. These orally administered chemotherapeutic agents prescribed in the treatment of numerous cancers are metabolized by hepatic cytochrome P450 enzymes (CYP). Therefore, drug interaction in cancer patients must be noted because it can lead to overdosing or undertreatment resulting in severe clinical consequences compared to traditional time-limited cytotoxic chemotherapy [3, 4]. In addition, most herbal medicines which are orally administered could affect the activity of the intestinal affecting bioavailability of coadministered drugs [5, 6]. In addition, several herbs can give rise to the potential of harmful interactions with the targeted agents [7].

Rhus verniciflua stokes (RVS) of the Anacardiaceae family, commonly known as the lacquer tree, have been used in Korean medicine for centuries to treat diseases of the digestive system, including tumors [8]. Several preclinical studies of its flavonoids and other extracts have reported that it has antioxidant, anti-inflammatory, antiproliferative, and anti-cancer activities [9–18]. However, the clinical application of RVS has been restricted because urushiol (Figure 1(a)) can cause an allergic skin rash on contact, known as urushiol-induced contact dermatitis in sensitive individuals [19]. Therefore, urushiol, which is a mixture of several derivatives of catechol, should be removed from RVS for pharmaceutical use. Recently, several clinical reports of a standardized extract with the allergen removed RVS (aRVS) based on traditional method showed promising outcomes in advanced cancer patients [20–25]. Therefore, the potential effects of aRVS-drug interactions should be tested for the patients with concurrent chemotherapy.



(a) (b) (c)

R: $(CH_2)_{14}CH_3$ or

R: $(CH_2)_7CH{=}CH(CH_2)_5CH_3$ or

R: $(CH_2)_7CH{=}CHCH_2CH{=}CH(CH_2)_2CH_3$ or

R: $(CH_2)_7CH{=}CHCH_2CH{=}CHCH{=}CHCH_3$ or

R: $(CH_2)_7CH{=}CHCH_2CH{=}CHCH_2CH{=}CH_2$ and others

Figure 1: Structures of *Rhus verniciflua* stokes constituents. (a) Urushiol: the likelihood and severity of allergic reaction to urushiol are dependent on the degree of unsaturation of the alkyl chain. (b) Fustin: 2-(3,4-dihydroxyphenyl)-3,7-dihydroxy-2,3-dihydrochromen-4-one. (c) Fisetin, 2-(3,4-dihydroxyphenyl)-3,7-dihydroxychromen-4-one.

In this study, aRVS standardized extract and some of its major and commercially available constituents, including fustin (>13.0% in aRVS) and fisetin (>2.0% in aRVS) (Figures 1(b) and 1(c)), were selected to study their inhibitory effects on the five major CYP isoforms (CYP1A2, CYP3A4, CYP2C9, CYP2C19, and CYP2D6) involved in the hepatic metabolism of most drugs.

2. Materials and Methods

2.1. Plant Materials. The standardized extract from RVS was prepared as follows. RVS stalk, which was 10 years old and grew in Wonju, Republic of Korea, was dried without exposure to direct sunlight and chopped up. The pieces were extracted two times with a 10-fold volume of water at 90°C to 95°C for 6 hours according to Korean patent no. 0504160. The extract was filtered with Whatman GF/B filter paper and concentrated under vacuum to remove water. The concentrate was lyophilized to a brownish powder. The extract yield from 100 g of chopped material was 3.3 g. A component analysis method using high performance liquid chromatography showed that the aRVS extract contained fustin, fisetin, sulfuretin, and butein, among others. The quality of the aRVS extract was tested and controlled according to the quality standards of the Korea Food & Drug Administration and our hospital's standards (fustin > 13.0%, fisetin > 2.0%, and urushiol not detected) [8]. The standard aRVS samples were comprised of 8 serial dilutions of aRVS, ranging between 0.001–10,000 μg/mL dissolved in 50% methanol. Fustin (3,3′,4′,7-Tetrahydroxyflavanone, $C_{15}H_{12}O_6$) is a flavonoid and constitutes 13–30% (w/w) of aRVS. The fustin extracted from aRVS by high-performance liquid chromatography mass spectroscopy had a purity of >95%. The standard samples were comprised of 8 serial dilutions of fustin, ranging between 0.001 and 10,000 μg/mL dissolved in 10% methanol. The fisetin (3,3′,4′,7-Tetrahydroxyflavone, $C_{15}H_{10}O_6$) is also a flavonoid and constitutes 2–5% (w/w) of aRVS. The fisetin was purchased from Sigma Chemical Co. (St. Louis, MO, USA) with a purity of >95%, because the fisetin extracted from aRVS was hard to ensure its purity. The standard samples were comprised of 8 serial dilutions of fisetin, ranging between 0.001 and 5,000 μg/mL dissolved in 70% methanol.

2.2. Luminogenic P450 Enzymes Inhibition Assay. The assay was carried out using P450-Glo Screening system from Promega (Promega Inc., Madison Wis., USA). It contains recombinant human CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 enzymes in the membranes produced by a baculovirus expression system, specific luminogenic cytochrome enzyme substrates (luciferin 6-methyl ether, luciferin 6-benzyl ether, 6-deoxyluciferin, ethylene glycol ester of 6-deoxyluciferin, and ethylene glycol ester of luciferin 6-methyl ether), negative control membranes, a nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) regeneration system containing NADP+, glucose-6-phosphate, magnesium chloride ($MgCl_2$) and glucose-6-phosphate dehydrogenase (functioning to initiate and sustain the CYP450 reaction by maintaining a non-limiting NADPH system), reaction buffer, luciferin detection reagent, and Luciferin-Free Water. The negative control membranes were devoid of CYP activity. The luminogenic inhibition assays were performed following the protocols from Promega Corp. (Technical Bulletin, P450-Glo Assays, Promega Corp., 2009) and previous studies [26, 27].

Samples of different concentrations of both the aRVS extract and the two compounds were prepared. Firstly, 12.5 μL

Table 1: The IC_{50} values of the aRVS extract, fustin, and fisetin (μg/mL).

	aRVS	Fustin	Fisetin
CYP1A2	10.0	4930.0	0.46
CYP2C9	<0.001	540.2	5.4
CYP2C19	9.68	64.3	<0.001
CYP2D6	503.3	5346.1	46.8
CYP3A4	391.8	463.0	309.0

of the 4× aRVS extract or the test compounds (appropriate for each enzyme) was added to the "treated" wells. The 12.5 μL of the luciferin-free water was added individually in both the "untreated" wells representing the values of total CYP activity and the "minus-P450 control" wells representing the values of the CYP-independent background luminescence. Secondly, 12.5 μL of the 4× reaction mixture (containing human CYP membrane preparations, the appropriate luminogenic substrate, and potassium phosphate buffer) was added to the "treated" and the "untreated" wells. The 12.5 μL of the 4× control reaction mixture (containing membrane preparations devoid of CYP enzymes, the appropriate luminogenic substrate, and potassium phosphate buffer) was added to the "minus-P450 control" wells. The plate was preincubated at room temperature (25°C) for 10 min, and then 25 μL of the 2× NADPH regeneration system was added to initiate the reaction. After this was incubated at room temperature for 30 min (45 min for CYP2D6), 50 μL of the reconstituted luciferin detection reagent was added to stop the reaction and produce the luminescent signal. The luminescence in all of the samples was measured using a microplate reader as relative light units (RLU) after 20 min incubation to stabilize the luminescent signal. The assay experiments were repeated three independent times.

2.3. Statistical Analysis. The amount of light produced is directly proportional to the CYP enzyme activity. The net signals from untreated CYP reactions represent total CYP activity (100%). The modulation of the CYP activity by the test compound was determined by comparing the changes from the average net signal of untreated CYP reactions with the changes observed from the test compound. The IC_{50} values showing more than 50% inhibition were calculated by plotting the percent inhibition of CYPs enzyme activities versus log concentration of the extract or compounds using GraphPad Prism (version 5.01. USA).

3. Results

The inhibitory potencies of aRVS extract and the constituents against CYP450 were determined by evaluating the IC_{50} values. The potency of a test compound can be classified by its IC_{50} value according to the following scale: potent if IC_{50} < 20 μg/mL, moderate if IC_{50} = 20–100 μg/mL, or weak if IC_{50} > 100 μg/mL [26]. The aRVS extract was found to exhibit potent inhibitory activity against CYP2C9, CYP2C19, and CYP1A2 (Table 1), with most potent inhibition on CYP2C9 (IC_{50} < 0.001 μg/mL). Among the components, fisetin showed much more inhibitory activity against all the CYPs compared to fustin. Like aRVS extract, fisetin was found to exhibit potent inhibitory activity against CYP2C19, CYP1A2, and CYP2C9 (Table 1), with most potent inhibition on CYP2C19 (IC_{50} < 0.001 μg/mL). However, like aRVS, fisetin showed weak inhibition towards the CYP3A4 and CYP2D6 (Table 1).

4. Discussion

The recombinant human CYP enzyme study found that aRVS extract potently inhibits CYP2C9 and so less CYP2C19 and CYP1A2 and weakly inhibits CYP3A4 and CYP2D6. Moreover, the major flavonoid, fisetin, potently inhibits CYP2C19 and so less CYP1A2 and CYP2C9, moderately inhibits CYP2D6, and weakly inhibits CYP3A4. On the other hand, fustin showed negligible inhibition towards the CYP enzymes except CYP2C19. The above result suggests that the tested fisetin is responsible for the inhibition of the CYP isoforms by aRVS extract.

CYP inhibition can lead to higher levels of the cytotoxic drug, causing recognizable, greater toxicity, while inactive prodrugs such as cyclophosphamide or ifosfamide often lead to therapeutic failure because of lower plasma levels of the chemotherapeutic drug [2, 6]. Among the CYP enzymes, CYP3A4 and CYP2D6 are the most important enzymes in the metabolism of anticancer drugs [1, 2]. CYP3A4, which is the most abundant enzyme (~80%) in the intestinal mucosa, should be the most important contributor in drug metabolism [28]. Since most herbal medicines including aRVS extract are administered orally, a high concentration in the gut might cause significant inhibition of CYP3A4. However, our results suggest that aRVS extract and the tested compounds are not likely to inhibit the metabolism of a chemotherapeutic drug whose primary route of elimination is through CYP3A4 or CYP2D6.

CYP2C9, which comprises about 15% of the CYP in the intestinal mucosa, is involved in the metabolism of some chemotherapeutic drugs such as capecitabine, cyclophosphamide, and ifosfamide, drugs with a narrow therapeutic index such as warfarin and phenytoin, and other drugs such as acenocoumarol, tolbutamide, losartan, glipizide, and some nonsteroidal anti-inflammatory drugs [2, 4, 29]. Flavones and flavonols are two major classes of flavonoids, polyphenolic secondary metabolites, which also act as potent inhibitors of CYP2C9 [30].

In our study, fustin (2-(3,4-dihydroxyphenyl)-3,7-dihydroxy-2,3-dihydrochromen-4-one) is a flavanonol, a type of flavonoid. The fisetin (2-(3,4-dihydroxyphenyl)-3,7-dihydroxychromen-4-one) is a flavonol, a structurally distinct chemical substance that belongs to the flavonoid group of polyphenols. It was reported to have multiple pharmaceutical properties such as antiaging, anti-inflammatory, anticancer, and antiviral effects in different lines of culture cells [11, 31]. Several herbs have been reported to show the inhibition of CYP2C19 as well as CYP2C9 [26, 32]. Therefore, inhibition of CYP2C9 and CYP2C19 by aRVS extract is mainly due to the fisetin.

Furthermore, the genetically polymorphic CYP2C9 and CYP2C19 show different drug excretion rates and final serum concentrations in different people. An extensive metabolizer has two copies of wild-type alleles, whereas poor metabolizers have two copies of variant alleles, causing reduced enzymatic activity [33, 34]. The CYP2C9*2 and CYP2C9*3, recognized as main CYP2C9 variants in humans, have reduced catalytic activity compared with the wild-type CYP2C9*1. The CYP2C19*2 and CYP2C19*3 variant alleles account for the majority of the poor metabolizer phenotypes. Considering that poor metabolizers with CYP2C9 and CYP2C19 variants are more prominent in Asian population (15–20%) than in Caucasians and African-American populations (3–5%) [35], further inhibition of CYP2C9 and CYP2C19 by herbal medicine, including aRVS extract, likely cause clinically significant herb-drug interactions in the Asian population.

CYP1A2 is able to metabolize some polycyclic aromatic hydrocarbons (PAHs), a procarcinogen, to carcinogenic intermediates, so that higher CYP1A2 activity may influence the risk of lung cancer and breast cancer [36, 37]. CYP1A2 is also involved in the metabolism of antidepressants drugs and antipsychotic drugs [29]. Dietary flavonoids are important contributors for cancer prevention, due to their inhibition of CYP1A2 activity [38]. Therefore, the mechanism of the anticancer effect from aRVS extract in practice might be explained in the view of CYP1A2 inhibition or its genetic polymorphism.

Our in vitro results suggested that aRVS extract potently inhibits CYP2C9, CYP2C19, and CYP1A2, which means aRVS extract in conjunction with chemotherapeutic drugs such as capecitabine, cyclophosphamide, or ifosfamide, or the drugs mentioned above could increase or decrease the plasma level of the drugs, which could result in significant clinical consequences. In contrast, the concurrent administration of aRVS and cisplatin was reported not to alter the antitumor efficacy of cisplatin. In addition, aRVS prevents cisplatin-induced toxicity in vitro and in vivo via an antioxidant activity [39].

Therefore, in vivo investigation of aRVS extract's inhibition of CYP2C9 and CYP2C19 is needed to study drug-herb interaction. The genetic polymorphism of CYP2C9 and CYP2C19 should also be studied, because a better understanding will instruct individuals with ultraextensive or poor metabolism on clinically important herb-drug interactions.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

The present research was conducted by the research fund of Dankook University in 2013.

References

[1] A. Thomas-Schoemann, B. Blanchet, C. Bardin et al., "Drug interactions with solid tumour-targeted therapies," *Critical Reviews in Oncology/Hematology*, vol. 89, no. 1, pp. 179–196, 2014.

[2] C. D. Scripture and W. D. Figg, "Drug interactions in cancer therapy," *Nature Reviews Cancer*, vol. 6, no. 7, pp. 546–558, 2006.

[3] R. W. F. van Leeuwen, E. L. Swart, E. Boven, F. A. Boom, M. G. Schuitenmaker, and J. G. Hugtenburg, "Potential drug interactions in cancer therapy: a prevalence study using an advanced screening method," *Annals of Oncology*, vol. 22, no. 10, pp. 2334–2341, 2011.

[4] G. Giunta, "Adverse interaction between capecitabine and warfarin resulting in altered coagulation parameters: a review of the literature starting from a case report," *Case Reports in Medicine*, vol. 2010, Article ID 426804, 4 pages, 2010.

[5] A. K. Goey, J. H. Beijnen, and J. H. Schellens, "Herb-drug interactions in oncology," *Clinical Pharmacology & Therapeutics*, vol. 95, no. 4, pp. 354–355, 2014.

[6] I. Meijerman, J. H. Beijnen, and J. H. M. Schellens, "Herb-drug interactions in oncology: focus on mechanisms of induction," *Oncologist*, vol. 11, no. 7, pp. 742–752, 2006.

[7] S. W. Hwang, H. S. Han, K. Y. Lim, and J. Y. Han, "Drug interaction between complementary herbal medicines and gefitinib," *Journal of Thoracic Oncology*, vol. 3, no. 8, pp. 942–943, 2008.

[8] W. Choi, H. Jung, K. Kim et al., "*Rhus verniciflua* stokes against advanced cancer: a perspective from the korean integrative cancer center," *Journal of Biomedicine and Biotechnology*, vol. 2012, Article ID 874276, 7 pages, 2012.

[9] J. S. Shin, Y. M. Park, J. H. Choi et al., "Sulfuretin isolated from heartwood of Rhus verniciflua inhibits LPS-induced inducible nitric oxide synthase, cyclooxygenase-2, and pro-inflammatory cytokines expression via the down-regulation of NF-κB in RAW 264.7 murine macrophage cells," *International Immunopharmacology*, vol. 10, no. 8, pp. 943–950, 2010.

[10] D. S. Lee, G. S. Jeong, B. Li, H. Park, and Y. Kim, "Anti-inflammatory effects of sulfuretin from *Rhus verniciflua* Stokes via the induction of heme oxygenase-1 expression in murine macrophages," *International Immunopharmacology*, vol. 10, no. 8, pp. 850–858, 2010.

[11] J. D. Lee, J. E. Huh, G. Jeon et al., "Flavonol-rich RVHxR from *Rhus verniciflua* Stokes and its major compound fisetin inhibits inflammation-related cytokines and angiogenic factor in rheumatoid arthritic fibroblast-like synovial cells and *in vivo* models," *International Immunopharmacology*, vol. 9, no. 3, pp. 268–276, 2009.

[12] J. H. Kim, C. H. Jung, B. Jang et al., "Selective cytotoxic effects on human cancer cell lines of phenolic-rich ethyl-acetate fraction from Rhus verniciflua stokes," *American Journal of Chinese Medicine*, vol. 37, no. 3, pp. 609–620, 2009.

[13] J. H. Kim, H. Y. Go, D. H. Jin et al., "Inhibition of the PI3K-Akt/PKB survival pathway enhanced an ethanol extract of *Rhus verniciflua* Stokes-induced apoptosis via a mitochondrial pathway in AGS gastric cancer cell lines," *Cancer Letters*, vol. 265, no. 2, pp. 197–205, 2008.

[14] C. H. Jung, J. H. Kim, M. H. Hong et al., "Phenolic-rich fraction from *Rhus verniciflua* Stokes (RVS) suppress inflammatory response via NF-κB and JNK pathway in lipopolysaccharide-induced RAW 264.7 macrophages," *Journal of Ethnopharmacology*, vol. 110, no. 3, pp. 490–497, 2007.

[15] Y. O. Son, K. Y. Lee, J. C. Lee et al., "Selective antiproliferative and apoptotic effects of flavonoids purified from Rhus verniciflua Stokes on normal versus transformed hepatic cell lines," *Toxicology Letters*, vol. 155, no. 1, pp. 115–125, 2005.

[16] H. S. Jang, S. H. Kook, Y. O. Son et al., "Flavonoids purified from Rhus verniciflua Stokes actively inhibit cell growth and induce apoptosis in human osteosarcoma cells," *Biochimica et Biophysica Acta*, vol. 1726, no. 3, pp. 309–316, 2005.

[17] I. T. Kim, Y. M. Park, K. M. Shin et al., "Anti-inflammatory and anti-nociceptive effects of the extract from Kalopanax pictus, Pueraria thunbergiana and Rhus verniciflua," *Journal of Ethnopharmacology*, vol. 94, no. 1, pp. 165–173, 2004.

[18] K. T. Lim, C. Hu, and D. D. Kitts, "Antioxidant activity of a *Rhus verniciflua* Stokes ethanol extract," *Food and Chemical Toxicology*, vol. 39, no. 3, pp. 229–237, 2001.

[19] S. D. Park, S. W. Lee, J. H. Chun, and S. H. Cha, "Clinical features of 31 patients with systemic contact dermatitis due to the ingestion of Rhus (lacquer)," *British Journal of Dermatology*, vol. 142, no. 5, pp. 937–942, 2000.

[20] W. Choi, S. An, E. Kwon, W. Eo, and S. Lee, "Impact of standardized allergen-removed Rhus verniciflua stokes extract on advanced adenocarcinoma of the ampulla of Vater: a case series," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 203168, 8 pages, 2013.

[21] S. Lee, K. Kim, H. Jung et al., "Efficacy and safety of standardized allergen-removed Rhus verniciflua Stokes extract in patients with advanced or metastatic pancreatic cancer: a Korean single-center experience," *Oncology*, vol. 81, no. 5-6, pp. 312–318, 2012.

[22] S. K. Lee, H. S. Jung, W. K. Eo, S. Y. Lee, S. H. Kim, and B. S. Shim, "Rhus verniciflua Stokes extract as a potential option for treatment of metastatic renal cell carcinoma: report of two cases," *Annals of Oncology*, vol. 21, no. 6, pp. 1383–1385, 2010.

[23] S. H. Lee, W. C. Choi, K. S. Kim, J. W. Park, and S. W. Yoon, "Shrinkage of gastric cancer in an elderly patient who received *Rhus verniciflua* stokes extract," *Journal of Alternative and Complementary Medicine*, vol. 16, no. 4, pp. 497–500, 2010.

[24] H. R. Kim, K. S. Kim, H. S. Jung, W. C. Choi, W. K. Eo, and S. H. Cheon, "A case of recurred hepatocellular carcinoma refractory to doxorubicin after liver transplantation showing response to herbal medicine product, *Rhus verniciflua* stokes extract," *Integrative Cancer Therapies*, vol. 9, no. 1, pp. 100–104, 2010.

[25] S. H. Lee, K. S. Kim, W. C. Choi, and S. W. Yoon, "Successful outcome of advanced pulmonary adenocarcinoma with malignant pleural effusion by the standardized rhus verniciflua stokes extract: a case study," *Explore*, vol. 5, no. 4, pp. 242–244, 2009.

[26] S. Ramasamy, L. V. Kiew, and L. Y. Chung, "Inhibition of human cytochrome P450 enzymes by Bacopa monnieri standardized extract and constituents," *Molecules*, vol. 19, no. 2, pp. 2588–2601, 2014.

[27] N. A. Hanapi, J. Azizi, S. Ismail, and S. M. Mansor, "Evaluation of selected Malaysian medicinal plants on phase I drug metabolizing enzymes, CYP2C9, CYP2D6 and CYP3A4 activities in vitro," *International Journal of Pharmacology*, vol. 6, no. 4, pp. 494–499, 2010.

[28] M. F. Paine, H. L. Hart, S. S. Ludington, R. L. Haining, A. E. Rettie, and D. C. Zeldin, "The human intestinal cytochrome P450 'pie'," *Drug Metabolism and Disposition*, vol. 34, no. 5, pp. 880–886, 2006.

[29] T. Lynch and A. Price, "The effect of cytochrome P450 metabolism on drug response, interactions, and adverse effects," *American Family Physician*, vol. 76, no. 3, pp. 391–396, 2007.

[30] D. Si, Y. Wang, Y. Zhou et al., "Mechanism of CYP2C9 inhibition by flavones and flavonols," *Drug Metabolism and Disposition*, vol. 37, no. 3, pp. 629–634, 2009.

[31] N. Khan, D. N. Syed, N. Ahmad, and H. Mukhtar, "Fisetin: a dietary antioxidant for health promotion," *Antioxidants and Redox Signaling*, vol. 19, no. 2, pp. 151–162, 2013.

[32] P. G. Sand, A. Dreiseitel, M. Stang et al., "Cytochrome P450 2C19 inhibitory activity of common berry constituents," *Phytotherapy Research*, vol. 24, no. 2, pp. 304–307, 2010.

[33] J. A. Goldstein, "Clinical relevance of genetic polymorphisms in the human CYP2C subfamily," *British Journal of Clinical Pharmacology*, vol. 52, no. 4, pp. 349–355, 2001.

[34] K. Kim, J. A. Johnson, and H. Derendorf, "Differences in drug pharmacokinetics between East Asians and Caucasians and the role of genetic polymorphisms," *Journal of Clinical Pharmacology*, vol. 44, no. 10, pp. 1083–1105, 2004.

[35] Z. F. Yang, H. W. Cui, T. Hasi, S. Q. Jia, M. L. Gong, and X. L. Su, "Genetic polymorphisms of cytochrome P450 enzymes 2C9 and 2C19 in a healthy Mongolian population in China," *Genetics and Molecular Research*, vol. 9, no. 3, pp. 1844–1851, 2010.

[36] Y. Osawa, K. K. Osawa, A. Miyaishi et al., "NAT2 and CYP1A2 polymorphisms and lung cancer risk in relation to smoking status," *Asian Pacific Journal of Cancer Prevention*, vol. 8, no. 1, pp. 103–108, 2007.

[37] I. Ayari, U. Fedeli, S. Saguem, S. Hidar, S. Khlifi, and S. Pavanello, "Role of CYP1A2 polymorphisms in breast cancer risk in women," *Molecular Medicine Reports*, vol. 7, no. 1, pp. 280–286, 2013.

[38] S. Lautraite, A. C. Musonda, J. Doehmer, G. O. Edwards, and J. K. Chipman, "Flavonoids inhibit genetic toxicity produced by carcinogens in cells expressing CYP1A2 and CYP1A1," *Mutagenesis*, vol. 17, no. 1, pp. 45–53, 2002.

[39] J. Lee, H. Lee, W. Choi et al., "*Rhus verniciflua* stokes prevents cisplatin-induced cytotoxicity and reactive oxygen species production in MDCK-I renal cells and intact mice," *Phytomedicine*, vol. 16, no. 2-3, pp. 188–197, 2009.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2014, Article ID 970385, 6 pages
http://dx.doi.org/10.1155/2014/970385

Research Article

Influence of Herbal Complexes Containing Licorice on Potassium Levels: A Retrospective Study

WooSang Jung,[1] SeungWon Kwon,[1] JinWook Im,[1] SeongUk Park,[2] SangKwan Moon,[1] JungMi Park,[2] ChangNam Ko,[2] and KiHo Cho[1]

[1] *Department of Cardiovascular and Neurologic Diseases, Kyung Hee University Oriental Medicine Hospital, 1 Hoegi-dong, Dongdaemun-gu, Seoul 130-702, Republic of Korea*
[2] *Department of Cardiovascular and Neurologic Diseases, Kyung Hee University Hospital at Gangdong, Seoul 134-727, Republic of Korea*

Correspondence should be addressed to SeungWon Kwon; kkokkottung@hanmail.net

Received 11 February 2014; Revised 1 May 2014; Accepted 19 May 2014; Published 19 June 2014

Academic Editor: Paul Posadzki

To observe the influence of these complexes on potassium levels in a clinical setting, we investigated the influence of herbal complexes containing licorice on potassium levels. We retrospectively examined the medical records of patients treated with herbal complexes containing licorice from January 1, 2010, to December 31, 2010. We recorded the changes in the levels of potassium, creatinine, and blood urea nitrogen and examined the differences between before and after herbal complexes intake using a paired t-test. In addition, we investigated the prevalence of hypokalemia among these patients and reviewed such patients. We identified 360 patients who did not show significant changes in the levels of potassium and creatinine ($P = 0.815, 0.289$). We observed hypokalemia in 6 patients. However, in 5 patients, the hypokalemia did not appear to be related to the licorice. Thus, we could suggest that herbal complexes containing licorice do not significantly influence the potassium levels in routine clinical herbal therapies. However, we propose that follow-up examination for potassium levels is required to prevent any unpredictable side effects of administration of licorice in routine herbal medicine care.

1. Introduction

Licorice has been used as a medicinal agent for patients of all ages and in East Asian countries. Licorice has been used for the treatment of peptic ulcer, as an anti-inflammatory agent, expectorant, drink, candy, and sweetener [1, 2]. In particular, in Korean medicine, licorice has been used in many herbal prescriptions for the treatment of sore throat, cough, and wounds and for neutralizing the poisonous effects of other herbs such as *Aconiti Ciliare Tuber*, *Ephedrae Herba,* or *Rhei Rhizoma*. Therefore, East Asian people who prefer taking herbal complexes for treating their various symptoms frequently consume licorice.

Licorice was reported to cause hypokalemia for the first time in 1950 [3]. Subsequently, many studies have shown that licorice can induce hypokalemia, muscle weakness, and hypertension [1, 4–17]. In serious situation, licorice consumptions can cause rhabdomyolysis with generalized muscle aches or weakness, hematuria, hypokalemia, and renal dysfunction [8–10]. These studies suggest that long-term use and overdose of licorice can lead to severe hypokalemia and can be life threatening. However, previous studies only included case reports.

In the present study, we investigated the influence of herbal complexes containing licorice on potassium levels in 360 cases. We aimed to observe the influence of such herbal complexes on potassium levels in a clinical situation.

2. Methods

We examined the medical records of patients who were treated with herbal complexes containing licorice from January 1,

2010, to December 31, 2010, at the Kyung Hee University Oriental Medicine Hospital, Seoul, Korea. From these patients, we selected subjects whose potassium levels were stable before the administration of herbal medication and whose potassium levels were in the normal range. Thus, we excluded patients with hypokalemia or hyperkalemia at the starting point of the study. Further, we selected subjects whose herbal medication and existing medications such as antiplatelet agents, antihypertensive agents, antidiabetic agents, statins, or nonsteroidal anti-inflammatory drugs had not changed during followup. If the composition of the herbal complexes or the dose of licorice or other medications changed, we assumed that followup was completed. We recorded the patients' characteristics, reasons for using herbal complexes, dose of licorice in each patient, duration of herbal medication intake, and changes in the levels of potassium, blood urea nitrogen (BUN), and creatinine. To measure the differences in potassium levels during administration of herbal medicines containing licorice (before and after herbal medicine intake), we used a paired t-test. Statistical analysis was performed using SPSS for Windows, version 10.0 (SPSS Inc., Chicago, IL, USA). Subsequently, we reviewed the cases of patients who showed hypokalemia during followup to assess the probable causes that affected the patients' potassium levels. When we reviewed the cases revealed hypokalemia, we used the Naranjo scale to estimate the influence of medication objectively.

3. Results

3.1. Patients' Characteristics. We identified 360 subjects fulfilling our criteria. The average dose of licorice was 8.7 ± 4.1 g/day (average ± standard deviation [SD]). Further, the average intake duration was 18.9 ± 19 days. The frequency of usage of herbal medicines was the highest for cerebral infarction (62.4%). Other characteristics of the patients at baseline are listed in Table 1.

3.2. Changes in the Levels of Potassium, BUN, and Creatinine. Although the potassium level decreased during follow-up examination, this decrease was not statistically significant (potassium levels before versus after, 4.00 ± 0.4 versus 3.99 ± 0.4; $P = 0.815$). BUN and creatinine levels also decreased. The levels of BUN showed a statistically significant decrease (before versus after, 13.91 ± 6.4 versus 12.64 ± 4.9; $P < 0.001$) (Table 2). However, all values were within the normal range (potassium 3.5~5.0 mEq/L; BUN 10~26 mg/dL; and creatinine 0.6~1.2 mg/dL).

3.3. Patients with Hypokalemia. Six patients (1.7%) had hy-pokalemia during the follow-up period (Table 3). The remaining 354 patients (98.3%) had normal levels of potassium during the intake of the herbal complexes.

The cases of patients with hypokalemia are as follows (Table 4).

Case 1 was a 78-year-old woman who presented dysarthria and right upper and lower extremities weakness with a history of cerebral infarction. Over a 22-day period, she received aspirin to prevent the recurrence of stroke and the herbal complex *Shipyukmiyouki-eum* to treat sequelae after cerebral infarction. During treatment with these agents, her potassium level decreased from 4.0 mEq/L to 2.5 mEq/L.

Case 2 was a 60-year-old woman diagnosed with cerebral hemorrhage, hypertension, and urinary tract infection. The patient was referred by the neurosurgery department to be treated with herbal complexes and acupuncture therapy to treat her left side paralysis and left shoulder pain caused by cerebral hemorrhage. She started treatment with antibiotics for the treatment of urinary tract infection that occurred during rehabilitation 5 days before administration of the herbal complex *Seogyung-tang*. Over a 6-day period, she received ciprofloxacin, ceftriaxone sodium, oxiracetam, and

Table 1: Baseline characteristics ($n = 360$).

Characteristics	Frequency	Percentage %
Male/female	182/178	50.6/49.4
Age, years (Mean ± SD)	65.1 ± 13.9	
Causes of herbal medicine intake		
Cerebral infarction	224	62.4
Intracerebral hemorrhage	88	24.5
Brain tumor	2	0.6
Moyamoya disease	4	
Parkinson disease	4	1.1
Parkinsonism	5	1.4
Anoxic brain damage	23	6.4
Tension-type headache	1	0.3
Transverse myelitis	1	0.3
C-HNP	6	1.7
T-HNP	1	0.3
L-HNP	7	1.9
Spinal stenosis	4	1.1
Facial palsy	11	3.1
Lung cancer	3	0.8
Esophagus cancer	1	0.3
Colon cancer	1	0.3
Liver cancer	3	0.8
Kidney cancer	1	0.3
Gastric cancer	1	0.3
Gall bladder cancer	1	0.3
GERD	1	0.3
DVT	1	0.3
Osteoarthritis	3	0.8
CRPS	2	0.6
Licorice dose (per day) in g (Mean ± SD)	8.7 ± 4.1	
Duration of administration in days (Mean ± SD)	18.9 ± 19	

GERD: gastroesophageal reflux disease; DVT: deep vein thrombosis; CRPS: complex regional pain syndrome type; C-HNP: cervical herniated nucleus pulposus; T-HNP: thoracic cervical herniated nucleus pulposus; L-HNP: lumbar herniated nucleus pulposus.

Table 2: Measurement of the levels of potassium, blood urea nitrogen, and creatinine ($n = 360$).

Laboratory findings	First	Second	P value
Potassium level (mEq/L)	4.00 ± 0.4	3.99 ± 0.4	0.815
Blood urea nitrogen level (mg/dL)	13.91 ± 6.4	12.64 ± 4.9	<0.001
Creatinine level (mg/dL)	1.04 ± 7.4	0.6 ± 0.3	0.289

Values are average (standard deviation [SD]) and were compared using paired t-test.

Table 3: Incidence of hypokalemia ($n = 360$).

	Numbers (%)
Hypokalemia	6 (1.7)
Nonhypokalemia	354 (98.3)

Hypokalemia in this table indicates that potassium levels (at end point) are below 3.0 mEq/L.

the herbal complex *Seogyung-tang*. During this series of treatments, her potassium level decreased from 3.5 mEq/L to 2.5 mEq/L.

Case 3 was a 56-year-old man diagnosed with cerebral hemorrhage and hypertension. The patient was admitted to treat hemiplegia and insomnia caused by cerebral hemorrhage. He had used lercanidipine HCl, atenolol, hydrochlorothiazide, and losartan potassium to control his hypertension and to prevent the recurrence of cerebral hemorrhage. After 14 days of onset of cerebral hemorrhage, he started treatment with *Gamiondamg-tang* to relieve insomnia over a 29-day period. During this treatment, his potassium level decreased from 3.9 mEq/L to 2.8 mEq/L.

Case 4 was a 77-year-old woman with cerebral infarction, hypertension, and diabetes. She was referred by the neurosurgery department for treatment of insomnia, palpitation, and hemiparesis caused by stroke. She had taken *S*-amlodipine, clopidogrel, calcium carbonate, pravastatin sodium, donepezil HCl, metformin HCl, and nicergoline to control the risk factors of stroke (hypertension, dyslipidemia, and diabetes mellitus). Over a 20-day period, she received the herbal complex *Gwibi-tang* to treat insomnia, palpitation, and sporadic diarrhea, which may be caused by metformin HCl. During this treatment, her potassium level decreased from 3.6 mEq/L to 2.6 mEq/L.

Case 5 was an 89-year-old woman with hypertension and coronary artery disease. After coronary angioplasty, she was referred by the cardiology department to treat the residual fatigue. At the time of admission, she had taken fluvastatin sodium, aspirin, nifedipine, atenolol, hydrochlorothiazide, tramadol HCl, acetaminophen, teprenone, and alfacalcidol. Over an 11-day period, she took the herbal complex *Bojungikki-tang* to treat her symptoms of fatigue. During this series of treatments, her potassium level decreased from 3.0 mEq/L to 2.2 mEq/L.

Case 6 was a 72-year-old woman diagnosed with cerebral infarction and hypertension, and she was referred by the neurosurgery department to treat sequelae of stroke after craniectomy. She had used quetiapine to treat delirium, gabapentin to treat neuralgia, aspirin to prevent stroke recurrence, and sodium valproate, dimethicone, hemicellulase, ox bile extract, and pancreatin. Over an 8-day period, she used the herbal complex *Banhasashim-tang* to treat her dyspepsia and abdominal discomfort. During this series of treatments, her potassium level decreased from 3.7 mEq/L to 2.8 mEq/L.

However, symptoms such as muscle weakness, hypertension, and renal failure were not observed in any of these 6 patients.

4. Discussion

In this study, we found no significant change in the potassium levels during the administration of herbal complexes containing licorice in 360 patients for 18.9 ± 19 days. Follow-up examination indicated that the potassium level decreased, but this decrease was not statistically significant ($P = 0.815$). The potassium levels were normal in 98.3% patients during the intake of herbal complexes. Only 1.7% patients had hypokalemia during followup. The average dose of licorice was 8.7 ± 4.1 g/day, and the average administration duration was 18.9 ± 19 days. Therefore, we suggested that administration of herbal complexes containing licorice could be safe when the dose of licorice was less than 8.7 ± 4.1 g/day and the duration of administration was less than 18.9 ± 19 days.

We observed 6 patients (1.7%) who developed hypokalemia after treatment with herbal medicine complexes containing licorice. However, we believed that the development of hypokalemia could not be attributed only to licorice in almost all patients (5 patients). We believe that concomitant administration of western medicines such as antihypertensive and antidiabetic agents and the medical condition of patients affected the development of hypokalemia.

Cases 3 and 5 took the antihypertensive agent hydrochlorothiazide. Diuretics such as thiazides are common cause of drug-induced hypokalemia [18]. Therefore, we assumed that long-term intake of hydrochlorothiazide can be a cause of hypokalemia in cases 3 and 5. Case 4 used metformin HCl to control her blood glucose levels. Before administration of the herbal medicine *Gwibi-tang*, the patient had sporadic diarrhea and soft stool. A previous case report indicated that a 57-year-old Caucasian male had hypokalemia, hypocalcemia, and hypomagnesemia induced by long-term administration of metformin HCl [19]. Therefore, we believe that long-term intake of metformin HCl can be attributed to the development of hypokalemia in case 4. Case 6 had used sodium valproate since craniectomy. A previous case report

Table 4: Occurrence of hypokalemia in patients after treatment with herbal complexes containing licorice.

Case number	Pot 1	Pot 2	Duration (days)	Causes of herbal medicine intake	Herbal complex taken, administration method (components in g/day)	Concomitant medications	Licorice doses per day	Estimate possibility/ Naranjo scale score
1	4.0	2.5	22	Cerebral infarction	*Shipyukmiyouki-eum*, oral administration (licorice 8 g, *Cinnamomum cassia* 6 g, *Platycodi radix* 6 g, *Angelica gigas* 12 g, *Aucklandia lappa* 6 g, *Saposhnikovia divaricata* 6 g, *Paeonia lactiflora* 6 g, *Angelica dahurica* 6 g, *Areca catechu* 6 g, *Perilla frutescens* var. acuta 18 g, *Lindera aggregata* 6 g, *Panax ginseng* 12 g, *Citrus aurantium* 6 g, *Cnidium officinale* 6 g, *Astragalus membranaceus* 6 g, and *Magnolia obovata* 6 g)	Aspirin 100 mg	**8**	*Shipyukmi youki-eum*/2
2	3.5	2.5	6	Cerebral hemorrhage, hypertension, and **urinary tract infection**	*Seogyung-tang*, oral administration (licorice 6 g, *Ostericum koreanum* 6 g, *Curcuma longa* 24 g, *Angelica gigas* 12 g, *Atractylodes japonica* 12 g, *Zingiber officinale* 12 g, *Paeonia lactiflora* 12 g, and *Kalopanax* species 12 g)	Ciprofloxacin 800 mg, ceftriaxone sodium 2 g, and oxiracetam 1600 mg	**6**	Urinary tract infection/—
3	3.9	2.8	29	Cerebral hemorrhage and hypertension	*Gamiondamg-tang*, oral administration (licorice 12 g, *Citrus unshiu* 15 g, *Platycodi radix* 8 g, *Liriope platyphylla* 8 g, *Pinellia ternata* 9 g, *Poria cocos* 8 g, *Bupleurum falcatum* 8 g, *Panax ginseng* 8 g, *Phyllostachys nigra* 9 g, *Citrus aurantium* 9 g, and *Cyperus rotundus* 9 g)	Lercanidipine HCl 10 mg, famotidine 40 mg, atenolol 25 mg, **hydrochlorothiazide** 12.5 mg, and losartan potassium 50 mg	**12**	Hydrochlorothiazide/2
4	3.6	2.6	20	Cerebral infarction, hypertension, and diabetes mellitus	*Gwibi-tang*, oral administration (licorice 6 g, *Angelica gigas* 6 g, *Aucklandia lappa* 12 g, *Poria cocos* 12 g, *Atractylodes japonica* 12 g, *Zizyphus jujuba* 12 g, *Dimocarpus longan* 12 g, *Polygala tenuifolia* 12 g, *Panax ginseng* 12 g, and *Astragalus membranaceus* 12 g)	S-Amlodipine 2.5 mg, clopidogrel 75 mg, Ca carbonate 500 mg, pravastatin sodium, 20 mg, donepezil HCl 5 mg, **metformin HCl,** 500 mg, and nicergoline 60 mg	**6**	Metformin HCl/2
5	3.0	2.2	11	Hypertension and coronary artery disease	*Bojungikki-tang*, oral administration (licorice 12 g, *Angelica gigas* 6 g, *Atractylodes japonica* 12 g, *Cimicifuga heracleifolia* 3 g, *Bupleurum falcatum* 3 g, *Panax ginseng* 12 g, *Citrus unshiu* 6 g, and *Astragalus membranaceus* 18 g)	Fluvastatin sodium 80 mg, aspirin 10 mg, nifedipine 33 mg, atenolol 25 mg, **hydrochlorothiazide** 12.5 mg, tramadol HCl 37.5 mg, acetaminophen 325 mg, teprenone 50 mg, and alfacalcidol 0.5 μg	**12**	Hydrochlorothiazide/2
6	3.7	2.8	8	Cerebral infarction and hypertension	*Banhasashim-tang*, oral administration (licorice 12 g, Zingiberis Rhizoma Siccus 15 g, *Zizyphus jujuba* 12 g, *Pinellia ternata* 30 g, *Panax ginseng* 15 g, *Scutellaria baicalensis* 15 g, *Coptis japonica* 6 g)	Quetiapine 25 mg, Gabapentin 900 mg, aspirin 100 mg, **sodiumvalproate** 1800 mg, dimethicone 75 mg, hemicellulase 150 mg, ox bile extract 75 mg, and pancreatin 525 mg	**12**	Sodium valproate/2

[20] indicated that a 14-year-old Japanese patient sometimes had fever and hypokalemia after intake of sodium valproate for 6 years. Therefore, we believe that consumption of sodium valproate can be a cause of hypokalemia in Case 6. In Case 2, fever caused by urinary tract infection may be attributed to the development of hypokalemia. Moreover, a previous study showed that the prevalence of hypokalemia in patients hospitalized for infection at an institution was 23% [21]. Thus, our results show that herbal complexes containing licorice cannot be a sole reason for the development of hypokalemia in these 5 patients.

Among the 360 patients, 1 patient (Case 1) had definite licorice-induced hypokalemia. In the case of this patient, no reasons other than the intake of *Shipyukmiyouki-eum* (licorice 8 g) could be attributed to the development of hypokalemia. This patient only used aspirin during *Shipyukmiyouki-eum* intake. No previous study to date suggests that aspirin may cause hypokalemia. Therefore, *Shipyukmiyouki-eum* can be a reason for hypokalemia.

Our study has the following limitations. We did not measure the serum levels of aldosterone, rennin, cortisol, and adrenocorticotropic hormone (ACTH). Therefore, we could not observe the influence of herbal complexes containing licorice on the endocrine system. Furthermore, because of retrospective nature of the study, we did not measure the amounts of glycyrrhetic acid which is the active component in licorice and duration of licorice contained herbal complexes intake and licorice dosage in each patients were not equal.

However, we believe that our findings can be a guide for safe use of licorice. We examined the average duration (18.9 ± 19 days) and dose of licorice (8.7 ± 4.1 g/day) use in these 360 patients. Our results revealed that severe hypokalemia did not occur during administration of herbal complexes containing licorice. Moreover, our study reflects the clinical circumstances better than those reported in previous studies. Doctors in Korea, Japan, and China who use herbal medicines to treat patients usually prescribe many types of herbs simultaneously. All patients in the present study consumed herbal complexes containing licorice. Previous studies have reported the cases of patients consuming only licorice [1, 4–17].

Our data suggest that administration of herbal complexes containing licorice does not affect the potassium levels when the dose of licorice is less than 8.7 ± 4.1 g/day and duration of administration is less than 18.9 ± 19 days. However, we think that hypokalemia in patients who use licorice can be made by interaction between licorice and drug which might cause electrolyte disturbances and renal function. Thus, we can assume that followup of potassium levels is required to prevent any unpredictable side effects of long-term administration of herbal complexes containing licorice in elderly people, especially, who used herbal medication with western medication.

Furthermore, we suggest that well designed prospective study, with standard administration (dosages of licorice and intake duration) and documented amount of glycyrrhetic acid, is necessary to solve the question of possible licorice induced side effects.

5. Conclusion

The present study assessed the influence of herbal complexes containing licorice on potassium levels. This study was conducted as a retrospective, chart review study. The results of this study are as follows.

(1) Three hundred and sixty patients did not show significant changes in the levels of potassium and creatinine ($P = 0.815$, 0.289).

(2) The average dose of licorice was 8.7 ± 4.1 g/day. Further, the average intake duration was 18.9 ± 19 days.

(3) Six patients revealed hypokalemia. However, in 5 patients, the hypokalemia did not appear to be related to the herbal complex therapy containing licorice.

Therefore, we could suggest that administration of herbal complexes containing licorice does not affect the potassium levels at low dose and for a short period of time. However, we suggest that followup of potassium levels is needed to prevent any unpredictable side effects of administration of herbal complexes containing licorice.

Disclosure

No financial support was received during the present study.

Conflict of Interests

The authors declare that they have no conflict of interests.

References

[1] S. Shintani, H. Murase, H. Tsukagoshi, and T. Shiigai, "Glycyrrhizin (licorice)-induced hypokalemic myopathy: report of 2 cases and review of the literature," *European Neurology*, vol. 32, no. 1, pp. 44–51, 1992.

[2] B. R. Walker and C. R. W. Edwards, "Licorice-induced hypertension and syndromes of apparent mineralocorticoid excess," *Endocrinology and Metabolism Clinics of North America*, vol. 23, no. 2, pp. 359–377, 1994.

[3] F. E. Cayley, "Potassium deficiency in p-aminosalicylic acid therapy: cardiac and paralytic effects," *The Lancet*, vol. 255, no. 6602, pp. 447–448, 1950.

[4] J. W. Conn, D. R. Rovner, and E. L. Cohen, "Licorice-induced pseudoaldosteronism. Hypertension, hypokalemia, aldosteronopenia, and suppressed plasma renin activity," *Journal of the American Medical Association*, vol. 205, no. 7, pp. 492–496, 1968.

[5] S. Cho, B. Lim, H. Cho et al., "Licorice Induced Hypokalemia," *Korean Journal of Nephrology*, vol. 20, pp. 1021–1025, 2001.

[6] H. I. Hong, K. H. Han, J. W. Hwang et al., "A case of licorice induced hypokalemic paralysis with rhabdomyolysis," *Journal of Korean Endocrine Society*, vol. 20, pp. 179–182, 2005.

[7] B. J. Kim, Y. H. Hong, J. J. Sung et al., "A Case of Glycyrrhizin(Licorice)-induced Hypokalemic Myopathy," *Journal of the Korean Neurological Association*, vol. 24, pp. 389–391, 2006.

[8] S. W. Kwon, H. Y. Jeong, S. K. Yoon, Y. R. Seo, J. Y. Park et al., "A Case of taking self prescribed Licorice Induced Hypokalemic

Paralysis," *Journal of the Korean Oriental Medicine*, vol. 32, pp. 134–138, 2011.

[9] H. Korri, A. Awada, W. Baajour, M. Beaini, and W. Nasreddine, "Rapidly progressing quadriparesis secondary to licorice (souss) intoxication," *Journal Medical Libanais*, vol. 60, no. 2, pp. 117–119, 2012.

[10] M. Shah, C. Williams, A. Aggarwal, and W. M. Choudhry, "Licorice-related rhabdomyolysis: a big price for a sweet tooth," *Clinical Nephrology*, vol. 77, no. 6, pp. 491–495, 2012.

[11] P. Pant, L. Nadimpalli, M. Singh, and J. C. Cheng, "A case of severe hypokalemic paralysis and hypertension. Licorice-induced hypokalemic paralysis," *The American Journal of Kidney Diseases*, vol. 55, no. 6, pp. A35–A37, 2010.

[12] A. C. Meltem, C. Figen, M. A. Nalan et al., "A hypokalemic muscular weakness after licorice ingestion: a case report," *Cases Journal*, vol. 2, no. 9, article 8053, 2009.

[13] T. Yamamoto, M. Hatanaka, J. Matsuda et al., "Clinical characteristics of five elderly patients with severe hypokalemia induced by glycyrrhizin derivatives," *Japanese Journal of Nephrology*, vol. 52, no. 1, pp. 80–85, 2010.

[14] C. Templin, M. Westhoff-Bleck, and J.-R. Ghadri, "Hypokalemic paralysis with rhabdomyolysis and arterial hypertension caused by liquorice ingestion," *Clinical Research in Cardiology*, vol. 98, no. 2, pp. 130–132, 2009.

[15] F. Francini-Pesenti, M. Puato, A. Piccoli, and F. Brocadello, "Liquorice-induced hypokalemia and water retention in the absence of hypertension," *Phytotherapy Research*, vol. 22, no. 4, pp. 563–565, 2008.

[16] G. C. Isaia, C. Pellissetto, M. Ravazzoli, and C. Tamone, "Acute adrenal crisis and hypercalcemia in a patient assuming high liquorice doses," *Minerva Medica*, vol. 99, no. 1, pp. 91–94, 2008.

[17] A. E. van den Bosch, J. M. van der Klooster, D. M. H. Zuidgeest, R. J. T. Ouwendijk, and A. Dees, "Severe hypokalaemic paralysis and rhabdomyolysis due to ingestion of liquorice," *Netherlands Journal of Medicine*, vol. 63, no. 4, pp. 146–148, 2005.

[18] F. J. Gennari, "Hypokalemia," *The New England Journal of Medicine*, vol. 339, no. 7, pp. 451–458, 1998.

[19] A. Svare, "A patient presenting with symptomatic hypomagnesemia caused by metformin-induced diarrhoea: a case report," *Cases Journal*, vol. 2, no. 10, article 156, 2009.

[20] H. Yoshikawa, T. Watanabe, and T. Abe, "Tubulo-interstitial nephritis caused by sodium valproate," *Brain and Development*, vol. 24, no. 2, pp. 102–105, 2002.

[21] D. Widodo, B. Setiawan, K. Chen, L. Nainggolan, and W. D. Santoso, "The prevalence of hypokalemia in hospitalized patients with infectious diseases problem at Cipto Mangunkusumo Hospital, Jakarta," *Acta Medica Indonesiana*, vol. 38, no. 4, pp. 202–205, 2006.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2014, Article ID 609064, 8 pages
http://dx.doi.org/10.1155/2014/609064

Research Article

Irritant Contact Dermatitis Risk of Common Topical Traditional Chinese Medicines Used for Skin-Lightening: A Pilot Clinical Trial with 30 Volunteers

Kao-Sung Tsai,[1,2] Tzu-Chun Lin,[1,2] Meng-Tse Wu,[2] Jui-Lung Shen,[3] Ming-Ya Mao,[2] Huey-Yi Chen,[1,2] Yung-Hsiang Chen,[1,2] and Wen-Chi Chen[1,2]

[1] *School of Chinese Medicine, Graduate Institute of Chinese Medicine, Graduate Institute of Integrated Medicine, College of Chinese Medicine, China Medical University, Taichung 40402, Taiwan*
[2] *Departments of Dermatology, Medical Research, Obstetrics and Gynecology, and Urology, China Medical University Hospital, Taichung 40402, Taiwan*
[3] *Department of Dermatology, Taichung Veteran General Hospital, Taichung 40705, Taiwan*

Correspondence should be addressed to Yung-Hsiang Chen; d87a03@ym.edu.tw and Wen-Chi Chen; wgchen@mail.cmu.edu.tw

Received 8 November 2013; Revised 24 February 2014; Accepted 7 March 2014; Published 10 April 2014

Academic Editor: Junhua Zhang

Topical traditional Chinese medicine- (TTCM-) related contact dermatitis is not uncommon but ignored. Patch and photopatch tests using 6 individual herbal ingredients and Bai-Zhi-Kao (BZK; 白芷膏), a skin-lightening TTCM preparation, were conducted on 30 participants. Twenty-five subjects showed at least 1 positive reaction, including 6 (20.0%) participants who reacted to BZK. The majority reacted to Radix *Ampelopsis japonica* (Bai-Lian; 白蘞) (60.0%), whereas few reacted to Rhizoma Bletilla striata (Bai-Ji; 白芨) (16.7%), Rhizoma *Atractylodis macrocephalae* (Bai-Zhu; 白朮) (10.0%), Radix *Angelicae dahuricae* (Bai-Zhi; 白芷) (3.3%), and Herba asari (Xi-Xin; 細辛) (3.3%). In the photopatch test, 3 participants (10.0%) reacted positively to BZK and 10 to ≥1 constituent; however, all reacted to Radix *Angelicae dahuricae* (26.7%), Radix *Ampelopsis japonica* (13.3%), and Rhizoma Bletilla striata (3.3%). In contrast, no subjects showed positive reactions to Sclerotium Poria cocos (Bai-Fu-Ling; 白茯苓). Thus, BZK and its constituents might present potential latent risk of contact dermatitis owing to the possible presence of Radix *Ampelopsis japonica* and Radix *Angelicae dahuricae*. Furthermore, TTCMs, particularly cosmetic products, must be used carefully, with ample warning of potential contact dermatitis risk.

1. Introduction

During the last few decades, cosmetics derived from or partially composed of topical traditional Chinese medicines (TTCMs) have been widely used in skin care, despite the lack of parallel human clinical trials [1–3]. However, research has revealed that allergic dermatitis and irritant contact dermatitis are the most common adverse events associated with TTCMs [4, 5]. Many variants of traditional Chinese medical formulas contain complex combinations of individual ingredients from multiple herbal plant constituents (in both crude and galenic extracted forms), thus limiting the scope of clinical investigation [6]. In addition, studies on TTCMs and other herbal medicine allergies are also problematic because of the limited number of commercially available standardised patch test substances and the danger of active sensitisation when testing with botanical medicines, their constituent parts or individual extracts.

Although TTCMs are regarded as critical allergens by dermatologists, the number of affected cases reported is relatively small. Furthermore, safety, adverse effects, standards, and quality control are issues of concern, and the evaluation of all of these factors should be mandatory in herbal medicine. For example, Bai-Zhi-Kao (BZK; 白芷膏), a currently commonly used TTCM for skin lightening in Chinese communities, including those in Taiwan and China, had been mentioned in an ancient official Chinese pharmacopoeia (Sheng-Ji-Zong-Lu; 聖濟總錄, A.D. 1,117). This

topical Chinese medicinal formula is composed of 7 crude botanical components, including Radix *Angelicae dahuricae* (Bai-Zhi; 白芷), Rhizoma *Atractylodis macrocephalae* (Bai-Zhu; 白朮), Sclerotium *Poria cocos* (Bai-Fu-Ling; 白茯苓), Rhizoma *Bletilla striata* (Bai-Ji; 白芨), Radix *Ampelopsis japonica* (Bai-Lian; 白蘞), *Herba asari* (Xi-Xin; 細辛), and Rhizoma *Typhonium giganteum* (Bai-Fu-Zi; 白附子). In the ancient pharmacopoeia, this mixture had been used for a relatively long period (used as a mask at night and rinsed off the next morning). The herbal constituents of this topical Chinese medicinal formula are known to cause appreciable antityrosinase activity and suppress tyrosinase synthesis [7–10]; thus, many BZK-based pharmaceutical and cosmetic products such as BZK medicinal powder and extracts mixed with aqueous, petrolatum or olive-oil vehicles are prescribed by traditional Chinese medicine practitioners in the Chinese community.

Herbal medicine is a widely used modality of complementary and alternative medicine. Along with the rapid growth of consumption, the safety of herbal medicines has become a highlighted topic. Thus, by employing patch and photopatch tests on healthy volunteers, this pilot clinical study was designed to evaluate the potential incidence of contact dermatitis resulting from some TTCMs commonly used.

2. Patients and Methods

2.1. Study Design and Subjects Selection. This pilot clinical study was conducted from April to May 2008, and volunteers were recruited from the outpatient clinic of the Dermatology Department of China Medical University Hospital in Taiwan. The inclusion criteria were age of 20–65 years and absence of any illnesses. The exclusion criteria were topical or systemic use of corticosteroids or antihistamines within 4 weeks of the start of the study, intake of immunosuppressive drugs, intense ultraviolet exposure on the back, prior eczematous dermatitis, actinic dermatitis or severe sunburn injury, and infectious disease at the patch test site on the back. In addition, pregnant and lactating women were also excluded. No patients were allowed to take corticosteroids or antihistamines during the study period. The Institutional Review Board (IRB) of the hospital approved the study (DMR96-IRB-186), and the subjects provided signed informed consent.

2.2. Herbal Materials and Preparation of Reagents. In the present study, the tested BZK was composed of 6 individual botanical components, including Radix *Angelicae dahuricae*, Rhizoma *Atractylodis macrocephalae*, Sclerotium *Poria cocos*, Rhizoma *Bletilla striata*, Radix *Ampelopsis japonica,* and *Herba asari*, which were further examined individually. Because of the potential preclinical toxic effects of Rhizoma *Typhonium giganteum* [11, 12], this component was excluded from the study, according to the IRB restriction.

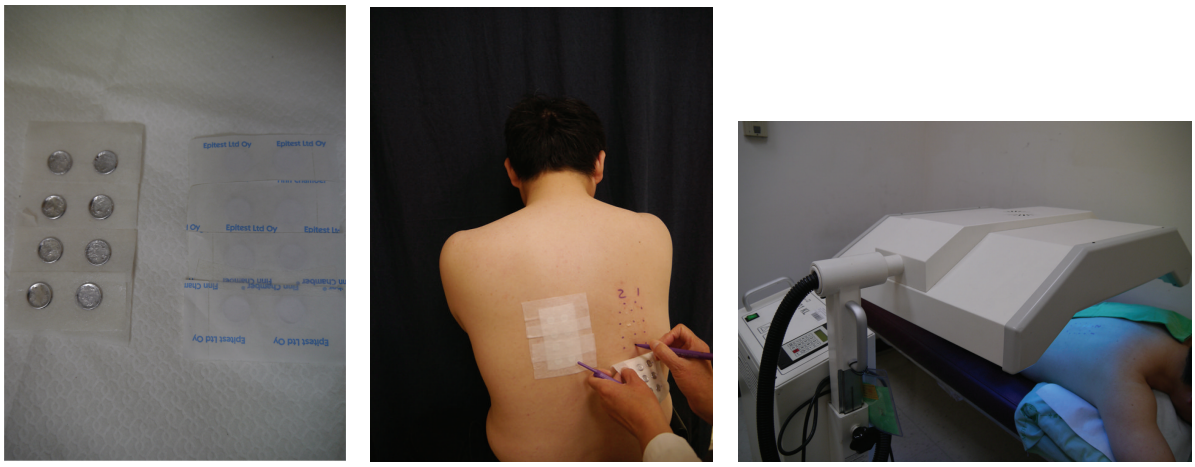
It must be noted that only a few references to the patch test concentration of "cosmetic" TTCM substances are available in the literature. Patch test is used in traditional medicine to confirm the cause of contact dermatitis in "as-is" preparations and diluted substances [13]. Many substances used in everyday life, such as cosmetics and medications, are conventionally used without dilution [14]. In the present study, BZK and its constituents were subjected to patch test at an undiluted concentration in a 50% petrolatum mix to mimic their traditional daily use, according to the ancient pharmacopoeia.

The authenticated (appearance, microscopic characteristics, and thin-layer chromatography) samples of the individual herbal constituents, complying with specifications given in the Chinese pharmacopoeia, were provided by Sheng Foong Pharmaceutical Co., Ltd (Proof certification according to ISO17025/TAF, Ilan County, Taiwan). Briefly, the 6 dried herbal constituents (450 g for each constituent) were ground (5 min) into powder of 80 meshes by using herb-grinding machines (IKA A11 grinder; IKA, Germany). Each of the ground substances was homogeneously mixed with the warm (60°C) petrolatum base at a 1 : 1 ratio by weight. Likewise, the BZK formula was prepared in a similar manner from 6 equivalent 50 g herbal constituents and a 300 g petrolatum base. All the petrolatum-based reagents were prepared by Sinphar Pharmaceutical Co., Ltd. (Ilan County, Taiwan) in accordance with Good Laboratory Practice and stored in a refrigerator below 4°C until use. As a negative control, a sample of petrolatum base was employed. All the reagents were labelled as per their registration number.

2.3. Patch and Photo Patch Testing Procedures. The subjects underwent patch and photopatch test on an unaffected area of the upper back. For patch tests, 50 mg of each reagent was placed within 8 mm Finn Chambers on Scanpor tape (Epitest Ltd., Tuusula, Finland), which were then fixed with Scanpor tape and secured with 3M tape (Figure 1(a)). Each reagent was applied in duplicate on the left and right side of the upper back of each subject (Figure 1(b)) for 24 h. The patches were then uniformly removed from side-to-side. Photopatch tests were applied on the right side of the backs of the subjects who were irradiated with 5 J/cm^2 UVA (Waldmann UV801KL; Villing-Schwenningen, Germany) on Day 1 (Figure 1(c)).

2.4. Assessment. The patches were removed on Day 1 and readings were taken on Days 1, 3, and 7 by 2 experienced dermatologists. The test reactions were graded according to the guidelines of the International Contact Dermatitis Research Group [15]. Throughout the study period, all the tested cutaneous conditions and adverse effects were noted and recorded by using a digital camera. The flowchart describing the protocol is given in Figure 2.

2.5. Statistical Analyses. The study participants were characterised by using descriptive statistics (mean ± standard deviation). The chi-square test and Fisher exact test were used to compare the reactions to BZK and its constituents. A phi coefficient was determined to estimate the relationship between the reactive patch tests produced by the formula, BZK, and its constituents. Statistical significance was indicated by $P < 0.05$. All statistical analyses were carried out



(a) (b) (c)

Figure 1: Patch and photopatch testing on an unaffected area of the upper back. (a) Each reagent placed within 8 mm Finn Chambers on Scanpor tape was then fixed with Scanpor tape and secured with 3M tape. (b) Each reagent was applied in duplicate to the left and right side of the upper back of each subject. (c) Photopatch tests were applied on the right side of the backs of subjects who were irradiated with 5 J/cm^2 UVA on Day 1.

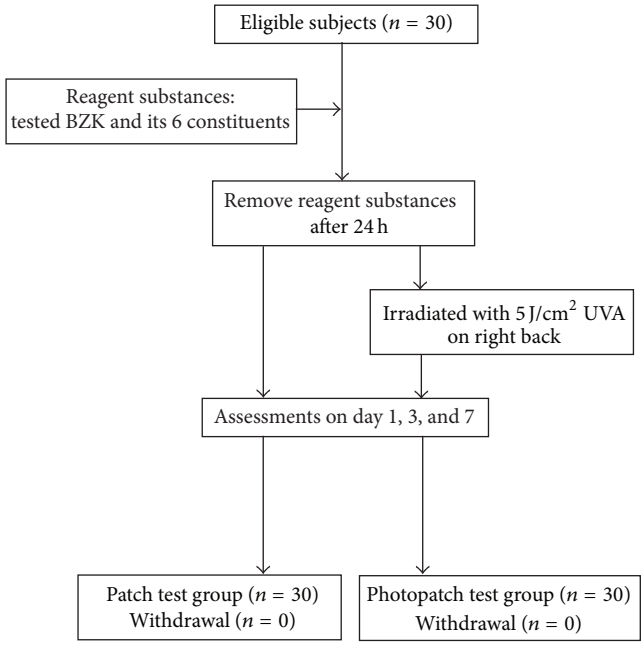


Figure 2: The flow chart of this study.

by using the software SPSS Statistics 17.0 (SPSS, Chicago, IL, USA).

3. Results

The average age of the participants was 35.2 ± 10.7 years (range, 21–64 years), with the majority being women (n = 25; 83.3%), and there were no dropouts. The detailed data obtained on all assessment days (Days 1, 3, and 7) are given in Figure 3, and the results of the patch and photopatch tests are summarised in Table 1.

Of the 30 subjects, 25 (5 men and 20 women) exhibited at least 1 positive reaction to the tested patches. Among them, 6 (20.0%) showed reactions to BZK. To identify the differences between the reactions to different BZK constituents, the individual reactions of the herbal constituents of this formula were analysed. The majority showed positive reactions to Radix *Ampelopsis japonica* (n = 18; 60.0%), followed by Rhizoma *Bletilla striata* (n = 5; 16.7%), Rhizoma *Atractylodis macrocephalae* (n = 3, 10.0%), Radix *Angelicae dahuricae* (n = 1; 3.3%), and *Herba asari* (n = 1; 3.3%). Furthermore, there were strong positive reactions (++) in 4 subjects, all of whom reacted to Radix *Ampelopsis japonica* (Figure 4). The positive patch test results between BZK and Radix *Ampelopsis japonica* were almost significant (P = 0.07; Fisher's exact test). The value of the phi coefficient was 0.408, indicating a moderate association of positive patch tests between BZK and Radix *Ampelopsis japonica*. In contrast, Sclerotium *Poria cocos* did not show any positive reaction in these subjects. All the control patch results with the petrolatum base were negative.

All the 9 subjects with positive reactions to the BZK patch or photopatch test also reacted to at least 1 of the constituents, including 4 subjects who were reactive to at least 2 constituents in the patch test. In contrast, the patch test did not reveal any positive reaction to the second-ranked reactive constituent Rhizoma *Bletilla striata*. Similarly, with regard to Radix *Angelicae dahuricae* and *Herba asari*, the number of reactive subjects was very small (for both, n = 1).

In the photopatch test series, 3 subjects (10.0%) showed positive reactions to BZK, 10 (33.3%) showed positive reactions to at least 1 constituent, and all showed positive reaction to Radix *Angelicae dahuricae* (n = 8; 26.7%), Radix *Ampelopsis japonicae* (n = 4; 13.3%), and Rhizoma *Bletilla striata* (n = 1;

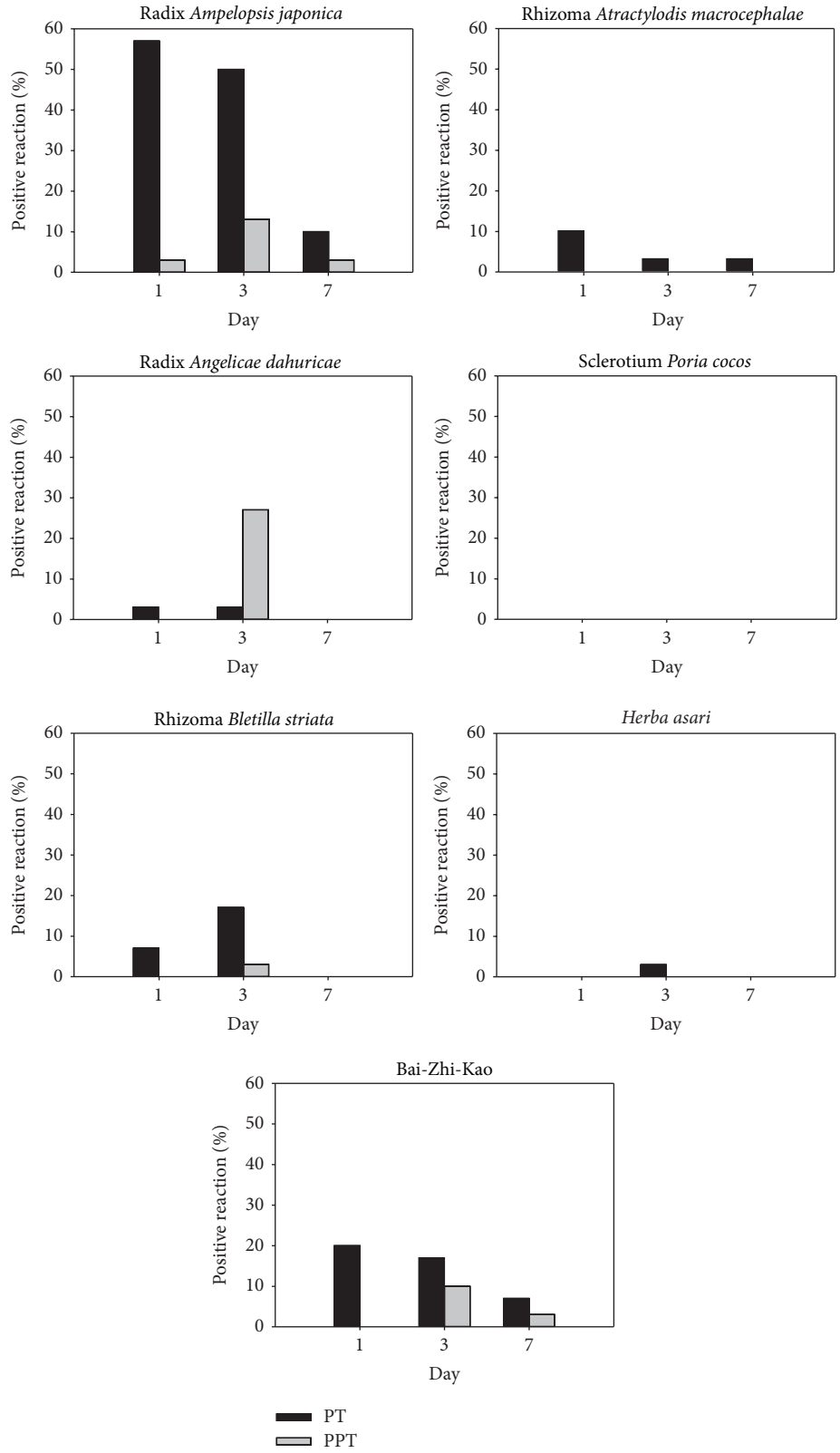


Figure 3: The positive reaction rate in all assessment days (Day 1, 3, and 7).

Table 1: Results of patch and photopatch tests from 30 subjects.

No.	Gender	Age	Radix *Ampelopsis japonica*		Rhizoma *Atractylodis macrocephalae*		Radix *Angelicae dahuricae*		Sclerotium *Poria cocos*		Rhizoma *Bletillae striatae*		*Herba asari*		Bai-Zhi-Kao		Petrolatum	
			PT	PPT	PT	PPT	PT	PPT	PT	PPT	PT	PPT	PT	PPT	PT	PPT	PT	PPT
1	M	38									+	+				+		
2	F	37	+	+												+		
3	F	27																
4	F	64		+			+				+							
5	F	52	+		+										+			
6	F	45	++					+							+			
7	F	40	+															
8	M	37	+					+										
9	F	43	++					+							+			
10	F	38	+															
11	F	36																
12	F	50	+	++												+		
13	M	35	++		+			+							+			
14	F	34	+															
15	F	56	+								+							
16	M	30						+										
17	F	23	++												+			
18	F	23																
19	F	32											+					
20	F	37									+							
21	F	24	+															
22	F	44	+					+										
23	M	29	+		+			+							+			
24	F	22	+															
25	F	21									+							
26	F	25		+				+										
27	F	26																
28	F	30																
29	F	24	+															
30	F	35	+															

Blank: represent negative reaction; PT: patch test; PPT: photo patch test.

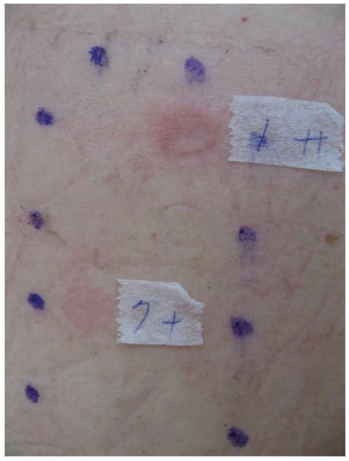


Figure 4: Patch test reactions in patient number 17 showed strong positive reaction (++) to the label number 1 substance and positive (+) to label number 7 substance.

3.3%). On the other hand, no positive reactions to the other 3 constituents were noted.

4. Discussion

The adverse effects caused by herbal medicines constitute an important, yet neglected, issue that deserves further investigation. Till date, a thorough method for the assessment of individual Chinese herb constituents, such as a patch test screening series for identifying certain contact sensitivities, has not yet been reported. Investigators often encounter problems when patch-testing TTCMs, because it is difficult to determine the correct reagent concentrations and vehicles. In the present study, the BZK constituent that caused maximum reactions was Radix *Ampelopsis japonica,* with approximately 60% subjects showing positive reactions to this compound. Among the patch-tested positive subjects, strong positive reactions (more than ++) to Radix *Ampelopsis japonica* were more frequent than those to the other constituents. This indicated that Radix *Ampelopsis japonica* has the greatest potential among all the constituents of BZK to induce contact sensitivities.

The natural compositions of herbal formula are comminuted, powdered, or galenic extracts of the whole or specific parts of a plant [16]. Recently, the potential use of TTCMs in developing new skin-care cosmetics has been emphasised [17]. The fact that women are exposed to more cosmetics and toiletries [18] explains their predominance among the subjects of the present study. Nonetheless, patch test results showed that 76.7% of our subjects were reactive to the formula or to its individual herbal constituents. This finding contradicts the common misconception that TTCM is harmless because of its natural composition and minimal side effects.

Further improvements in the safety of TTCM products can be achieved by replacing or removing the constituents that are associated with a higher risk of contact dermatitis, thus simplifying the composition of TTCM by decreasing the concentration of those constituents or by stating the potential adverse effects of TTCM products on their packages [13]. The fact that contact dermatitis resulting from the use of TTCMs is associated with a particularly high rate of patch test-positive reactions to individual constituents suggests that patients with positive patch test reactions to TTCM constituents or formulas should be advised to avoid or be more cautious when using them.

The BZK reagent tested in our study was composed of 6 individual/single botanical components with proven sun-protective qualities (data not shown), and as many as 40.0% subjects who underwent photopatch test showed reactions to the formula or its individual constituents. Therefore, we believe that these herbal remedies might have the potential to enhance photosensitisation [19]. In the photopatch test of individual constituents, a positive reaction was observed in 33.3% subjects. Among the constituents examined, Radix *Angelicae dahuricae* is known to contain furocoumarins such as imperatorin, isoimperatorin, and alloimperatorin, which are potentially strong photocontact sensitizers [20–22] that also confer photoallergenic capability. This individual constituent exhibited the highest positive rate in the photopatch test. It is known that the action spectrum of contact photodermatitis is related to the level of exposure to ultraviolet radiation and the concentration of harmful agents [23]. Hence, the adverse effect of photosensitivity should be more carefully considered when defining TTCM application.

The present study was observed to have several limitations. First, the study is limited by the relatively small number of subjects. This is a pilot study with a small sample size, thereby limiting the applicability to populations in Asian countries. However, as different TTCMs are commonly prescribed as skin-whitening agents by traditional Chinese medicine practitioners and are frequently purchased over the counter, our sample size may be sufficient to address issues regarding the frequent adverse effects of TTCMs, particularly, in cosmetics. Second, this study was conducted on healthy volunteers rather than on those with a history of previous sensitisation to TTCMs. Although similar studies had been conducted using subjects with a history of sensitisation, it was not possible in our setting. Third, with regard to plants or herbs with unknown nature or uncertain concentration (many plants or fruits contain crystals and impurities), open application tests, such as patch test or series dilution patch test, may be considered, which may decrease the false positivity of patch test.

Furthermore, to detect low levels of sensitisation, it is suggested to test the individual constituents of a mixture at a higher concentration than those present in the mixture [24]. However, the higher concentration of individual constituent reagents could also result in irritant patch test reaction and deviation from the suggested constituents. In the present study, the positive reaction rate of Radix *Ampelopsis japonica* in the patch test, which was higher than that of BZK, may be owing to the high concentration of the individual reagent (50.0% in petrolatum), which was different from the actual content in BZK (approximately 8.3% of each ingredient in petrolatum). Moreover, patch test using individual constituents was performed at 50% concentration in petrolatum,

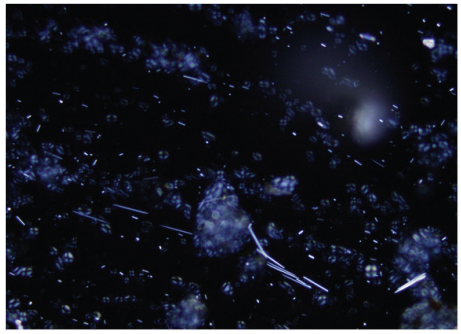


Figure 5: Needle shaped impurities and raphides in Radix *Ampelopsis japonica* preparation under polarizing microscope (400x).

which was higher than that used in the formula. In the screening series aimed to identify contact sensitivities, a positive reaction to the mixture formula is usually associated with a positive reaction to at least 1 of the individual constituents that rarely gives a false-negative reaction if there is contact sensitivity to the mixture. Thus, it is logical to conclude that reactivity to ≥1 individual constituents contributes to reactivity to the mixture. Nevertheless, in the present study, a positive reaction to the individual constituents might not have necessarily led to reactivity to the mixture, which may be owing to the diluted concentration of the individual constituents in the mixture. This dilution effect may explain the more frequent strong positive reactivity of the subjects to individual constituents than to the mixture.

In modern times, herbals are usually used in drugs or cosmetics after extraction. In the present study, we examined the reagents under polarising microscope and found the presence of high quantities of needle-shaped impurities and raphides of Radix *Ampelopsis japonica* rather than BZK (Figure 5). A subsequent patch test experiment with ethanol extraction of Radix *Ampelopsis japonica* (1.0% in petrolatum), which was used to eliminate the impurities and raphides, was conducted 3 months later and was repeated in the 13 subjects who tested positive to Radix *Ampelopsis japonica* or BZK. Interestingly, all the subjects failed to exhibit positive results in the subsequent experiment, suggesting that the needle-shaped impurities and raphides from the herbal powder of Radix *Ampelopsis japonica* might have caused irritant contact dermatitis. Thus, an understanding of these limitations may help to improve future study design. Although it is likely that our findings can be extrapolated to the general population, further studies are needed to confirm our findings, especially with TTCMs after extraction.

5. Conclusions

Recently, the potential use of TTCMs in the development of new skincare cosmetics has been emphasised. In the present study, among the constituents of BZK, Radix *Ampelopsis japonica* and Radix *Angelicae dahuricae* appeared to be the sensitisers. Given the vast variety of TTCMs available in the market, ascertaining the safety and therapeutic effects of cosmetic TTCM products and their constituents should be regarded as mandatory. Furthermore, careful use of TTCMs and ample warnings about the risk of contact dermatitis are necessary, particularly, for cosmetic products.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by the Taiwan National Science Council (NSC102-2314-B-039-025) and in part by the Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH102-TD-B-111-004). The authors would like to thank Enago for the English language review.

References

[1] T. Pluemsamran, P. Tripatara, R. Phadungrakwittaya et al., "Redox mechanisms of AVS022, an oriental polyherbal formula, and its component herbs in protection against induction of matrix metalloproteinase-1 in UVA-irradiated keratinocyte HaCaT cells," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 739473, 10 pages, 2013.

[2] F. Hajiaghaalipour, M. S. Kanthimathi, M. A. Abdulla, and J. Sanusi, "The effect of *Camellia sinensis* on wound healing potential in an animal model," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 386734, 7 pages, 2013.

[3] I. Binic, V. Lazarevic, M. Ljubenovic, J. Mojsa, and D. Sokolovic, "Skin ageing: natural weapons and strategies," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 827248, 10 pages, 2013.

[4] P. Sen, M. S. L. Ho, S.-K. Ng, and G. Yosipovitch, "Contact dermatitis: a common adverse reaction to topical traditional Chinese medicine," *International Journal of Dermatology*, vol. 49, no. 11, pp. 1255–1260, 2010.

[5] Y. H. Leow, "Contact dermatitis due to topical traditional Chinese medication," *Clinics in Dermatology*, vol. 15, no. 4, pp. 601–605, 1997.

[6] K. S. Lim, M. B. Y. Tang, A. T. J. Goon, and H. L. Yung, "The role of topical traditional Chinese medicaments as contact sensitisers in chronic venous leg ulcer patients," *Annals of the Academy of Medicine Singapore*, vol. 36, no. 11, pp. 942–946, 2007.

[7] M. S. Chang, M. J. Choi, S. Y. Park, and S. K. Park, "Inhibitory effects of Hoelen extract on melanogenesis in B16/F1 melanoma cells," *Phytotherapy Research*, vol. 24, no. 9, pp. 1359–1364, 2010.

[8] Y. H. Chang, C. T. Kim, M. Jung, Y.-H. Lim, S. Lee, and S. Kang, "Inhibition of melanogenesis by selina-4(14),7(11)-dien-8-one isolated from Atractylodis Rhizoma Alba," *Biological and Pharmaceutical Bulletin*, vol. 30, no. 4, pp. 719–723, 2007.

[9] Y. H. Cho, J. H. Kim, S. M. Park, B. C. Lee, H. B. Pyo, and H. D. Park, "New cosmetic agents for skin whitening from *Angelica dahurica*," *Journal of Cosmetic Science*, vol. 57, no. 1, pp. 11–21, 2006.

[10] X. L. Piao, S. H. Baek, M. K. Park, and J. H. Park, "Tyrosinase-inhibitory furanocoumarin from *Angelica dahurica*," *Biological and Pharmaceutical Bulletin*, vol. 27, no. 7, pp. 1144–1146, 2004.

[11] M. Carles, M. K. L. Cheung, S. Moganti et al., "A DNA microarray for the authentication of toxic traditional Chinese medicinal plants," *Planta Medica*, vol. 71, no. 6, pp. 580–584, 2005.

[12] X. S. Chen, D. H. Chen, J. Y. Si, and G. Z. Tu, "Chemical constituents of *Typhonium giganteum* Engl," *Journal of Asian Natural Products Research*, vol. 3, no. 4, pp. 277–283, 2001.

[13] H. H. Chen, C. C. Sun, M. P. Tseng, and C. J. Hsu, "A patch test study of 27 crude drugs commonly used in Chinese topical medicaments," *Contact Dermatitis*, vol. 49, no. 1, pp. 8–14, 2003.

[14] S. J. Hooper, M. A. O. Lewis, M. J. Wilson, and D. W. Williams, "Antimicrobial activity of Citrox bioflavonoid preparations against oral microorganisms," *British Dental Journal*, vol. 210, no. 1, article E22, 2011.

[15] T. H. Larsen, P. Gregersen, and G. B. E. Jemec, "Skin irritation and exposure to diisocyanates in orthopedic nurses working with soft casts," *The American Journal of Contact Dermatitis*, vol. 12, no. 4, pp. 211–214, 2001.

[16] C. Thornfeldt, "Cosmeceuticals containing herbs: fact, fiction, and future," *Dermatologic Surgery*, vol. 31, no. 7, pp. 873–880, 2005.

[17] D. A. Kiken and D. E. Cohen, "Contact dermatitis to botanical extracts," *The American Journal of Contact Dermatitis*, vol. 13, no. 3, pp. 148–152, 2002.

[18] D. A. Buckley, S. H. Wakelin, P. T. Seed et al., "The frequency of fragrance allergy in a patch-test population over a 17-year period," *British Journal of Dermatology*, vol. 142, no. 2, pp. 279–283, 2000.

[19] E. Ernst, "Adverse effects of herbal drugs in dermatology," *British Journal of Dermatology*, vol. 143, no. 5, pp. 923–929, 2000.

[20] Y. Xie, W. Zhao, T. Zhou, G. Fan, and Y. Wu, "An efficient strategy based on MAE, HPLC-DAD-ESI-MS/MS and 2D-prep-HPLC-DAD for the rapid extraction, separation, identification and purification of five active coumarin components from radix *Angelicae dahuricae*," *Phytochemical Analysis*, vol. 21, no. 5, pp. 473–482, 2010.

[21] X. L. Piao, I. H. Park, S. H. Baek, H. Y. Kim, M. K. Park, and J. H. Park, "Antioxidative activity of furanocoumarins isolated from *Angelicae dahuricae*," *Journal of Ethnopharmacology*, vol. 93, no. 2-3, pp. 243–246, 2004.

[22] J. Koo and R. Desai, "Traditional Chinese medicine in dermatology," *Dermatologic Therapy*, vol. 16, no. 2, pp. 98–105, 2003.

[23] K. H. Kaidbey and A. M. Kligman, "Photosensitization by coumarin derivatives. Structure-activity relationships," *Archives of Dermatology*, vol. 117, no. 5, pp. 258–263, 1981.

[24] P. J. Frosch, S. C. Rastogi, C. Pirker et al., "Patch testing with a new fragrance mix—reactivity to the individual constituents and chemical detection in relevant cosmetic products," *Contact Dermatitis*, vol. 52, no. 4, pp. 216–225, 2005.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2014, Article ID 634592, 6 pages
http://dx.doi.org/10.1155/2014/634592

Research Article

Effect of Garden Cress Seeds Powder and Its Alcoholic Extract on the Metabolic Activity of CYP2D6 and CYP3A4

Fahad I. Al-Jenoobi,[1] Areej A. Al-Thukair,[1] Mohd Aftab Alam,[1] Fawkeya A. Abbas,[2] Abdullah M. Al-Mohizea,[1] Khalid M. Alkharfy,[3] and Saleh A. Al-Suwayeh[1]

[1] *Department of Pharmaceutics, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia*
[2] *Department of Pharmacognosy, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia*
[3] *Department of Clinical Pharmacy, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia*

Correspondence should be addressed to Fahad I. Al-Jenoobi; aljenobi@ksu.edu.sa and Mohd Aftab Alam; afealam@rediffmail.com

Received 5 November 2013; Accepted 21 January 2014; Published 10 March 2014

Academic Editor: Mohamed Eddouks

The powder and alcoholic extract of dried seeds of garden cress were investigated for their effect on metabolic activity of CYP2D6 and CYP3A4 enzymes. In vitro and clinical studies were conducted on human liver microsomes and healthy human subjects, respectively. Dextromethorphan was used as a common marker for measuring metabolic activity of CYP2D6 and CYP3A4 enzymes. In in vitro studies, microsomes were incubated with NADPH in presence and absence of different concentrations of seeds extract. Clinical investigations were performed in two phases. In phase I, six healthy female volunteers were administered a single dose of dextromethorphan and in phase II volunteers were treated with seeds powder for seven days and dextromethorphan was administered with last dose. The O-demethylated and N-demethylated metabolites of dextromethorphan were measured as dextrorphan (DOR) and 3-methoxymorphinan (3-MM), respectively. Observations suggested that garden cress inhibits the formation of DOR and 3-MM metabolites. This inhibition of metabolite level was attributed to the inhibition of CYP2D6 and CYP3A4 activity. Garden cress decreases the level of DOR and 3-MM in urine and significantly increases the urinary metabolic ratio of DEX/DOR and DEX/3-MM. The findings suggested that garden cress seeds powder and ethanolic extract have the potential to interact with CYP2D6 and CYP3A4 substrates.

1. Introduction

The consumption of herbs is common either in the form of food or as traditional medicine. Since decades, the use of herbs was considered safe. However, in recent years, there are large numbers of reports related to herbal toxicity and herb's effects on basic activities such as modulation of drug metabolizing enzymes and drug transporters and interference with bioavailability and pharmacokinetics of concomitantly administered therapeutic substances [1–3]. The metabolic enzyme modulation also leads to incidences of altered bioavailability and pharmacokinetics of therapeutic substrates. The consequences will be more serious with substrates having narrow therapeutic index [4–6]. In human, most of the drugs are metabolized by CYP3A4, CYP2D6, CYP2C9/10, CYP2C19, CYP2E1, and CYPlA2 [7–9]. Han et al. reported that Rhizoma Coptidis showed in vitro inhibition of CYP2D6 [10]. Corynoline, an isoquinoline alkaloid, showed strong inhibitory effects on the activities of CYP3A4 and CYP2C9 [11]. Myricetin inhibited the liver metabolizing enzymes CYP3A4 and CYP2C9 [12]. St. John's wort induces CYP3A4 and P-glycoprotein and reduces the blood level of cyclosporine, tacrolimus, digoxin, amitriptyline, midazolam, warfarin, indinavir, phenprocoumon, and theophylline [6, 13, 14].

Garden cress (*Lepidium sativum* L.) belongs to the family Cruciferae [15]. The seeds comprise 33–54% of carbohydrates, 25% of protein, 14–24% of lipids, and 8% of crude fiber. The major abundant amino acids of seeds were aspartic and glutamic acids. Potassium was the most abundant mineral [16]. Garden cress seed also contains 20–25% yellowish semidrying oil. The major fatty acid of oil is alpha-linolenic acid

(34.0%). The oil also comprises polyunsaturated fatty acids (46.8%) and monounsaturated fatty acids (37.6%) and antioxidants, such as tocopherols and carotenoids [17, 18]. Seven imidazole alkaloids; lepidine B, C, D, E, and F (dimeric); and two new monomeric alkaloids semilepidinoside A and B were reported in seeds of *L. sativum* [18, 19]. The herb is used for cough, vitamin C deficiency, constipation, and poor immunity and as a diuretic. The modern practitioners of Indian medicine consider the seeds useful in dysenteric diarrhea as well as in febrile and catarrhal infections. The seeds are also considered to be lactagogue [18, 20].

The objective of present investigation was to evaluate the metabolizing enzyme modulating potential of *Lepidium sativum*. The metabolic activities of enzymes CYP3A4 and CYP2D6 were assessed. The human liver microsomes were employed for in vitro investigation, while in vivo study was conducted on healthy human subjects. Dextromethorphan (DEX) was used as common marker for both enzymes. The DEX and its metabolites were estimated in the urine and urinary metabolic ratios of dextromethorphan and its metabolites were calculated. DEX is extensively metabolized in the liver by N-demethylation and O-demethylation. The O-demethylation of DEX is primarily catalyzed by CYP2D6 and forms dextrorphan (DOR) [21]. The N-demethylation of DEX is mediated by CYP3A4 to form 3-methoxymorphinan (3-MM) [22].

2. Materials and Methods

Dextromethorphan hydrobromide, dextrorphan hydrobromide, and 3-methoxymorphinan hydrobromide were purchased from ICN Biomedicals Inc., Warrenale, USA. Dextromethorphan hydrobromide syrup was obtained from Riyadh Pharma Medical and Cosmetic Products Co. Ltd., Riyadh, Saudi Arabia. Human liver microsomes (protein concentration of 20 mg/mL) were purchased from Human Biologics International LLC (HBI, Scpttsdale, USA), shipped in small vials on dry ice, and stored at −80°C. Nicotinamide adenine dinucleotide phosphate (NADPH) and β-glucuronidase (76,800 U/mL) were purchased from Helix Pomatia, ICN Biomedicals Inc., Costa Mesa, AC, USA. Garden cress seeds were purchased from local Saudi Market. Codeine and betaxolol were of USP reference standard. General purpose reagents (GPR) were used for extraction processes, while HPLC grade solvents were used for HPLC determinations. All other materials are of analytical grade.

2.1. Extract of Plant Material. Dried garden cress seeds were finely powered and exhaustively macerated. The cold maceration was carried out for five days with ethanol. Extract was filtered and concentrated under reduced pressure at 40°C by using rotatory evaporator. Weighed concentrated extract was serially diluted with ethanol (96%) to give stock solutions of concentrations of 1.25, 2.5, 5, 25, and 50 mg/mL. These diluted ethanolic stock solutions were stored in refrigerator.

2.2. Microsomal Incubation. Dextromethorphan (DEX) dissolved in methanol was transferred into dried and clean Eppendorf tubes at final reaction concentration of 25 μM. The methanol was evaporated by using nitrogen. Human liver microsomes (0.25 mg/mL protein conc.) and appropriate volume of potassium phosphate buffer (0.1 M, pH 7.4) were transferred into DEX loaded tubes. The loaded composition (DEX, microsomes, and buffer) was gently mixed and preincubated in a shaker water bath at 37°C for 3 minutes. The in vitro metabolic reaction was initiated in a final volume of 0.5 mL by adding 1 mM NADPH, in absence and presence of garden cress seeds extract (at concentrations of 10, 25, 50, and 100 μg/mL). In vitro metabolic reaction was run for about 30 minutes. Reaction was terminated by adding 70% perchloric acid (10 μL) with vigorous shaking for 2-3 minutes. The experiments were repeated in triplicate at each concentration of extract. Codeine (25 μL) was added as an internal standard to each tube. The mixture was centrifuged at 10000 rpm for about 15 minutes. Supernatant was separated and transferred to a clean vial and injected for HPLC analysis of metabolites.

2.3. Clinical Study. Nonsmoking, healthy female volunteers ($n = 6$) of 18–35 years of age were selected to participate in this study. Details of clinical study protocol were explained to the volunteers and written informed consent was received from each one of them. Study protocol was approved by Ethics Committee at College of Medicine, King Saud University, Riyadh. Subjects were asked to refrain from caffeine and caffeine containing products, for at least 24 hours before study. Furthermore, the volunteers were also asked not to take any other traditional/conventional/herbal medications or grapefruit/grapefruit comprising foodstuff for at least two weeks before and during the study. The study was conducted in two phases with two weeks washout period. In phase I, all subjects received a single oral dose of DEX (30 mg) by administering 10 mL of 15 mg/5 mL DEX HBr syrup. The subjects were not allowed to eat for two hours before and after dosing. Subjects were instructed to empty their bladder before dosing. The urine samples were collected for eight hours after DEX administration, and urine aliquots were stored at −20°C until analyzed. In phase II, the powder of garden cress seed was administered in a dose of 7.5 gm twice daily for seven consecutive days. On the last dosing day, subjects received DEX (10 mL of 15 mg/5 mL syrup) and powder of garden cress seeds, concurrently. The subjects were not allowed to eat for two hours before and after dosing. Subjects were instructed to empty their bladder before dosing. The urine samples were collected and stored at −20°C until analyzed. The concentrations of DEX and its metabolites were estimated by using HPLC methods. The urinary metabolic ratios of DEX/3-MM and DEX/DOR were used as indices to metabolic activities of CYP3A4 and CYP2D6 enzymes, respectively.

2.4. Preparation of Urine Samples. The human urine samples were hydrolyzed by using β-glucuronidase (19200 U/mL, incubated for 18 hours) to obtain unconjugated form of DEX and its metabolites. Hydrolyzed urine sample (1 mL) was vigorously mixed with 5 mL organic extracting solvent system

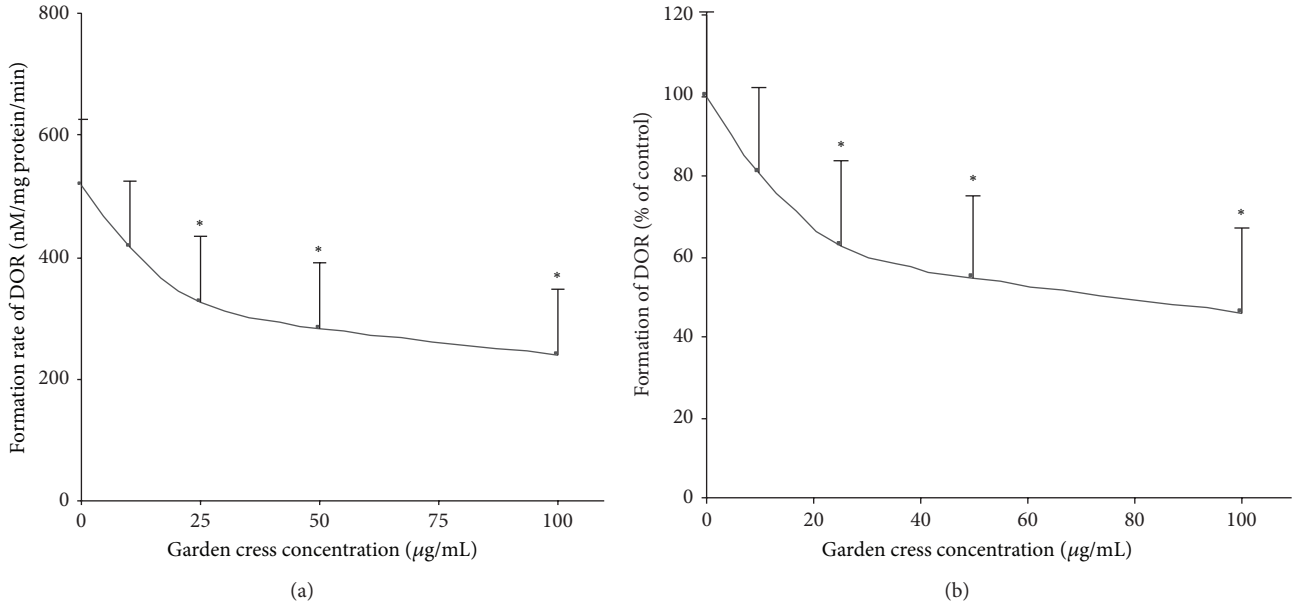


Figure 1: Effect of garden cress on the formation of DOR from DEX in human liver microsomes ($n = 3$, mean ± SD). Formation of the metabolite is expressed as (nM/mg protein/min) in (a) and percent of control in (b). $^{*}P \leq 0.05$.

(diethyl ether/chloroform/propranolol, 20 : 9 : 1 v/v/v). This vigorously mixed mixture was centrifuged at 14500 g for 10 minutes. The organic layer was separated and again vigorously mixed with 300 μL of 0.1 N hydrochloric acid and centrifuged at 14500 g for 10 minutes. The aqueous layer was separated and transferred into HPLC vials for analysis.

2.5. Determination of DEX and Its Metabolites. The analysis of DEX metabolites was performed on Shimadzu Class-VPV 5.02 instrument. Two distinct HPLC methods were adopted for analysis of DEX and its metabolites in human liver microsomes and the urine of human volunteers [23, 24]. In vitro microsomal analytes were eluted on a Nova-Pak phenyl column (5 μm, 150 × 3.9 mm) by using a mobile phase composed of a 75 : 25 mixture of acetonitrile and water (1.5% glacial acetic acid and 0.1% triethylamine), flowing at 1 mL/min. The urine samples were analyzed by using a Zorbax SB-CN column (5 μm, 250 × 4.6 mm). The mobile phase for urine samples was a mixture of water (1.5% glacial acetic acid and 0.1% triethylamine) and acetonitrile (87.5 : 12.5 v/v). The pH of the mobile phase was adjusted to 3 using orthophosphoric acid. Analytes were monitored using a fluorescence detector at excitation and emission wavelengths of 280 and 330 nm, respectively. The calibration curves for microsomal metabolite and dextromethorphan were constructed.

2.6. Statistical Analysis. For in vitro microsomal study, formation of metabolites from DEX in presence of garden cress extract was compared to that of control by using one-way analysis of variance (ANOVA) and a post hoc Scheffe's multiple comparison test with a significant P value ≤0.05. Statistical analysis of results obtained from clinical study was performed by using paired Student's t-test. Differences were considered statistically significant when P values were ≤ 0.05. Statistical analysis was conducted using Graph-Pad Prism version 3.0 for Windows (San Diego, CA, USA).

3. Results

The effect of garden cress seeds powder and its ethanolic extract was investigated on CYP2D6 and CYP3A4 mediated metabolism of dextromethorphan. The level of DEX metabolites dextrorphan (DOR) and 3-methoxymorphinan (3-MM) was determined in the absence and presence of garden cress. The investigations were carried out in vitro by using human liver microsomes and healthy volunteers were recruited for in vivo study. Ethanolic extract of garden cress seeds showed concentration-dependent inhibition of DOR and 3-MM formation. In vitro results are illustrated in Figures 1 and 2. Figure 1(a) represents the formation of DOR (nM/mg protein/min). Figure 2(a) represents the formation of 3-MM (mM/mg protein/min). At lower concentration (10 μg/mL), ethanolic extract produced insignificant effect, and about 20% inhibition of DOR and 3-MM level was observed. Ethanolic extract at concentrations of 25 and 50 μg/mL produced statistically significant inhibition of about 37–45% DOR and about 42–52% 3-MM levels, respectively. The maximum inhibition (about 50%) of DOR formation was observed at highest tested concentration of garden cress extract (100 μg/mL). Ethanolic extract also produced a statistically significant inhibition of 3-MM level (by 60%) at 100 μg/mL.

Clinical observations showed that garden cress seeds powder produced a remarkable inhibitory effect on the DOR level in urine. The quantitative reduction in the amount of DOR excreted in urine in presence of herb was about 30%. The urine metabolic ration of DEX/DOR was increased

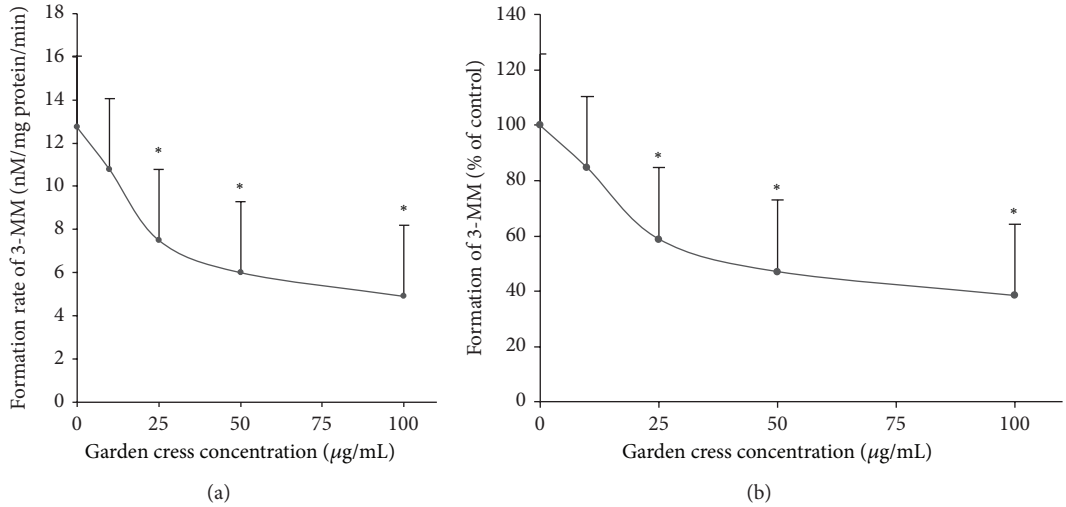


Figure 2: Effect of garden cress on the formation of 3-MM from DEX in human liver microsomes ($n = 3$, mean ± SD). Formation of the metabolite is expressed as (nM/mg protein/min) in (a) and percent of control in (b). $^{*}P \leq 0.05$.

Table 1: Urinary metabolic ratio (MR) of DEX with its metabolites ($n = 6$).

Subjects	Urinary metabolic ratio DEX/DOR		Urinary metabolic ratio DEX/3-MM	
	MR, control	MR, phase-II	MR, control	MR, phase-II
1	0.034	0.049	2.387	3.388
2	0.067	0.086	4.382	8.0586
3	0.030	0.060	1.382	2.781
4	0.028	0.038	0.379	0.519
5	0.032	0.044	2.118	3.666
6	0.073	0.090	3.770	5.160
Mean	0.044	0.061	2.403	3.929
SD	0.020	0.022	1.483	2.524
P	0.002		0.025	

significantly in presence of garden cress seed powder (see Table 1). Table 1 summarizes the ratio of DEX and its metabolites excreted in urine before and after administration of garden cress seed powder. As presented in Table 1, the metabolic ratio of DEX/3-MM was also increased significantly in presence of garden cress seeds powder. The inhibitory effect of garden cress on 3-MM level in urine was statistically significant; about 40% inhibition was recorded.

4. Discussion

The in vitro and in vivo metabolic activities of enzymes CYP2D6 and CYP3A4 were assessed in presence and absence of garden cress seed powder and ethanolic extract. These investigations were carried out to demonstrate the enzymes modulating potential of garden cress seeds powder and ethanolic extract. Dextromethorphan was used as a common probe for CYP2D6 and CYP3A4 enzymes [25–28]. The O-demethylation of dextromethorphan was used to assess the in vitro and in vivo metabolic activity of CYP2D6. The N-demethylation of dextromethorphan was used to assess the activity of CYP3A4. Garden cress ethanolic extract significantly inhibited the activity of hepatic microsomal enzymes CYP2D6 and CYP3A4, which was represented by decreased level of metabolites DOR and 3-MM in in vitro experiments. The effect was observed as concentration dependent. The highest decrease in the metabolite levels was observed at the highest tested concentration of extract. These findings suggested that garden cress have potential to inhibit metabolic activity of CYP2D6 and 3A4. A clinical study was designed to evaluate the effect of garden cress on metabolic activity of CYP2D6 and 3A4. Therefore, the effect was evaluated in two phases on healthy volunteers. The garden cress significantly diminishes the level of DOR and 3-MM metabolites excreted in urine. Furthermore, the urinary metabolic ratio of DEX/DOR and DEX/3-MM was also enhanced. The high intraindividual variability of DEX/3-MM urinary metabolic ratio was observed, which may be because of partial N-demethylation of DEX by CYP3A4. These observations indicated that garden cress has remarkable inhibitory effect on the activities of human CYP2D6 and

CYP3A4 enzymes. The results of clinical study were generally consistent with the results obtained from in vitro microsomal investigations.

5. Conclusion

Caution should be warranted when garden cress seeds are consecutively administered with therapeutics medicines metabolized by CYP2D6 and CYP3A4 enzymes. Special attention is required if the substrate is of narrow therapeutic index such as carbamazepine and cyclosporine. Further investigations are suggested to isolate the active constituents responsible for this inhibitory effect and to find exact mechanism of interaction between garden cress and CYP2D6 and 3A4 substrates.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors acknowledge the financial and logistic support by College of Pharmacy Research Center and the Deanship of Scientific Research in King Saud University.

References

[1] T. Efferth and B. Kaina, "Toxicities by herbal medicines with emphasis to traditional Chinese medicine," *Current Drug Metabolism*, vol. 12, no. 10, pp. 989–996, 2011.

[2] C.-F. Chien, Y.-T. Wu, W.-C. Lee, L.-C. Lin, and T.-H. Tsai, "Herb-drug interaction of *Andrographis paniculata* extract and andrographolide on the pharmacokinetics of theophylline in rats," *Chemico-Biological Interactions*, vol. 184, no. 3, pp. 458–465, 2010.

[3] X.-X. Yang, Z.-P. Hu, W. Duan, Y.-Z. Zhu, and S.-F. Zhou, "Drug-herb interactions: eliminating toxicity with hard drug design," *Current Pharmaceutical Design*, vol. 12, no. 35, pp. 4649–4664, 2006.

[4] A. C. Müller and I. Kanfer, "Potential pharmacokinetic interactions between antiretrovirals and medicinal plants used as complementary and African traditional medicines," *Biopharmaceutics and Drug Disposition*, vol. 32, no. 8, pp. 458–470, 2011.

[5] A. A. Izzo and E. Ernst, "Interactions between herbal medicines and prescribed drugs: a systematic review," *Drugs*, vol. 61, no. 15, pp. 2163–2175, 2001.

[6] G. W. Barone, B. J. Gurley, B. L. Ketel, and S. R. Abul-Ezz, "Herbal supplements: a potential for drug interactions in transplant recipients," *Transplantation*, vol. 71, no. 2, pp. 239–241, 2001.

[7] S.-F. Zhou, "Drugs behave as substrates, inhibitors and inducers of human cytochrome P450 3A4," *Current Drug Metabolism*, vol. 9, no. 4, pp. 310–322, 2008.

[8] S. Y. Lee, J. Y. Lee, W. Kang et al., "Cytochrome P450-mediated herb-drug interaction potential of Galgeun-tang," *Food and Chemical Toxicology*, vol. 51, pp. 343–349, 2013.

[9] S. Y. Lee, J. Y. Lee, W. Kang et al., "*In vitro* and *in vivo* assessment of cytochrome P450-mediated herb-drug interaction of Ssanghwa-tang," *Food Chemistry*, vol. 136, no. 2, pp. 450–457, 2013.

[10] Y.-L. Han, H.-L. Yu, D. Li et al., "*In vitro* inhibition of huanglian [*Rhizoma coptidis* (L.)] and its six active alkaloids on six cytochrome P450 isoforms in human liver microsomes," *Phytotherapy Research*, vol. 25, no. 11, pp. 1660–1665, 2011.

[11] Z.-Z. Fang, Y.-Y. Zhang, G.-B. Ge et al., "Identification of cytochrome P450 (CYP) isoforms involved in the metabolism of corynoline, and assessment of its herb-drug interactions," *Phytotherapy Research*, vol. 25, no. 2, pp. 256–263, 2011.

[12] D.-H. Choi, C. Li, and J.-S. Choi, "Effects of myricetin, an antioxidant, on the pharmacokinetics of losartan and its active metabolite, EXP-3174, in rats: possible role of cytochrome P450 3A4, cytochrome P450 2C9 and P-glycoprotein inhibition by myricetin," *Journal of Pharmacy and Pharmacology*, vol. 62, no. 7, pp. 908–914, 2010.

[13] Z. Hu, X. Yang, P. C. L. Ho et al., "Herb-drug interactions: a literature review," *Drugs*, vol. 65, no. 9, pp. 1239–1282, 2005.

[14] A. A. Izzo, "Herb-drug interactions: an overview of the clinical evidence," *Fundamental and Clinical Pharmacology*, vol. 19, no. 1, pp. 1–16, 2005.

[15] N. D. Raval and T. N. Pandya, "Pharmacognostic study of *Lepidium sativum* Linn (*Chandrashura*)," *Ayu*, vol. 32, no. 1, pp. 116–119, 2011.

[16] S. S. Gokavi, N. G. Malleshi, and M. Guo, "Chemical composition of garden cress (*Lepidium sativum*) seeds and its fractions and use of bran as a functional ingredient," *Plant Foods for Human Nutrition*, vol. 59, no. 3, pp. 105–111, 2004.

[17] B. T. Diwakar, P. K. Dutta, B. R. Lokesh, and K. A. Naidu, "Physicochemical properties of garden cress (*lepidium sativum* l.) seed oil," *Journal of the American Oil Chemists' Society*, vol. 87, no. 5, pp. 539–548, 2010.

[18] P. K. Datta, B. T. Diwakar, S. Viswanatha, K. N. Murthy, and K. A. Naidu, "Safety evaluation studies on Garden cress (*Lepidium sativum* L.) seeds in Wistar rats," *International Journal of Applied Research in Natural Products*, vol. 4, no. 1, pp. 37–42, 2011.

[19] U. H. Maier, H. Gundlach, and M. H. Zenk, "Seven imidazole alkaloids from *Lepidium sativum*," *Phytochemistry*, vol. 49, no. 6, pp. 1791–1795, 1998.

[20] N.-U. Rehman, A.-U. Khan, K. M. Alkharfy, and A.-H. Gilani, "Pharmacological basis for the medicinal use of *Lepidium sativum* in airways disorders," *Evidence-based Complementary and Alternative Medicine*, vol. 2012, Article ID 596524, 8 pages, 2012.

[21] J. W. Barnhart, "The urinary excretion of dextromethorphan and three metabolites in dogs and humans," *Toxicology and Applied Pharmacology*, vol. 55, no. 1, pp. 43–48, 1980.

[22] J. C. Gorski, D. R. Jones, S. A. Wrighton, and S. D. Hall, "Characterization of dextromethorphan N-demethylation by human liver microsomes: contribution of the cytochrome P450 3A (CYP3A) subfamily," *Biochemical Pharmacology*, vol. 48, no. 1, pp. 173–182, 1994.

[23] E.-K. Bendriss, N. Markoglou, and I. W. Wainer, "High-performance liquid chromatography assay for simultaneous determination of dextromethorphan and its main metabolites in urine and in microsomal preparations," *Journal of Chromatography B*, vol. 754, no. 1, pp. 209–215, 2001.

[24] D. I. Min, Y.-M. Ku, A. Vichiendilokkul, and L. L. Fleckenstein, "A urine metabolic ratio of dextromethorphan and 3-methoxymorphinan as a probe for CYP3A activity and

prediction of cyclosporine clearance in healthy volunteers," *Pharmacotherapy*, vol. 19, no. 6, pp. 753–759, 1999.

[25] A. Yu and R. L. Haining, "Comparative contribution to dextromethorphan metabolism by cytochrome P450 isoforms *in vitro*: can dextromethorphan be used as a dual probe for both CYP2D6 and CYP3A activities?" *Drug Metabolism and Disposition*, vol. 29, no. 11, pp. 1514–1520, 2001.

[26] J. Ducharme, S. Abdullah, and I. W. Wainer, "Dextromethorphan as an *in vivo* probe for the simultaneous determination of CYP2D6 and CYP3A activity," *Journal of Chromatography B*, vol. 678, no. 1, pp. 113–128, 1996.

[27] C. Funck-Brentano, P.-Y. Boëlle, C. Verstuyft, C. Bornert, L. Becquemont, and J.-M. Poirier, "Measurement of CYP2D6 and CYP3A4 activity *in vivo* with dextromethorphan: sources of variability and predictors of adverse effects in 419 healthy subjects," *European Journal of Clinical Pharmacology*, vol. 61, no. 11, pp. 821–829, 2005.

[28] D. R. Jones, J. C. Gorski, M. A. Hamman, and S. D. Hall, "Quantification of dextromethorphan and metabolites: a dual phenotypic marker for cytochrome P450 3A4/5 and 2D6 activity," *Journal of Chromatography B*, vol. 678, no. 1, pp. 105–111, 1996.