



Research Paper

Evaluation of the indicative roles of seven potential biomarkers on hepato-nephrotoxicity induced by *Genkwa Flos*



Yu Jiang^a, Liqiang Gu^a, Ruowen Zhang^b, Yuanyuan Zhang^a, Lunhui Zhang^a, Ping Ju^a,
Bingjie Ma^a, Kexia Zhang^a, Kaishun Bi^a, Xiaohui Chen^{a,*}

^a School of Pharmacy, Shenyang Pharmaceutical University, Shenyang 110016, China

^b Department of Pathology, School of Medicine and Health Sciences, University of North Dakota, 501 North Columbia Rd., Grand Forks, ND 58202 USA

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ABSTRACT

Ethnopharmacological relevance: *Genkwa Flos*, a classical traditional Chinese medicine, is used for the definite antitumor activity and tends to be taken overdose or long term in these years. While the excessive application can result in damage to liver and kidney. In this study, the indicative roles of seven potential biomarkers were evaluated to investigate hepato-nephrotoxicity in the early stages after oral administration of *Genkwa Flos* for 14 days.

Materials and methods: Histopathology, serum biochemistry and seven potential biomarkers in serum or urine from male Sprague–Dawley rats were monitored. Hepatic and renal tissues were histopathologically examined to identify specific changes occurring. Routine serum biochemical parameters were tested by using standard clinical laboratory methods. Seven biomarkers including cholic acid, taurine, 5-oxoproline, hippuric acid, uric acid, 3-indoxyl sulfate and kynurenic acid were detected by a developed LC–MS method.

Results: The histopathological alterations and the increased levels of serum biochemistry were detected on the 8th day after *Genkwa Flos* treated. The seven analytes were also found significantly changed in *Genkwa Flos* treated group, especially cholic acid, taurine, 5-oxoproline and hippuric acid which were changed on the 2nd or 4th day.

Conclusions: Although serum biochemistry and histopathology suggested that *Genkwa Flos* was responsible for the hepato-nephrotoxicity that occurred following the ingestion of this medicinal herb, evaluation of these biomarkers might be more beneficial for the early detection of liver and kidney injuries. This study could be further used in hepatic and renal failures caused by other reasons in the following research works.

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1. Introduction

Genkwa Flos (GF), the flower bud of *Daphne genkwa* Sieb. et Zucc. (Thymelaeaceae), known as a valuable traditional Chinese medicine, is well known for the treatment of edema, ascites, sudden cough, asthma and cancer (Hong et al., 2011; Zhan et al., 2005). The herbal medicine has been officially listed in the Chinese

Pharmacopoeia for its definite pharmaceutical activity and wide use (Chinese Pharmacopoeia Commission, 2010). However, it was limited to use due to its toxicity which has been known for a long time. GF was classified low grade with mild toxicity existed in Shen Nong's Herbal Classic. There were also evidences that excessive and chronic use of the raw herb will finally result in serious damages to liver and kidney (Yang et al., 1989). It is widely recognized that the identification of hepato-nephrotoxicity induced by GF are particularly essential for the safe and effective use of medicinal herbs in clinical practice.

Hepatotoxicity and nephrotoxicity are important causes of human ill health (Beger et al., 2010a), while early detection of herb-induced hepato-nephrotoxicity is still quite difficult. Routine measurements, such as serum biochemistry and histopathology, are efficient in evaluating liver and kidney damages, while the two measurements were effective when severe liver and kidney impairments happened (Geng et al., 2013a, 2013b; Zhou et al., 2008; Gibbs, 2005). Hence, the reliable and efficient indices are

Abbreviations: GF, *Genkwa Flos*; LC–MS, Liquid chromatography–mass spectrometry; CA, cholic acid; TA, taurine; 5-OP, 5-oxoproline; HIP, hippuric acid; UA, uric acid; 3-IS, 3-indoxyl sulfate; DB, *Rhizoma Dioscoreae bulbiferae*; AM, *Aristolochia manshuriensis*; IS, internal standard; HPLC–DAD, High performance liquid chromatography–diode array detector; HCG, Healthy control group; HPCG, Hepatotoxic positive control group; NPCG, Nephrotoxic positive control group; RGFG, raw *Genkwa Flos* group; AST, aspartate aminotransferase; ALT, alanine aminotransferase (ALT); BUN, urea nitrogen; CR, creatinine; ESI, electrospray ionization; KYN, the kynurenine pathway; IDO, indoleamine 2,3-dioxygenase

* Corresponding author. Tel./fax: +86 2423986259.

E-mail address: cxh_syphu@hotmail.com (X. Chen).

critical for monitoring hepato-nephrotoxicity induced by GF in the early stages of drug treated.

Endogenous metabolites with low molecular weight play important roles in various metabolic processes, and levels of some metabolites in body fluids or tissues are liable to change when the internal environment alters. Thus, the significantly changed endogenous metabolites were regarded as biomarkers for monitoring different diseases (Beger et al., 2010a). Recently, some metabonomics studies have been done to discover novel biomarkers for drug-induced liver and kidney injuries (Boudonck et al., 2009; Kumar et al., 2012; Ma et al., 2010a, 2010b; Weiss and Kim 2011; Yang et al., 2012). According to the reported studies (Chan et al., 2008; Geenen et al., 2011; Kumar et al., 2012; Lenz et al., 2004; Uehara et al., 2014; Xu et al., 2011; Zhang et al., 2014; Zhao et al., 2012), several potential hepato-nephrotoxicity biomarkers come up, such as cholic acid (CA), taurine (TA), 5-oxoproline (5-OP), hippuric acid (HIP), uric acid (UA), 3-indoxyl sulfate (3-IS) and kynurenic acid (KA).

Bile acids have many physiological roles and exhibit both toxic and protective influences within the liver. Alterations in the BA profile may be the result of disease induced liver injury (Lake et al. 2013). CA, one of the primary bile acids produced by liver, is widely recognized as a biomarker of hepatotoxicity (Geng et al., 2013a, 2013b). So it is typical that CA as a hepatotoxicity indicator was tested in this study. TA is a sulfur-containing amino acid and mainly as bile acid conjugates in mammal liver. When liver disease takes place, TA could not combine with bile acids and the increased free TA might come into being. Thus, TA is also regarded as a potential hepatotoxicity biomarker (Yang et al., 2008). Glutathione biosynthesis occurs via the gamma-glutamyl cycle and a number of studies have shown increases in the concentration of 5-OP, an intermediate in this pathway, in various biofluids/tissues, following the administration of hepatic toxicants (Geenen et al., 2011; Xu et al., 2011). HIP is the catabolic product of phenylalanine by the gut microflora (Ohno et al., 2001), and the intestinal flora disturbance is closely related with the impairing degree of the liver function. Thus, HIP might also be used as a biomarker for monitoring liver injury. UA is the major end product of purine metabolism and purine metabolism might be a disorder following kidney damage. It has been widely used as a nephrotoxicity biomarker in the clinical diagnose (Levey et al., 2006; Levey et al., 2007). According to the previous studies (Xie et al., 2010), the metabolic pathways would be significantly perturbed if kidney damage happened, which mainly involve amino acid metabolism. While 3-IS and KA are mainly from these amino acid metabolism pathways, indicating that the two biomarkers may be used to reflect renal failure.

In this study, seven potential hepato-nephrotoxicity biomarkers were simultaneously determined and evaluated for GF-induced liver and renal failures during the early stages of herb treated. In order to investigate GF-induced hepato-nephrotoxicity resulting from short-term dosing for 14 days, four serum biochemical parameters, liver and kidney histopathological alterations were applied as routine measurements. As the levels of these biomarkers are low and they are hard to be separated with other endogenous metabolites in the body fluids, routine method such as ultraviolet method is not suitable to detect these biomarkers in this study. Thus, seven metabolite biomarkers in rats serum and urine were also quantified by a simple LC–MS method, including CA, TA, 5-OP and HIP as liver function markers, UA, 3-IS and KA as kidney function markers. *Rhizoma Dioscoreae bulbiferae* (DB) and *Aristolochia manshuriensis* (AM) were selected as hepatotoxic and nephrotoxic positive control herbs respectively for obvious hepatotoxicity and nephrotoxicity showed in the reported studies (Tan et al. 2003; Liu et al. 2003). The main purpose of this study is to evaluate the indicative roles of the seven potential biomarkers, whose levels might be significantly changed in early detection of GF toxicity. The newly found biomarkers could be used not only in monitoring hepato-nephrotoxicity induced by GF, which might be conducive to GF's clinical application,

but also in monitoring hepatic and renal failures caused by other herbs or drugs in early stages.

2. Experimental

2.1. Chemicals, reagents and materials

GF and DB were purchased from the Liaoning Chinese Herbal Medicine Factory (Shenyang, Liaoning, China). AM was supplied by Shenyang Pharmaceutical University. The herbs were all authenticated by Professor Jincai Lu of Traditional Chinese Medicine College, Shenyang Pharmaceutical University. According to Chinese pharmacopoeia (Chinese Pharmacopoeia Commission, 2010), the quality of GF is controlled by the level of genkwanin (1.86 mg/g), which was detected by liquid chromatography external standard method.

Standard compounds of CA, TA, 5-OP and UA were supplied from Sigma-Aldrich (MO, USA). Standard compounds of 3-IS, HIP and KA were obtained from J&K Co. Ltd. (Beijing, China). Bendrofluazide as the internal standard (IS) were purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). The purities of the reference standards were determined not less than 98% by HPLC-DAD (Shimadzu, Tokyo, Japan). The structures of the analytes and IS are shown in Fig. 1.

Acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific (Nanjing, China). Fomic acid (HPLC grade) was obtained from Yuwang Industrial Co. Ltd. (Shandong, China). Redistilled and deionized water was used for all aqueous solutions throughout the study. Fermented rice vinegar (pH 2.92) was purchased from Hongmei Corporation (Shenyang, Liaoning, China).

Preparation of herb extract: the dried and powdered GF (1 kg) were extracted three times by macerating with 8 L of 95% ethanol for 12 h each time. DB (1 kg) and AM (500 g) were extracted three times by refluxing with water (1:10, w/v) for one hour each time (Geng et al., 2013a, 2013b). All the solvents were collected and then removed under reduced pressure. Then, the extracts of raw GF (RGF), DB and AM were redissolved with 0.5% CMC-Na solution and diluted to a volume equivalent to 2 g RGF/mL, 4 g DB/mL and 0.5 g AM/mL. Solutions were stored at 4 °C before administration to animals.

2.2. Animal treatment

A total of 72 male pathogen-free Sprague–Dawley rats (230 ± 20 g) were kindly supplied by the Experimental Animal Center of Shenyang Pharmaceutical University. (Shenyang, Liaoning, China). Animals were housed in a room with an ambient temperature of 20–25 °C, 12 h light and dark cycles, and relative humidity of 45–55%. All rats were allowed to acclimate for a period of one week with free access to water and standard rodent food. All animal studies were carried out in accordance with the Guideline for Animal Experimentation of Shenyang Pharmaceutical University, and the protocol was approved by the Animal Ethics Committee of the Institution (No. SYP-UIACUC-2014-0005). All rats were randomly divided into four groups ($n=18$ /group) as follows:

Healthy control group (HCG): Rats were orally administrated the approximately same volume of 0.5% CMC-Na solution as rats in RGF for 14 days.

Hepatotoxic positive control group (HPCG): Rats were orally administrated DB for 14 days at a dose of 25 g/kg/day.

Nephrotoxic positive control group (NPCG): Rats were orally administrated AM for 14 days at a dose of 7.5 g/kg/day.

RGF group (RGFG): Rats were orally administrated RGF for 14 days at a dose of 12 g/kg/day.

The dose of GF was determined according to the previous studies and based on the results of our preliminary experiments, in which four different doses (6, 12, 18, 24 g/kg/d) were designed

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