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Toxicity of daphnane-type diterpenoids from Genkwa Flos and their pharmacokinetic profile in rat

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ABSTRACT

Daphnane-type diterpenoids (DDs) are the main types of plant diterpene orthoesters known and have remarkable biological activities. However, the *in vivo* toxicity and pharmacokinetic profile of DDs remains unkonwn. The aim of this study was to investigate the toxicity and pharmacokinetic profile of DDs from Genkwa Flos (Thymelaeaceae). The toxicity of diterpenoids was evaluated after oral administration of total diterpenoids extract from Genkwa Flos to rats, and the blood concentration of diterpenoids was analyzed by ultra performance liquid chromatography tandem triple-quadrupole mass spectrometry (UPLC–TQ-MS). The diterpenoids were confirmed to be the toxic components of Genkwa Flos. The pharmacokinetic profile of these diterpenoids was quite different due to their different structures. Although the contents of yuanhuafine and yuanhuapine were low in the extract, the blood concentrations were extremely high. In contrary, the contents of genkwanine F and Wikstroemia factor M_1 in the extract were much higher, but they could not be detected in the blood. This result implied that yuanhuafine and yuanhuapine but not genkwanine F and Wikstroemia factor M_1 were the potentail toxic components of Genkwa Flos *in vivo*. This paper shows for the first time the toxicity of diterpenoids from Genkwa Flos was correlated with their blood concentration and when DDs were used for medicinal purposes, their contents in herb as well as their blood concentrations should be considered.

Introduction

Herbal medicines are widely used around the world, which have a history of several thousands of years for the prevention, diagnosis and treatment of diseases. Since early times, toxicity has been recognized as an intrinsic property of herbal medicines. The intrinsic toxicity of herbal plants generally results from the toxic chemical constituents in herbs (Efferth and Kaina, 2011). The best strategy to minimize the risk posed by toxic herbs is zero exposure. However, it is sometimes inevitable because the herbs are beneficial and necessary for a specific treatment due to the lack of alternatives. So the dosage is the key issue for both therapeutic efficacy and safety. Ideally any herbal medicine, especially those containing toxic components should be used under well-controlled conditions.

As a well-known traditional Chinese medicine (TCM), Genkwa Flos (GF), the dried flower buds of *Daphne genkwa* Sieb. et Zucc. (Thymelaeaceae), has been used for the diuretic, antitussive, expectorant, abortifacient, and antitumor purposes for centuries (Medicine, 2006). In Shen Nong's Herbal Classic, China's oldest pharmacy monograph, it was classified as "Xiapin" (low grade) which means mild toxicity existed. Even in the Pharmacopeia of the People's Republic of China (Committee, 2010), it is recorded and described as slightly toxic. There is evidence that excessive and chronic use of GF will finally result in serious damage to liver, lung, kidney, brain and heart (Xiang et al., 2006; Yang et al., 1989), and it was publicly known to have irritation to mucous and skin (Xia, 2005). Recently, a metabonomic approach was applied to evaluate GF-induced hepatotoxicity and the mechanism was considered to be related to the disturbance of amino acid metabolism, gut microflora and bile acid biosynthesis (Geng et al., 2013).

Previous phytochemical studies have indicated that GF contains different types of chemical components, including flavonoids, diterpenoids and coumarins (Akhtar et al., 2006; Hong et al., 2010; Li et al., 2010; Zhan et al., 2005), and among which, daphnane-type diterpenoids (DDs) are main active constituents. DDs are believed to be derived from a tigliane precursor and have an orthoester motif, though a very large number of DDs were identified, they occurred only in the plant families of Thymelaeaceae and Euphorbiaceae (Evans and Soper, 1978). DDs not only have remarkable biological activities such as antitumor (Badawi et al., 1983; Jo et al., 2012), antifertility (Hu et al., 1985; Wang et al., 1981; Ying et al., 1977), antihyperglycemic (Carney et al., 1999), antiviral (Allard et al., 2012), anti-bladder-hyper-reflexia (Appendino and Szallasi, 1997), anti-HIV (Asada et al., 2011; Huang et al., 2012)

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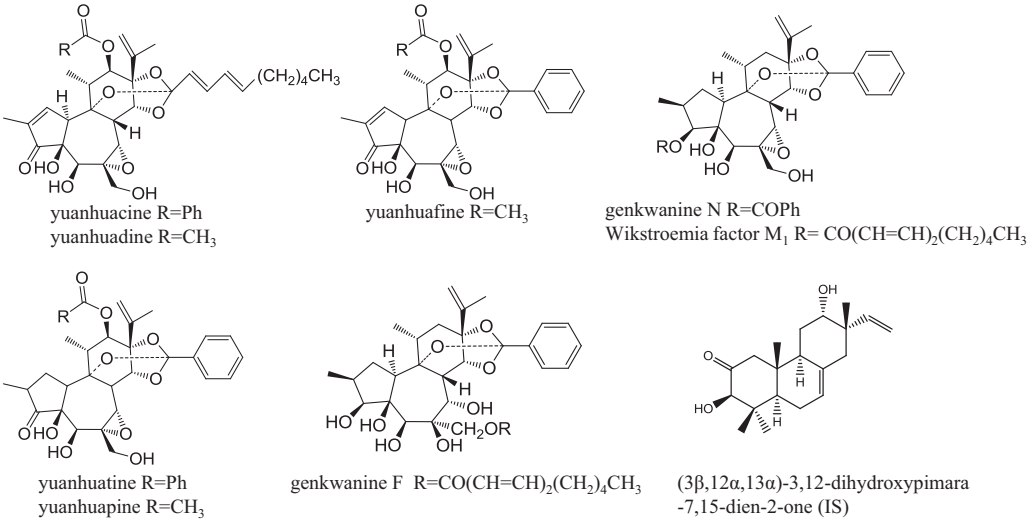

Fig. 1. Chemical structure of diterpenoids and IS.

and neurotrophy (He et al., 2002b), but also exhibit toxicity such as cytotoxic (Zhan et al., 2005), pesticidal (Sakata et al., 1971) and irritant (Evans et al., 1992).

DDs are a class of important natural compounds with a non-negligible toxicity, however, as far as we are concerned, there have been few papers published on the *in vivo* toxicity and pharmacokineitcs studies for DDs. Since the relationship between the toxicity and toxic chemical components of herbal medicines as well as their pharmacokineitc profile has been paid more and more attention, this study was undertaken to investigate the toxicity and pharmacokinetic profile of DDs from GF in rats by evaluating the toxicological effects and determinating the blood concentration of each diterpenoid.

Materials and methods

Plant materials and chemicals

The dried flower buds of *Daphne genkwa* Sieb. et Zucc. were collected from Liu'an city, Anhui province, China. The material was authenticated by the corresponding author, and the voucher specimen (No. 110326) was deposited at the Herbarium in Jiangsu Key Laboratory for High Technology Research of TCM Formulae, Nanjing University of Chinese Medicine. Yuanhuacine, yuanhuadine, yuanhuafine, yuanhuatine, yuanhuapine, genkwanine F, genkwanine N and Wikstroemia factor M_1 and (3β,12α,13α)-3,12-dihydroxypimara-7,15-dien-2-one (internal standard, IS) were isolated and purified in our laboratory. On the basis of UV, NMR and MS analysis, their structures were confirmed, and their purities determined using UPLC–PDA-MS were over 98.0%. Their structures are presented in Fig. 1. Acetonitrile and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany) and deionized water was purified by an EPED super purification system (Eped, Nanjing, China). Other chemicals and solvents used in this study were of analytical grade (Nanjing Chemical Plant, Nanjing, China).

Preparation of total diterpenoids extract from GF (TDG)

One thousand grams of GF was soaked in petroleum and extracted three times by decocting with petroleum (1:10, 1:10, and then 1:8, w/v) for 2 h per time. The extracts were combined and petroleum was removed under reduced pressure, the residue was then dissolved and precipitated with dehydrated ethanol, stored at room temperature till cold. After the precipitation was filtered, the ethanol was removed under reduced pressure and 16 g total diterpenoids extract was obtained. The contents of eight diterpenoids in total diterpenoids extract from GF (TDG) were measured quantitatively by external standard method using the same chromatography conditions as described above. The contents of yuanhuacine, yuanhuadine, yuanhuafine, yuanhuadine, yuanhuapine, genkwanine F, genkwanine N and Wikstroemia factor M_1 in the extract were 7.24, 10.70, 1.97, 1.04, 1.31, 11.90, 4.03 and 23.31 mg/g, respectively.

Animals

Male Sprague-Dawley rats weighing 180–200 g were purchased from Shanghai Slac Laboratory Animal Co. Ltd., China. The animals were housed under controlled temperature (25 ± 1 °C), relative humidity (40–70%), and a 12-h light/dark cycle for minimum of 7 days before use and fed with food and water ad libitum. All the procedures were in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council.

Oral toxicity

Twenty-four rats were divided into four groups ($n = 6$) at random. After fasting over night, TDG solution (dissolved in physiological saline solution with 0.5% Tween Monostearate (Tween 80)) was orally administrated to rats at doses of 0.1, 0.25, 0.5 and 1 g/kg. In order to evaluate the toxicity, blood samples were analyzed. Blood samples were collected from each rat by retro-orbital puncture at a predetermined time interval of pre-dose, 0.17, 0.33, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 10, 12 and 24 h into the tubes containing EDTA-2K. Plasma was separated by centrifuging the blood samples at 13 000 rpm and finally stored by freezing at −80 °C until analysis.

LC–MS/MS analysis

LC–MS/MS conditions

Chromatographic analysis was performed on a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA). A Thermo Syncronic C_{18} column (100 mm × 2.1 mm, 1.7 μm) was employed and the column temperature was maintained at 35 °C. The mobile phase was composed of A (0.1% formic acid in water) and B (acetonitrile) using a gradient elution of 40–95% B at 0–7 min with a flow rate set at 0.40 ml/min. The auto-sampler was conditioned at 4 °C and the injection volume was 5 μL. Mass spectrometry detection was