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Evaluation of subchronic (13 week) toxicity and genotoxicity potential of vinegar-processed *Genkwa Flos*

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ABSTRACT

Genkwa Flos (GF) is a well-known traditional medicine that is used to treat tumors and to relieve inflammation-related symptoms. GF tends to be taken in repeated doses for a long period of time, and although many reports on the toxicity of raw GF have led to a processing method to remove the toxicity, little information is currently available with regards to the toxic effects of subchronic exposure to processed GF (PGF). The aim of this study was to assess the possible genotoxicity and subchronic toxicity of PGF extract in accordance with the test guidelines published by the Organization for Economic Cooperation and Development. A 13-week repeat-dose oral toxicity study was carried out with rats, and the change in body weight observed in rats receiving PGF extract was normal. It is worth noting that the PGF extract groups exhibited an obvious increase in liver weight along with a significant increase in serum alkaline phosphatase activity at doses of 667 and 2000 mg/kg, providing evidence of hepatotoxic potential. More importantly, the results of the Ames test indicated that the PGF extract presented a mutagenic potential. Altogether, these results are the first to determine the subchronic toxicity and genotoxicity of the PGF extract, indicating that when GF is used for medicinal purposes, the period of use should be considered despite the manner in which the extract is processed.

1. Introduction

Herbs are widely used around the world as therapeutic materials to improve health (Cheung, 2011), and in the United States herbal medicines are also available from health food stores without a prescription (Dasgupta, 2003). *Genkwa Flos* (GF) are the flower buds of wild *Daphne genkwa* Sieb. et Zucc., which belongs to the Thymelaeaceae family. GF are collected in the spring before the blossom, and they are distributed mainly in East Asia, including regions of the Yangtze River and the Yellow River in China (Jiang et al., 2014). GF were introduced in the oldest book on the foundation of traditional Chinese medicine, "Shen Nong's Herbal Classic", which was published over two thousand years ago (Li et al., 2007). GF possess common characteristics with a pungent/bitter flavor and a warm nature (Zhang et al., 1988) and have been used as a traditional oriental medicine for centuries to treat edema, ascites, sudden cough, asthma and cancer (Zhan et al., 2005; Hong et al., 2011).

Previous phytochemical studies on GF have led to the isolation of pharmacologically active flavonoids, daphnane diterpenoids, and coumarins (Hall et al., 1982; Zhan et al., 2005; Akhtar et al., 2006). The flavonoids isolated from GF are known to have remarkable biological activities, such as anti-inflammatory (Lee et al.,

Abbreviations: GF, *Genkwa Flos*; OECD, Organization for Economic Co-operation and Development; ALP, alkaline phosphatase; CHL, Chinese hamster lung; MNPCEs, micronucleated polychromatic erythrocytes; NCEs, normochromatic erythrocytes.

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2009), analgesic (Wang et al., 2005) and immunoregulatory action (Gao et al., 2006). In addition, daphnane diterpenoids isolated from GF possess antitumor (Badawi et al., 1983; Jo et al., 2012), antifertility (Hu et al., 1985), antihyperglycemic (Carney et al., 1999), and antiviral (Allard et al., 2012) properties. In particular, daphnane diterpenoids exhibit potent anti-proliferative effects against A549 human lung cancer cells in contrast to normal lung epithelial cells (Hong et al., 2010), indicating the antitumor properties of GF.

In recent studies, we demonstrated that effective herbs, including *Paecilomyces tenuipes* and *Sophorae radix*, have toxic side effects (Che et al., 2014; Che et al., 2015). Raw GF have also been commonly recognized to be toxic, and for this reason, raw GF are traditionally processed with vinegar to remove the toxicity (Geng et al., 2013). Although herbal medicines with both raw and vinegar-processed GF (PGF) are currently available on the market (Geng et al., 2013), the safety of PGF has not yet been comprehensively determined. In this study, we conducted a subchronic toxicity and genotoxicity test to evaluate the potential hazards of PGF.

2. Materials and methods

2.1. Test substance and animals

GF were obtained from the National Institute of Food and Drug Safety Evaluation (Osong, Korea) and were processed using the same method described by Geng et al. (2013). In brief, the GF, the flower buds of wild *Daphne genkwa* Sieb. et Zucc., were collected in the spring in China before blossoming. The dried GF (1 kg) were submerged in 0.3 kg of vinegar diluted in 0.6 kg of water, moistened in a closed container overnight and dried with gentle heat. They were then powdered and macerated with distilled water (DW) at 110 °C. After filtration, the filtrate was freeze-dried. Under these conditions, the extraction yields of the hot water PGF extract were 0.116 g/g. The freeze-dried PGF extract were then resuspended in DW for the experiments.

F344 rats (SLC, Hamamatsu, Japan) and ICR mice (Orient Bio, Seongnam, Korea) were maintained under standard conditions (22 ± 2 °C, 40–60% humidity, and 12 h light/dark cycle). The animals were permitted free access to rodent diet (LabDiet 5002 Certified Rodent Diet, Purina, Seoul, Korea) and tap water. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of the Biomedical Research Institute at the Seoul National University Hospital, and this study was performed in compliance with the guidelines published by the Organization for Economic Cooperation and Development (OECD) as well as the guidance for Good Laboratory Practices for toxicity tests issued by the Ministry of Food and Drug Safety (MFDS, 2005).

2.2. Experimental design for the oral toxicity study

For the 14-day repeat-dose toxicity study, hot water PGF extract was administered to F344 rats (5/sex/group) by oral gavage at doses of 0, 125, 250, 500, 1000, and 2000 mg/kg of body weight once daily for 14 days. For the 13-week repeat-dose toxicity study, the hot water PGF extract was administered to F344 rats (10/sex/group) by oral gavage at doses of 0, 25, 74, 222, 667, and 2000 mg/kg of body weight once daily for 13 weeks, in accordance with OECD guideline 408 (OECD, 1998). The rats were observed daily for clinical signs and mortality. The body weights and food/water consumption were measured weekly during the study period, and the rats were anesthetized with isoflurane one day after the final gavage.

2.3. Urinalysis, hematology and serum biochemistry

During the last week of treatment, fresh urine samples were taken from 10 rats per group (5 males and 5 females) to perform a urinalysis with a urine analyzer (Miditron Junior II, Roche, Mannheim, Germany) according to the manufacturer's instructions. Blood samples were collected *via* the posterior vena cava to extract routine hematology and clinical chemistry parameters. The standard hematology parameters were measured using an automatic hematology analyzer MS9-5 Hematology Counter (Melet Schloesing Laboratories, Osny, France), and the standard serum biochemistry parameters were analyzed with an automatic chemistry analyzer 7070 (Hitachi, Tokyo, Japan).

2.4. Gross findings, organ weights, and histopathological assessments

During necropsy, the organs and tissues were removed and subjected to a detailed macroscopic examination. The testis and epididymis were fixed in Bouin's solution, the eyes with Harderian glands were fixed in Davidson solution, the remaining organs were weighed and fixed in 10% neutral formalin. The nasal cavity, spinal cords with bones, sternum, and femora were treated with a decalcification solution for up to 3 weeks. Slices from all tissues were routinely processed for paraffin embedding and sectioning. The samples were subjected to hematoxylin and eosin staining to examine the histological changes *via* light microscopy.

2.5. Genotoxicity study

A bacterial reverse mutation assay (Ames test) was conducted in accordance with OECD guideline 471 (OECD, 1997a). *Salmonella typhimurium* strains TA98, 100, 102, 1535, and 1537 (MFDS, Osong, Korea) were incubated with PGF extract with or without an S9 mix in the dark at 37 °C for 48 h. 2-nitrofluorene, sodium azide, mitomycin C, 9-aminoacridine, and 2-aminoanthracene (Sigma–Aldrich, St. Louis, MO, USA) were used as positive controls. The extract was considered to be positive if there was a twofold increase relative to negative control or a dose-dependent increase in the number of revertant colonies.

An *in vitro* chromosomal aberration test was conducted in accordance with OECD guideline 473 (OECD, 1997b). Chinese hamster lung (CHL) fibroblast cells were incubated in a CO_2 incubator (5% CO_2, 37 °C, high humidity) with PGF extract in the presence or absence of an S9 mix for 6 h or 24 h. Mitomycin C and cyclophosphoamide (Sigma–Aldrich) were used as positive controls. After adding colcemid (0.2 μg/ml, GIBCO, Carlsbad, CA, USA) for 2 h, the cells were treated with hypotonic solution, fixed in 3:1 methanol/glacial acetic acid, and stained with 4% Giemsa.

An *in vivo* bone marrow micronucleus test was conducted in accordance with OECD guideline 474 (OECD, 1997c). 8-week-old male ICR mice were orally gavaged daily for 4 d with 0, 500, 1000, and 2000 mg/kg PGF extract. Mitomycin C (2 mg/kg) served as a positive control and was intraperitoneally injected. The mice were sacrificed at 24 h after the last dose of PGF extract had been administered. The femoral bone marrow cells were isolated, centrifuged, smeared onto slides, and dried. The slides were fixed in methanol and were stained with 5% Giemsa. The results are expressed as the number of micronucleated polychromatic erythrocytes (MNPCEs) per 2000 PCEs. In addition, the PCE/(PCE + NCE) ratio, where the NCEs indicate the normochromatic erythrocytes, was calculated to detect the possibility of cytotoxicity (Heddle et al., 1984).