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Toxicological evaluation of the flavonoid-rich extract from *Maydis stigma*: Subchronic toxicity and genotoxicity studies in mice

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Rutin (PubChem CID: 5280805)
Quercetin (PubChem CID: 5280343)
Luteolin (PubChem CID: 5280445)
Kaepmferol (PubChem CID: 5280863)
Apigenin (PubChem CID: 5280443)
Diosmetin (PubChem CID: 5281612)
Formononetin (PubChem CID: 5280378)

ABSTRACT

Ethnopharmacological relevance: *Maydis stigma* (corn silk) has a long history of use as a traditional herbal medicine or functional food in China and many other countries and has been listed in the Chinese Pharmacopea. However, little data about its potential toxicity is available.
Aim of the study: In this study, we evaluated the subchronic toxicity and genotoxicity of the flavonoid-rich extract from *Maydis stigma* (FMS) in mice.
Materials and methods: In the subchronic toxicity study, the FMS was administered orally to mice at doses of 2.50, 5.00 and 10.00 g/kg/day for 28 consecutive days. At the end of experiment, general clinical signs, mortality, haematological, biochemical and histopathological parameters were examined. The genotoxicity of FMS was also evaluated by the micronucleus assay and the sperm malformation assay.
Results: All animals survived until the scheduled necropsy, and no statistically significant or toxicologically relevant differences were observed in any of the FMS-treatment groups, compared with the control group. The no-observed-adverse-effect level (NOAEL) was determined as 10.00 g/kg/day. Based on the results of the micronucleus assay and the sperm malformation assay, no evidence of genotoxicity was found either in somatic cells or germ cells even at an experimental upper limit dose (10.00 g/kg/day).
Conclusions: The results of the present studies might support the safe use of FMS as a functional food, food additive and natural remedy.

1. Introduction

Maydis stigma (corn silk), the stigma and style of *Zea mays L.* (corn), consists of numerous bioactive components, including flavonoids (Liu et al., 2011b), polysaccharides (Chen et al., 2013), steroids (Abdel-Wahab et al., 2002), tannins, alkaloids, proteins and vitamins. It has been reported widely to possess antioxidant (Liu et al., 2011a), anti-diabetic (Guo et al., 2009), diuretic (Velazquez et al., 2005), anti-fatigue (Hu et al., 2010), anti-depressant (Ebrahimzadeh et al., 2009), anti-inflammation (Wang et al., 2012a), antibacterial (Eman, 2011; Widstrom and Snook, 1998), antifungal (Miller et al., 2003) and anti-tumor (Habtemariam et al., 1998) activities.

M. stigma is also a traditional herbal medicine in China, which has also been used as a natural remedy or functional food for a long time in many countries like United States and France (Hasanudin et al., 2012). Traditionally, *M. stigma* is clinically used for the treatment of such ailments as hypertension, diabetes, edema, prostatitis, cystitis, nephritis, gout and renal calculus, which has been listed in the Chinese Pharmacopeia (1977). In recent years, it has been developed as food additive and flavoring agents to improve food taste (Rosli et al., 2011).

Recently, many researches have indicated that flavonoids widely exist in plants and have high antioxidant activity (Li et al., 2015; Lou et al., 2014; Wang et al., 2012b). Meanwhile, it has been reported that *M. stigma* was rich in flavonoids and possessed potent antioxidant activity *in vitro* and *in vivo* (Ren et al., 2013; Hu et al., 2011; El-Ghorab et al., 2007). Our previous studies (Peng et al., 2015) have demonstrated the flavonoid-rich extract from *M. stigma* (FMS) show potent scavenging activity against DPPH and ABTS radical *in vitro*. Moreover, it significantly diminished the protein and lipid peroxidation induced by ethanol and reverse the ethanol-diminished superoxide dismutase (SOD) and glutathione (GSH) content in ethanol-treated mice. Therefore, FMS as a bioactive source of natural antioxidants has attracted increasing attention, for use as a natural remedy to prevent some diseases caused by oxidative stress.

Although, *M. stigma* is widespread and has a long history of use

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in China and many other countries, its completely natural origin and long history of utilization cannot guarantee its safety. An earlier study has shown that the crude extract of *M. stigma* was not toxic to rats via a 90-day repeated dose toxicity study (Wang et al., 2011). Our previous studies (Peng et al., 2015) on acute toxicity of flavonoid-rich extract from *M. stigma* (FMS) indicated that the LD_{50} value for oral administration in mice is higher than 30 g/kg bw. However, the subchronic toxicity and genotoxicity of FMS have not yet been scientifically evaluated. Hence, additional preclinical toxicity studies were carried out to evaluate the subchronic toxicity and genotoxicity of FMS in mice via a 28-day repeated dose toxicity study, the *in vivo* mouse micronucleus and sperm malformation assays. These studies can provide information on the safety of this new natural remedy.

2. Materials and methods

2.1. Materials

Maize plants were harvested from corn fields in Jilin province, China. *Maydis stigma* samples were collected and then deposited in a well-ventilated and dry place. Standard animal feed was purchased from the Experimental Animal Center of Jilin University (Changchun, China). All other chemicals and reagents were purchased from Sigma Aldrich Chemical Co., Ltd (St. Louis, USA) and were of analytical grade.

2.2. Sample preparation and total flavonoids determination

The pulverized *M. stigma* (300 g) was extracted with water at 80 °C (9 L water, 1 h for the first time, 4.5 L water, 0.5 h for the second time). The extract was filtered through a Whatman No. 1 filter paper to remove the debris and the filtrate was then concentrated to 3 L with a rotary flash evaporator at 40 °C under vacuum (RE-52A, Shanghai Yarong Biochemical Instruments Co. Shanghai, China). Then, the water extract of *M. stigma* was precipitated by the addition of anhydrous ethanol to a final concentration of 70% (*v*/*v*). The mixture was maintained overnight at room temperature. The supernatant was obtained by centrifugation (3500 rpm, 15 min), and then concentrated using a rotary flash evaporator and freeze-dried to furnish the flavonoid-rich extract from *M. stigma* (FMS).

A colorimetric aluminum chloride method was used for determination of the contents of flavonoid (Chang et al., 2002) with some modification. A dilute solution of FMS in methanol (0.5 mL in 50 mL) was separately mixed with 4.5 mL of methanol and 5.0 mL of 0.01 mol/L aluminum chloride in methanol. The reaction mixture remained at room temperature for 10 min. Then, the absorbance of the reaction mixture was measured at 400 nm using an ultraviolet visible spectrophotometer (UV-2550, Shimadzu Corporation, Kyoto, Japan). The calibration curve was established by preparing rutin solutions at concentrations ranging from 0.005 to 0.125 mg/mL in methanol. The yield of the flavonoids was expressed as mg of rutin equivalents per gram of *M. stigma* on a dry weight basis. The purity of total flavonoids in the extracts of *M. stigma* was 10.45%.

2.3. Identification of flavonoids

The above flavonoids-rich extract was re-dissolved with water and thereafter extracted with petroleum ether (two fold volumes). The mixture was kept at room temperature for 4 h, and the water solution was further partitioned with two fold volumes of ethyl acetate (EtOAc). The EtOAc solution was concentrated with a rotary evaporator under reduced pressure. Then, the extract solution was subjected to a silica gel column chromatography by elution with chloroform-methanol (from 5:1 to 0:1) to yield 10 fractions. All the fractions were analyzed by TLC. Two fractions which contain the more constituent were used for the analysis of flavonoids by high performance liquid chromatography. The HPLC system (LC-20AT, Shimadzu, Kyoto, Japan) equipped with a binary solvent delivery module (LC-20AT), a PDA detector (SPD-M20A) were applied to identify the flavonoids. Each 20 μL of the filtered samples was separated on a VP-ODS (4.6 × 250 mm, *i.d.*, 5.0 μm) at 30 °C, with a controlled flow rate of 1 mL/min and set wavelengths of 350 nm. Linear gradient elution was employed using 0.1% phosphoric acid (A) and methanol (B) as mobile phases and processed as follows: 0–1 min, 40% B; 1–10 min, 50% B; 10–30 min, 60% B; 30–40 min, 75% B; 40–50 min, 75% B; 50–60 min, 95% B. Identification of flavonoid compounds in Q1 and Q2 were performed based on the retention times and the spectral characteristics of peaks with those of the authentic reference standards. The standard solution which consisted of rutin, quercetin, luteolin, kaepmferol, apigenin, diosmetin and formononetin was analysis in the same condition with that of the samples.

In the sample of Q1, the retention time of peak 1 was 12.03, which is identical to that of rutin. The retention time of peak 2 was 28.47. Its UV spectra showed that the maximum absorption wavelength was 340 nm and was identical to that of apigenin. The retention time of peak 3 was 28.99. Its UV spectrum showed that the maximum absorbance was 344 nm and was identical to that of diosmetin. In the sample of Q2, the retention time of peak 1 was 28.18, its UV spectrum showed that the maximum absorbance was 340 nm and was identical to that of apigenin. The retention time of peak 2 was 28.47. Its UV spectrum showed that the maximum absorbance was 340 nm and was identical to that of diosmetin (Fig. 1). Therefore, the main components in the EtOAc extract of *M. stigma* were identified as rutin, apigenin and diosmetin.

2.4. Experimental animals

Kunming mice (SPF grade, 8 weeks old, weighing from 25 to 30 g) were purchased from the Experimental Animal Center of Jilin University (Changchun, China). The mice were housed in polycarbonate cages, and provided with normal diet and purified water *ad libitum*. All animals were reared at a room temperature of 20 ± 2 °C, with 60 ± 10% relative humidity and a 12 h light/dark regime. Before the experiments, all animals were acclimatized to the laboratory environments for at least 7 days. All experimental procedures used in this study had been approved by the ethics committee in this institute, and all animal experiments was conducted in accordance to the "Guide for the Care and Use of Laboratory Animals" (National Research Council, 2010).

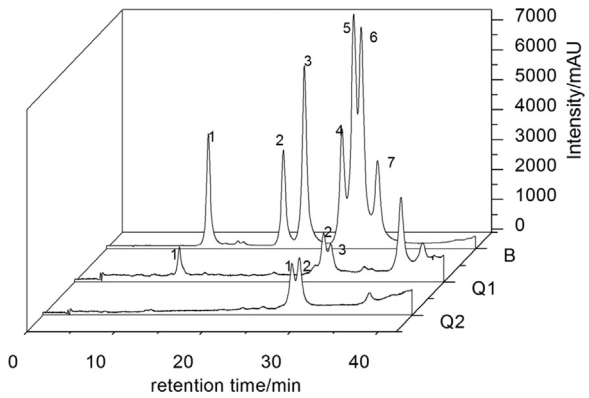

Fig. 1. HPLC chromatogram of the standard solution (B), Q1 and Q2.