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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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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).

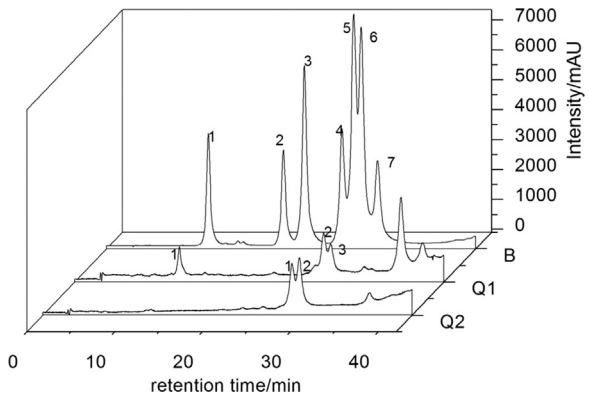

Fig. 1. HPLC chromatogram of the standard solution (B), Q1 and Q2.

Table 1
General clinical observations for the FMS treated mice.

Observation	Every day after administration	
	0.5 h	7 h
Skin and fur	Normal	Normal
Eyes	Normal	Normal
Locomotor activity	Increased	Normal
Sleep	Normal	Normal
Salivation	Normal	Normal
Lethargy	Normal	Normal
Diarrhea	Normal	Normal
Tremor	Normal	Normal

2.5. Experimental dose design

According to the guidelines of Procedure and Methods of Food Safety Toxicological Assessment, GB15193-2014 (China's Ministry of Health, 2014), doses were determined based on the result of acute oral toxicity. Besides, our previous studies on acute oral toxicity indicated that LD_{50} of FMS was higher than 30 g/kg in mice (Peng et al., 2015). Hence, three doses of 2.50 g/kg bw d (Low dose), 5.00 g/kg bw d (Mid dose) and 10.00 g/kg bw d (High dose) were selected for the subchronic toxicity and genotoxicity studies.

2.6. Subchronic toxicity study

2.6.1. Study design

This study was performed in accordance with the GB15193.22-2014 Test Guidelines on 28-Day Repeated Dose Toxicity Study (China's Ministry of Health, 2014) and the OECD Guideline no. 407 (OECD, 1995) for principles of Good Laboratory Practices (FDA, 2010) with minimal modification (Arbo et al., 2009).

Eighty mice were randomly divided into three treatment groups (2.50, 5.00 and 10.00 g/kg bw d) and a control group, and each group had 20 mice with half of the number being males and other half females. FMS was dissolved in distilled water and administered by oral gavage on a daily basis for 28 consecutive days. The control group was orally administered with equivalent volume of distilled water. Food and water were available *ad libitum* throughout the experiment. All mice were observed twice daily for general clinical signs and mortality and these were recorded. Body weights and food consumption of all mice were measured and recorded weekly.

2.6.2. Toxicological evaluations

On day 29, all mice were anesthetized with carbon dioxide after 18 h overnight fast (water allowed) and blood samples were collected from mice orbit. Routine haematological parameters were measured by an automatic hematology analyzer (KX-21, Sysmex Co., Japan) using whole blood stabilized by EDTA and sodium citrate, and the parameters analyzed included white blood cells (WBC), neutrophils (Nut), lymphocytes (Lym), monocytes (Mon), eosinophils (Eos), basophils (Bas), red blood cells (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocytes (RTC), platelets (PLT), mean platelet volume (MPV), platelet distribution width (PWD), prothrombin times (PT) and activated partial thromboplastin times (APTT).

The serum was separated from blood by centrifugation at 4 °C, 2000 rpm for 15 min. And the serum biochemical parameters were analyzed with an automatic biochemistry analyzer (CHEMIX-180, Sysmex Co., Japan). The parameters analyzed included total protein (TP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), albumin/globulin ratio (A/G ratio), alkaline phosphatase (ALP), total cholesterol (TCHO), triglyceride (TG), blood urea nitrogen (BUN), glucose (GLU), creatinine (CRE).

2.6.3. Necropsy and histopathological observation

At study termination, all animals were sacrificed by exsanguination. During necropsy, all the organs were examined and observed carefully, and the macroscopic pathological changes were recorded. The brain, thyroid, thymus, heart, liver, spleen, kidney, adrenals, stomach, duodenum, colon, pancreas, mesenteric lymph node, bladder and testes/ovaries were collected and weighed separately. The relative organ weight were calculated based on the fasted animal's body weight (Relative organ weight (%)=organ weight/body weight × 100).

These organs obtained from each animal were fixed in 10% neutral buffered formalin for histopathology examination. If any treatment-related changes were observed in the high dose group, the organs obtained from the low and mid dose groups were to be investigated. The fixed-organs were embedded in paraffin, processed to produce 5 μm tissue sections and stained with hematoxylin and eosin. Then the slides were examined under a microscope. Finally, the no-observed-adverse-effect levels (NOAEL) of FMS were determined.

Table 3
Effects of FMS on weekly feed consumption of mice.

Sex	Dose (g/kg bw d)	Week 1 (g)	Week 2 (g)	Week 3 (g)	Week 4 (g)
Male	0	5.16 ± 0.48	6.06 ± 0.44	6.91 ± 0.44	7.82 ± 0.52
	2.50	5.20 ± 0.51	5.73 ± 0.45	6.55 ± 0.55	7.33 ± 0.47
	5.00	5.06 ± 0.56	5.78 ± 0.53	6.44 ± 0.60	7.21 ± 0.52
	10.00	5.25 ± 0.44	5.89 ± 0.47	6.71 ± 0.60	7.45 ± 0.50
Female	0	4.20 ± 0.46	5.39 ± 0.51	6.21 ± 0.45	6.92 ± 0.44
	2.50	4.04 ± 0.46	4.98 ± 0.36	5.88 ± 0.61	6.52 ± 0.60
	5.00	3.95 ± 0.42	4.87 ± 0.56	6.00 ± 0.56	6.83 ± 0.59
	10.00	4.01 ± 0.53	4.93 ± 0.59	5.76 ± 0.51	6.68 ± 0.60

No statistically significant differences were found.

Table 2
Effects of FMS on weekly body weights of mice.

Sex	Dose (g/kg bw d)	Initial weight (g)	Week 1 (g)	Week 2 (g)	Week 3 (g)	Week 4 (g)
Male	0	28.38 ± 0.89	30.39 ± 0.92	32.35 ± 0.85	34.51 ± 0.69	36.60 ± 0.85
	2.50	28.52 ± 0.84	30.49 ± 0.99	32.48 ± 0.85	34.56 ± 0.90	36.29 ± 0.86
	5.00	28.55 ± 0.89	30.79 ± 0.86	32.51 ± 0.80	34.49 ± 0.81	36.41 ± 0.97
	10.00	28.45 ± 0.93	30.40 ± 0.88	32.34 ± 0.74	34.10 ± 0.89	36.01 ± 0.79
Female	0	26.09 ± 0.61	28.01 ± 0.77	30.29 ± 0.88	32.33 ± 0.83	34.21 ± 0.78
	2.50	25.95 ± 0.64	27.76 ± 0.68	29.62 ± 0.57	31.71 ± 0.67	33.63 ± 0.75
	5.00	26.15 ± 0.73	28.05 ± 0.80	30.14 ± 0.66	32.00 ± 0.78	34.03 ± 0.74
	10.00	26.14 ± 0.64	27.99 ± 0.80	29.95 ± 0.72	31.86 ± 0.73	33.94 ± 0.56

No statistically significant differences were found.

Table 4
Effects of FMS on haematological parameters of mice.

Parameter	Sex	Dose (g/kg bw d)			
		0 (Control)	2.5 (Low dose)	5.0 (Mid dose)	10.0 (High dose)
WBC ($\times 10^9$/L)	Male	5.82 ± 1.07	5.76 ± 1.10	5.72 ± 1.13	6.03 ± 1.44
	Female	5.52 ± 1.28	5.48 ± 1.46	5.65 ± 1.50	5.54 ± 1.39
Nut (%WBC)	Male	2.72 ± 1.10	2.98 ± 0.69	2.56 ± 1.35	2.30 ± 0.98
	Female	2.66 ± 1.15	2.32 ± 0.90	2.79 ± 1.16	2.50 ± 0.50
Lym (%WBC)	Male	80.26 ± 5.58	76.90 ± 9.43	76.48 ± 4.07	77.51 ± 10.46
	Female	75.84 ± 7.73	78.32 ± 4.92	75.33 ± 10.87	79.29 ± 5.29
Mon (%WBC)	Male	4.57 ± 0.80	4.85 ± 1.44	4.65 ± 1.09	5.02 ± 1.25
	Female	4.64 ± 0.99	4.94 ± 1.08	4.47 ± 1.45	4.63 ± 1.05
Eos (%WBC)	Male	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
	Female	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.02
Bas (%WBC)	Male	9.42 ± 1.46	9.70 ± 3.02	10.58 ± 5.51	8.93 ± 3.03
	Female	10.84 ± 3.24	9.21 ± 1.73	9.91 ± 3.54	9.21 ± 4.08
RBC ($\times 10^{12}$/L)	Male	9.33 ± 1.51	9.71 ± 1.26	9.41 ± 1.38	8.96 ± 1.31
	Female	9.87 ± 1.42	10.02 ± 1.06	9.50 ± 1.26	10.40 ± 1.35
HGB (g/L)	Male	144.46 ± 19.01	138.24 ± 14.65	132.28 ± 18.83	146.26 ± 15.94
	Female	151.27 ± 13.36	147.81 ± 10.38	158.37 ± 12.82	150.75 ± 12.97
HCT (%)	Male	0.49 ± 0.13	0.46 ± 0.13	0.50 ± 0.11	0.51 ± 0.11
	Female	0.47 ± 0.11	0.49 ± 0.12	0.43 ± 0.12	0.50 ± 0.13
MCV (fL)	Male	49.52 ± 3.82	49.89 ± 3.14	49.35 ± 4.12	48.86 ± 2.94
	Female	52.44 ± 2.74	53.24 ± 2.95	53.80 ± 2.82	51.63 ± 2.80
MCH (pg)	Male	15.26 ± 1.71	15.17 ± 1.51	15.38 ± 1.73	14.72 ± 2.00
	Female	15.73 ± 2.01	15.92 ± 1.59	15.63 ± 1.21	16.46 ± 1.83
MCHC (g/L)	Male	310.52 ± 7.68	311.04 ± 6.45	309.37 ± 6.13	311.32 ± 5.46
	Female	319.78 ± 6.49	320.73 ± 6.41	319.75 ± 6.58	321.45 ± 7.16
RTC (%)	Male	2.70 ± 0.40	2.60 ± 0.63	2.62 ± 0.68	2.75 ± 0.40
	Female	2.23 ± 0.43	2.28 ± 0.46	2.17 ± 0.54	2.38 ± 0.56
PLT ($\times 10^9$/L)	Male	930.60 ± 163.34	891.96 ± 147.13	994.15 ± 139.37	962.98 ± 168.64
	Female	759.97 ± 141.84	720.11 ± 134.71	776.66 ± 139.15	699.08 ± 114.03
MPV (fL)	Male	6.33 ± 0.71	6.42 ± 0.64	6.41 ± 0.53	6.28 ± 0.54
	Female	6.69 ± 0.69	6.82 ± 0.59	6.49 ± 0.61	6.68 ± 0.40
PWD (fL)	Male	7.33 ± 0.94	7.04 ± 1.16	7.23 ± 1.08	7.54 ± 0.80
	Female	7.92 ± 0.80	7.46 ± 1.31	7.81 ± 1.03	7.69 ± 1.21
PT (s)	Male	12.5 ± 0.6	12.4 ± 0.8	12.0 ± 0.7	12.1 ± 0.8
	Female	13.3 ± 0.9	13.6 ± 0.7	13.0 ± 0.8	13.3 ± 0.8
APTT (s)	Male	25.0 ± 2.1	24.5 ± 3.5	26.5 ± 2.3	25.5 ± 2.7
	Female	19.6 ± 3.1	18.9 ± 2.2	20.9 ± 3.0	20.6 ± 3.3

No statistically significant differences were found.

2.7. Genotoxicity studies

2.7.1. Micronucleus assay

This assay was performed in accordance with the GB15193.5-2003 Test Guidelines on micronucleus Assay (China's Ministry of Health, 2003) and the OECD Guidelines no. 475 (OECD, 1997) for principles of Good Laboratory Practices (FDA, 2010) with minimal modification.

Fifty mice, with half male and female, were randomly divided into five groups. FMS was dissolved in distilled water and administered by oral gavage at doses of 2.50, 5.00 and 10.00 g/kg bw d for 2 days at 24-h intervals. Freshly prepared cyclophosphamide (CCP, 40 mg/kg bw d, dissolved in distilled water) and distilled water were given to mice as a positive control and a negative control, respectively.

All animals were anesthetized and euthanized at 6 h after the last treatment. The bone marrow cells were collected from the sternum bone marrow and diluted with newborn calf serum to obtain cell suspensions. The cell suspensions were smeared onto slides and dried in air. After that, the slides were fixed with methanol for 10 min and stained with Giemsa for 15 min. The smears were flushed with distilled water softly, dried in air and coded. The number of micronucleated polychromatic erythrocytes (MNPCE) was counted based on an examination of 1000 polychromatic erythrocytes (PCE) each animal and the frequencies of micronucleus per one thousand PCE was calculated. The ratio of PCE to red blood cells (RBC) was calculated based on an examination of 1000 RBC per mouse.

2.7.2. Sperm malformation assay

This assay was performed in accordance with the GB15193.7-2003 Test Guidelines on Sperm Malformation Assay (China's Ministry of Health, 2003) and the principles of Good Laboratory Practices (FDA, 2010) with minimal modification.

Fifty adult male mice were randomly divided into five groups. FMS was dissolved in distilled water and administered by oral gavage at doses of 2.50, 5.00 and 10.00 g/kg bw d for 5 days with a 24-h interval. Mitomycin C (MMC, 1.5 mg/kg bw d, dissolved in distilled water) and distilled water were given to mice for five days as a positive control and a negative control, respectively.

Table 5
Effects of FMS on serum biochemical parameters of mice.

Parameter	Sex	Dose (g/kg bw d)			
		0 (Control)	2.5 (Low dose)	5.0 (Mid dose)	10.0 (High dose)
TP (g/L)	Male	47.46 ± 6.23	47.76 ± 6.78	45.58 ± 5.92	49.72 ± 6.56
	Female	48.36 ± 4.99	50.83 ± 5.65	51.37 ± 6.41	48.72 ± 5.08
AST (U/L)	Male	140.21 ± 13.92	145.31 ± 10.89	137.95 ± 10.16	142.18 ± 8.36
	Female	114.33 ± 11.10	118.98 ± 11.69	117.75 ± 16.21	115.27 ± 11.07
ALT (U/L)	Male	31.70 ± 3.74	33.54 ± 3.97	31.19 ± 2.29	30.55 ± 4.16
	Female	26.25 ± 3.53	24.71 ± 2.72	28.18 ± 3.10	25.14 ± 3.72
AST/ALT ratio	Male	4.47 ± 0.62	4.38 ± 0.55	4.45 ± 0.46	4.73 ± 0.69
	Female	4.42 ± 0.62	4.87 ± 0.70	4.24 ± 0.79	4.66 ± 0.68
ALB (g/L)	Male	27.13 ± 2.89	28.35 ± 2.42	26.62 ± 2.33	27.37 ± 1.92
	Female	25.60 ± 2.97	25.37 ± 1.91	25.83 ± 2.62	24.53 ± 2.83
A/G ratio	Male	1.15 ± 0.07	1.17 ± 0.06	1.19 ± 0.04	1.14 ± 0.05
	Female	1.47 ± 0.03	1.48 ± 0.04	1.46 ± 0.05	1.47 ± 0.05
ALP (U/L)	Male	104.07 ± 14.11	103.27 ± 11.48	100.89 ± 9.86	102.36 ± 12.41
	Female	125.78 ± 5.38	122.47 ± 6.64	127.19 ± 6.09	123.85 ± 7.11
TCHO (mmol/L)	Male	2.45 ± 0.27	2.50 ± 0.33	2.39 ± 0.35	2.53 ± 0.36
	Female	2.01 ± 0.28	1.92 ± 0.28	1.96 ± 0.25	2.07 ± 0.22
TG (mmol/L)	Male	1.98 ± 0.13	2.00 ± 0.14	1.95 ± 0.11	1.98 ± 0.13
	Female	1.82 ± 0.11	1.78 ± 0.09	1.85 ± 0.11	1.86 ± 0.09
BUN (mmol/L)	Male	6.48 ± 0.53	6.41 ± 0.67	6.53 ± 0.48	6.56 ± 0.66
	Female	6.89 ± 0.61	7.00 ± 0.60	7.04 ± 0.46	6.91 ± 0.52
GLU (mmol/L)	Male	4.52 ± 0.57	4.32 ± 0.53	4.61 ± 0.53	4.54 ± 0.67
	Female	3.73 ± 0.47	3.89 ± 0.67	4.13 ± 0.51	4.04 ± 0.41
CRE (μmol/L)	Male	48.09 ± 6.25	47.18 ± 6.14	46.86 ± 5.91	50.11 ± 6.24
	Female	39.40 ± 6.17	37.30 ± 4.62	41.04 ± 5.51	40.66 ± 5.71

No statistically significant differences were found.

All animals were maintained on basal diet for 34 days and sacrificed by cervical dislocation on the 35th day after the first treatment. The bilateral epididymides were dissected out and placed in a plate with 1 mL normal saline, and cut into pieces with ophthalmic scissors. After stirring for 5 min, samples were centrifuged at 1000 rpm. The tissue supernatants were smeared onto glass slides and dried in air. The dried slides were fixed with methanol for 10 min and stained with 1% eosin for 60 min. The smears were flushed with distilled water softly, air-dried and coded. The number of spermatozoa with morphological abnormalities was counted based on an examination of 1000 spermatozoa per animal under a microscope, and the sperm malformation rate was calculated.

2.8. Statistical analyses

Statistical analysis of the experimental data was conducted using SPSS 19.0 software (SPSS Inc., Chicago, USA). Levene's test was performed to analyze the homogeneity of variances. When the variances were homogeneous, one-way analysis of variance (ANOVA) was carried out. Dunnett's test was used when the variance was significant. Histopathological findings and the data from the sperm abnormality assay and the micronucleus assay were subjected to Fisher's exact probability test. All values were expressed as mean ± standard deviation. A value of $p < 0.05$ was taken as statistically significant.

3. Results

3.1. Subchronic toxicity study

3.1.1. General clinical signs and mortality

All the animals survived throughout the experimental period of 28 days even at an experimental upper limit dose (10.0 g/kg bw d). Table 1 shows the general clinical signs usually associated with toxicity. The results showed that some mice presented an increased locomotor activity during the first 30 min after administration, and returned to normal state within 30 min, which might have been caused by gavage administration stress (Brown et al., 2000). Beside this observation, no other obvious general clinical signs or changes were found in any of the groups of animals throughout the monitoring period.

3.1.2. Body weights and food consumption

The body weight changes and food consumption were summarized in Tables 2 and 3. There were no statistically significant differences ($p > 0.05$) in body weights between the FMS-treated and control groups. The feed consumption was slightly decreased in three FMS-treated groups, compared with the control group, but this difference was not significant ($p > 0.05$).

3.1.3. Haematological and serum biochemical parameters

Haematological and serum biochemical parameters are presented in Tables 4 and 5, respectively. When compared with the control group, no statistically significant differences ($p > 0.05$) were observed in haematological and serum biochemical parameters on the day 29, for each of the FMS-treated groups. In addition, some parameters for each individual animal presented slightly increase or decrease changes in FMS-treated groups compared with the control group, including lymphocytes in males,

Table 6
Effects of FMS on relative organ weights (%) of mice.

Organ	Sex	Dose (g/kg bw d)			
		0 (Control)	2.5 (Low dose)	5.0 (Mid dose)	10.0 (High dose)
Brain	Male	1.235 ± 0.051	1.228 ± 0.054	1.242 ± 0.065	1.239 ± 0.041
	Female	1.427 ± 0.050	1.446 ± 0.075	1.425 ± 0.040	1.431 ± 0.034
Thymus	Male	0.332 ± 0.047	0.335 ± 0.046	0.318 ± 0.062	0.341 ± 0.074
	Female	0.429 ± 0.052	0.437 ± 0.058	0.428 ± 0.059	0.440 ± 0.066
Thyroid	Male	0.059 ± 0.024	0.061 ± 0.026	0.054 ± 0.027	0.059 ± 0.022
	Female	0.069 ± 0.021	0.073 ± 0.020	0.072 ± 0.024	0.068 ± 0.020
Heart	Male	0.614 ± 0.063	0.600 ± 0.061	0.630 ± 0.055	0.619 ± 0.070
	Female	0.518 ± 0.081	0.516 ± 0.077	0.526 ± 0.075	0.510 ± 0.074
Liver	Male	5.870 ± 0.624	5.816 ± 0.554	5.834 ± 0.519	5.989 ± 0.554
	Female	5.531 ± 0.478	5.582 ± 0.640	5.452 ± 0.530	5.509 ± 0.572
Spleen	Male	0.204 ± 0.040	0.203 ± 0.041	0.206 ± 0.047	0.195 ± 0.023
	Female	0.280 ± 0.041	0.287 ± 0.023	0.274 ± 0.041	0.277 ± 0.031
Kidneys	Male	1.687 ± 0.267	1.724 ± 0.282	1.649 ± 0.219	1.704 ± 0.217
	Female	1.167 ± 0.151	1.163 ± 0.187	1.173 ± 0.158	1.154 ± 0.137
Adrenals	Male	0.014 ± 0.003	0.013 ± 0.003	0.013 ± 0.002	0.014 ± 0.003
	Female	0.020 ± 0.002	0.021 ± 0.001	0.021 ± 0.002	0.019 ± 0.003
Stomach	Male	1.226 ± 0.211	1.311 ± 0.248	1.204 ± 0.211	1.297 ± 0.206
	Female	1.048 ± 0.151	1.075 ± 0.140	1.027 ± 0.151	1.052 ± 0.134
Duodenum	Male	0.534 ± 0.091	0.541 ± 0.086	0.527 ± 0.081	0.538 ± 0.117
	Female	0.585 ± 0.077	0.580 ± 0.092	0.595 ± 0.091	0.579 ± 0.110
Colon	Male	1.595 ± 0.266	1.576 ± 0.238	1.554 ± 0.201	1.607 ± 0.232
	Female	1.672 ± 0.125	1.685 ± 0.147	1.692 ± 0.206	1.680 ± 0.155
Pancreas	Male	0.375 ± 0.074	0.383 ± 0.067	0.388 ± 0.072	0.374 ± 0.074
	Female	0.410 ± 0.045	0.438 ± 0.056	0.400 ± 0.028	0.412 ± 0.061
Mesenteric lymph node	Male	0.609 ± 0.080	0.617 ± 0.084	0.600 ± 0.088	0.613 ± 0.098
	Female	0.689 ± 0.071	0.679 ± 0.064	0.686 ± 0.077	0.680 ± 0.069
Bladder	Male	0.073 ± 0.013	0.075 ± 0.015	0.072 ± 0.012	0.078 ± 0.014
	Female	0.058 ± 0.006	0.060 ± 0.007	0.061 ± 0.005	0.058 ± 0.005
Testis/Ovary	Male	0.602 ± 0.043	0.625 ± 0.052	0.599 ± 0.056	0.622 ± 0.050
	Female	0.071 ± 0.008	0.074 ± 0.007	0.069 ± 0.009	0.072 ± 0.009

No statistically significant differences were found.

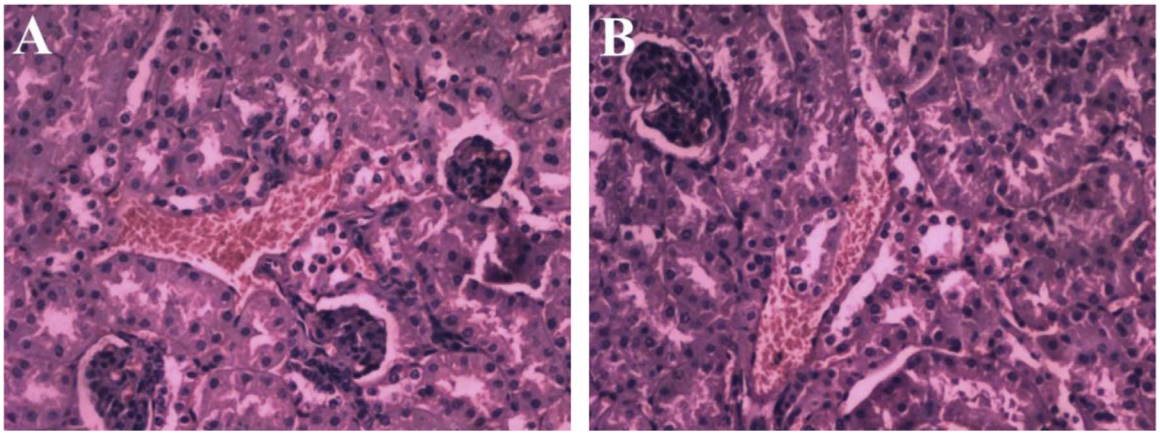

Fig. 2. Microphotographs of kidney at 100 ×. The different treatments of mice are shown in (A) Control; (B) 10.00 g/kg bw d FMS.

basophils in females, total protein in females, aspartate aminotransferase in females, alkaline phosphatase in males, blood urea nitrogen in females, glucose in females and prothrombin times in males. These data showed no clear dose-response relationships, and all of the minor fluctuations in these parameters were within the normal range of the testing laboratory.

3.1.4. Relative organ weights and macroscopic observations

The relative organ weights are summarized in Table 6. No statistically significant differences were found in relative organ weights of the organs and tissues, including brain, thyroid, thymus, heart, liver, spleen, kidney, adrenals, stomach, duodenum, colon, pancreas, mesenteric lymph node, bladder and testes/ovaries in both male and female groups at any dose level. All the

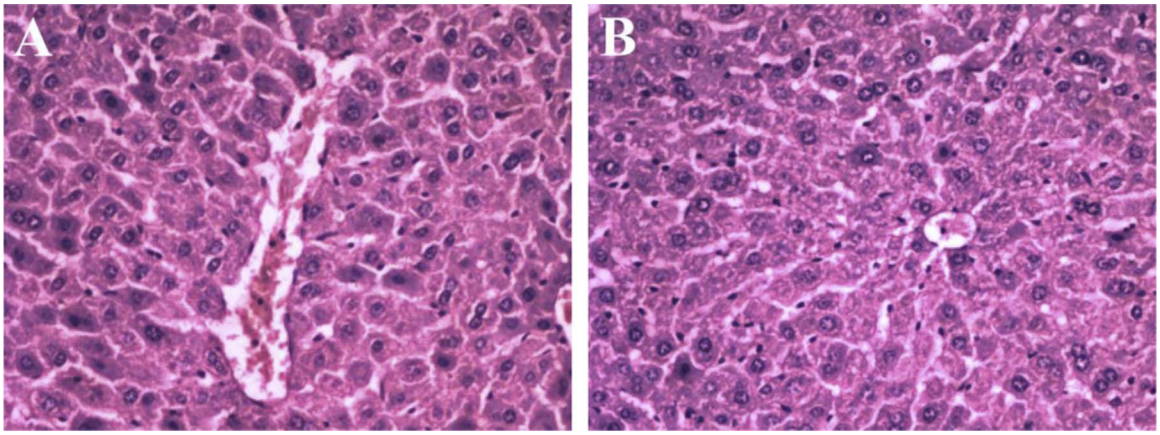

Fig. 3. Microphotographs of liver at 100 × . The different treatments of mice are shown in (A) Control; (B) 10.00 g/kg bw d FMS.

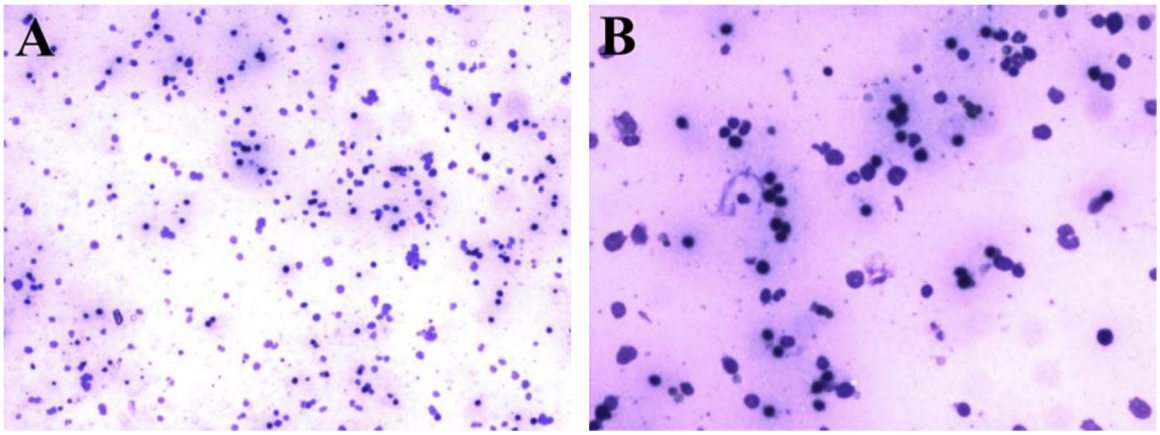

Fig. 4. Microphotographs of micronucleated polychromatic erythrocytes 40 × (A) and 100 × (B).

minor fluctuations in these relative organ weight values fell within the normal range. During necropsy, microscopic observations revealed no obvious pathological changes associated with the administration of FMS.

3.1.5. Histopathological observation

The microphotographs of typically histopathological observation of kidney and liver in control group and FMS-treated groups are shown in Figs. 2 and 3, respectively. No changes were observed in the livers and kidney from any of the groups. Similarly, no changes were found in the heart, spleen, lung, stomach, testis and ovaries (data not shown).

3.2. Genotoxicity studies

3.2.1. Micronucleus assay

Some selected microphotographs of micronucleated polychromatic erythrocytes (MNPCE) are shown in Fig. 4. The results from micronucleus assay are shown in Table 7. There were no statistically significant differences in the PCE/RBC ratio and the micronucleus frequency between each FMS-treated groups and negative control group ($p > 0.05$), which indicated that the FMS shows no genotoxic activity in bone marrow stem cells at doses of up to 10 g/kg bw d.

3.2.2. Sperm malformation assay

Sperm head defects include double-headed, banana-shaped, amorphous and hookless features, while sperm tail defects include double-tailed and tail folding. Some selected microphotographs of sperm malformation observed were showed in Fig. 5.

Table 7
Results of micronucleus assay in mice.

Treatment (g/kg bw d)		PCE		Micronucleus	
		PCE (counts/each)	PCE/RBC (%)	MNPCE (counts/each)	Micronucleus frequency (‰)
FMS	2.50	539.3 ± 2.8	53.9	1.2 ± 0.9	1.2
	5.00	538.3 ± 2.9	53.8	1.0 ± 1.1	1.0
	10.00	540.1 ± 3.6	54.0	1.1 ± 1.0	1.1
Water		537.5 ± 3.6	53.8	1.0 ± 0.8	1.0
CCP		443.2 ± 5.7	44.3	26.9 ± 4.2	26.9*

* $p < 0.05$.

The results from sperm malformation assay are shown in Table 8. There were no statistically significant differences in sperm malformation rate between the FMS-treated groups and negative control group ($p > 0.05$), which indicated that the sperm malformation rate were not significantly affected by FMS.

4. Discussion

Our previous studies have demonstrated the antioxidant activity of FMS and its acute toxicity in mice (Peng et al., 2015). The present study was conducted to evaluate the subchronic toxicity and genotoxicity of FMS in mice, which is essential to ensure its safe application and provide information on long term toxic and

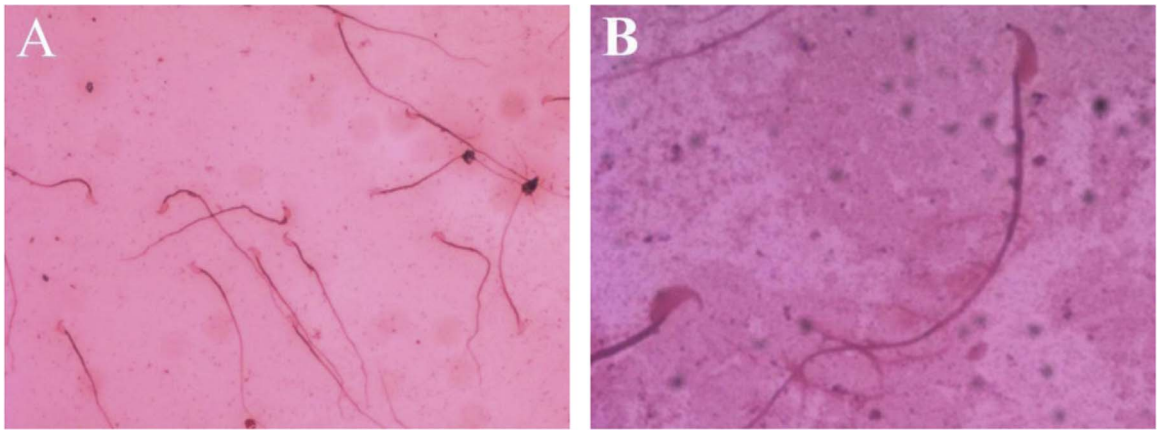

Fig. 5. Microphotographs of sperm malformation at 40 × (A) and 100 × (B).

Table 8
Results of sperm malformation assay in mice.

Treatment (g/kg bw d)		Number of spermatozoa	Number of sperm malformation			Malformation rate (%)
			Head defects	Tail defects	Total	
FMS	2.50	10 × 1000	126	17	143	1.43 ± 0.22
	5.00	10 × 1000	119	11	130	1.30 ± 0.37
	10.00	10 × 1000	127	15	142	1.42 ± 0.26
Water		10 × 1000	117	17	134	1.34 ± 0.24
Mitomycin C		10 × 1000	535	99	634	6.34 ± 1.37*

* $p < 0.05$.

genotoxic effects which are needed for further clinical studies.

Evaluation of subchronic toxicity via a 28-day repeated dose toxicity study has been applied in many safety assessment studies and plays a key role in chemical toxicity assessment (Tatefuji et al., 2014; Mohamed et al., 2011). In the present study, all animals survived until the scheduled necropsy without any obvious general clinical signs or changes. Generally, the increase or decrease in the body weight of animal can indicate an adverse effect caused by drugs and chemicals (Teo et al., 2002). In the present study, no significant changes were observed in body weight and food consumption in FMS-treated mice after 28 days of repeated administration. Relative organ weight values have been shown to reflect the pathological changes in impaired organs (Li et al., 2014; Mabeku et al., 2007). However, no treatment-related alterations in relative organ weights were found in any groups and no obvious pathological changes were also observed during necropsy in these main organs or tissues.

Results on the examination of haematological and serum biochemical parameters could demonstrate potential lesions in liver and kidney. No statistically significant differences were observed for each FMS-treated group with respect to these parameters after they were sacrificed. However, some parameters for some individual animals were slightly increased or decreased in FMS-treated groups. These changes were considered as toxicologically irrelevant because these changes did not appear in both sexes and no statistically significant differences were observed between each FMS-treated group and control group. Additionally, these data were within the normal range and showed no clear dose-response relationships (Evan, 2009). Moreover, this was further confirmed by histopathological observation of the liver and kidney in FMS-treated and control mice. In summary, subchronic oral administration of FMS did not have any observable adverse effects. Therefore, no-observed-adverse-effect level (NOAEL) of FMS in mice was 10.00 g/kg/day.

The micronucleus assay and sperm malformation assay has been applied in many food or chemical toxicity assessments (Wan et al., 2015; Zhou et al., 2015). In the present studies, these assays were conducted to evaluate the genotoxicity of FMS in mice. There were no statistically significant differences in PCE/RBC ratio, micronucleus frequency and sperm malformation rate between each FMS-treated groups and negative control group. The results indicated that the FMS is not a genotoxic substance either in somatic cells or germ cells even at an experimental upper limit dose (10.00 g/kg/day).

According to the results, FMS is relatively safe when given orally to mice. Besides, a previous 90-day repeated dose toxicity study of crude extract of *M. Stigma* showed that the NOAEL was 9.354 and 10.308 g/kg/day for male and female rats, respectively (Wang et al., 2011). However, toxicity of the food or chemical in human body cannot be entirely extrapolated from animal toxicological studies (Kandhare et al., 2015). Therefore, additional preclinical toxicity studies will be necessary to confirm the safety of FMS for long-term usage and verify the toxicological effect on human body. Nevertheless, the present study is still useful to demonstrate the subchronic toxicity and genotoxicity of FMS.

5. Conclusion

In conclusion, subchronic oral administration of FMS showed the no-observed-adverse-effect level (NOAEL) of FMS is 10.00 g/kg/day for both male and female mice. The results of micronucleus assay and sperm malformation assay indicated that FMS is not a genotoxic substance. The results of present studies demonstrated that the FMS could be safe as a functional food, food additive or natural remedy. However, this study is probably the first study on safety assessment of FMS, and additional pre-clinical and clinical studies are essential to ensure its safe application.

Conflicts of interest

The authors declare no conflict of interest.

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