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Full Length Research Paper

Toxicological evaluation of carotenoid-type extracts from *Flos Chrysanthemi Indici*

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Flos Chrysanthemi Indici **(FCI) was considered as a traditional medicine and regarded as a conventional food in China, Korea and Japan. Toxicological evaluation of carotenoid-type extracts derived from FCI was investigated in the present paper. Acute toxicity of the extracts was evaluated with a single dose administered by the intragastric route to SD rats in dosages of 15 g/kg. After an observation period of 2 weeks after intragastric administration, no mortality or any changes in appearance, behavior occurred for both female and male rats in the high dosages mentioned. Macroscopic and microscopic studies of the internal organs revealed no pathological changes. The observations of acute toxicity test indicated that the extracts were nontoxic. Genetic toxicity of the extracts was assessed by Ames assay in *Salmonella typhimurium* strains (TA97, TA98, TA100 and TA102) with and without metabolic activation (S9), by bone marrow micronucleus test and sperm abnormality test in ICR mice. The examinations of genotoxicity assay indicated that the extracts lack genotoxic potential. Although, these relatively short-term studies document no toxicity, longer-term use could result in serious toxicity. Further studies are therefore required to study long-term toxicity.**

Key words: Acute oral toxicity, carotenoid-type extracts, *Flos Chrysanthemi Indici*, genetic toxicity.

INTRODUCTION

Chrysanthemum indicum L. (*C. indicum*), spreading widely in China (Cheng et al., 2005), is a significant medicinal plant with small yellow flowers (capitula, FCI) (Zhu et al., 2005; Shen et al., 2004; Deng et al., 2006). Yellow flowers most commonly derive their color from carotenoid pigmentation; however there are examples in which yellow colouration results from the presence of flavonoids (Kenneth et al., 2001). The same flower can contain carotenoids and flavonoids (Miller et al., 2009). Phytochemical profile of the herb had shown the presence of flavonoids, terpenoids and phenolic compounds (Yoshikawa et al., 1999).

Flavonoid-type extracts isolated from FCI have been well reported in historical literatures (Cheng et al., 2005; Cheon et al., 2009; Lee et al., 2009), they are safe and possess many pharmacological activities, such as immunomodulatory activities (Cheng et al., 2005) and anti-inflammatory properties (Cheng et al., 2005; Cheon et al., 2009; Lee et al., 2009). However, carotenoid-type extracts from this plant remain are to be investigated. Carotenoids are a group of colored terpenoids (Kishimoto et al., 2004), they are usually used as additives in food and cosmetic industries (Aksu and Tuğba, 2005; Vila et al., 2008).

Previous studies have shown that diethyl ether was the customary extracting solvent for carotenoids from plant materials (Lewis et al., 1998; Kishimoto et al., 2004; Guerra et al., 2005). Here, carotenoid-type extracts from FCI were extracted with diethyl ether. The aim of the present study was to evaluate if the carotenoid-type extracts (etheric extracts) from the herb were potential source of natural safe extracts.

MATERIALS AND METHODS

Plant material and extraction procedures

The samples were collected from Jinzhai country in Anhui Province and during the same season by traditional collection (that is, mainly during the flowering season), and were identified as capitulum of *C. indicum* by Prof. Dr. Guo Qiao Sheng working in Nanjing Agricultural University, Nanjing, China.

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The air-dried samples were entirely extracted until they became colorless by stirring them with diethyl ether at ambient temperature (below 20°C), the crude extracts was filtered and rotary evaporated at 40°C with vacuum, followed by lyophilization to dryness and kept in the dark at -18°C until further tested. The yield of dried extracts from starting crude materials (dry weight) was 2.15%. Carotenoid-type extracts (etheric extracts) used for different investigations originated from a single extraction batch.

Characterizations of the extracts

The concentration of total carotenoids and β-carotene in dried prepared extracts were 165.51±1.42 and 2.54±0.01 mg/g. Characteristic of the extracts solutions (dissolved in diethyl ether) was yellow-to-red range with the concentration increased. Color parameters of 1% solution of the extracts, that is L*, a*, b* and ΔE were 32.32, 0.20, 21.36 and 22.57, respectively. Loss on drying, total ash, water-insoluble ash, acid-insoluble ash and sulfated ash of the extracts were 7.03±0.26%, 0.22±0.04%, 0.11±0.03%, 0.08±0.01% and 9.09±0.07%, respectively. Characteristic UV-Vis absorption bands of the extracts in diethyl ether were 412, 436 and 468 nm. Characteristic IR absorption bands of the extracts in diethyl ether were 3442, 1636, 1136 and 654 cm^{-1}. Maximum fluorescence excitation and emission wavelength of the extracts in diethyl ether were 509 and 525 nm.

Animals housing conditions and experimental protocols

All animals were maintained under controlled conditions in a room ventilated with fresh air, with at least 10 to 20 air changes per hour. The room temperature was maintained at 22°C (range from 20 to 23°C) with relative humidity 55% (range from 40 to 60%) and a 12 h light/dark cycle. All animals were allowed free access to food and water.

All the animal experiment protocols were approved by the Institutional Animal Ethics Committee (IAEC) and the experimentation on animals was done in accordance with the CPCSEA (Committee for the Purpose of Control and Supervision of Experimentation on Animals) guidelines.

Acute oral toxicity test

SD rats, with an average body weight of 182 to 200 g, were received from Medical animal experimental center, Nanjing General Hospital of Nanjing Military Command, PLA. A single dose of this extracts (60 g extracts dissolved in 80 mL corn oil) was given intragastrically to both male and female rats (n = 20 per group, 10 per sex) at a dosage of 15 g extracts/kg of body weight. Animals were sacrificed, necropsied and submitted to macroscopic and microscopic examination of tissues at the end of the 14-day observation period, as requested by internationally accepted acute toxicity study protocols. They were closely observed for gross morphological, physiological, behavioral changes and mortality.

Genetic toxicity study

Ames assay

The assay comprised two independent parts: *Salmonella typhimurium* (strains TA97, TA98, TA100 and TA102) and metabolic activation (S9) were obtained from School of Public Health, Fudan University. Mutagenic effect was examined by Salmonella/ microsome assay according to Maron and Ames (1983). Different concentrations of the extracts were incubated with special genotypic variants of *Salmonella* strains within and without S9. The test was assessed based on the number of revertant colonies. For analyses, specific quantities of the extracts were dissolved in dimethyl sulfoxide (DMSO), and the concentrations were 10, 2, 0.4, 0.02 mg/ml. Five doses of the extracts (that is, 5000, 1000, 40, 2 and 0 µg/plate) were plated in triplicate with 0.1 ml of the bacterial culture. After incubating the test sample and bacterial culture at 37°C for 30 min, 2.0 ml top agar containing traces of histidine and biotin were added and contents were poured on minimal glucose agar plates. Plates were incubated at 37°C for 48 h. Bacterial colonies were counted manually after 48 h. Positive controls (Dexon; Sodium azide; 2-aminofluorene, 2-AF; and 1,8-dihydroxyanthraquinone, 1,8-DA) and negative controls (DMSO and distilled water) were concurrently maintained.

Bone marrow cell micronucleus test

Healthy ICR mice (8 to 12 weeks old, purchased from Shanghai laboratory animal center, Chinese Academy Sciences, National Rodent Laboratory Animal Resources, Shanghai Branch), with an average body weight of 25.5 to 28.9 g, were randomly divided into five groups (n = 10 per group, 5 per sex) and used for the experiment. Mice in three extracts-treated groups were treated daily at doses of 10000, 2500, 625 mg/kg of body weight (the extracts were dissolved in corn oil and the volume of corn oil was 20 ml/kg of body weight) for 2 days by gavage. The negative and positive control groups were given corn oil and cyclophosphamide (CP, 40 mg/kg of body weight), respectively, also administered for 2 consecutive days.

Animals were sacrificed 24 h after treatment. Two hours before sacrifice, the mice were injected with 0.3 ml of freshly prepared colchicine solution (4 mg/kg of body weight). Bone-marrow preparations for the analysis of chromosome aberrations in metaphase cells were obtained by the technique of Yosida and Amano (1965). Slides were stained with 7% Giemsa stain in phosphate buffer (pH 6.8). About 5000 erythrocytes were scored for the presence of micronuclei for each animal. To evaluate bone marrow toxicity, the ratio polychromatic erythrocytes/ normochromatic erythrocytes (PCE/NCE) was calculated by counting 500 erythrocytes.

Sperm abnormality assay

Male ICR mice (29.2 to 31.8 g, supplied by Shanghai laboratory animal center, Chinese Academy Sciences, National Rodent Laboratory Animal Resources, Shanghai Branch) were randomly divided into five groups (n = 5 per group). Mice, in three extracts-treated groups, were given daily at doses of 10000, 2500, 625 mg/kg of body weight (the extracts were dissolved in corn oil and the given dose volume of corn oil was 20 ml/kg of body weight) via oral gavage for 5 consecutive days. The negative and positive control groups were given corn oil and CP (40 mg/kg of body weight), respectively, also administered for 5 consecutive days. The sperm morphology test was performed at 35 days after exposure.

The cauda epididymis were surgically removed after sacrificing (cervical dislocation) the animals and placed in a Petri dish containing 2 to 3 ml of HBSS at room temperature. The epididymis was minced into small pieces to allow the sperm to swim out. The sperm suspension thus obtained was centrifuged at 1000 rpm for 5 min. Then, 1 ml of the supernatant was taken and the epididymal sperm count was determined using Neubauer's hemocytometer. Data were expressed as the number of sperms per mg wt of epididymis. For sperm morphology, the sperm suspension in HBSS was stained with 1% eosin solution and kept undisturbed for 1 h. Smears were prepared using the foregoing solution, air dried and fixed with absolute methanol for 5 min. Two hundred sperms per

Table 1. Effect of carotenoid-type extracts from FCI on body weight of rats.

Sex	Treated animals	Initial body weight (g)	Final body weight (g)	Chemical dose (g/kg·bw)	Number of death
Female	10	186.3±3.7	224.8±7.6	15	0
Male	10	190.2±5.2	297.0±18.4	15	0

[a]Data were expressed as the mean ± standard deviation.

animal were examined to determine the morphological abnormalities at 1000× magnification (Brown et al., 1994; Kishikawa et al., 1999). Sperm head morphology was scored under the category of normal, sperm without hook, amorphous head, banana head and triangular head essentially as described (Wyrobek and Bruce, 1975). Sperm tail morphology was counted under the category of bifurcated tail and folded tail. Data were shown in terms of normal to abnormal ratio of sperms.

Statistical analysis

For statistical analysis on toxicity tests, differences between the experimental and the control groups were calculated by one tailed Student's *t*-test. A probability value of $P<0.01$ was considered significant.

RESULTS AND DISCUSSION

Acute toxicity

Single oral (that is, intragastric) administration of the extracts in a dose of 15 g/kg of body weight for rats produced zero mortality over an observation period of 2 weeks. During the 2-week observation period, appearance, behavioral peculiarities, eating and drinking habits remained within the normal values. A small increase in the body weights was recorded for all test animals with no abnormalities at necropsy on day 14 (Table 1).

Morphological macroscopic and microscopic studies of the internal organs (brain, heart, lungs, stomach and intestines, pancreas, liver, kidneys, spleen, thymus, etc.) revealed no changes characteristic of acute intoxication. Labeling and classification of acute systemic toxicity of chemicals based on oral 50% lethal dose values are recommended by the organization for economic co-operation and development (OECD, 1996). These recommendations are: very toxic, <5 mg/kg of body weight; toxic, >5 to <50 mg/kg of body weight; harmful, >50 to <500 mg/kg of body weight; and no label, >500 to <2,000 mg/kg of body weight (OECD, 1996). In accordance with this classification system the substances with 50% lethal doses higher than 2 g/kg of body weight do not fit into even the lowest "no label" toxicity class and should, therefore, be considered nontoxic with respect to potential acute toxicity. The acute oral toxicity study not only confirms the lack of acute toxicity in rates after oral administration of carotenoid-type extracts derived from FCI, but also shows that even with a higher concentration range, no acute toxic effects are observed.

Genotoxicity

In the genotoxicity studies, including the Ames assay, bone marrow cell micronucleus test and sperm abnormality assay were elucidated. The Ames test was performed to detect genetic damage, induced directly or indirectly using *S. typhimurium* tester strains (TA97, TA98, TA100 and TA102) with extracts (5000, 1000, 40, 2 and 0 µg/plate). Results revealed that there were no substantial increases in the mean numbers of revertant colonies, in the presence or absence of S9 metabolic activation. The experiment was confirmed in an independent repeat under the same study conditions. The results displayed in Table 2 suggested that the extracts were not mutagenic.

The results of Ames test showed that carotenoid-type extracts derived from FCI do not induce mutations in a set of five tested Salmonella strains in doses up to and including 5000 g/plate, both without and within metabolic activation (addition of S9 mix). The extracts did not induce bacteriotoxicity. The results of micronucleus test were exhibited in Table 3. It was analyzed a basal level of 1.8±1.1 (female) and 1.8±0.8 (male) micronucleated erythrocytes (MNE)/1000 analyzed cells in the animals of the negative control group without statistical differences ($P>0.01$) with the treated animals at each dose of extracts. The NCE/PCE ratio in all the extracts which treated groups of mice were not increased from that of the negative control mice and not found to be disturbed during the analysis of PCEs.In bone marrow cell micronucleus test, we did not observe a statistically significant increase in MN frequency with any of the test concentrations of the extracts.

The results suggest that at doses <10000 mg/kg of body weight and under the test conditions of these experiments, carotenoid-type extracts derived from FCI is not genotoxic to mice in *vivo*. Sperms without hook, banana head sperms and sperms with triangular and amorphous heads were classified as sperm head abnormalities. The different types of murine sperm abnormalities observed were summarized in Table 4, no considerable dose related increase was observed in the percentage of abnormal sperms. The average percentage of abnormal sperm was 2.8±0.4 animals. in the negative control mice. The analysis and it of sperm number in the epididymis increased significantly

Table 2. Mutagenic activity of carotenoid-type extracts from FCI on *Salmonella typhimurium* TA97, TA98, TA100 and TA102 with or without metabolic activation S9.

Position	Repeat group	Chemical dose (µg/plate)	Revertants/plate with each bacterial test strain[a]							
			+S9				-S9			
			TA97	TA98	TA100	TA102	TA97	TA98	TA100	TA102
First	Etheric extracts	5000	107±8	39±3	154±6	274±21	114±11	37±3	167±12	263±16
		1000	109±8	35±2	171±3	274±12	112±15	34±2	154±22	261±14
		200	115±23	36±2	172±27	289±22	108±13	38±3	176±8	289±8
		40	121±18	37±6	170±17	264±22	119±8	36±2	171±22	258±12
		2	113±15	35±3	179±13	278±28	106±12	34±4	166±11	261±13
	Spontaneous revertants (H_2O)	0.1 (ml)	110±22	40±7	151±26	279±17	117±21	36±8	155±22	283±8
	DMSO	0.1 (ml)	111±15	35±5	174±18	291±28	111±25	37±9	178±18	253±15
	2-AF	10	1201±147[b]	3690±155[b]	2905±178[b]					
	1,8-DA	50				1129±203[b]				
	Dexon	50					2208±222[b]	1209±158[b]		1164±83[b]
	Sodium azide	1.5							2204±148[b]	
Second	Etheric extracts	5000	111±8	35±2	153±6	272±25	119±12	33±2	174±21	275±29
		1000	120±15	37±2	169±14	267±27	112±13	34±1	187±3	280±16
		200	121±15	34±1	154±5	276±17	119±16	33±3	168±5	284±15
		40	122±21	34±3	171±21	275±26	107±11	37±4	171±25	254±13
		2	123±12	39±2	182±14	283±20	118±13	37±4	172±21	255±15
	Spontaneous revertants (H_2O)	0.1 (ml)	107±14	35±3	175±18	280±25	117±9	35±5	170±27	262±21
	DMSO	0.1 (ml)	123±10	34±3	166±15	285±18	123±20	36±3	156±18	288±18
	2-AF	10	1190±196[b]	3492±151[b]	2638±97[b]					
	1,8-DA	50				1078±124[b]				
	Dexon	50					2123±218[b]	1344±107[b]		1134±117[b]
	Sodium azide	1.5							2057±128[b]	

[a] Data were expressed as the mean ± standard deviation of three replicates. b Expressed significant difference (P<0.01), *t*-test for comparison between treatment and control in two times.

to (P<0.01) 10.2±0.7 in the positive control mice. There were no statistical differences (P>0.01) in the percentage frequency of abnormal sperm between the negative control group and the treated animals. In Sperm abnormality experiment, carotenoid-type extracts derived from FCI did not show any effects on sperm differentiation in mice. In fact, the sperm morphology test revealed that the frequency of

Table 3. Effect of carotenoid-type extracts from FCI on formation of micronucleated polychromatic erythroblasts in bone marrow cells of mice.

Sex	Chemical dose (mg/kg·bw)	Total examined PCE	Micronucleated PCE	MNE per 1000[a] (‰)	PCE/NCE[a]
Female	10000	5000	9	1.8±0.4	1.09±0.03
	2500	5000	8	1.6±1.1	1.11±0.06
	625	5000	6	1.2±0.4	1.10±0.09
	0	5000	9	1.8±1.1	1.12±0.06
	40 (CP)	5000	146	29.2±1.9[b]	0.92±0.03
Male	10000	5000	10	2.0±0.7	1.06±0.05
	2500	5000	9	1.8±0.8	1.07±0.05
	625	5000	7	1.4±0.5	1.11±0.06
	0	5000	9	1.8±0.8	1.07±0.06
	40 (CP)	5000	167	33.4±1.7[b]	0.89±0.03

[a] Data were expressed as the mean ± standard deviation of five animals. [b] expressed significant difference (P<0.01), *t*-test for comparison between treatment and control.

Table 4. Sperm morphology and frequency of different types in mice treated with different doses of carotenoid-type extracts from FCI.

Chemical dose (mg/kg·bw)	Treated animals	Total counted sperms	Head										Tail				Total abnormal sperms	Total sperm abnormality[a] (%)
			No hook		Amorphous		Megacephaly		Banana		Double		Bifurcated		Folded			
			n	%	n	%	n	%	n	%	n	%	n	%	n	%		
10000	5	5000	40	29.4	83	61.0	9	6.6	2	1.5	0	0.0	0	0.0	2	1.5	136	2.7±0.5
2500	5	5000	34	29.1	73	62.4	7	6.0	1	0.9	0	0.0	0	0.0	2	1.7	117	2.3±0.4
625	5	5000	39	32.0	76	62.3	5	4.1	1	0.8	0	0.0	0	0.0	1	0.8	122	2.4±0.2
0	5	5000	42	30.2	89	64.0	6	4.3	1	0.7	0	0.0	0	0.0	1	0.7	139	2.8±0.4
40 (CP)	5	5000	159	31.2	289	56.8	32	6.3	6	1.2	6	1.2	7	1.4	10	2.0	509	10.2±0.7[b]

[a] Data were expressed as the mean ± standard deviation of three replicates. The % values presented correspond to the relative contribution of each particular abnormality to the total abnormal sperms. [b] Expressed significant difference (P<0.01), *t*-test for comparison between treatment and control.

head, tail or multiple abnormalities in treated animals is not higher than that in control did not evidence any differences between control and experimental groups. FCI is a drug that has been in clinical use as a heat clearing agent for thousands of years, but previous uses were aqueous and/or ethanolic extracts from the herb (Cheng et al., 2005; Cheon et al., 2009; Lee et al., 2009). Very few published reports could be found on the potential toxicity of etheric extracts. The solvent systems for the preparation of the extracts used in the present and the previous study differed in polarity (diethyl ether vs. water or water/ethanol mixture), which could result in a qualitatively and/or quantitatively different pattern of natural compounds in the extracts. The etheric extracts from FCI are carotenoid-type extracts. Carotenoids may be considered micronutrients, because of their relationship to vitamin A (Rock, 1997). Studies on safety of carotenoids have been well documented, such as, Leal et al (1998) lectured that lutein derived from marigold and lycopene obtained from tomato showed cytotoxicity when they were in contact with

chicken hepatocytes, in high concentrations (>10^{-5} M) and when carotenoids were used in low concentrations (10^{-6} M) during long incubation periods; but Garcia-Gasca et al. (1998) reported that carotenoids from pepper (*Capsicum* spp.) provide a dose-dependent protective effect against genotoxicity induced by diethylnitrosamine in isolated rat hepatocytes. In our test, the examinations of genotoxicity assay indicated that the extracts lack genotoxic potential.

Although, these relatively short-term studies document no toxicity, the results obtained in the present study might be limited, longer-term use could result in serious toxicity. Further studies are therefore required to study long-term toxicity.

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