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Safety Evaluation of *Chrysanthemum indicum* L. Flower Oil by Assessing Acute Oral Toxicity, Micronucleus Abnormalities, and Mutagenicity

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ABSTRACT: *Chrysanthemum indicum* is widely used to treat immune-related and infectious disorders in East Asia. *C. indicum* flower oil contains 1,8-cineole, germacrene D, camphor, α-cadinol, camphene, pinocarvone, β-caryophyllene, 3-cyclohexen-1-ol, and γ-curcumene. We evaluated the safety of *C. indicum* flower oil by conducting acute oral toxicity, bone marrow micronucleus, and bacterial reverse mutation tests. Mortality, clinical signs and gross findings of mice were measured for 15 days after the oral single gavage administration of *C. indicum* flower oil. There were no mortality and clinical signs of toxicity at 2,000 mg/kg body weight/day of *C. indicum* flower oil throughout the 15 day period. Micronucleated erythrocyte cell counts for all treated groups were not significantly different between test and control groups. Levels of 15.63∼500 μg *C. indicum* flower oil/plate did not induce mutagenicity in *S.* Typhimurium and *E. coli*, with or without the introduction of a metabolic activation system. These results indicate that ingesting *C. indicum* flower oil produces no acute oral toxicity, bone marrow micronucleus, and bacterial reverse mutation.

Keywords: *Chrysanthemum indicum* flower oil, acute oral toxicity, bone marrow micronucleus, bacterial reverse mutation

INTRODUCTION

Chrysanthemum indicum L. (*C. indicum*), belonging to the Compositae family, is a perennial garden herb in many countries. *C. indicum* has been used to treat various immune-related disorders, hypertension symptoms, and several infectious diseases in Korean and Chinese medicine (1-3). *C. indicum* flowers are also commonly used as tea to treat antiinflammation, headache, and eye diseases (2,4). The pharmaceutical benefits of *C. indicum* were reportedly due to its antioxidant, antiviral, antibacterial, and immunomodulatory properties (5,6). In previous reports, the major volatile compounds of *Chrysanthemum* sp. were 1,8-cineole, germacrene D, camphor, α-cadinol, camphene, pinocarvone, β-caryophyllene, 3-cyclohexen-1-ol, and γ-curcumene (7-10).

The micronucleus test, an *in vivo* short-term screening test developed by Schmid (11) and Heddle (12), is useful in investigating the toxicity of compounds. The frequency of micronucleated polychromatic erythrocytes (MPCE) induced by mutagens increases after exposure to carbon monoxide (13), bleeding (14), or anoxia (15). Well-known, carbon monoxide causes tissue anoxia by producing a carbon monoxide-hemoglobin complex.

Abnormalities in chromosomal structures such as increased chromosomal breakage or chromosomal loss are associated with enhanced risk of carcinogenesis and in the progression of neoplastic transformation (16). Detection of chromosomal aberrations has been widely used as a tool to indicate carcinogen induced DNA damage as well as to assess the antigenotoxic effect of natural or synthetic chemopreventive agents (11,17,18).

The available toxicological information on *C. indicum* flower oil is insufficient to assure its safe use in dietary supplements or functional materials. We evaluated the mutagenicity and toxicity of *C. indicum* flower oil based on acute oral toxicity, bone marrow micronucleus, and bacterial reverse mutation tests.

MATERIALS AND METHODS

Extraction of *C. indicum* flower oil

Flowers of *C. indicum* were harvested in the fall of 2006 from Namwon (Jeollabuk-do) province, in western Korea, and purchased at Gyeongdong herbal market (Seoul, Korea)

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in the spring of 2007. Samples were stored at −70°C in airtight bags for later analysis. Oils of *C. indicum* flowers were extracted by steam distillation method (19). The obtained oils were dried over anhydrous sodium sulfate for 24 hr, and then stored in hermetically sealed glass containers at −4°C for later testing.

Acute oral toxicity study in mice

Both 10 female and 10 male ICR mice (specific pathogen free, 6 weeks of age) were purchased from Orient Bio (Orient Bio Inc., Seongnam, South Korea). Mice were assigned to each group (n=10) and acclimated for a week in the housing. Temperature was maintained at 23±3°C and relative humidity was 50±10%. A 12 hr light/dark cycle was observed and air was changed 10~20 times per day. Mice were freely accessed to stock rodent pellet (PMI Nutritional International, Richmond, IN, USA) and tap water. The study protocol was reviewed by IACUC (Institutional Animal Care and Use Committee, Daejeon, South Korea) and conducted in compliance with the Association for Assessment and internationally recognized Accreditation of Laboratory Animal Care (AAALAC, Daejeon, South Korea) (20). Body weight of the mice at the beginning of the study was 29.6±1.1 g for males and 21.5±1.3 g for females. Mice were fasted for 4 hr prior to oral administration of *C. indicum* flower oil dissolved in carboxymethyl cellulose (CMC). Constant volume (10 mL/kg body weight) containing 200 mg/mL of *C. indicum* flower oil was orally administered to each of the five mice per sex to achieve a dose of 2,000 mg/kg body weight. CMC was used as negative control. Feeds and water were provided after 4 hr of oral administration. Overall symptoms, including mortality, clinical signs, and gross findings, were observed once a day for 15 days. Body weight was weighed just before oral administration (day 1) and after dosing (days 2, 4, 8, and 15). On day 15, all animals were euthanized under CO_2 gas overdose and examined for internal organ abnormalities.

Animal test subjects for bone marrow micronucleus test

Female and male Institute for Cancer Research (ICR) mice (30 each, specific pathogen free, 6 weeks old) were obtained (Orient Bio Inc.) and divided into 10 groups (five male, five female) of six mice each. Average body weight at the beginning of the study was about 35.5 g for males and 25.7 g for females. Six of the mice groups (three male, three female) were administered *C. indicum* flower oils orally at doses of 500, 1,000 and 2,000 mg/kg body weight for 2 days. Two groups (one male, one female) were given oral doses of corn oil as a negative control, and the remaining two groups were given cyclophosphamide monohydrate as a positive control. Animals were euthanized within 24 hr of final dose administration by an overdose of CO_2 gas.

Bone marrow micronucleus test

Micronucleus slides were prepared using the method described by Schmid (21). The femur bones of each mouse were separated and cleaned of surrounding muscle tissue. The upper end of the femur was cut until a small opening was visible. About 0.5 mL bovine albumin was injected into the opening of the bone via syringe, flushing the bone marrow into a clean, dry centrifuge tube. The flushing was repeated to ensure a homogeneous cell suspension. The suspension volume was increased to 3 mL and then centrifuged at 1,000 rpm for 5 min. The supernatant was discarded and the collected cells were disturbed by gently tapping the tube, then mixed well in a minimum quantity of bovine albumin.

One drop of the cell suspension was placed on a clean, dry slide and spread with another slide held at a 45° angle. Both slides were air dried and fixed in absolute methanol for 5 min. The slides were placed for 20 min in 5% Giemsa staining solution in 0.01 M phosphate buffer, with pH adjusted to 6.8. The slides were washed in running tap water, air dried, mounted with DPX (synthetic resin of distrene plasticiser xylene), then examined under oil immersion at 1,000× magnification using bright-field illumination. All slides were randomly coded prior to microscopic analysis, and 2,000 bone marrow polychromatic erythrocytes (PCE) were examined from each animal, with the number of micronucleated PCE recorded. The percentage of PCE among 500 normochromatic erythrocytes (NCE) was determined for a given sample to evaluate treatment cytotoxicity. Mann-Whitney's U-test was used to evaluate results from the treatment and positive control groups with respect to micronucleus formation and PCE/(PCE+NCE) ratios.

Bacterial reverse mutation assay

Mutagenic activity of *C. indicum* flower oil was evaluated using the Ames protocol (22). Histidine-requiring *Salmonella* Typhimurium (TA100, TA1535, TA98, and TA1537) and tryptophan-requiring *Escherichia coli* (WP2uvrA) strains were obtained from Molecular Toxicology Inc. (Boone, NC, USA). *C. indicum* flower oil was dissolved at a concentration of 2,000 μg/mL in dimethyl sulfoxide (DMSO, 99.99% pure, Sigma-Aldrich Chemical Co., St. Louis, MO, USA). This base-concentration solution was further diluted with DMSO into a series of lower concentration test solutions (i.e., 500, 250, 125, 62.5, 31.25 and 15.63 μg/mL) in accordance with Organization for Economic Cooperation and Development (OECD) guidelines (23). Triplicate mutagenicity assays were conducted with and without the rat liver S9 mix (Molecular Toxicology Inc.) metabolic activation system, and with negative and positive controls in accordance with OECD TG 471

guidelines (24). The sources and grades of positive control materials were as follows: sodium azide (100.1% pure, Sigma-Aldrich Chemical Co.), 2-nitrofluorene (98.1% pure, Aldrich Chemical Co., Milwaukee, WI, USA), 9-aminoacridine (97.7% pure, Merck Chemical Co., Whitehouse Station, NJ, USA), 4-nitroquinoline 1-oxide (99% pure, Sigma Chemical Co., St. Louis, MO, USA), 2-aminoanthracene (99.8% pure, Aldrich Chemical Co.), and benzo[a]pyrene (99.8% pure, Sigma-Aldrich Chemical Co.).

Statistical analysis

Changes in body weight and clinical signs observed for 15 days after oral administration were analyzed using the Path/Tox system (V4.2.2; Xybion Medical Systems Corp., Cedar Knolls, NJ, USA). All data were evaluated using the Statistical Analysis System (SAS, v8.2; SAS Institute Inc., Cary, NC, USA). Differences between treatment and control group averages were assessed using a *t*-test. Differences in the frequency of chromosome aberration between negative control and treatment groups were evaluated using chi-square and Fisher's exact tests. The Cochran-Armitage trend test was used to assess dose-response relationships. Differences between negative and positive control group means were assessed using the Fisher's exact test.

Table 1. Major components (%) of *Chrysanthemum indicum* flower oil

Components	R.I.[1)]	Contents (Relative %)
α-Pinene	940	14.63
1,8-Cineol	1,020	10.71
Camphor	1,152	2.64
Borneol	1,186	3.02
Bornyl acetate	1,289	3.64
β-Elemene	1,382	3.18
Germacrene D	1,502	5.25
(-)-Sinularene	1,514	3.95
β-Bisabolene	1,516	3.95
β-Sesquiphellandrene	1,532	1.19

[1)]Retention indices (R.I.) were calculated using n-alkanes (C_8-C_{22}) as external references on an HP-5MS capillary column.

RESTULS AND DISCUSSION

We previously determined the main components of *C. indicum* flower oil (Table 1) (7). Flower oil of *C. indicum* was characterized as having prominent (>3%) contents of α-pinene (14.63%), 1,8-cineol (10.71%), germacrene D (5.25%), (−)-sinularene (3.95%), β-bisabolene (3.95%), bornyl acetate (3.64%), β-elemene (3.18%), and borneol (3.02%). Zhang et al. (8) analyzed the volatile composition of *C. indicum* essential oils by GC/MS from eight populations in China. They found a total of 169 compounds and the predominant components were 1,8-cineole (0.62～7.34%), (+)-(1R, 4R)-camphor (0.17～27.56%), caryophyllene oxide (0.54～5.8%), β-phellandrene (0.72～1.87%), 2-methyl-6-(p-tolyl)hept-2-ene (0.3～8.6%), and hexadecanoic acid (0.72～15.97%). These results indicate that major compounds in *C. indicum* were varied with growing region, species, analytical tools, etc.

Acute oral toxicity test

Mortality, clinical signs and gross findings of mice were measured for 15 days after the oral single gavage admin-

Table 2. Mortality of mice during 15 days after oral administration of *Chrysanthemum indicum* flower oil

Dose (mg/kg)	Dosing phase				Mortality
	1 day	≤5 day	≤10 day	≤15 day	
Male					
0	0	0	0	0	0/5
2,000	0	0	0	0	0/5
Female					
0	0	0	0	0	0/5
2,000	0	0	0	0	0/5

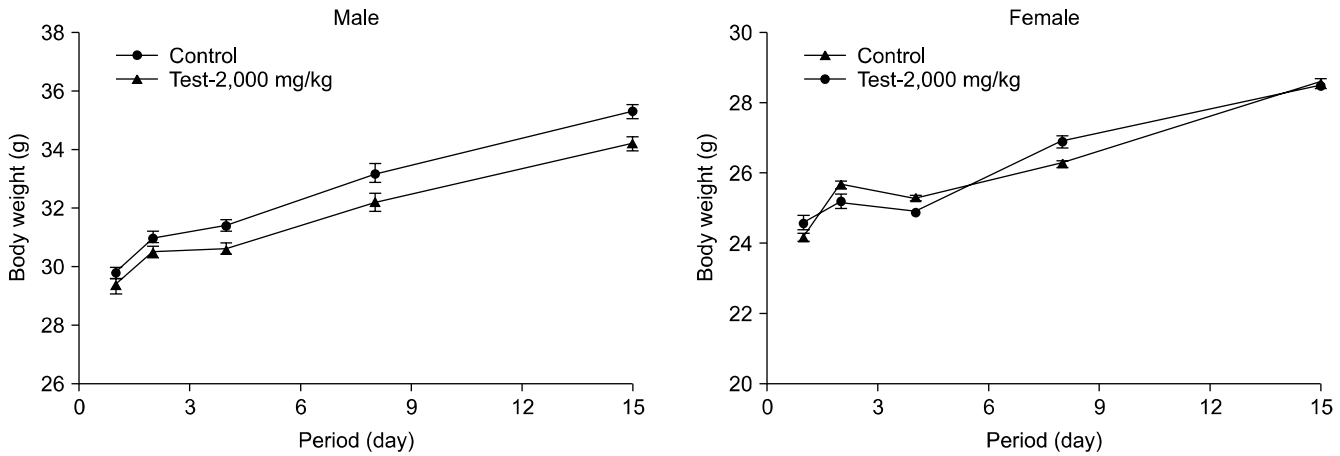

Fig. 1. Body weight changes for male and female rats after administered by *C. indicum* flower oil at 2,000 mg/kg body weight. Each value is expressed as mean±SD of measurement.

istration of *C. indicum* flower oil. No mortality and clinical signs of toxicity were observed throughout the 15 day period (Table 2). Therefore, the approximate lethal doses of *C. indicum* flower oil in male and female mice are higher than 2,000 mg/kg. Mean body weights for male and female rats at 2,000 mg/kg of *C. indicum* flower oil group were compared to control groups (Fig. 1). Slight body weight loss was observed from male mice in test group during the experimental periods but not statistically different from the control group. Slight body weight loss occurred among female mice at 4 days in both control and 2,000 mg/kg of *C. indicum* flower oil group but recovered after 8 days. After fifteen days of study, the mean body weights of control and *C. indicum* flower oil groups there were not significantly different.

Bone marrow micronucleus test

Micronucleated erythrocyte cell counts were not significantly different between test groups and negative control groups (Table 3). PCE content in relation to total erythrocytes was evaluated to assess erythropoiesis rate, a measure of cytotoxicity. The estimated PCE : NCE ratio in the bone marrow preparations showed no statistically significant difference in hematopoiesis alterations as a result of extract treatment, indicating no cytotoxic effects.

Bacterial reverse mutation assay

Mutagenicity of *C. indicum* flower derived essential oil was evaluated in a bacterial reverse mutation assay using histidine requiring *S.* Typhimurium (TA100, TA1535, TA98, and TA1537) and tryptophan-requiring *E. coli* (WP2uvrA) (Table 4). Regardless of S9 mix presence or absence, the number of reverting colonies for all treatment concentrations was not significantly different from negative control. No cytotoxicity was observed in any bacterial systems used in the mutation assay. A marked increase in the number of revertant colonies was observed in the positive controls compared to the negative control. No mutagenic activity was observed for any test concentration of the *C. indicum* flower oil. According to a previous study, *d*-limonene does not elicit mutagenicity in four strains of *S.* Typhimurium (25), nor chromosomal aberrations or sister chromatid exchange in cultured Chinese hamster ovary cells, or cell mutations in the livers or kidneys of rats (26).

Our results suggest that *C. indicum* flower oil produces no bone marrow micronucleus abnormalities, mutagenicity, or chromosomal aberrations, and thus might be considered a functional food or medicinal ingredient. However, further detailed studies, such as *in vivo* animal studies that further define toxicological properties, are required to understand the potential of adverse health effects from routine ingestion by humans.

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Table 3. Micronucleus test of *Chrysanthemum indicum* flower oil in mice

Chemical tested	Dose (mg/kg)	No. of animals	MNPCE/2000 PCEs (mean±SD)	PCE/(PCE+NCE) (mean±SD)
Male				
Vehicle[1)]	0	6	0.67±0.82	0.49±0.01
Test item[2)]	500	6	0.83±1.17	0.48±0.03
Test item	1,000	6	0.67±0.82	0.48±0.04
Test item	2,000	6	1.17±0.75	0.46±0.05
CPA	70	6	67.00±7.24*	0.43±0.02*
Female				
Vehicle	0	6	1.17±0.75	0.54±0.03
Test item	500	6	0.67±1.03	0.54±0.03
Test item	1,000	6	0.50±0.55	0.51±0.03
Test item	2,000	6	0.67±0.52	0.47±0.01*
CPA	70	6	52.00±5.22*	0.47±0.03*

*Significantly different from the control at $P<0.01$.
[1)]Vehicle: corn oil.
[2)]Test item: *C. indicum* flower oil.
MNPCE, PCE with one or more micronuclei; PCE, polychromatic erythrocyte; NCE, normochromatic erythrocyte; CPA, cyclophosphamide monohydrate (positive control article).

Table 4. Bacterial reverse mutation assay with *Chrysanthemum indicum* flower oil

Test strain	Chemical treated	Dose (μg/plate)	Revertant colonies/plate (mean) [Factor][1)]	
			Without S9 mix	With S9 mix
TA 100	Test item[2)]	0	120±9	133±6
		15.63	131±11 [1.1]	
		31.25	118±3 [1.0]	132±19 [1.0]
		62.5	121±7 [1.0]	145±5 [1.1]
		125	116±13 [1.0]	144±12 [1.1]
		250	112±17 [0.9]	133±15 [1.0]
		500	76±5 [0.6]	137±6 [1.0]
		750		133±12 [1.0]
TA 1535	Test item	0	13±3	12±1
		15.63	13±2 [1.0]	
		31.25	13±2 [1.0]	9±2 [0.8]
		62.6	15±1 [1.2]	10±1 [0.8]
		125	15±2 [1.2]	10±3 [0.8]
		250	12±2 [0.9]	9±1 [0.8]
		500	10±1 [0.8]	10±3 [0.8]
		750		9±7 [0.8]
TA 98	Test item	0	29±5	35±3
		15.63	23±3 [0.8]	
		31.25	29±6 [1.0]	36±1 [1.0]
		62.5	22±3 [0.8]	34±3 [1.0]
		125	24±2 [0.8]	42±1 [1.2]
		250	24±1 [0.8]	34±3 [1.0]
		500	17±2 [0.6]	33±6 [0.9]
		1,000		37±5 [1.1]
TA 1537	Test item	0	5±1	18±3
		15.63	7±1 [1.4]	
		31.25	6±2 [1.2]	17±2 [0.9]
		62.5	6±2 [1.2]	17±3 [0.9]
		125	6±1 [1.2]	21±3 [1.2]
		250	6±0 [1.2]	16±2 [0.9]
		500	3±2 [0.6]	14±3 [0.8]
		750		7±2 [0.4]
E. coli WP2uvrA	Test item	0	32±4	35±7
		125	34±1 [1.1]	40±3 [1.1]
		250	31±3 [1.0]	35±1 [1.0]
		500	29±5 [0.9]	32±3 [0.9]
		1,000	28±4 [0.9]	33±3 [0.9]
		2,000	19±1 [0.6]	23±3 [0.7]
		2,500	15±2 [0.5]	18±0 [0.5]
Positive controls				
TA 100	SA	0.5	426±11 [3.6]	
TA 1535	SA	0.5	232±23 [17.8]	
TA 98	2-NF	2	443±40 [15.3]	
TA 1537	9-AA	50	467±42 [93.4]	
WP2uvrA	4NQO	0.5	13±16 [4.3]	
TA 100	BP	2		1,081±48 [8.1]
TA 1535	2-AA	2	11±2 [0.8]	110±35 [9.2]
TA 98	BP	2	24±3 [0.8]	457±11 [13.1]
TA 1537	BP	2		112±17 [6.2]
WP2uvrA	2-AA	4		185±13 [5.3]

[1)]Number of relevant colonies on treated plate/ Number of relevant colonies on vehicle control plate.
[2)]Test item: C. *indicum* flower oil.
SA, sodium azide; 2-NF, 2-nitrofluorene; 4NQO, 4-nitroquinoline 1-oxide; 2-AA, 2-aminoanthracene; BP, benzyl[a]pyrene.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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