SAFETY EVALUATIONS OF DAVALLIA FORMOSANA EXTRACTION

Jiunn-Wang Liao[1], Jhaol-Huei Wu[1], Wen-Chuan Lin[2]

[1]*Graduate Institute of Veterinary Pathobiology, National Chung Hsing University, Taichung, Taiwan*
[2]*School of Medicine and Graduate Institute of Basic Medical Science, China Medical University, Taichung, Taiwan*

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In this study, we evaluated the safety of 70% ethanolic extract of *Davallia formosana* (DFE). The LD_{50} value for oral administration of DFE was greater than 10 g/kg. Repeated dosing toxicity study was carried out by orally administering daily doses of 0.28, 0.83, and 2.5 g/kg of DFE to groups of male and female rats for 13 weeks. The mean body weights at weekly intervals were not affected by the administration of DFE during the 13-weeks dosing period. Scattered significant differences in WBC differential count, plasma alanine aminotranferase, alkaline phosphatase, sodium and chloride were found, but all these were still within the range of normal physiological reference or had no dose-correlation. A significant increase was seen in the absolute and relative weight of spleen treated with DFE 0.28 g/kg in female rats, but without dose-correlation. From the microscopic evaluations, there were no treatment-related pathological lesions in the tissue/organs. The no-observed adverse effect level is higher than 2.5 g/kg in rats. Reproductive test study indicated no-effect dose level of DFE was greater than 3.8 g/kg for dams, fetuses, and apparent teratogenicity of offspring. But, DFE markedly reduced the body weight of dams and offsprings in lactating period. The results of three genotoxicity studies, including Ames test, CHO-K1 chromosomal aberration test, and micronucleus test, demonstrated that DFE had no genotoxicity. Our findings provided scientific evidence for the safe use of DFE. But it should be contraindicated in lactating female.

Key words: *Davallia formosana*, acute toxicity, thirteen-week repeated dose toxicity, reproductive toxicity, genotoxicity

Introduction

According to Chinese medicine theories, herbs that are "Yang-tonifying" would tonify shen (kidney) and then strengthen the bones. Gusuibu is one of the "Yang-tonifying" herbs for strengthening bones. It has been claimed to have therapeutic effects on bone healing and osteoporosis. The rhizome of *Drynaria fortunei* (Polypodiaceae) is adopted in Chinese Pharmacopeia as the authentic origin of Gusuibu. In the herbal markets in Taiwan, the *D. fortunei* was substituted by *Davallia formosana* (Davalliaceae)[1]. One chemical study has shown that davallic acid is present in the rhizome of *D. formosana*[2]. However,

Correspondence to: Wen-Chuan Lin, School of medicine, China Medical University, No.91, Hsueh-Shih road, Taichung 404, Taiwan, Tel: 04-22053366 ext. 2229, Fax: 04-22053764, E-mail: wclin@mail.cmu.edu.tw

the pharmacological effect of davallic acid has not been reported. The pharmacological studies of *D. formosana* are limited. Recently, we observed that *D. formosana* extract could decrease ovariectomy-induced osteopenia in rats (unpublished data). Therefore, it may be a useful remedy for treating bone-resorption diseases such as osteoporosis.

There is a need to establish the safety levels of *D. formosana* as information on this is very little. The purposes of this study were to evaluate the safety levels of the ethanolic extract of *D. formosana* including genotoxicity study, acute and subacute toxicity studies in rats, and reproductive toxicity study in mice.

Materials and Methods

Preparation of the *Davallia formosana*

Rhizoma of *D. formosana* were purchased from a local market in Taichung, Taiwan. The plants were identified by the School of Chinese Medicine Resources, China Medical University, where voucher specimens have been deposited.

The roots of *D. formosana* were extracted twice with 70% ethanol, followed by evaporation of the solvent under reduced pressure at 50°C. The yield of the ethanolic extract (DFE) was 4%. The dosage of DFE used in the experiments was based on the dry weight of the extract. DFE was suspended in distilled water and administered orally to each rat in a volume of 1 ml/100 g body weight.

Animals

Wistar rats and ICR mice were obtained from BioLASCO Co. Ltd. (Taipei, Taiwan), and housed in an air-conditioned room at 21–24°C with 12 h of light. The rats and mice had free access to food pellets and water throughout the study period. All the animal experiments were conducted in accordance with the guidelines established by the Animal Care and Use Committee of China Medical University and were approved by this committee.

Acute toxicity study

Single dose toxicity of DFE by oral administration was evaluated in both male and female rats. Using 12 animals per dose level, the animals were equally divided in terms of sex. The 16 h fasted rats were administered DFE at doses of 5 and 10 g/kg. Deionized water was administered to the control group. Inspections for mortality and clinical signs were made frequently on the day of administration (day 0) and then twice a day up to the end of the 14-day post-treatment observation period. Body weights were recorded at day 0, 1, 7, and 14.

Thirteen-weeks repeated dose toxicity study

Rats, 5–6 weeks old, were employed. The dosage levels for this 13-weeks repeated dose toxicity study were derived from an acute toxicity study. In acute toxicity study, the maximum tolerated dose (MTD) of DFE was set at 10 g/kg in rats. Thus, the high-dose group was set at 1/4 (2.5 g/kg) of the established MTD. Likewise, the middle dose group was at 1/12 (0.83 g/kg) of the established MTD and the lowest-dose group was 1/36 (0.28 g/kg) of the established MTD. The control group received deionized water only. Both sexes received the same dose levels assigned to their group. Each dose group comprised 10 males and 10 females. Dosing was performed 7 days a week for a period of 13 weeks.

1) Observation

All animals were examined twice daily to determine if any were dead or were moribund. Animals were observed continuously for 30 min after dosing, for signs of ill health or obvious toxicity. Each rat was weighed prior to the treatment period and then at weekly intervals throughout the study period. The doses were adjusted weekly according to changes in body weight.

2) Laboratory investigations

The hematological examinations, blood chemistry tests, and urinalysis listed below were performed on all animals in all groups. In order to collect urine sample, on the day before the scheduled analysis the animals were kept in metabolism cages for about 16 hours. On the scheduled analysis day, blood samples were collected from the fasted rats subdued with CO_2 anesthesia. The determinations under each item were as follows. Hematology: erythrocyte count, hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), leukocyte count, WBC differential count, platelets, prothrombin time (PT), activated partial thromboplastin time (APTT).

Blood chemistry: plasma alanine aminotranferase (ALT), plasma aspirate aminotransferase (AST), gamma glutamyl transpeptidase (γ-GT), total protein, albumin, globulin, total bilirubin, glucose, blood urea nitrogen (BUN), creatinine, sodium, potassium, chloride, calcium, inorganic phosphorus.

Urinalysis: urine volume, pH, specific gravity, glucose, bilirubin, protein, ketones, and occult blood.

3) Organ weight

At the end of the dosing period, the animals were fasted overnight and then killed. Sacrificing was carried out by exsanguinations via the abdominal aorta under CO_2 anesthesia. Each animal was subjected to a detailed gross necropsy. The following organs were dissected free of contiguous tissue and weighed: adrenals, brain, heart, kidneys, liver, ovaries, pituitary, spleen, and testes.

4) Pathology

Samples of the following tissue were fixed in 10% neutral buffered formalin: adrenal, aorta, brain, eye, heart, intestine, kidney, liver, lung, ovary, parathyroid, prostate, sciatic nerve, seminal vesicle, spinal cord, stomach, spleen, testis, thymus, thyroid, urinary bladder, uterus, and vagina. Samples of all listed tissues from control and highest dose group were processed, sectioned, and stained with hematoxylin and eosin. All prepared sections were examined light microscopically.

Reproductive toxicity study

Three groups of 36 pregnant mice received by gavage either deionized water or 1.2, 3.8 g/kg DFE from days 7 of gestation through days 21 after parturition. During gestation, the doses were according to the body weight at day 7 of gestation. During lactating, the doses for F0 females were adjusted weekly according to changes in body weight.

Pregnant mice were allowed to deliver their offspring naturally. The offspring were examined with respect to the following parameters: total litter size, number of stillborn, and the presence of any obvious external congenital anomalies. Litter sizes were standardized on day 4 after birth. The size of each litter was adjusted to four males and females. This

was done by eliminating extra pups through random selection. Weighing and counting were continued on days 7, 14, and 21 after parturition. On day 21 of lactation all dams and their litters were sacrificed. The numbers of implant in dams were counted.

The reproductive parameters from this study were expressed in terms of indices, ratios and weights that considered all stages from conception to weanling[3]. These parameters were defined below:

Gestation index (%) = (number of delivering dam / number of pregnant dams) × 100

Birth index (%) = (number of pups / number of implants) × 100

4-day survival index (%) = (number of live pups on day 4/number of pups alive on day 0) × 100

Weanling index (%) = (number of pups alive on day 21/number of pups alive on day 4) × 100

Genotoxicity study

1) Ames test

The *Salmonella typhimurium* tester strains, including TA98 and TA100, were purchased from Food Industry Research and Development Institution (Hsinchu, Taiwan). In addition, TA102, TA1535, and TA1537 were purchased from Discovery Partners International (CA, USA). S-9 mix was obtained as a metabolic activation system consisting of the postmitochondrial fraction of the livers from rats (MA Bioservice, Rockville, Md, USA).

If the chemicals tested for Ames test is toxic to the tester strains, it could lead to an erroneous result. It is recommended that a preliminary toxic dosage range experiment be performed to determine an appropriate dose range for the Ames test. Bactericidal toxicity tests for DFE were performed according to the procedure described in our previous work[4]. Within the tested dose range (0.3–5.0 mg/plate), the DFE did not show toxicity toward the tester strains with or without the presence of S-9 mix.

Mutagenicity was assayed by the standard plate incorporation assay[5,6]. A mixture containing 0.1 ml of the DFE (0.3–5.0 mg/plate), 0.5 ml of S-9 mix or phosphate buffer, 0.2 ml of 0.5 mM histidine/biotin, and 0.1 ml of fresh culture of the tester strain (approximately 10^8 cells/ml) was added to a tube containing 2 ml of Top agar (contained 0.75% agar and 0.5% NaCl). The tube was then gently vortexed and poured onto glucose minimal agar plate (MA plate) contained 1.5% agar, 0.02% $MgSO_4 \cdot 7H_2O$, 0.2% citric acid, 1% K_2HPO_4, 0.35% $NaHNH_4PO_4 \cdot 4H_2O$, and 2% glucose. The compound was tested with and without S-9 mix and triplicate plates were poured for each dose of mutagen. Diagnostic mutagens including sodium azide, 4-nitroquinoline-N-oxide, mitomycin, 9-aminoacridine, and 2-antthramine were served as positive control chemicals. These five positive control drugs were purchased from Sigma-Aldrich (St Louis, MO, USA). After incubation at 37^0C for 48 h, the number of revertant colonies was scored. A tested substance was considered a mutagen if there was a twofold increase in the number of revertants compared with spontaneous revertants (negative control) or a dose-related increase in the number of revertant in one or more strains.

2) Chromosomal aberrations assay

CHO-K1 cells were purchased from Food Industry Research and Development Institution (Hsinchu, Taiwan). CHO-K1 cells were grown in Ham's medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100

μg/ml) at 37^0C in a 5% CO_2 atmosphere. DFE was first dissolved in dimethyl sulfoxide (DMSO) as a stock solution and then diluted with the medium. The viability of cells was determined by trypan blue exclusion test. The LD_{50} for DFE was 0.8 mg/ml.

Chromosomal aberrations assay was performed essentially according to the procedure described by Sayer *et al*.[7] All treatments were carried out both in the presence and absence of S-9. Cyclophosphamide and mitomycin C were used in the presence and absence of S-9 as positive control, respectively, and the solvent blank contained 50 mg/l DMSO. The cells were incubated with solvent, DFE (0.2, 0.4, 0.8 mg/ml) or cyclophosphamide (25 μg/ml) and mitomycin C (2.5 μg/ml) for 48 h. In all experiments, colcemid (0.1 μg/ml) was added to the Petri dish 24 h before harvest. The cells were dislodged with 0.05% trypsin and 0.53 mM EDTA in PBS-A, and transferred to slides. The slides were stained with 5% Giemsa solution for 4 min and a total number of 100 metaphases were counted for each dosage. All results were expressed in number of colonies per plate. The number of cells with broken chromosomes was recorded from which the rate of mutation was calculated:

Mutation rate (%) = (number of cells with broken chromosomes/100) × 100.

3) Micronuclei tests

Micronuclei tests were performed essentially according to the procedure described by De Boeck[8]. Five mice per group received a single treatment with H_2O, DFE or cyclophosphamide. H_2O and DFE (1.2, 3.8 g/kg) were given orally. Cyclophosphamide (0.1 g/kg), a positive control drug, was injected intraperitoneally. After 48 h treatments, blood samples were collected into tubes containing an anti-coagulant, through the retro-orbital sinus. These samples were fixed for flow cytometric analysis of reticulocytes (RETs) and micronucleus (MN)-RET frequencies according to procedures described in the Mouse MicroFlow® PLUS Micronucleus Analysis Kit (Litron laboratories, Rochester, NY). The MicroFlow® PLUS method for MN analysis by flow cytometry is based on the binding of fluorescein isothiocyanate (FITC)-labeled antibodies to the CD71 trasferrin receptor of immature RETs. Parallel RNA degradation in RETs and propidium iodide staining of DNA present as MN discriminates between different cell types: normochromatic erythrocytes (FITC-negative/PI negative), RETs (FITC-positive/PI-negative), MN-RETs (FITC-positive/PI-negative) (De Boeck *et al*., 2005). As an index for cytotoxicity, the percentage of RETs (%RETs) was recorded. The frequency of micronucleated RETs (%MN-RETs) was registered as a measure for induction of chromosome/genome alterations by the test compound.

Statistical analysis

Results were expressed as mean ± SD. All experimental data were analyzed using one-way analysis of variance with Dunnett's test. Values of $P < 0.05$ were considered statistically significant.

Results and Discussion

Acute toxicity study

At a single dose of 5, 10 g/kg, all the treated rats appeared normal during the observation period, and all the animals survived during the experimental period. No abnormal changes in the body weight of the treated rats were noted. The mean body weight

for control male group were 143.8 ± 4.0 g, 165.6 ± 5.9 g, 227.2 ± 8.9g, and 292.0 ± 6.7 g at day 0, 1, 7, and 14, respectively. The mean body weight for control female group were 136.4 ± 5.0 g, 157.0 ± 8.4 g, 187.4 ± 9.9 g, and 217.2 ± 13.8 g at day 0, 1, 7, and 14, respectively. There were no significant clinical signs and death of rats that were attributed to DFE treatment. Thus, the acute oral LD_{50} of DFE was greater than 10 g/kg in rats. The maximum tolerated dose (MTD) of DFE was 10 g/kg in rats.

Thirteen-week repeated dose toxicity study

No death and no clinical signs attributable to treatment with any dosage of the DFE were noted. There also was no effect on body weight changes (Table 1).

The following variations were observed in male rats given DFE at middle dose, without dose-correlation: decrease in the ratio of lymphocytes and an increase in the ratio of segmented neutrophils (Table 2). Decreased levels of ALT were observed in male rats at two higher doses (Table 3). A decreased level of sodium was also observed in male rats at highest dose (Table 3). An increased level of chloride was observed in female rats at middle dose (Table 3), without dose-correlation. Scattered significant values in ALT, sodium, and chloride were found, but all these values were still within the range of normal physiological reference[9]. No effects were noted in either sex at any dose of DFE, in either urinalysis studies (Table 4).

A significant increase was seen in the absolute and relative weight of spleen treated with DFE 0.28 g/kg in female rats, but without dose-correlation (Table 5). The macroscopic appearance of all the organs observed was found to be normal. Histological examination of the tissues showed no marked differences between the control group and highest dose group of DFE (Table 6).

Based on the results of this study, orally administered DFE for 13 weeks exerts the no-

Table 1. Body weight changes in rats orally treated with DFE for 90 days.

Weeks	Body weight (g)			
	Control	DFE 0.28 g/kg	DFE 0.83 g/kg	DFE 2.50 g/kg
Male				
0	176.1 ± 4.5	177.2 ± 5.9	178.6 ± 4.5	176.1 ± 6.3
1	251.5 ± 8.2	251.2 ± 10.3	254.6 ± 7.9	254.1 ± 7.9
4	362.3 ± 19.4	369.7 ± 23.0	376.5 ± 24.3	383.8 ± 23.5
8	453.0 ± 25.9	448.1 ± 28.5	471.7 ± 36.8	474.1 ± 24.5
13	494.5 ± 30.6	475.6 ± 49.4	497.0 ± 41.8	504.3 ± 39.3
Female				
0	148.7 ± 8.7	147.9 ± 4.1	146.4 ± 5.2	147.5 ± 11.6
1	183.3 ± 11.3	178.6 ± 4.5	178.7 ± 11.4	179.5 ± 15.8
4	246.8 ± 15.7	244.9 ± 7.5	251.4 ± 18.6	240.8 ± 18.8
8	287.1 ± 15.9	287.8 ± 11.8	295.9 ± 16.4	282.2 ± 26.9
13	304.7 ± 19.2	298.1 ± 16.2	311.8 ± 16.2	291.4 ± 25.7

All values are means ± S.D. (n = 10).

Table 2. Hematological findings in rats treated orally with DFE for 90 days.

Parameters		Control	DFE 0.28 g/kg	DFE 0.83 g/kg	DFE 2.50 g/kg
Erythrocytes	M	8.7 ± 0.4	8.6 ± 0.4	8.6 ± 0.2	8.6 ± 0.4
(10^6/μ L)	F	9.7 ± 1.3	10.3 ± 0.8	9.6 ± 0.9	9.2 ± 0.8
Hemoglobin	M	15.1 ± 0.5	14.9 ± 0.5	14.9 ± 0.5	15.1 ± 0.6
(g/dL)	F	18.4 ± 2.4	19.3 ± 2.3	18.5 ± 2.0	16.9 ± 1.5
Hematocrit	M	47.7 ± 1.7	47.1 ± 1.5	47.0 ± 1.4	47.6 ± 2.1
(%)	F	55.0 ± 7.3	57.1 ± 3.5	54.0 ± 4.8	52.0 ± 4.0
MCV	M	55.1 ± 1.8	54.6 ± 2.1	54.6 ± 1.7	55.0 ± 1.5
(μ^3)	F	56.6 ± 1.2	55.6 ± 1.3	56.5 ± 1.0	56.4 ± 1.2
MCH	M	17.5 ± 0.7	17.3 ± 0.7	17.3 ± 0.7	17.5 ± 0.7
(pg)	F	19.0 ± 0.9	18.7 ± 1.2	19.4 ± 1.1	18.4 ± 1.1
MCHC	M	31.7 ± 0.4	31.7 ± 0.4	31.8 ± 0.6	31.8 ± 0.7
(g/dL)	F	33.5 ± 1.5	33.7 ± 2.6	34.3 ± 2.0	32.5 ± 1.5
Platelets	M	1124.4 ± 1114.4	1114.4 ± 105.9	1208.0 ± 138.8	1161.7 ± 54.2
(10^3/μL)	F	729.3 ± 158.2	622.6 ± 206.9	864.8 ± 138.1	884.5 ± 167.4
Leukocytes	M	12.7 ± 2.3	14.8 ± 3.2	13.8 ± 3.4	14.6 ± 3.4
(10^3/μL)	F	8.4 ± 1.4	7.5 ± 1.4	8.7 ± 0.7	9.2 ± 1.8
Lymphocytes	M	79.9 ± 6.3	68.5 ± 11.5*	75.5 ± 6.4	75.1 ± 9.4
(%)	F	78.2 ± 4.0	74.9 ± 7.2	70.3 ± 11.0	73.4 ± 8.9
Seg. Neu	M	20.2 ± 6.3	31.5 ± 11.5*	24.5 ± 6.4	24.9 ± 9.4
(%)	F	21.9 ± 4.0	25.1 ± 7.2	29.7 ± 11.0	26.6 ± 8.9
PTT	M	12.0 ± 1.4	13.4 ± 3.9	15.4 ± 4.3	12.3 ± 4.5
(sec)	F	11.7 ± 3.1	12.8 ± 4.5	10.9 ± 1.2	11.1 ± 1.5
APTT	M	36.5 ± 8.2	36.3 ± 8.5	37.9 ± 7.2	36.9 ± 7.7
(sec)	F	45.5 ± 17.4	38.6 ± 10.6	44.9 ± 12.9	49.1 ± 22.7

All values are means ± S.D. *P < 0.05 compared with control group.
M: male; F: female; Seg. Neu: segmented neutrophil

observed adverse effect level (NOAEL) is 2.5 g/kg. The NOAEL serves as the basis for risk assessment calculations such as reference doses (RfDs). RfDs are estimates of a daily exposure to an agent that is assumed to be without adverse health impact in humans.

Typically, a safety factor would be used for reference dose calculations. In this study, safety factor of 100 was employed for extrapolation from a well-conducted animal bioassay (10-fold factor animal to human) and to account for human variability in response (10-fold factor human-to-human variability)[10]. RfD is derived by applying safety factor to NOAEL. Thus, the RfD of DFE for human is 0.025 g/kg, and the RfD for an adult man with 60 kg body weight is 1.5 g per day.

Reproductive toxicity study

Because small species require higher doses of drugs[11], the DFE dose of 0.83, 2.5 g/kg for rats were increased to 1.2, 3.8 g/kg for mice. DFE (1.2, 3.8 g/kg) did not affect the body weight gains in pregnant mice, but significantly decreased body weight gain in lactating mice (Table 7). The delivery

Table 3. Blood chemistry in rats treated orally with DFE for 90 days.

Parameters		Control	DFE 0.28 g/kg	DFE 0.83 g/kg	DFE 2.50 g/kg
AST	M	80.8 ± 10.4	72.2 ± 11.7	75.1 ± 12.2	73.7 ± 16.3
(U/L)	F	76.4 ± 18.0	81.9 ± 17.2	77.9 ± 14.6	75.2 ± 9.8
ALT	M	41.5 ± 9.1	33.9 ± 7.3	30.0 ± 4.0**	31.2 ± 7.7**
(U/L)	F	23.8 ± 2.4	27.3 ± 5.1	26.4 ± 4.6	23.6 ± 5.0
γ-GT	M	1.7 ± 1.4	1.1 ± 1.0	0.7 ± 0.6	1.6 ± 0.9
(IU/L)	F	0.8 ± 0.6	2.3 ± 2.3	1.9 ± 0.7	1.7 ± 1.7
T-protein	M	6.2 ± 0.2	6.0 ± 0.1	6.0 ± 0.1	6.0 ± 0.2
(g/dL)	F	6.2 ± 0.2	6.5 ± 0.2	6.4 ± 0.4	6.0 ± 0.3
Albumin	M	3.5 ± 0.1	3.4 ± 0.1	3.4 ± 0.2	3.4 ± 0.1
(g/dL)	F	3.9 ± 0.1	4.0 ± 0.2	3.8 ± 0.4	3.8 ± 0.2
Globulin	M	2.7 ± 0.2	2.6 ± 0.2	2.6 ± 0.1	2.6 ± 0.1
(g/dL)	F	2.3 ± 0.2	2.5 ± 0.2	2.7 ± 0.2	2.2 ± 0.3
T-Bili	M	0.23 ± 0.01	0.23 ± 0.01	0.24 ± 0.01	0.24 ± 0.01
(mg/dL)	F	0.15 ± 0.08	0.15 ± 0.02	0.17 ± 0.12	0.17 ± 0.03
Glucose	M	75.2 ± 4.5	77.8 ± 9.8	74.6 ± 10.4	78.2 ± 9.1
(mg/dL)	F	65.1 ± 14.4	67.3 ± 6.4	70.9 ± 11.6	58.3 ± 6.4
BUN	M	19.0 ± 2.2	19.1 ± 1.1	18.4 ± 2.1	19.0 ± 1.9
(mg/dL)	F	16.0 ± 2.1	17.1 ± 2.2	16.7 ± 2.4	16.0 ± 2.2
Crea	M	0.17 ± 0.02	0.16 ± 0.01	0.17 ± 0.01	0.17 ± 0.02
(mg/dL)	F	0.18 ± 0.01	0.18 ± 0.2	0.18 ± 0.02	0.19 ± 0.02
Na	M	144.0 ± 1.2	143.5 ± 1.7	143.7 ± 0.8	141.7 ± 0.9***
(mEq/dL)	F	142.4 ± 2.4	140.2 ± 2.9	141.2 ± 1.5	140.5 ± 1.6
K	M	3.6 ± 0.5	3.5 ± 0.3	3.5 ± 0.4	3.7 ± 0.5
(mEq/dL)	F	3.3 ± 0.5	3.0 ± 0.2	3.0 ± 0.2	3.0 ± 0.3
Cl	M	107.4 ± 2.6	106.4 ± 1.8	108.7 ± 1.4	107.2 ± 1.8
(mEq/dL)	F	105.6 ± 2.3	104.5 ± 2.7	108.2 ± 1.5*	107.5 ± 1.1
Ca	M	9.7 ± 0.2	9.7 ± 0.2	9.7 ± 0.2	9.6 ± 0.2
(mg/dL)	F	9.8 ± 0.4	9.8 ± 0.4	9.6 ± 0.3	9.5 ± 0.2
P	M	6.1 ± 0.8	5.7 ± 0.7	6.3 ± 0.4	6.0 ± 0.4
(mg/dL)	F	5.0 ± 1.2	4.8 ± 0.9	4.7 ± 0.7	4.7 ± 0.8

T-Bili: total bilirubin; Crea: creatinine

All values are means ± S.D. (n = 10). *P<0.05, ***P<0.001 compared with control group.
M: male; F: female.

Table 4. Urinary finding in rats treated orally with DFE for 90 days.

Drug	Doses (g/kg)	Volume (ml)	pH	Specific gravity
Male				
Control		57.2 ± 17.9	7.3 ± 0.5	1.005 ± 0.004
DFE	0.28	48.6 ± 21.1	7.2 ± 0.2	1.008 ± 0.008
	0.83	51.4 ± 18.5	7.1 ± 0.2	1.006 ± 0.004
	2.50	41.2 ± 19.7	7.2 ± 0.2	1.007 ± 0.006
Female				
Control		31.4 ± 14.5	7.1 ± 0.6	1.008 ± 0.008
DFE	0.28	48.6 ± 21.1	7.2 ± 0.3	1.010 ± 0.008
	0.83	35.8 ± 13.3	7.3 ± 0.5	1.005 ± 0.006
	2.50	46.8 ± 17.8	7.0 ± 0.0	1.006 ± 0.006

All values are means ± S.D. (n = 10).

Table 4. (continued)

Drug	Doses	Urobilinogen (mg / dL)					Protein (mg / dL)					
	(g/kg)	0.2	1	2	4	8	−	15	30	100	300	2000
Male rats												
Control		10	0	0	0	0	9	1	0	0	0	0
DFE	0.28	10	0	0	0	0	8	2	0	0	0	0
	0.83	10	0	0	0	0	9	1	0	0	0	0
	2.50	10	0	0	0	0	9	1	0	0	0	0
Female rats												
Control		10	0	0	0	0	10	0	0	0	0	0
DFE	0.28	10	0	0	0	0	10	0	0	0	0	0
	0.83	10	0	0	0	0	10	0	0	0	0	0
	2.50	10	0	0	0	0	9	1	0	0	0	0

Table 4. (continued)

Drug	Doses	Bilirubin (mg / dL)				Glucose (mg / dL)					
	(g/kg)	−	1	2	4	-	100	250	500	1000	>2000
Male rats											
Control		7	3	0	0	10	0	0	0	0	0
DFE	0.28	10	0	0	0	10	0	0	0	0	0
	0.83	10	0	0	0	10	0	0	0	0	0
	2.50	10	0	0	0	10	0	0	0	0	0
Female rats											
Control		9	1	0	0	10	0	0	0	0	0
DFE	0.28	9	1	0	0	10	0	0	0	0	0
	0.83	10	0	0	0	10	0	0	0	0	0
	2.50	10	0	0	0	10	0	0	0	0	0

Table 4. (continued)

Drug	Doses	Occult blood					Ketones (mg / dL)					
	(g/kg)	−	±	+	++	+++	−	5	15	40	80	160
Male rats												
Control		9	1	0	0	0	3	7	0	0	0	0
DFE	0.28	8	2	0	0	0	3	7	0	0	0	0
	0.83	9	1	0	0	0	2	8	0	0	0	0
	2.50	8	2	0	0	0	3	7	0	0	0	0
Female rats												
Control		10	0	0	0	0	4	6	0	0	0	0
DFE	0.28	10	0	0	0	0	1	9	0	0	0	0
	0.83	10	0	0	0	0	4	6	0	0	0	0
	2.50	10	0	0	0	0	4	6	0	0	0	0

Table 5. Absolute and relative organ weights in rats treated orally with DEF or 90 days.

Item		Control	DFE0.28g/kg	DFE0.83g/kg	DFE2.50g/kg
Brain(g)	M	2.00 ± 0.11	1.97 ± 0.09	2.00 ± 0.04	2.00 ± 0.04
(g/100 g b.w.)		0.41 ± 0.04	0.42 ± 0.04	0.41 ± 0.04	0.40 ± 0.04
	F	1.86 ± 0.11	1.91 ± 0.08	1.85 ± 0.14	1.93 ± 0.14
		(0.63 ± 0.05)	(0.66 ± 0.05)	(0.62 ± 0.06)	(0.68 ± 0.08)
Pituitary (mg)	M	10.9 ± 1.9	11.4 ± 1.1	12.1 ± 1.8	12.0 ± 1.3
(mg/100 g b.w.)		(2.3 ± 0.5)	(2.4 ± 0.4)	(2.4 ± 0.4)	(2.4 ± 0.2)
	F	13.4 ± 1.8	15.1 ± 1.7	15.6 ± 2.3	14.2 ± 2.2
		(4.5 ± 0.5)	(5.2 ± 0.7)	(5.2 ± 0.8)	(5.0 ± 0.9)
Heart (g)	M	1.29 ± 0.08	1.24 ± 0.09	1.30 ± 0.10	1.30 ± 0.09
(g/100 g b.w.)		(0.27 ± 0.02)	(0.26 ± 0.02)	(0.26 ± 0.01)	(0.26 ± 0.01)
	F	0.88 ± 0.06	0.90 ± 0.05	0.94 ± 0.06	0.84 ± 0.06
		(0.30 ± 0.02)	(0.31 ± 0.02)	(0.31 ± 0.03)	(0.29 ± 0.02)
Liver (g)	M	11.9 ± 0.9	11.9 ± 1.7	12.4 ± 1.2	12.3 ± 1.6
(g/100 g b.w.)		(2.4 ± 0.1)	(2.5 ± 0.2)	(2.5 ± 0.2)	(2.5 ± 0.2)
	F	7.0 ± 0.6	7.2 ± 0.3	7.6 ± 0.6	6.8 ± 0.6
		(2.4 ± 0.1)	(2.5 ± 0.2)	(2.5 ± 0.2)	(2.4 ± 0.2)
Spleen (g)	M	0.98 ± 0.07	0.98 ± 0.14	1.03 ± 0.13	1.00 ± 0.12
(g/100 g b.w.)		(0.20 ± 0.02)	(0.21 ± 0.02)	(0.21 ± 0.03)	(0.20 ± 0.02)
	F	0.66 ± 0.06	0.77 ± 0.08**	0.70 ± 0.07	0.68 ± 0.08
		(0.22 ± 0.02)	(0.26 ± 0.03**)	(0.23 ± 0.02)	(0.24 ± 0.02)
Kidney (L; g)	M	1.46 ± 0.1	11.39 ± 0.15	1.48 ± 0.14	1.49 ± 0.22
(g/100 g b.w.)		(0.30 ± 0.03)	(0.29 ± 0.02)	(0.30 ± 0.04)	(0.30 ± 0.03)
	F	0.85 ± 0.08	0.88 ± 0.05	0.88 ± 0.07	0.86 ± 0.08
		(0.29 ± 0.02)	(0.30 ± 0.01)	(0.29 ± 0.02)	(0.30 ± 0.03)
Kidney (R; g)	M	1.50 ± 0.12	1.40 ± 0.16	1.53 ± 0.17	1.51 ± 0.20
(g/100 g b.w.)		(0.31 ± 0.02)	(0.29 ± 0.02)	(0.31 ± 0.04)	(0.30 ± 0.02)
	F	0.83 ± 0.09	0.86 ± 0.07	0.88 ± 0.07	0.84 ± 0.09
		(0.28 ± 0.02)	(0.30 ± 0.03)	(0.29 ± 0.02)	(0.29 ± 0.02)
Adrenal (L; mg)	M	30.1 ± 3.0	31.4 ± 5.5	32.2 ± 2.5	33.7 ± 3.5
(mg/100 g b.w.)		(6.2 ± 0.7)	(6.6 ± 1.1)	(6.5 ± 1.0)	(6.8 ± 0.7)
	F	43.1 ± 4.7	44.6 ± 2.5	45.2 ± 9.6	44.9 ± 8.2
		(14.6 ± 1.8)	(15.3 ± 1.4)	(14.9 ± 2.9)	(15.7 ± 3.1)
Adrenal (R; mg)	M	29.7 ± 4.7	29.4 ± 4.5	32.2 ± 2.5	31.5 ± 3.8
(mg/100 g b.w.)		(6.1 ± 1.0)	(6.2 ± 1.2)	(6.1 ± 0.9)	(6.4 ± 0.9)
	F	40.9 ± 4.4	43.3 ± 3.7	43.2 ± 6.4	43.3 ± 7.1
		(13.9 ± 1.6)	(14.8 ± 1.5)	(14.3 ± 2.3)	(15.1 ± 2.5)
Testis (L; g)	M	1.88 ± 0.24	1.86 ± 0.12	1.67 ± 0.36	1.75 ± 0.24
(g/100 g b.w.)		(0.39 ± 0.06)	(0.39 ± 0.04)	(0.34 ± 0.08)	(0.35 ± 0.05)
(R; g)	M	1.88 ± 0.23	1.86 ± 0.12	1.64 ± 0.35	1.77 ± 0.23
(g/100 g b.w.)		(0.39 ± 0.06)	(0.40 ± 0.04)	(0.34 ± 0.08)	(0.36 ± 0.06)
Ovary (L; mg)	F	74.8 ± 13.1	77.2 ± 13.6	81.4 ± 12.0	78.0 ± 9.6
(mg/100 g b.w.)		(25.3 ± 4.7)	(26.4 ± 4.5)	(27.0 ± 4.6)	(27.3 ± 4.0)
(R; mg)	F	77.8 ± 14.0	80.8 ± 19.7	78.4 ± 13.8	81.4 ± 10.5
(mg/100 g b.w.)		(26.3 ± 4.9)	(27.7 ± 6.6)	(26.0 ± 4.8)	(28.5 ± 4.7)

All values are means ± S.D. (n=10).

( ): relative weight; b.w.: body weight; M: male; F: female; L: left; R: right

Table 6. Pathological findings in rats treated orally with DFE (2.50 g/kg) for 90 days.

Organ	Lesions	Male		Female	
		H_2O	DFE 2.50g/kg	H_2O	DFE 2.50g/kg
Adrenal		0/10[1]	0/10	0/10	0/10
Aorta		0/10	0/10	0/10	0/10
Brain		0/10	0/10	0/10	0/10
eye		0/10	0/10	0/10	0/10
Heart	Infiltration, mononuclear cell, focal	0/10	0/10	1/10	0/10
	Pericarditis, focal	0/10	0/10	1/10	0/10
Intestine		1/10	0/10	0/10	0/10
Kidney	Cyst, focal, minimal	1/10	2/10	0/10	0/10
	Hemorrhage, focal, slight	0/10	0/10	0/10	0/10
	Hydronephrosis, severe	1/10	0/10	0/10	0/10
	Regeneration, tubule, focal, minimal	2/10	1/10	0/10	0/10
	Necrotic cells accumulation, pelvis	0/10	0/10	1/10	0/10
Liver		0/10	0/10	0/10	0/10
Lung	Necrosis, focal, slight	1/10	0/10	0/10	0/10
Ovary				0/10	0/10
Parathyroid				0/10	0/10
Prostate	Infiltration, monouclear cell, minimal to slight	2/10	2/10		
Sciatic nerve		0/10	0/10	0/10	0/10
Seminal vesicle		0/10	0/10		
Spinal cord		0/10	0/10	0/10	0/10
Stomach		0/10	0/10	0/10	0/10
Spleen		0/10	0/10	0/10	0/10
Testis		0/10	0/10		
Thymus		0/10	0/10	0/10	0/10
Thyroid		0/8	0/10	0/10	0/10
Urinary bladder		0/10	0/10	0/10	0/10
Uterus	hydrouterus			1/10	3/10
vagina				0/10	0/10

[1] Incidence: no. of affected rats/ no. of rats were examined.

status in F0 dams, as well as the number of stillborn pups, number of live pups, and birth index were not affected by DFE (Table 8). DFE did not affect the four-day survival index and weanling index in F1 mice (Table 8). Body weight gains were decreased in F1 mice (Table 9). Apparent teratogenicity of DFE could not be detected. These results indicate that the no-observed adverse effect dose level of DFE is 3.8 g/kg for dams, fetuses, and for the apparent teratogenicity of offsprings. As the body weight of dams and offsprings was markedly decreased in the lactating period, DFE was contraindicated in a lactating female.

Table 7. Body weight changes in pregnant and lactating F0 mice treated with DFE orally during the fetal organogenesis and lactation periods.

	Body weight (g)		
	Control (n =12)	DFE 1.2 g/kg (n = 12)	DFE 3.8 g/kg (n = 12)
Gestation 7 day	27.9 ± 2.0	27.4 ± 3.0	27.4 ± 2.0
10	30.8 ± 2.1	30.4 ± 2.6	30.5 ± 2.5
13	36.4 ± 3.3	34.1 ± 3.1	35.9 ± 2.1
16	45.1 ± 4.9	41.6 ± 4.3	44.4 ± 2.5
Postpartum 0 day	33.1 ± 2.5	30.4 ± 3.4	32.1 ± 2.2
7	37.9 ± 2.5	32.1 ± 3.8**	33.6 ± 2.9**
14	39.6 ± 3.4	33.3 ± 3.7**	34.8 ± 2.8**
21	35.4 ± 3.1	31.2 ± 4.3*	31.6 ± 2.2*

All values are means ± S.D. *$P < 0.05$, **$P < 0.01$ compared with control group at the same period.

Table 8. Delivery status in F_0 mice treated with DFE orally during the fetal organogenesis and lactation periods, and postnatal viability in F_1 offspring.

Groups		Control	DFE 1.2 g/kg	DFE 3.8 g/kg
No. of pregent dams		12	12	12
No. of dams with live offspring		12	12	12
Gestation index (%)		100	100	100
Gestation period (day)	Mean ± SD	18.2 ± 0.4	18.1 ± 0.3	18.2 ± 0.5
No. of implants	Total	136	131	138
	Mean ± SD	11.3 ± 2.5	10.9 ± 2.5	11.5 ± 0.9
No. of stillborn pups (%)		0	0	0
No. of offspring born alive	Total	133	127	128
	Mean ± SD	11.1 ± 2.5	10.6 ± 1.9	10.7 ± 0.9
Birth index (%)		99.2	96.9	92.7
No. of offspring alive on postnatal day 4		132	125	121
4-day survival index (%)		99.2	98.4	94.5
No. of offspring alive immediately after culling		96	96	96
No. of live wealings		94	91	91
Weanling index (%)		97.9	94.8	94.8
External malformations:		0	0	0
No. of offspring with External malformations (%)		0	0	0

Genotoxicity study

1) Ames tests

The Ames test is a widely accepted method to identify various chemicals and drugs that can cause gene mutations[6], and it has a high predictive value for *in vivo* carcinogenicity. TA98 and TA1537 have

Table 9. Body weight changes in male and female F1 mice delivered from F0 dams treated with DFE orally during the fetal organogenesis and lactation periods.

	Drugs Dose	Body weight (g)			No. of
	(g/kg)	Day 7	Day 14	Day 21	mice
Male					
Control		5.5 ± 0.5	9.3 ± 0.5	15.7 ± 0.9	48
DFE	1.2	3.9 ± 0.8***	6.5 ± 1.3***	11.5 ± 2.4***	45
	3.8	4.4 ± 1.0***	7.5 ± 1.2***	12.5 ± 3.0***	45
Female					
Control		5.4 ± 0.4	9.1 ± 0.7	14.9 ± 1.1	46
DFE	1.2	3.9 ± 0.7***	7.0 ± 1.3***	10.8 ± 2.3***	46
	3.8	4.4 ± 0.9***	7.2 ± 1.7***	11.9 ± 3.3***	46

All values are means ± S.D. ***$P < 0.001$ compared with control group at the same period.

been used to detect mutagens causing DNA base frameshifts (addition or deletions of one or more bases), and TA100, TA102, and TA1535 to detect mutagens that cause DNA-base-pair substitution (single base changes)[6].

As some compounds can become mutagens during metabolism, yet bacteria, such as, strains of *S. typhimurium* used in this study do not have a cytochrome-base P450 metabolic oxidation system, the S9 fraction (activation enzyme) of the rat liver microsome is often added to the Petri dish together with a test substance and tester strains[6].

In the Ames test (Table 10 and 11), the revertant numbers induced by DFE (0.3 – 5.0 mg/plate) for all tester strains were close to that of the negative control (spontaneous revertant, without DFE) and much lower than that of the positive control (with diagnostic mutagens). The result of the Ames test demonstrated that DFE had no mutagenicity effect under the tested dose range.

2) Chromosomal aberrations test

Table 10. Revertant changes of DFE in *Salmonella* TA102, TA1535, TA1537, TA98 and TA100 mutagenicity test (without S9).

Treatments	Conc.	Number of revertants (colony/plate)				
	(mg/plate)	TA98	TA100	TA102	TA1535	TA1537
Control		26.3 ± 2.1	198.3 ± 4.9	275.7 ± 8.6	13.7 ± 2.5	7.0 ± 0.8
PC[1]		2264.0 ± 36.4*	1988.3 ± 96.2*	659.0 ± 15.1*	336.0 ± 31.8*	989.0 ± 126.0*
DFE	5.0	29.9 ± 1.8	217.8 ± 11.3	245.7 ± 0.5	11.0 ± 1.6	7.7 ± 1.2
	2.5	29.7 ± 1.7	215.7 ± 10.2	257.0 ± 18.8	11.7 ± 3.9	7.7 ± 2.1
	1.2	27.0 ± 2.4	208.0 ± 3.6	257.7 ± 5.2	8.3 ± 1.2	9.0 ± 0.8
	0.6	31.7 ± 3.1	202.3 ± 12.7	250.7 ± 5.2	11.3 ± 2.1	9.0 ± 2.2
	0.3	30.3 ± 2.5	186.7 ± 4.0	243.0 ± 9.4	11.0 ± 0.8	8.0 ± 0.8

[1]Positive reagents were 1 μg/plate 4-nitroquinoline-N-oxide for TA98, 5 μg/plate sodium azide for TA 100 and TA1537, 0.5 μg/plate mitomycin C for TA102, 50 μg/plate 9-aminoacridine for TA1535.
Data was presented as mean ± SD. *Significant difference of colonies more than two folds of control and treated groups.
Conc.: concentration

Table 11. Revertant changes of DFE in *Salmonella* TA102, TA1535, TA1537, TA98 and TA100 mutagenicity test (with S9).

Treatments	Conc.	Number of revertants (colony/plate)				
	(mg/plate)	TA98	TA100	TA102	TA1535	TA1537
Control		40.7 ± 1.7	159.7 ± 3.3	199.7 ± 4.5	9.7 ± 0.5	6.0 ± 0.0
PC[1]		2597.3 ± 156.0*	2818.3 ± 122.0*	820.0 ± 18.8*	280.0 ± 7.7*	121.0 ± 19.2*
DFE	5.0	37.6 ± 3.0	180.6 ± 11.2	187.7 ± 5.7	12.3 ± 0.9	9.0 ± 1.6
	2.5	34.7 ± 3.7	164.0 ± 10.6	187.7 ± 3.4	11.0 ± 0.9	10.0 ± 1.6
	1.2	36.3 ± 2.1	182. ± 11.7	187.7 ± 13.6	14.7 ± 3.3	8.3 ± 1.2
	0.6	36.0 ± 2.9	178.7 ± 11.5	194.7 ± 7.9	11.7 ± 1.9	8.3 ± 0.9
	0.3	35.7 ± 2.9	174.3 ± 7.6	203.3 ± 7.4	11.0 ± 0.0	12.3 ± 1.7

[1] Positive reagent was 5 μg/plate 2-aminoanthracene for all *Salmonella* strains.
Data was presented as mean±SD. *Significant difference of colonies more than two folds of control and treated groups.
Conc.: concentration

The CHO-K1 chromosomal aberration test has also been a widely used test for the clastogenicity of various chemicals. When the known castogenic compounds (mitomycin C and cyclophosphamide) were added to the CHO-K1 cells in the absence or presence of S9, respectively, there was a threefold or fourfold increase in the ratio of castogenicity compared to the solvent group. The rate of clastogenicity for all three doses of DFE was the same as observed in the solvent control (Table 12). Thus, DFE did not show significant clastogenicity at all dosages, both in the absence and presence of S9 in the CHO-K1 cells.

Table 12. Chromosomal aberration test with mammalian cell in cultured CHO-K1 cells of DFE.

Group	Concentration	Frequency of chromosomal aberration (%)[1]	
		-S9	+S9
Control		6.0 ± 1.0	4.3 ± 1.5
Mitomycin C	2.5 μg/ml	22.3 ± 1.5*	-
Cyclophosphamide	25 μg/ml	–	27.3 ± 3.1*
DFE	0.2 mg/ml	4.3 ± 0.6	5.0 ± 1.0
	0.4 mg/ml	4.0 ± 1.0	5.7 ± 1.2
	0.8 mg/ml	4.0 ± 1.0	5.7 ± 0.6

[1] Two slides were prepared and stained with 5% Giemsa solution for 5 min and a total number of 100 metaphases were counted for each dosage. All results were expressed in number of aberration per plate.
[2] The number of cells with damage chromosomes was recorded from which the rate of mutation was calculated. Aberration rate (%) = (number of cells with damage chromosomes/100) × 100.
– Not done.
* Significant difference between the control and treated groups at $P < 0.05$.

3) Micronucleus test

The MN test is widely used in research and regulatory safety assessment to evaluate the potential of chemical and physical agents that cause chromosomal damage *in vivo*. After a single intraperitoneal injection, cyclophosphamide (0.1 g/kg), a positive control compound, caused an increase in %MN-RETs. A significant reduction of %RETs was also observed. DFE (1.2, 3.8 g/kg), after a single oral administration, did not have any effect on both %MN-RETs and %RETs (Table 13). These results demonstrated that DFE had no chromosomal damage *in vivo*.

Table 13. The frequency of micronucleated reticulocytes in peripheral blood in mice induced by DFE and cyclophosphamide (CP).

Treatment	Dose (g/kg)	No. of RE	% MN-RETs	% RETs
Control		1034	3.2 ± 1.9	7.2 ± 0.9
DFE	1.2	1023	4.4 ± 1.5	6.0 ± 1.7
	3.6	1017	4.8 ± 0.9	6.0 ± 1.4
cyclophosphamide	0.1[a]	1006	11.2 ± 2.3**	3.4 ± 0.6**

All values are means ± SD (n = 5). ***P*< 0.01, ****P*<0.001 compared with control group.
a: i.p.
RE: reticulocytes; MN: micronucleus

The Ames test, CHO-K1 chromosomal aberration test, and micronucleus test are the three most commonly used methods for the evaluation of genetic mutation and chromosome damages caused by mutagens and clastogenic compounds. DFE showed negative results in these tests, suggesting that it had no genotoxicity.

In conclusion, The LD_{50} of DFE was greater than 10 g/kg in rats. The no-observed-adverse-effect levels of DFE were greater than 2.5 g/kg in the 13-week repeated dose toxicity study in rats. The no-effect dose level of DFE was 3.8 g/kg for dams, fetuses, and offspring. The results of the Ames test, chromosomal aberrations test and micronucleus test showed that DFE had no genotoxicity. According to these results, it is potentially safe for DFE to be used. However, the body weight of dams and offsprings were markedly decreased in the lactating period, DFE is contraindicated in a lactating female.

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台灣骨碎補粗萃物安全性評估

廖俊旺[1]、吳昭慧[1]、林文川[2]

[1] 國立中興大學獸醫病理生物學研究所，台中，台灣

[2] 中國醫藥大學醫學系暨基礎醫學研究所，台中，台灣

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本試驗進行台灣骨碎補（*Davallia formosana*；DFE）70%酒精萃取物安全性評估。急性毒性試驗結果顯示，DFE酒精萃取物對大鼠口服一半致死劑量大於10 g/kg。90天餵食毒性試驗，雌、雄大鼠每天分別經口投與DFE 0.28、0.83及2.5 g/kg連續90天，結果顯示DFE投與13週期間，對大鼠體重並無影響。DFE對於白血球分類計數、麩氨酸丙氨基轉氨、鹼性磷酸酶、鈉和氯雖有統計上的差異，但仍在正常生理值範圍內或無劑量反應關係。DFE (0.28 g/kg) 使雌鼠脾臟的絕對和相對重量增加，但無劑量反應關係。組織病理檢查沒有發現因DFE的處理引起的組織或器官病理變化。本實驗確立DFE 90天餵食毒性試驗的無明顯效應劑量大於2.5 g/kg。

生殖試驗顯示對於母鼠、胎鼠及仔鼠外觀畸型的無明顯效應劑量大於3.8 g/kg。但 DFE明顯減少仔鼠及哺乳母鼠體重的增加。基因毒性試驗包含Ames測試、CHO-K1細胞株染色體異常試驗及微核試驗，三種測試的結果皆顯示DFE並無基因毒性。這些試驗結果提供了DFE安全使用的證據，但禁用於於哺乳婦女。

關鍵字：台灣骨碎補、急性毒性、13週重複劑量毒性、生殖毒性、基因毒性

聯絡人 ： 林文川，中國醫藥大學醫學系藥理學科，404 台中市學士路 91 號，電話 ：04-22053366 分機 2229，傳真 ：04-22053764，電子郵件信箱 ： wclin@mail.cmu.edu.tw