Original Article

In vitro and *in vivo* toxicological assessments of *Antrodia cinnamomea* health food product (Leader *Antrodia cinnamomea* Capsule)

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ABSTRACT — A unique medicinal mushroom *Antrodia cinnamomea* has been used for centuries to treat various human diseases. Recent studies revealed its potent pharmacological effects including anti-cancer, anti-inflammation, anti-oxidant, anti-diabetic, neuroprotection and hepatoprotection. The present study was aimed to investigate the toxicological effects of *A. cinnamomea* health food product "Leader *Antrodia cinnamomea* Capsule (LACC)" by measuring its genotoxic, oral toxic and teratotoxic effects *in vitro* and *in vivo*. Result of Ames test with 5 strains of *Salmonella typhimurium* shows no sign of increase in the numbers of revertant colonies upon exposure to LACC. Treatment of Chinese Hamster Ovary cells (CHO-K1) with LACC did not affect increase in the frequency of chromosomal aberration *in vitro*. In addition, treatment with LACC did not affect the proportions of immature to total erythrocytes and the number of micronuclei in the immature erythrocytes of ICR mice. Moreover, acute oral toxicity (14-days single-dose) or prolonged oral toxicity (28- and 90-days repeated oral dose) tests with rats showed that there were no observable adverse effects were found. Furthermore, teratological studies with LACC (500-2500 mg/kg/day) for 20 days, shows no observable segment II reproductive and developmental toxic evidences in pregnant SD rats and their fetus. These toxicological assessments strongly support the safety efficacy of LACC for human consumption.

Key words: *Antrodia cinnamome*, Leader *Antrodia cinnamomea* Capsule, genotoxicity, mutagenicity, teratotoxicity

INTRODUCTION

Antrodia cinnamomea (syn. *Antrodia camphorata* or *Taiwanofungus camphoratus*) is a unique medicinal mushroom endemic to Taiwan. The fruiting bodies and mycelia of *A. cinnamomea* (AC) are ground into dry powder or stewed with other herbal drugs for oral uptake for the treatment of liver diseases, twisted tendons, muscle damage, terrified mental state, influenza, cold, headache diarrhea, abdominal pain, food and drug intoxication, skin diseases, hypertension, and tumorigenic diseases (Ao *et al*., 2009; Geethangili and Tzeng, 2011). AC is now believed one of the most liver protecting natural medicinal ingredients in Taiwan. Accumulating evidences from scientific studies indicate that the pharmacological applications of this mushroom goes beyond its traditional usage (Lu *et al*., 2013). Recent studies have shown that AC possesses various pharmacological properties including anti-oxidant, anti-inflammatory, anti-cancer, anti-metastatic, anti-hyperlipemic, anti-diabetic, hepato-protective, neuro-protective, cardio-protective and immunomodulatory effects (Geethangili and Tzeng, 2011; Liu *et al*., 2012; Lu *et al*., 2013; Yue *et al*., 2012) . The pharmacological efficacy of AC have been accredit-

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ed by high content of bioactive components such as terpinoids, benzenoids, benzoquinone derivatives, maleic/succinic acid derivatives, lignans, polysaccharides, sterols, nucleotides and fatty acids (Lu *et al*., 2013; Yue *et al*., 2012). Predominant bioactive components such as triterpinoids were found in fruiting bodies of AC compared with mycelia (Geethangili and Tzeng, 2011). Therefore, demand for the fruiting bodies of AC has far exceeded the supply. However, to compensate the demand, researchers developed other cultivation methods for the mass production such as wood or solid-state cultivation and liquid or submerged cultivation.

Considering the potential health benefits, the fruiting bodies and mycelia of AC is widely used as a health food supplement in Taiwan and available in the form of tablet, capsules and tonic. Therefore, the safety issues of AC is the primary concern. A previous study shows that AC products have low oral toxicity, with an oral medial lethal dose (LD_{50}) > 1.5 g/kg body weight in CD mice (Chang *et al*., 2013). Although hundreds of AC products are sold, only six products have been awarded a "National Health Food" certification by Taiwan's Department of Health. For this study, we selected one certificated AC product, Leader *Antrodia cinnamomea* Capsule (LACC), as study material. To assess the safety of LACC, we examined genotoxicity, oral toxicity and teratogenicity *in vitro* and *in vivo*.

MATERIALS AND METHODS

Test substance

The health food supplement LACC was manufactured by Taiwan Leader Biotech Corp, Taipei City, Taiwan. LACC is a brown powder of mycelium of *Antrodia cinnamomea* from high-efficient solid-state cultivation.

Chemicals

Ham's F-12 medium, heat inactivated fetal bovine serum (FBS), L-Glutamine, Penicillin and Streptomycin were obtained from Biological Industries Israel Beit-Haemek Ltd., Israel. Mitomycin C, benzo(a)pyrene, 2- Nitrofluorene, sodium azide, 9-aminoanthracene, histidine, biotin, Giemsa stain, acridine orange, cyclophosphamide and Methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Sigma- Aldrich, St. Louis, MO, USA. Colcemid was obtained from Life Technologies, Carlsbad, CA, USA.

Bacterial reverse mutation test (Ames test)

The histidine-requiring *Salmonella typhimurium* strains TA97a, TA98, TA100, TA102 and TA1535 were obtained from Molecular Toxicology Inc., Bonne, NC, USA. The genotypes of the bacterial strains were confirmed by histidine mutation, *rfa* mutation, Δ*uvrB* repair and ampicillin and tetracycline resistance before the assay. Prior to the assay, a dose range finding test was performed with five different doses of LACC (0.313-5 mg/plate) in the TA98 strain according to standard operating procedures (SOP): M5051-03 protocol. A plate incorporation assay was employed and performed to detect reverse mutation in bacterial strains (Ames *et al*., 1975). LACC was dissolved in sterile water at a concentration of 5 mg/mL, and then centrifuged at 1200 × *g* for 5 min. The supernatant was filtered through a 0.22 μm filter and used for subsequent studies. Briefly, 0.05 mL of aqueous solution of LACC (0,313, 0.625, 1.25, 2.5 and 5 mg/plate) was mixed with 0.1 mL of overnight culture of *Salmonella typhimurium* strains (2×10^9 cells/mL) in either 0.5 mL of 0.2 M phosphate buffer (without S9 metabolic activation group) or 0.5 mL S9 mixture (S9 metabolic activation group). The composition of S9 mixture was 5% v/v Aroclor-1254 induced Sprague Dawley (SD) rat liver S9 (Molecular Toxicology Inc., Bonne, NC, USA) and 0.15 M KCl. The mixture was subsequently mixed with 2 mL of molten top agar solution with 0.5 mM histidine/biotin. The cultures were incubated at 50 ± 1ºC before transferring to minimal glucose agar plates. The solidified agar plates were inverted and incubated at 37 ± 1ºC for 48-72 hr. Following incubation, the revertant colonies were counted. In all experiments, vehicle control (sterile water) and positive controls (Table S1) were also tested under similar conditions. Triplicate experiments were performed throughout the study.

Mammalian chromosomal aberration test

For the *in vitro* chromosomal aberration test was performed with OECD protocols. Chinese hamster ovary cell line (CHO-K1) was obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) were cultured in Ham F-12 medium supplemented with 10% heat inactivated FBS, 2 mM L-Glutamine and 100 U/L Penicillin and Streptomycin in a humidified atmosphere containing 5% CO_2 at 37 ± 1ºC. LACC was dissolved in sterile water at a concentration of 5 mg/mL, and then centrifuged at 1200 × *g* for 5 min. The supernatant was filtered through a 0.22 μm filter and used for subsequent studies. LACC 5 mg/mL was used as the highest dose and 0.313 mg/mL as lowest concentration for cytotoxicity assay. Plain culture media served as the negative control, and the positive controls were 0.5 μg/mL mitomycin C for the group without S9 and 25 μg/mL benzo(a) pyrene for the S9 group.

CHO-K1 cells at a density of 4×10^5 cells/well were seeded in 6-well culture plates and incubated for 24 hr before treatment. LACC and controls were administered in three conditions. For short-term treatment, test samples were exposed for 3 hr followed by a recovery period of 6 hr. For metabolic activation, test samples were exposed with S9 for 3 hr. For long-exposure, the test samples were kept in culture for 22 hr without S9. After the designated treatment duration, cytotoxicity was determined by MTT assay using an ELISA microplate reader (μ-Quant, Bio-Tek Instruments Inc., Winooski, VT, USA). The morphology of the cells was observed and recorded by microscope at 100 x magnification. In parallel, specimens were prepared for the chromosomal aberration test. Colcemid solution was added to the culture medium at a final concentration of 0.1 μg/mL for 2 hr before harvest the cells. Collected cells were treated with a hypotonic solution (0.75 mM KCl) and fixed with a mixture of ice-cold methanol/glacial acetic acid at a ratio of 3:1 (v/v). Cell smear on clean glass slides were air-dried and stained with Giemsa solution.

The frequency of the cells with chromosome structural aberration was scored in 200 well-spread metaphase cells with a number of centromeres equal to the model number (20 ± 2) scored for each dose in duplicate. The structural chromosome aberrations were classified into 5 groups: chromosome breakage (csb), chromosome exchange (cse), chromatid breakage (ctb), chromatid exchange (cte), and other abnormalities such as polyploidy, these were scored and recorded by photographing.

Animals

Seven week old male and female ICR mice and 8-12 week old male and female SD rats were obtained from BioLasco Taiwan Co. Ltd, Taipei, Taiwan. Animals were housed in pathogen-free cages (5-6 mice/cage and 2 rats/cage of the same gender) in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility of Level Biotech. Inc., Taipei, Taiwan. The temperature was set at 21 ± 2ºC, relative humidity 55 ± 20%, and lighting was 12 hr per day. Autoclaved reverse osmosis (RO) treated water was supplied *ad libitum* and laboratory rodent diet (LabDiet, PMI Nutation International, Brentwood, MO) supplied for all animals. The bedding was composed of coarse grade Aspen Chip (Tapvei. Oy, Kavi, Finland) and was changed once in a week.

Mammalian micronucleus test

The micronucleus test was performed following the Organization for Economic Cooperation and Development (OECD) guidelines. 7 weeks old male ICR mice were used in this study. The negative control, sterile water and LACC were administered 10 mL/kg at doses of 0.5, 1 and 2 g/kg by stainless feeding needles. Positive control group mice were administered 80 mg/kg cyclophosphamide (Sigma Aldrich) *via* intraperitoneal injection (using a 10 mg/mL solution dosed at 8 mL/kg b.w). Mice were monitored daily for any post-treatment clinical symptoms, and their body weight was noted before treatment (day 0) and 5 days after treatment. At 24, 48 and 72 hr post-treatment, peripheral blood samples (2 μL) were obtained from the tail vein and smeared on microscope slide coated with acridine orange (Sigma Aldrich). The smeared slides were incubated at room temperature for 2-3 hr prior to fluorescence microscopic examination. A fluorescence microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) with 488 nm excitation and 515 nm long pass filter was used for polychromatic erythrocytes and micronucleus identification and counting. The percentage of polychromatic erythrocytes (PCE) in 1000 erythrocytes was quantified. At least 2000 PCE/animal were scored for the incidence of PCE with micronucleus (MN ‰ PCE).

Acute oral toxicity study

An acute oral toxicity (14 day) study was performed to examine the possible adverse effects of the test sample LACC in rats *via* oral administration. After acclimatization for 6 days, 48 rats were divided into 4 groups (Group I, II, III and IV) 12 in each group of 6 male and 6 female rats. Group 1 served as a control group received sterile water (Taiyu Chemicals & Pharmaceuticals Co. Ltd., Hsinchu, Taiwan) *via* oral gavage in a volume of 10 mL/kg b.w, whereas Group II, III and IV received 1000, 3000 and 5000 mg/kg b.w LACC in water solution respectively in a volume of 10 mL/kg b.w. These doses were 25, 50 and 100 times the human recommended daily intake based on a body weight conversion basis. All animals were fasted overnight (16 hr) prior to dosing. The animals in each group were dosed twice on Day 1 with the control or LACC. The second dose was performed within 6 hr of the first. The animal feed was supplied 3-4 hr after second dosing. The dosing day was denoted as study day 1 (Day 1). On Day 1, the animal feed was re-supplied after the second dose. Mortality and moribundity were recorded every 12 hr interval. All rats were observed individually for any clinical signs at 0-4 hr after dosing on Day 1, thereafter once daily during the study period. Any abnormal findings, local/systemic and behavioral abnormalities were recorded and documented. The body weight of each rat was recorded prior to dosing and at 4, 8 and 15 days post dosing. Animals were sacrificed with overdose

of CO_2 on Day 15. The gross necropsy performed included examination of the external surfaces, the thoracic and abdominal cavities, including the intestine as the dosing site.

Repeated dose 28 and 90-days oral toxicity study

A repeated dose toxicity (28 and 90 days) study was conducted to evaluate the possible health hazards likely to arise from repeated exposure of LACC in rats *via* oral administration in accordance with OECD guidelines. To test the 28-days repeated oral toxicity of LACC, 80 rats were dived into 4 groups (Group I, II, III and IV), 20 rats in each group of 10 male and 10 females. Group I served as a control group and received sterile water at a volume of 10 mL/kg b.w, whereas Groups II, III and IV were LACC treatment groups and received 500, 1000 and 3000 mg/kg b.w, respectively in a volume of 10 mL/kg b.w in water. Meanwhile, for 90-days oral toxicity assay, 96 rats were divided into 4 groups (Group I, II, III and IV), 24 in each group of 12 male and 12 female rats. Group I served as a control group and received sterile water at a volume of 10 mL/kg b.w, whereas Groups II, III and IV were sample treatment groups and received 500, 1500 and 2500 mg/kg b.w, respectively in a volume of 10 mL/kg b.w in water. All were monitored daily in the same manner as described in the acute toxicity study to observe signs of toxicity. Ophthalmologic examination was performed for all animals before treatment commenced and before terminal sacrifice. Vaginal smear was examined once for each female before necropsy. Clinical pathology examinations including hematology, serum chemistry and urine analysis were performed for all surviving animals after the 28 and 90-day dosing period. On the necropsy day, blood samples were obtained from the abdominal aorta and collected into three tubes: 1) containing K_2EDTA for complete blood count analysis; 2) containing sodium citrate for coagulation factor analysis; and 3) without anti-coagulant for serum chemistry analysis. Urine samples were collected approximately 12-16 hr using metabolism cages prior to terminal sacrifice. Animals were received water and food while in metabolic cages. Immediately after blood collection, all rats were sacrificed using a ketamine (80 mg/mL) and Xylazine (8 mg/mL) anesthesia mixture. The gross necropsies included examination of the external surface of the body, all thoracic and abdominal cavities, intestines and visceral organs. Tissue/organ samples were fixed and preserved in 10% neutral buffered formalin. Histopathological examinations were performed only in the control (Group I) and the highest dose group (Group IV). The formalin fixed tissues were trimmed, embedded, sectioned and stained with hematoxylin and eosin (H&E) before microscopic examination.

Oral reproductive and developmental toxicity study

A reproductive and developmental toxicity study was conducted in accordance with the "Safety Evaluation Method for Health Food" by Department of Health, Taiwan. Male and female virgin CD (SD) IGS rats were purchased from BioLasco, Taiwan. After acclimatization for a week, individual breeding pairs were co-habited overnight in a suspended stainless steel cage. Impregnation was verified each morning by detection of vaginal sperm and/or vaginal copulation plug and was designated as gestation day 0 (G 0) (Cope *et al*., 2015). Impregnation verified animals were assigned into four groups (Group I, II, III and IV) by randomization by at least 20 females in each group. After mating, the female rats were transferred to polycarbonate cages. Confirmed-mated females were assigned to the four study groups. Group 1 served as a control group received sterile water in a volume of 10 mL/kg b.w, whereas Group II, III and IV were LACC treated groups and received 500, 1500 and 2500 mg/kg b.w, respectively in a volume of 10 mL/kg b.w in water during the major embryonic organogenesis period (G6-G15).

The maternal mortality and moribundity were observed twice a day for 20 days. Clinical observations including changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions, autonomic activity were recorded. Behavioral observations such as changes in gait, posture to response to handling as well as the presence of colonic and tonic movements, stereotypies (e.g., excessive grooming and repetitive circling), difficult or prolonged parturition or bizarre behavior (e.g., self- mutilation and walking backward) were noted. During the gestation period, all the animals were weighed on G0, G6, G9, G12, G15, G18 and G20. Feeding and water consumption was monitored during study period. On G20, rats were euthanized by CO_2 inhalation followed by exsanguination and immediately subjected to a laparohysterectomy. Necropsy including examination of external surface of the body, all orifices, thoracic, abdominal and cranial cavities and their content. Internally, the skin was reflected from a ventral midline incision to examine mammary tissue and locate any subcutaneous masses. The uterus was excised and gravid uterine weight was recorded. Beginning at the distal end of the right uterine horn, extending caudally across the cervix to the left uterine horn, position of the cervix, and the number of total

implantations were recorded. Each litter was categorized according to the known criteria such as viable fetus, non-viable fetus, late resorption, early resorption, corpora luteal count and gravid uterus weight.

Following caesarean section, fetuses were examined for viability. All surviving fetuses were individually weighed, sexed and examined external malformations and variations. Crown-rump length (mm) of each fetus was recorded. After the external examination, each fetus was euthanized via intraperitoneal injection of sodium pentobarbital and alternately assigned by number and position for either visceral or skeletal examination. Approximately one-half of the fetuses in each litter were placed in Bouin's solution for a week for skull and visceral examination. All fetuses fixed in Bouin's solution were subjected for soft tissue defects using the modified Wilson razor-blade technique for any internal organ abnormalities. Prior to skeletal staining, all fetuses assigned for skeletal examination were eviscerated according to standard method following preservation in 95% ethyl alcohol fixative. The eviscerated skeleton was macerated with potassium hydroxide, stained with Alizarin Red S and Alcian Blue, and cleared with glycerin for subsequent skeletal studies. The skeleton of each fetus were examined for completeness of bone ossification and malformations or variations in the skeleton.

Statistical analysis

All data obtained in this study were expressed in mean ± S.D. The micronucleus frequency and chromosomal aberration test were analyzed by the model of Poisson distribution. The p value less than 0.05 ($p < 0.05$) was considered statistically significant. Ames test, acute toxicity, repeated oral dose toxicity and reproductive and developmental toxicity tests were analyzed by One-Way ANOVA and Dunnett's tests by SPSS ver 12.0 software (IBM, Armonk, NY, USA). Heterogenous data were analyzed with the Kruskal-Wallis non- parametric ANOVA method. Probability of 0.05 ($p < 0.05$) was used as the significance criterion.

RESULTS AND DISCUSSION

Bacterial reverse mutation test

Initially we determined the genotypes of five *Salmonella typhimurium* bacterial strains (TA97a, TA98, TA100, TA102 and TA1535) using histidine mutation, rfa mutation and uvrB repair assay. The genotype of all *S. typhimurium* strains were identified and met the standard as described in Table S2. Next, the cytotoxicity assay suggests that LACC is not cytotoxic to the bacterial strain TA98 at dose of 0.313, 0.625, 1.25, 2.5 and 5 mg/plate Table S3. Thus, we set these doses 0.313, 0.625, 1.25, 2.5 and 5 mg/plate are test doses and performed Ames test. As shown in Table 1, compared to the negative control groups (sterile water), the positive control group caused more than two-fold increase in revertant colonies on TA97a, TA98, TA100 and TA102; more than three-fold increase in revertants colonies on TA1535. Also, we found that LACC did not significant increase the mean number of reverse mutation at dose levels between 0.313 to 5 mg/plate in both normal and metabolically activated bacterial strains (Table 1). These results suggest that LACC does not induce bacterial reverse mutation within the test doses.

Mammalian chromosome aberration test

A. cinnamome is a well-known anti-tumor agent currently in clinical trials (Lu *et al*., 2013). Most of the anti-tumor agents are known to interact with specific biological molecules. Previous studies have reported that treatment with anti-tumor agents from different categories induce free radicals in non-tumor cells in both *in vitro* and *in vivo* (Weijl *et al*., 1997). Extracts of *A. cinnamomea* or its derived compounds induce apoptosis in cancer cells through reactive oxygen species (ROS) gen-

Table 1. Results of bacterial reverse mutation test.

Group		Number of revertants/plate (without S9 activator)					Number of revertants/plate (with S9 activator)				
		TA97a	TA98	TA100	TA102	TA1532	TA97a	TA98	TA100	TA102	TA1532
Negative		45.3 ± 8.1	25.3 ± 3.5	104.3 ± 16.0	184.7 ± 15.6	11.7 ± 5.5	60.3 ± 7.2	30.3 ± 4.2	102.7 ± 9.7	201.7 ± 13.3	15.0 ± 6.1
Positive		516.7 ± 26.1*	251.3 ± 26.5*	634.0 ± 31.4*	2477.3 ± 591.0*	315.3 ± 98.5*	631.3 ± 24.2*	228.3 ± 17.6*	700.0 ± 32.0*	440.0 ± 26.5*	291.0 ± 29.4*
LACC (mg/plate)	5	42.0 ± 2.0	26.7 ± 8.4	105.3 ± 16.7	239.3 ± 5.1	17.3 ± 7.6	54.7 ± 6.5	18.3 ± 2.5	121.7 ± 14.3	208.0 ± 15.7	14.0 ± 5.3
	2.5	53.7 ± 7.8	19.7 ± 4.0	111.0 ± 21.7	217.3 ± 4.0	17.7 ± 2.9	61.0 ± 6.2	15.0 ± 2.0	129.3 ± 13.6	210.0 ± 4.6	16.3 ± 4.0
	1.25	47.3 ± 3.2	24.3 ± 3.2	117.7 ± 15.0	242.7 ± 24.1	18.0 ± 4.6	53.7 ± 4.2	29.7 ± 5.0	111.3 ± 5.5	208.3 ± 7.2	18.0 ± 1.7
	0.625	45.0 ± 5.6	20.0 ± 6.2	138.0 ± 6.2	202.0 ± 31.8	18.0 ± 1.0	51.3 ± 1.5	23.7 ± 3.1	136.7 ± 7.5	212.0 ± 7.5	18.7 ± 4.5
	0.313	43.7 ± 10.0	19.0 ± 6.2	127.0 ± 8.2	168.7 ± 13.2	17.0 ± 1.7	55.3 ± 8.0	32.7 ± 3.1	118.7 ± 13.3	204.0 ± 11.1	15.7 ± 3.5

All values presented as mean ± S.D. *significantly different compared to all dose of test compounds.

eration following DNA damage (Chung *et al*., 2014; Yang *et al*., 2013). Thus, prior to the *in vitro* assay, the cytotoxic effect of LACC on CHO-K1 cells was examined by MTT assay. Cells were incubated with LACC or positive controls (mitomycin C and benzo(a)pyrene) in the presence or absence of S9 mixture for 3 and 18 hr. Result from MTT assay showed that incubation with LACC in the absence of S9 significant decreased cell viability in a dose-dependent manner, whereas a the cell viability was unaffected in the presence of S9 mixture (Table S4). Based on the results, dosages with over 50% cell viability, selected for use in the chromosome aberration test were 0.625, 1.25 and 2.5 mg/mL for 3 hr treatment group without S9 and those used in the 3 hr treatment group with S9 were 1.25, 2.5 and 5 mg/mL. In the 18 hr treatment group without S9, the doses used in the chromosome aberration test were 0.313, 0.625 and 1.25 mg/mL.

The chromosome aberration frequency were summarized in Table 2. In comparison of short-term (3 hr) testing scheme with negative control, the frequencies of chromosome aberration observed in positive control were significantly higher (p value < 0.05) at conditions of both with or without S9 activation. Whereas, there were no significant increases in the frequency of metaphases with aberrant chromosomes at 3 hr or 18 hr with or without the S9 mixture in low doses of LACC-treated group compared with the vehicle control group (Table 2). The chromosome aberrations in LACC treated cells were 2, 2 and 7 at 0.625, 1.25 and 2.5 mg/mL under 3 hr without S9 and 4, 1 and 5 at 1.25, 2.5 and 5 mg/mL, respectively under 3 hr with S9 metabolic activation. Moreover, the chromosome aberration in 200 observed metaphase cells were 1, 2 and 5 by 0.313, 0.625 and 1.25 mg/mL LACC, respectively under 18 hr without S9 metabolic activation (Table 2). The frequency of chromosome aberration were subjected to Poisson and Cochran-Armitage trend test, the results indicated the LACC did show any genotoxicity. In long-term testing scheme, the LACC testing group was without significantly response by Poisson distribution analysis (p value > 0.05). In summary, data indicate that exposure to LACC does not significantly induce chromosome aberration in cultured mammalian somatic cells under the test conditions.

Mammalian micronucleus test

Besides the possible use of LACC as a health food supplement, knowledge about its genotoxic potential is also of interest from the point of human consumption. Therefore, next we examined whether treatment with LACC resulted in chromosome damage in mice using an *in vivo* micronucleus test. There were no abnormal changes were observed in mortality or body weight between the first (day 0) and final administrations (day 5) in the vehicle control group, positive control group, or the groups treated with 0.5, 1 and 2 g/kg/day of LACC (Table S5 and S6).

The PCE percentage of negative control group at 24, 48 and 72 hr was 3.47 ± 0.25%, 3.54 ± 0.42% and 3.43 ± 0.41%, respectively. The PCE percentage of positive control group was decreased with time and 20% lower than

Table 2. Effect of LACC on mammalian chromosome aberration in cultured CHO-K1 cells.

Treatment period	Metabolic activation	Test sample	Aberration frequency[1]	p value[2]
Short-term treatment (3 hr)	Without S9	Negative control	0	NA
		Mitomicin C (0.5 μg/mL)	29	0.0000*
		LACC (0.625 mg/mL)	2	0.1353
		LACC (1.25 mg/mL)	2	0.1353
		LACC (2.5 mg/mL)	7	0.0009*
	With S9	Negative control	1	NA
		Benzo(a)pyrene (25 μg/mL)	23	0.0000*
		LACC (1.25 mg/mL)	4	0.0916
		LACC (2.5 mg/mL)	1	0.7358
		LACC (5 mg/mL)	5	0.0404*
Long term treatment (18 hr)	Without S9	Negative control	0	NA
		Mitomicin C (0.5 μg/mL)	30	0.0000*
		LACC (0.313 mg/mL)	1	0.9810
		LACC (0.625 mg/mL)	2	0.8571
		LACC (1.25 mg/mL)	5	0.2650

[1]The aberration frequency was displayed in the manner of number of cells with chromosome aberration in 200 observed metaphase cells (n/200). [2]The statistical analysis was performed by Poisson distribution in comparison with negative control. The "*" represents the statistical significance (p < 0.05).

the PCE percentage of negative control group, indicated that cyclophosphamide inhibited erythropoiesis. All testing LACC groups were not significantly different from negative control group, indicated that LACC did not affect erythropoiesis (Table 3). In addition, we further examined the micronucleus frequency in 1000 PCE using fluorescence microscope. The micronucleus frequencies in PCE of negative control group at 24, 48 and 72 hr (1.17 ± 0.98, 1.00 ± 0.89 and 0.83 ± 0.98 ‰ $_{PCE}$) and positive control group at 24, 48 and 72 hr (7.67 ± 1.03, 20.00 ± 1.67 and 11.33 ± 1.63 ‰ $_{PCE}$) were both confirmed to the criteria in section 7.4. After statistical analysis with Poisson distribution methods, all three LACC testing groups were not statistically significant from negative control group (Table 3). Based on these observations, we conclude that all the testing doses of LACC does not increase micronucleated PCE in the test condition.

Acute (14-day) oral toxicology study

Treatment of rats with LACC (1000, 3000 and 5000 mg/kg b.w) for 14 days produced neither death nor treatment-related signs of toxicity in any of the treatment groups during the study (Table S7). In addition, no weight loss resulted from the LACC treatment compare to the control groups in both genders throughout the treatment period. Moreover, there were no abnormal clinical findings from external observations which were attributable to LACC treatment. Furthermore, there were no abnormal findings from the gross pathological examination of internal organs including thoracic and abdominal cavities, intestines, or visceral organs at necropsy in all groups of animals. Based on these results, the oral LD_{50} of LACC is found to be greater than 5 g/kg b.w for both genders. Data generated from this study provide safety information for human exposure and also provide information to establish a dose regimen in further studies.

Repeated dose (90 days) oral toxicity study

The 90 days repeated oral dose toxicity study showed that there no mortalities or ophthalmologic and treatment related signs of toxicity were observed in any of the treatment groups (Table S8). In both genders, there were no statistically significant differences in the mean body weight and mean body weight gain between vehicle control and LACC treatment groups (Table S9-S12). In addition, there were no statistically significant variations in food consumption in all test groups (data not presented). Moreover, no treatment related severe clinical signs were observed in all test animals throughout the study period (Table 4). However, some clinical signs were observed due to housing behavior (wound) or individual animal differences (hair loss). In male rats, audible respiration was observed 1 in 12 rats of 1500 mg/kg LACC treated group and 2 in 12 was noted in 2500 mg/kg LACC treatment group. Wound was noted in control, 1500 mg/kg and 2500 mg/kg LACC treated group are 1 in 12, 2 in 12 and 1 in 12 rats, respectively. They were caused by housing behavior and the severities were slight. Hair loss was recorded in control, 1500 mg/kg and 2500 mg/kg LACC treated group as 1 in 12, 2 in 12 and 3 in 12 rats, respectively (Table 4). In female rats, wound was observed in control, 500 mg/kg and 2500 mg/kg LACC treated group are 1 in 12, 2 in 12 and 1 in 12 rats, respectively. Hair loss was recorded in control, 500 mg/kg, 1500 mg/kg and 2500 mg/kg LACC treated group as 2 in 12, 1 in 12 and 3 in 12, 3 in 12 rats, respectively (Table 5).

There were no statistically significant differences were observed from the results of hematological parameters of male rats, whereas some statistically significant differences were observed in female rats treated with LACC. Particularly, the RBC levels in 2500 mg/kg LACC treatment group was statistically ($p < 0.05$) lower than that of vehicle control group (Table 6). Results from individual animal serum chemistry analysis showed some statistically significant differences in both genders as summarized in Table 7. In male rats, the aspartate aminotransferase (AST) levels in high-dose (1500 mg/kg) treated group was statistically higher than that of control group. The total protein level in the 1500 and 2500 mg/kg LACC treated group was significantly

Table 3. Effect of LACC on percentage of PCE in erythrocytes and micronucleus frequency in PCE.

Treatment group	PCE % (mean ± S.D, n = 6)			MN‰$_{PCE}$ (mean ± S.D, n = 6)		
	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
Control	3.47 ± 0.25	3.54 ± 0.42	3.43 ± 0.41	1.17 ± 0.98	1.00 ± 0.89	0.83 ± 0.98
Cyclop. (80 mg/kg)	2.83 ± 0.23	1.43 ± 0.17	0.50 ± 0.01	7.67 ± 1.03*	20.00 ± 1.67*	11.33 ± 1.63*
LACC (500 mg/kg)	3.27 ± 0.15	3.39 ± 0.34	3.35 ± 0.16	1.33 ± 1.03	1.00 ± 0.63	1.67 ± 0.52
LACC (1000 mg/kg)	3.23 ± 0.12	3.22 ± 0.30	3.31 ± 0.20	0.67 ± 0.52	1.33 ± 1.03	0.83 ± 0.75
LACC (2000 mg/kg)	3.23 ± 0.31	3.16 ± 0.27	3.24 ± 0.24	1.17 ± 0.98	1.33 ± 0.52	0.83 ± 0.41

*Significant difference ($p < 0.05$) from negative control group analyzed by Poisson distribution model.

Table 4. Effect of repeated oral dose (90 days) of LACC on rats: Clinical observation in male animals.

		Clinical signs											
		Audible respiration				Wounds				Hair loss			
		LACC (mg/kg b.w)				LACC (mg/kg b.w)				LACC (mg/kg b.w)			
		0	500	1500	2500	0	500	1500	2500	0	500	1500	2500
Incidence during study period	Day 3	0/12	0/12	0/12	1/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
	Day 21-28	0/12	0/12	0/12	0/12	1/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
	Day 29-35	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12	1/12	1/12	0/12
	Day 36-44	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12	1/12	1/12	1/12
	Day 45-48	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12	1/12	1/12	1/12
	Day 49	0/12	0/12	0/12	0/12	0/12	0/12	1/12	0/12	1/12	1/12	1/12	1/12
	Day 50	0/12	0/12	0/12	0/12	0/12	0/12	1/12	0/12	1/12	1/12	0/12	0/12
	Day 51-52	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12	1/12	1/12	1/12
	Day 53-56	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12	1/12	0/12	1/12
	Day 57-59	0/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12	0/12	0/12	1/12	1/12
	Day 60-63	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12	1/12
	Day 64-65	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12	1/12
	Day 66	0/12	0/12	0/12	1/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12	0/12
	Day 67-73	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12	0/12
	Day 74	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12	0/12	1/12	0/12
	Day 75-84	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12	0/12	0/12	1/12
	Day 85	0/12	0/12	1/12	0/12	0/12	0/12	0/12	0/12	1/12	0/12	0/12	1/12
	Day 86-89	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12	0/12	0/12	1/12
	Day 90	0/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12	1/12	0/12	0/12	1/12
	Day 91	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12	0/12	1/12	1/12
Total incidence (n/n)		0/12	0/12	1/12	2/12	1/12	0/12	2/12	1/12	1/12	2/12	3/12	2/12

Table 5. Effect of repeated oral dose (90 days) of LACC on rats: Clinical observation in female animals.

		Clinical signs							
		Wounds				Hair loss			
		LACC (mg/kg b.w)				LACC (mg/kg b.w)			
		0	500	1500	2500	0	500	1500	2500
Incidence during study period	Day 36-40	0/12	0/12	0/12	0/12	0/12	1/12	0/12	1/12
	Day 41-42	1/12	0/12	0/12	0/12	0/12	1/12	1/12	1/12
	Day 43-45	1/12	0/12	0/12	1/12	0/12	1/12	1/12	0/12
	Day 46-47	0/12	0/12	0/12	1/12	0/12	1/12	0/12	0/12
	Day 48-54	0/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12
	Day 55	0/12	0/12	0/12	1/12	0/12	0/12	0/12	1/12
	Day 56-58	0/12	0/12	0/12	1/12	0/12	0/12	1/12	1/12
	Day 59-63	0/12	0/12	0/12	0/12	0/12	0/12	1/12	1/12
	Day 64-65	0/12	1/12	0/12	0/12	0/12	1/12	1/12	1/12
	Day 66-69	0/12	0/12	0/12	0/12	0/12	1/12	1/12	1/12
	Day 70	0/12	0/12	0/12	0/12	1/12	0/12	1/12	1/12
	Day 71-72	0/12	0/12	0/12	0/12	1/12	0/12	1/12	0/12
	Day 73	0/12	1/12	0/12	0/12	1/12	0/12	1/12	0/12
	Day 74-75	0/12	1/12	0/12	0/12	1/12	0/12	1/12	0/12
	Day 76-77	0/12	1/12	0/12	0/12	1/12	0/12	1/12	0/12
	Day 78	0/12	1/12	0/12	0/12	1/12	0/12	1/12	1/12
	Day 79-80	0/12	1/12	0/12	0/12	1/12	0/12	1/12	1/12
	Day 81-86	0/12	1/12	0/12	0/12	1/12	0/12	1/12	1/12
	Day 87-91	0/12	1/12	0/12	0/12	1/12	0/12	1/12	1/12
Total incidence (n/n)		1/12	0/12	2/12	1/12	1/12	2/12	3/12	2/12

[1] n'/n': Number of animals with observable sign/Number of animals alive.

[2] n/n: Total number of animals with observable sign/Total number of animals examined.

Table 6. Effect of repeated oral dose (90 days) of LACC on rats: Hematological findings.

Parameters	Hematology (Mean ± S.D., n = 12)							
	Male				Female			
		LACC (mg/kg b.w)				LACC (mg/kg b.w)		
	Control	500	1500	2500	Control	500	1500	2500
WBC (10^3/μL)	10.2 ± 2.0	11.3 ± 2.4	8.0 ± 2.8	9.2 ± 2.1	7.2 ± 3.6	6.2 ± 2.7	6.5 ± 2.6	6.5 ± 2.4
RBC (10^6/μL)	9.3 ± 0.5	9.5 ± 0.3	9.4 ± 0.4	9.4 ± 0.5	8.4 ± 0.4	8.1 ± 0.4	8.1 ± 0.2	8.0 ± 0.4*
HGB (g/dL)	16.0 ± 0.8	16.3 ± 0.6	16.1 ± 0.6	16.2 ± 0.6	15.6 ± 0.7	15.2 ± 0.4	15.0 ± 0.7	14.7 ± 0.5*
HCT (%)	44.8 ± 2.2	45.3 ± 1.8	44.8 ± 1.4	45.1 ± 1.6	44.1 ± 2.4	43.3 ± 1.5	42.4 ± 2.0	41.5 ± 1.5*
MCV (fL)	48.3 ± 1.9	46.7 ± 1.6	47.6 ± 1.2	47.9 ± 2.6	52.0 ± 1.8	53.2 ± 2.3	51.9 ± 1.8	51.9 ± 1.6
MCH (pg)	1145.1 ± 154.2	1178.4 ± 151.0	1094 ± 120.9	1155.5 ± 129.8	18.4 ± 0.5	18.7 ± 0.5	18.3 ± 0.5	18.5 ± 0.6
MCHC (g/dL)	16.9 ± 5.1	22.4 ± 11.0	20.8 ± 5.7	19.5 ± 7.3	35.4 ± 0.4	35.2 ± 0.5	35.3 ± 0.4	35.5 ± 0.8
PLT (10^3/μL)	78.7 ± 5.5	73.0 ± 10.9	74.8 ± 5.5	76.0 ± 7.5	1011.0 ± 109.4	998.3 ± 130.1	1100.7 ± 151.2	980.9 ± 110.7
NEUT (%)	3.9 ± 0.9	4.2 ± 0.7	4.1 ± 1.0	4.0 ± 1.3	13.7 ± 6.3	16.5 ± 3.3	20.0 ± 11.8	17.6 ± 9.9
LYMPH (%)	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.09	82.8 ± 6.5	79.5 ± 3.7	75.5 ± 12.1	78.4 ± 10.6
MONO (%)	0.10	0.10	ND	0.10	3.2 ± 1.0	3.6 ± 0.9	4.0 ± 1.2	3.7 ± 1.2
EOSIN (%)	13.0 ± 2.4	12.5 ± 1.7	12.0 ± 1.5	11.9 ± 1.5	0.25 ± 0.1	0.3 ± 0.1	0.4 ± 0.3	0.2 ± 0.1
BASO (%)	18.3 ± 1.5	18.4 ± 1.5	17.2 ± 2.3	18.1 ± 1.2	0.10	0.20	ND	ND
PT (sec)	10.1 ± 2.0	11.3 ± 2.4	8.0 ± 2.8	9.2 ± 2.1	9.1 ± 0.1	9.2 ± 0.1	9.1 ± 0.1	9.1 ± 0.2
APTT (sec)	9.2 ± 0.5	9.5 ± 0.3	9.4 ± 0.4	9.4 ± 0.5	14.5 ± 1.6	15.5 ± 1.0	15.6 ± 1.9	15.3 ± 0.5

*Statistically significant ($p < 0.05$). WBC: white blood cells; RBC: red blood cells; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet; NEUT: neutrophil; LYMPH: lymphocyte; MONO: monocyte; EOSIN: eosinophil; BASO: basophil; PT: prothrombin; APTT: activated thromboplastin time.

Table 7. Effect of repeated oral dose (90 days) of LACC on rats: Serum chemical analysis.

	Serum Chemistry (Mean ± S.D., n = 12)							
	Male				Female			
		LACC (mg/kg)				LACC (mg/kg)		
	Control	500	1500	2500	Control	500	1500	2500
AST (U/L)	105.8 ± 14.3	104.2 ± 27.6	126.1 ± 13.8*	118.9 ± 17.04	119.1 ± 27.3	132.4 ± 48.4	109.5 ± 25.2	118.7 ± 33.3
ALT (U/L)	27.1 ± 4.9	27.2 ± 4.2	29.2 ± 3.5	22.4 ± 4.03	28.8 ± 16.7	30.3 ± 13.9	26.8 ± 12.3	30.3 ± 12.6
Glucose (mg/dL)	181.2 ± 20.6	205.6 ± 26.6	173.5 ± 24.2	194.8 ± 27.3	173.4 ± 24.6	163.2 ± 24.1	188.8 ± 20.2	170.1 ± 19.7
TP (g/dL)	5.2 ± 0.2	5.9 ± 0.1	5.9 ± 0.2	5.9 ± 0.3	6.5 ± 0.3	6.2 ± 0.4	6.5 ± 0.4	6.6 ± 0.4
ALB (g/dL)	4.1 ± 0.1	3.9 ± 0.1	3.9 ± 0.1*	3.1 ± 0.2*	4.8 ± 0.4	4.6 ± 0.3	4.8 ± 0.4	4.9 ± 0.3
TBIL (mg/dL)	0.02 ± 0.01	0.01 ± 0.01	0.013 ± 0.01	0.01 ± 0.0	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.03	0.04 ± 0.02
BUN (mg/dL)	14.4 ± 1.4	14.4 ± 1.3	13.9 ± 1.8	14.7 ± 1.4	15.9 ± 2.3	16.0 ± 2.2	14.9 ± 1.6	15.3 ± 2.2
CR (mg/dL)	0.42 ± 0.04	0.4 ± 0.05	0.4 ± 0.05	0.4 ± 0.07	0.6 ± 0.1	0.6 ± 0.08	0.6 ± 0.1	0.54 ± 0.07
γGT (U/L)	0.8	ND	0.3	0.4	0.9	0.3	0.4	0.4
ALP (U/L)	238.3 ± 49.1	255.7 ± 43.3	256.4 ± 45.6	267.4 ± 34.6	130.0 ± 25.4	153.8 ± 52.8	109.2 ± 26.0	120.4 ± 42.1
CHL (mg/dL)	59.5 ± 9.2	68.0 ± 11.3	55.2 ± 10.3	62.3 ± 12.8	71.3 ± 16.1	73.1 ± 13.01	83.8 ± 12.6	89.8 ± 14.4*
TG (mg/dL)	38.1 ± 10.6	44.7 ± 22.97	31.4 ± 17.2	39.8 ± 20.04	28.2 ± 9.6	24.9 ± 8.8	26.1 ± 6.7	27.7 ± 7.5
Ca (mg/dL)	9.7 ± 0.2	9.9 ± 0.3	9.6 ± 0.2	9.6 ± 0.2	9.9 ± 0.5	9.8 ± 0.3	9.9 ± 0.4	10.1 ± 0.2
P (mg/dL)	6.8 ± 0.5	6.9 ± 0.5	6.5 ± 0.6	6.8 ± 0.5	6.3 ± 2.4	5.7 ± 1.1	5.9 ± 0.8	5.6 ± 1.1
CRK (U/L)	547.3 ± 153.3	527.9 ± 258.01	682.7 ± 1.89.6	747.7 ± 197.7	567.9 ± 192.9	586.5 ± 109.1	494.7 ± 170.0	488.5 ± 191.9
Am (U/L)	1309.6 ± 175	1405.9 ± 148.0	1390.3 ± 242.5	1443.2 ± 161.9	974.5 ± 132.1	1044.1 ± 175.3	1068.2 ± 154.3	979.8 ± 193.2
Na (mM)	143.7 ± 1.4	144.2 ± 1.3	145.2 ± 1.5	144.3 ± 1.8	143.8 ± 2.4	145.0 ± 1.7	145.1 ± 1.8	145.3 ± 1.4
K (mM)	4.7 ± 0.3	4.6 ± 0.4	4.8 ± 0.4	4.7 ± 0.3	5.09 ± 3.5	4.2 ± 0.3	4.3 ± 0.4	4.3 ± 0.3
Cl (mM)	106.6 ± 2.1	107.9 ± 1.91	105.7 ± 1.5	104.2 ± 1.97*	104.8 ± 2.4	105.0 ± 1.7	104.9 ± 1.9	105.2 ± 1.8

*Statistically significant from LACCC treatment group vs negative control group. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TP: total protein; ALB: albumin; TBIL: total bilirubin; BUN: blood urea nitrogen; GGT: gamma-glutamyl transferees; CHL: cholesterol; ALP: alkaline phosphatase; TG: triglyceride; Ca: calcium; P: phosphorus; CRK: creatinine kinase; Am: amylase; Na: Sodium; K: potassium; Cl: chloride.

lower than vehicle control group. The chloride level in the 2500 mg/kg treated group was statistically lower than that of control group. In female rats, compared to vehicle control group a significant increase of total cholesterol was observed in 2500 mg/kg treatment groups. However, there was no statistically significant difference in triglyceride levels. Urine analysis showed there was no significant difference in volume, specific gravity and urobilinogen in all tested groups, whereas the pH of high-dose (2500 mg/kg) treated rats were statistically lower (6.71 ± 0.33) than vehicle control group (7.04 ± 0.14) in male animals (Table S13). However, despite those statistical differences, the data were within the normal historical range and without physiological abnormalities. The internal organ weights in all treated groups of both genders were not significantly different from those of the vehicle control groups with the exception of the adrenal weight of those female animals of 2500 mg/kg LACC treatment group (Table 8). This statistical difference was within normal historical control range and without physiological abnormalities. In males, there was no statistical difference between the vehicle control and LACC treated groups (Table 8).

Moreover, results of gross necropsy findings revealed that there were neither signs of toxicity noted with respect to gross examination of all organs examined (Table S14). However, one male animal of each group was observed to have swelling in the liver. One male animal in the highest dose of LACC (2500 mg/kg) treatment group showed swelling in liver, redness in cecum, atrophy in thymus and bilateral edema in epididymides. A white macula in the left kidney of a female was observed in an animal of 2500 mg/kg treatment group. Histopathologically, nephrosclerosis, myocarditis and adrenal atrophy were observed in both control and LACC treatment group (Table S15). The microscopic observations in the LACC group were considered to be spontaneous due to incidence, significant and severity. These changes were observed across all groups and with no dose related response. They were nonspecific in natural. Therefore it was inferred that there were no pathological changes in the organs, studied which could be attributed to the test article administrated. All lesions showed moderate mononuclear cell leukemia. According to the severity and incidence in histopathological evaluation, this lesion was considered to be a spontaneous abnormality and not related to the LACC exposure.

Reproductive and developmental toxicity assessment

Male and female rats were cohabitated with a ratio of 1:1 for overnight. Impregnation was verified each morning by detection of the vaginal plug in vagina or on cage board or the presence of spermatozoa by vaginal smear. The vaginal smear was performed in animals without vaginal plug. The animal numbers of impregnation verified in the vehicle control, 500, 1500 and 2500 mg/kg LACC groups were 25, 23, 22 and 22, respectively. The pregnant animal numbers verified in the C vehicle control, 500, 1500 and 2500 mg/kg LACC groups after necropsy were 21, 20, 20 and 21, respectively. The fertility index were 84.00% (21/25), 86.96% (20/23), 90.91% (20/22) and 95.45% (21/22) of the vehicle control, 500, 1500 and 2500 mg/kg LACC groups, respectively. The maternal mortality and moribundity were observed twice daily during G0- G19. No animal death occurred up to the dose of 2500 mg/kg/day throughout the study period. There was no statistically significant maternal body weight and

Table 8. Effect of repeated oral dose (90 days) of LACC on rats: Internal organ weight.

	Organ weight (Mean ± S.D., n = 12)								
	Male					Female			
		LACC (mg/kg b.w)					LACC (mg/kg b.w)		
Organs	Control (WFI)	500	1500	2500	Organs	Control (WFI)	500	1500	2500
Adrenals	0.06 ± 0.00	0.05 ± 0.00	0.06 ± 0.01	0.06 ± 0.01	Adrenals	0.06 ± 0.00	0.07 ± 0.00	0.08 ± 0.01	0.08 ± 0.01
Pituitary	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	Pituitary	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Brain	2.11 ± 0.09	2.23 ± 0.01	2.14 ± 0.07	2.10 ± 0.11	Ovaries	0.13 ± 0.02	0.13 ± 0.02	0.15 ± 0.03	0.15 ± 0.03
Epididymides	1.39 ± 0.14	1.43 ± 0.11	1.39 ± 0.19	1.44 ± 0.13	Brain	1.9 ± 0.07	1.9 ± 0.10	1.9 ± 0.06	1.9 ± 0.07
Heart	1.53 ± 0.13	1.54 ± 0.16	1.51 ± 0.07	1.61 ± 0.13	Heart	0.96 ± 0.08	0.94 ± 0.10	0.97 ± 0.11	1.03 ± 0.10
Kidneys	3.66 ± 0.29	3.56 ± 0.29	3.52 ± 0.33	3.73 ± 0.45	Kidneys	1.99 ± 0.22	1.91 ± 0.18	2.03 ± 0.18	2.03 ± 0.57
Liver	14.7 ± 1.73	14.5 ± 1.85	13.5 ± 1.41	15.1 ± 1.54	Liver	7.96 ± 0.71	7.65 ± 0.59	8.50 ± 1.08	8.63 ± 0.54
Spleen	0.84 ± 0.12	0.85 ± 0.17	0.78 ± 0.11	0.83 ± 0.08	Spleen	0.58 ± 0.65	0.53 ± 0.08	0.55 ± 0.09	0.53 ± 0.08
Testes	3.33 ± 0.22	3.49 ± 0.29	3.47 ± 0.33	3.47 ± 0.22	Thymus	0.30 ± 0.07	0.26 ± 0.05	0.30 ± 0.05	0.28 ± 0.04
Thymus	0.37 ± 0.05	0.44 ± 0.16	0.32 ± 0.08	0.35 ± 0.08	Uterus with cervix	0.43 ± 0.05	0.48 ± 0.05	0.47 ± 0.12	0.52 ± 0.16

*Statistically significant ($p < 0.05$) from LACCC treatment group vs vehicle control group.

Table 9. Effect of LACC on fetal development.

	LACC (mg/kg)							
	Control		500		1500		2500	
Fetal body weight (g)	3.75 ± 0.33		3.70 ± 0.31		3.86 ± 0.31*		3.99 ± 0.33*	
Fetal body length (mm)	35.10 ± 2.16		34.57 ± 1.83*		35.70 ± 2.46*		32.09 ± 3.75*	
Total examined number	314		271		291		296	
External examination number	314		271		291		296	
Visceral examination number	153		130		142		142	
Skeletal examination number	161		141		149		154	
External examination (%)	L	F	L	F	L	F	L	F
Hematoma	9.52	0.96	10.00	0.74	0.00	0.00	4.76	0.34
Visceral examination (%)								
Distended renal pelvis	0.00	0.00	0.00	0.00	0.00	0.00	9.52	1.41
Ureter distended	23.81	3.27	15.00	4.62	45.00	13.38	23.81	6.34
Skeletal examination (%)								
Minor abnormality								
Dumbbell-shape thoracic central	14.29	4.35	15.00	2.13	20.00	3.36	47.62	11.04
Split thoracic central	19.05	3.73	20.00	4.96	40.00	8.05	23.81	5.84
Supernumerary ribs	9.52	2.48	5.00	0.71	20.00	6.04	47.62	15.58

*Statistically significant with vehicle control group ($P < 0.05$). L: Litter incidence (%); F: Fetal incidence (%).

weight gain among the study groups (data not presented). Maternal food and water consumption showed no consistent dose-related differences during the study period (data not presented).

The clinical observation was performed once daily during G0-G19. Slight to moderate hair loss was observed in two vehicle control female and one 2500 mg/kg LACC female group (Table S16). The clinical sign was caused by the nesting behavior of pregnant animals and not related to LACC administration. Results of maternal evaluation showed that there were no statistical significances noted in gravid uterus weight, corpora lutea number, implantation number, litter size, live or dead fetal number, resorption number, fetal sex ratio (M/F) and post-implantation loss. The statistical difference was noted in pre-implantation loss between vehicle control and 2500 mg/kg LACC group (Table S17). However, the difference was not out of normal reference ranges. And no maternal toxicity is noted in all groups.

As shown in Table 9, fetal body weight was statistically higher than vehicle control group was noted in 1500 and 2500 mg/kg LACC groups. Statistical difference was noted in fetal body length in 500, 1500 and 2500 mg/kg LACC groups. However, the fetal body weight and body length was within normal reference range in all groups. Hematoma was observed in vehicle control, 500 and 2500 mg/kg LACC groups (litter incidence: 9.52%, 10.00% and 4.76%. Fetal incidence: 0.96%, 0.74% and 0.34%, respectively). The incidence of the fetal external abnormality was not dose dependent and within normal reference range. And no fetal toxicity is noted in fetal external examination. Skeletal examination of 50% fetuses of each litter was performed with Alizarin Red S and Alcian Blue staining. Dumbbell-shape of thoracic central was observed in all groups (Litter Incidence: 14.29%, 15.00%, 20.00% and 47.62%. Fetal Incidence: 4.35%, 2.13%, 3.36% and 11.04%, respectively). Split thoracic central was observed in all groups (Litter Incidence: 19.05%, 20.00%, 40.00% and 23.81%. Fetal Incidence: 3.73%, 4.96%, 8.05% and 5.84%, respectively). Supernumerary of ribs were observed in all groups (Litter Incidence: 9.52%, 5.00%, 20.00% and 47.62%. Fetal Incidence: 2.48%, 0.71%, 6.04% and 15.58%, respectively) (Table 9). Those findings were minor skeletal abnormalities and within normal reference ranges. These data are well correlated with a previous study which reports that the mycelial extract of *A. cinnamomea* (50-500 mg/kg b.w) does not showed any teratogenic effects in female SD rats. (Chen *et al*., 2011) However, the highest oral dose of the present study was 5-fold higher than the previous report.

In conclusion, *Antrodia cinnamomea* based health food product Li Te *Antrodia cinnamomea* (LACC) showed no mutagenic activity in the bacterial reverse mutation Ames test, also did not induce micronuclei in mammalian erythrocytes or increase the rates of structural and numerical chromosome aberration of CD mice. 14 days acute and

90 days oral toxicity studies with LACC in rats (LD_{50} is greater than 5 g/kg b.w and there was no evident toxicity at 2500 mg/kg/day) confirm in part, safety of LACC for oral consumption. Based on the results of reproductive and developmental toxicity study, there were no observable segment II reproductive and developmental evidences of LACC. Moreover, there was no observable adverse effect dose level (NOAEL) under the conditions of this study was 2500 mg/kg. Taken together, the present studies demonstrate that LACC has a very low order of toxicity, which supports the safety of LACC for human consumption.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975): Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat. Res., **31**, 347-364.

Ao, Z.H., Xu, Z.H., Lu, Z.M., Xu, H.Y., Zhang, X.M. and Dou, W.F. (2009): Niuchangchih (*Antrodia camphorata*) and its potential in treating liver diseases. J. Ethnapharmacol., **121**, 194-212.

Chang, J.B., Wu, M.F., Lu, H.F., *et al*. (2013): Toxicological evaluation of *Antrodia cinnamomea* in BALB/c mice. In Vivo, **27**, 739-745.

Chen, T.I., Chen, C.W., Lin, T.W., Wang, D.S. and Chen, C.C. (2011): Developmental toxicity assessment of medicinal mushroom *Antrodia cinnamomea* T.T. Chang et W.N. Chou (higher Basidiomycetes) submerged culture mycelium in rats. Int. J. Med. Mushrooms, **13**, 505-511.

Chung, C.H., Yeh, S.C., Chen, C.J. and Lee, K.T. (2014): Coenzyme Q0 from *Antrodia cinnamomea* in submerged cultures induces reactive oxygen species-mediated apoptosis in A549 human lung cancer cells. Evid. Based Complement. Alternat. Med., **2014**, 246748.

Cope, R.B., Kacew, S. and Dourson, M. (2015): A reproductive, developmental and neurobehavioral study following oral exposure of tetrabromobisphenol A on Sprague-Dawley rats. Toxicology, **329**, 49-59.

Geethangili, M. and Tzeng, Y.M. (2011): Review of pharmacological effects of *Antrodia camphorata* and its bioactive compounds. Evid. Based Complement Alternat. Med., **2011**, 212641.

Liu, Y.W., Lu, K.H., Ho, C.T. and Sheen, L.Y. (2012): Protective effects of *Antrodia cinnamomea* against liver injury. J. Tradit. Complement. Med., **2**, 284-294.

Lu, M.C., El-Shazly, M., Wu, T.Y., *et al*. (2013): Recent research and development of *Antrodia cinnamomea*. Pharmacol. Ther., **139**, 124-156.

Weijl, N.I., Cleton, F.J. and Osanto, S. (1997): Free radicals and antioxidants in chemotherapy-induced toxicity. Cancer Treat Rev., **23**, 209-240.

Yang, H.L., Lin, K.Y., Juan, Y.C., *et al*. (2013): The anti-cancer activity of *Antrodia camphorata* against human ovarian carcinoma (SKOV-3) cells *via* modulation of HER-2/neu signaling pathway. J. Ethnophamacol., **148**, 254-265.

Yue, P.Y., Wong, Y.Y., Chan, T.Y., Law, C.K., Tsoi, Y.K. and Leung, K.S. (2012): Review of biological and pharmacological activities of the endemic Taiwanese bitter medicinal mushroom, *Antrodia camphorata* (M. Zang et C. H. Su) Sh. H. Wu *et al*. (higher Basidiomycetes). Int. J. Med. Mushrooms, **14**, 241-256.