Genotoxic, teratotoxic and oral toxic assessments of Antrodia cinnamomea health food product (Leader Deluxe Antrodia cinnamomea®)

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ABSTRACT

Antrodia cinnamomea is a rare and endemic medicinal mushroom native to Taiwan. The pharmacological effects of Antrodia cinnamomea have been extensively studied. The aim of the present study was to assess the genotoxic, oral toxic and teratotoxic effects of Antrodia cinnamomea health food product “Leader Deluxe Antrodia cinnamomea (LDAC)” using in vitro and in vivo tests. The Ames test with 5 strains of Salmonella typhimurium showed no signs of increased reverse mutation upon exposure to LDAC up to concentration of 5 mg/plate. Exposure of Chinese Hamster Ovary cells (CHO-K1) to LDAC did not produce an increase in the frequency of chromosomal aberration in vitro. In addition, LDAC treatment did not affect the proportions of immature to total erythrocytes and the number of micronuclei in the immature erythrocytes of ICR mice. Moreover, 14-days single-dose acute toxicity and 90-days repeated oral dose toxicity tests with rats showed that no observable adverse effects were found. Furthermore, after treatment with LDAC (700–2800 mg/kg/day) there was no evidence of observable segment II reproductive and developmental toxic effects in pregnant SD rats and their fetuses. These toxicological assessments support the safety of LDAC for human consumption.

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1. Introduction

Antrodia cinnamomea (AC) is a unique medicinal mushroom, which is rare and endemic to Taiwan. In traditional Chinese medicine, this mushroom was used to treat various illnesses including food poisoning, drug intoxication, abdominal pain, hypertension, diarrhea, skin irritation and liver and tumorgenic diseases [2,8]. After being used as a traditional Chinese medicine, AC is now believed one of the most liver protecting natural products in Taiwan. Recent studies have indicated that the pharmacological application of this mushroom goes beyond traditional knowledge [10]. An increasing number of studies support the contention that AC possesses various pharmacological effects including anti-oxidant, anti-inflammatory, anti-cancer, anti-metastatic, anti-hyperlipemic, anti-diabetic, hepatoprotective, neuro-protective, cardio-protective and immunomodulatory effects [8,9,10,14]. The potent pharmacological effects of AC have been attributed to its high content of bioactive components such as terpinoids, benzenoids, benzoquinone derivatives, maleic/succinic acid derivatives, lignans, polysaccharides, sterols, nucleotides and fatty acids [10,14]. Predominantly these bioactive compounds, especially triterpinoids, are found in the fruiting bodies [8]. Therefore, demand for the fruiting bodies of AC has far exceeded the supply. However, to compensate the demand, researchers developed other techniques for the mass production such as wood or solid-state cultivation and liquid or submerged cultivation.

Allied to these potential health benefits, AC is widely used as a health food supplement in Taiwan and available in the form of powder, tablet and capsules. To this end, the safety issues of AC must be examined. Previous studies have indicated that AC
products are of low oral toxicity, with an oral medial lethal dose (LD50) >1.5 g/kg body weight in CD mice [5]. Although hundreds of AC products are sold, only three products have been awarded a “National Health Food” certification by Taiwan’s Department of Health. For this study, we selected one certified AC product, Leader Deluxe Antrodia cinnamomea (LDAC), as study material. To assess the safety of LDAC, the present study performs genotoxic, oral toxicity and teratogenicity assessments of LDAC.

Genotoxicity studies of herbal products are of great interest because of the widespread and long-term use of herbal remedies, as well as health food supplements [11]. Many herbal products contain compounds known to cause severe side effects or even death in animals and humans. In addition, many natural products have been reported to act as a mutagens and/or carcinogens. A variety of in vitro genotoxicity test systems were developed including the bacterial reverse mutation test (Ames test), cultured mammalian cell systems such as Chinese hamster ovary (CHO) cells or human peripheral blood lymphocytes (PBL). The in vitro rodent bone marrow erythrocyte micronucleus test is the most widely used short-term test to identify the genotoxic effects of test samples such as chromosomal aberration and aneuploidy.

In the present study, the mutagenic potential of LDAC was evaluated by a bacterial reverse mutation assay, while genotoxicity was examined by a mammalian chromosomal aberration test and mammalian erythrocyte micronuclei test using CHO-K1 cells and rat bone marrow erythrocytes, respectively. Acute and repeated dose oral toxicity studies were performed with rats. The reproductive and developmental assessment was carried out using pregnant female rats.

2. Materials and methods

2.1. Test substance

The health food supplement LDAC was manufactured by Taiwan Leader Biotech Corp, Taipei City, Taiwan. LDAC powder consists of 5% extract of fruiting bodies from cut-log cultivation, 94% mycelium from high-efficient solid state cultivation of A. cinnamomea and 1% magnesium stearate.

2.2. 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-aminoacridine, histidine, biotin, Giemsa stain, acridine orange, cyclophosphamide and Methylthiazolyl diphenyl-tetrazolium bromide (MTT) were purchased from Sigma–Aldrich, St. Louis, MO. Colcemid was obtained from Life Technologies, Carlsbad, CA.

2.3. Bacterial reverse mutation test (Ames test)

The histidine-requiring Salmonella typhimurium strains TA98, TA100, TA102, TA1535 and TA1537 were obtained from Molecular Toxicology Inc., Bonne, NC. The genotypes of the bacterial strains were confirmed by histidine mutation, fra mutation, ΔuvrB repair and ampicillin resistance before the assay.

Prior to the assay, a dose range finding test was performed with LDAC (0.313–5 mg/plate) in the TA100 strain. A plate incorporation assay was employed and performed to detect reverse mutation in bacterial strains [11]. Briefly, 0.05 mL of aqueous solution of LDAC (0.013, 0.025, 0.125, 0.25 and 5 mg/plate) was mixed with 0.1 mL of overnight culture of S. typhimurium strains (2 × 10^8 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 SD rat liver S9 (Molecular Toxicology Inc) 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 ± 1 °C before transferring to minimal glucose agar plates. The solidified agar plates were inverted and incubated at 37 ± 1 °C for 48–72 h. Then the colonies were counted. Double distilled water was served as negative control, whereas for positive controls, test substance and corresponding concentrations were summarized in Table 1.

2.4. Mammalian chromosomal aberration test

For the in vitro chromosomal aberration test, the Chinese hamster ovary cell line (CHO-K1) was obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). CHO-K1 cells 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% CO2 at 37 ± 1 °C. LDAC was dissolved in the culture media at a concentration of 10 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. 5 mg/mL was used as the highest dose for cytotoxicity assay. The other testing doses were serial dilutions of 5 mg/mL to give 0.313, 0.625, 1.25, 2.5 and 5 mg/mL. 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 h before treatment. LDAC and controls were administered in three test systems. For short-term treatment, test samples were exposed for 3 h followed by a recovery period of 6 h. For metabolic activation, test samples were exposed with S9 for 3 h. For long-exposure, the test samples were kept in culture for 22 h 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). The morphology of the cells was observed and recorded by microscope at 100 × magnification. In parallel, specimens were prepared for the chromosomal aberration test. In brief, 100 ng/mL of Colcemid solution was added to the culture and incubated for 2 h. Cells were harvested, 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 (ece), chromatid breakage (ctb), chromatid exchange (cte), and other abnormalities such as polyploidy; these were scored and recorded by photographing.

2.5. Animals

Seven week old male and female ICR mice and 6–8 week old male and female Sprague-Dawley CD (SD) IGS 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 ± 2 °C, relative humidity 55 ± 20%, and lighting was 12 h per day. Autoclaved reverse osmosis (RO) treated water was supplied ad libitum and laboratory rodent diet (LabDiet, PMI Nutrition International, Richmond, IN) supplied for all animals. The bedding was composed of coarse grade Aspen Chip (Northeastern Products Corp, Warrens Burg, NY) and was changed weekly.

2.6. Mammalian micronucleus test

The micronucleus test was performed following the Organization for Economic Cooperation and Development (OECD) guidelines. Positive control group mice were administered 80 mg/kg cyclophosphamide via intraperitoneal injection (using a 10 mg/mL solution dosed at 8 mL/kg b.w.); the test sample groups received LDAC orally at 700, 1400 or 2800 mg/kg (using 70 mg, 140 and 280 mg/mL in water and dosed at 10 mL/kg b.w) and a negative control group received sterile water at 10 mL/kg. All doses were administered using a stainless steel feeding needle. Mice were monitored daily for any post-treatment clinical symptoms, and their body weight was noted before and after treatment. 48 and 72 h post-treatment, peripheral blood samples (2–3 µL) were obtained from the tail vein and smeared on acridine orange coated microscopic slides. The smeared slides were incubated at room temperature for 2–3 h prior to 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).

2.7. Acute oral toxicity study

An acute oral toxicity (14 day) study was performed to examine the possible adverse effects of the test sample LDAC in rats via oral administration. After acclimatization for 6 days, 48 rats were divided into 4 groups (Group I–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–IV received 1400, 2800 and 5600 mg/kg b.w LDAC 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 h) prior to dosing. The animals in each group were dosed twice on Day 1 with the control or LDAC. The second dose was performed within 6 h of the first. 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 morbidity were recorded every 12 h interval. All rats were observed individually for any clinical signs at 0–4 h 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 CO2 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.

2.8. Repeated dose 90-day oral toxicity study

A repeated dose toxicity (90 days) study was conducted to evaluate the possible health hazards likely to arise from repeated exposure of LDAC in rats via oral administration in accordance with OECD guidelines. After acclimatization for a week, 96 rats were divided into 4 groups (Group I–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–IV were sample treatment groups and received 700, 1400 and 2800 mg/kg b.w, respectively in a volume of 10 mL/kg b.w in water. All animals in each group were dosed once a day for 90 days and they 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. Cornea, conjunctiva, anterior chamber, iris, and lens were examined by an ophthalmoscope. 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 90-day dosing period. On the necropsy day, blood samples were obtained from the abdominal aorta and collected into three tubes: (1) containing K2 EDTA 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 h 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 necropsy 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.

2.9. 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. Sixty male virgin CD (SD) IGS rats and 120 female virgin rats were purchased from BioLasco, Taiwan. Animals were individually identified by ear notch. After acclimatization for a week, individual breeding pairs were co-habited overnight in a suspended stainless steel cage. Notably, in first set, 60 male rats were co-habited with 60 female rats and the second set, the same 60 male rats were again co-habited with another 60 female rats. After mating, the female rats were transferred to polycarbonate cages. The mating was confirmed with vaginal sperm and/or vaginal copulation plug [7] and was designated as gestation day 0 (G 0). Confirmed-mated females were
assigned to the four study groups. Eighty confirmed pregnant female rats were divided into 4 groups (Group I–IV) 20 rats in each group at least. Group 1 served as a control group received sterile water in a volume of 10 ml/kg b.w, whereas Group II–IV were LDAC treated groups and received 700, 1400 and 2800 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 morbidity 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, G3, G6, G9, G12, G15, G18 and G20. Feeding and water consumption was monitored during study period. On G20, rats were euthanized by CO2 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.

2.10. 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). Heterogeneous data were analyzed with the Kruskal–Wallis non-parametric ANOVA method. Probability of 0.05 (p < 0.05) was used as the significance criterion.

3. Results and discussion

3.1. Bacterial reverse mutation test

Initially we validated the genotypes of five S. typhimurium bacterial strains (TA98, TA100, TA102, TA1535 and TA1537) including histidine mutation, rfa mutation and uvrB repair (Table 2). TA98 and TA100 possessed all endogenous characteristics, whereas TA102 had no mutation on uvrB (ΔuvrB). TA1535 and TA1537 contained no plasmid that rendered ampicillin and tetracycline resistance, which were all consistent with previous reports [5,15]. Next, the cytotoxicity and range finding study suggest that the LDAC is not toxic to the bacterial strain TA100 at dose of 0.313 to 5 mg/plate (Table S1). Thus, we set these doses 0.313, 0.625, 1.25, 2.5 and 5 mg/plate are test doses and performed Ames test with five bacterial strains. As shown in Table 3, compared to the negative control groups (sterile water), the positive control substances induced generally at least 3-fold increase of the number of reverse mutation colonies, confirming the validity of the test. The mean number of revertant colonies of the negative control was within the historical range. Moreover, we found that LDAC did not increase the mean number of reverse mutation at dose levels between 0.313 and 5 mg/plate in both normal and metabolically activated bacterial strains (Table 3). These results suggest that LDAC does not induce bacterial reverse mutation within the test doses.

3.2. Mammalian chromosome aberration test

A. cinnamomea has been reported to be a potent anti-tumor agent [10]. Most of the antitumor 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 [12]. Extracts of A. cinnamomea or its derived compounds induce apoptosis in cancer cells through reactive oxygen species (ROS) generation following DNA damage [6,13]. Thus, prior to the in vitro assay, the cytotoxic effect of LDAC on CHO-K1 cells was examined by MTT assay. According to the results of MTT assay (Table S2), the cell viability was markedly decreased to the highest dose of LDAC (5 mg/mL). After exposure of CHO-K1 cells to LDAC for 3 h in the absence of S9 metabolic activation, the viability of was decreased to 94.71 ± 1.00%, 83.49 ± 2.81%, 89.76 ± 2.84%, 83.57 ± 1.99% and 39.96 ± 4.55% by 0.313, 0.625, 1.25, 2.5 and 5 mg/mL LDAC, respectively. The positive control mitomycin C (0.5 μg/mL) showed 95.78 ± 1.44% of cell viability after 3 h in the absence of S9 metabolic activation. In addition, exposure of CHO-K1 cells to LDAC for 3 h in the presence of S9 metabolic activation, the cell viability of LDAC was found as 124.22 ± 1.74%, 111.86 ± 2.25%, 90.20 ± 7.90%, 79.54 ± 4.04% and 58.70 ± 8.88% by 0.313, 0.625, 1.25, 2.5 and 5 mg/mL. The positive control benzo(a) pyrene at 25 μg/mL showed 86.19 ± 4.82% of cell viability after 3 h in the presence of S9. Moreover, exposure of CHO-K1 cells to LDAC for 18 h in the absence of S9 metabolic activation cell viability was recorded as 81.76 ± 5.94%, 83.53 ± 5.37%, 63.10 ± 4.50%, 48.62 ± 4.07% and 27.36 ± 1.29% by 0.313, 0.625, 1.25, 2.5 and 5 mg/mL. After 18 h exposure of mitomycin (0.5 μg/mL) without S9 mixture showed 94.18 ± 4.15% of cell viability. Based on the results of the viability test, 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 h treatment group without S9 and those used in the 3 h treatment group with S9 were 1.25, 2.5 and 5 mg/mL. In the 18 h treatment group without S9, the doses used in the chromosome aberration test were 0.313, 0.625 and 1.25 mg/mL.

The result of the chromosome aberration test are summarized in Table 4. The number of cells with chromosome aberration observed in the negative control group under different test schemes were
Table 2
Genotyping of the test of Salmonella typhimurium bacterial strains.

| Strains   | Histidine requirement | ΔuvB             | rfa   | Ampicillin | Tetracycline | Spontaneous Revertants |
|-----------|-----------------------|------------------|-------|------------|--------------|------------------------|
|           | His+ Bio+ plate       | His- Bio+ plate  | Mutation | Mutation | Resistance | Resistance | Revertants |
| TA98      | +                     | –                | –      | +         | +           | –                      | 34.7 ± 4.0 |
| TA100     | +                     | –                | –      | +         | +           | –                      | 142.0 ± 12.1 |
| TA102     | +                     | –                | +      | +         | +           | +                      | 318.0 ± 18.3 |
| TA1535    | +                     | –                | –      | +         | +           | –                      | 14.0 ± 2.0 |
| TA1537    | +                     | –                | –      | +         | +           | –                      | 14.3 ± 4.2 |

Table 3
Results of bacterial reverse mutation test.

| Group      | Number of revertants/plate (without S9 activator) | Number of revertants/plate (without S9 activator) |
|------------|--------------------------------------------------|--------------------------------------------------|
| (mg/plate) | TA98                                             | TA100                                            |
|            | TA102                                            | TA1535                                          |
| Negative   | 34.7 ± 5.0                                       | 173.0 ± 8.7                                     |
|            | 334.0 ± 24.0                                     | 8.3 ± 1.2                                        |
|            | 11.3 ± 4.0                                       | 34.0 ± 4.4                                       |
|            | 141.7 ± 18.1                                     | 377.3 ± 26.0                                     |
| Positive   | 224.7 ± 40.6**                                  | 624.7 ± 2.0                                     |
| LDAC       | 1376.0 ± 144.2*                                 | 402.7 ± 48.4*                                   |
| 5          | 96.3 ± 16.2                                       | 965.3 ± 53.3*                                   |
| 2.5        | 6.0 ± 3.0                                        | 1027.2 ± 14.6                                   |
| 1.25       | 10.0 ± 3.0                                       | 348.7 ± 15.1                                    |
| 0.625      | 12.3 ± 6.0                                       | 8.3 ± 1.5                                        |
| 0.313      | 21.0 ± 4.4                                       | 17.3 ± 6.0                                       |
|            | 341.3 ± 54.9                                     | 16.2*                                            |
|            | 188.0 ± 19.5                                     | 565.3 ± 63.5*                                   |
| 1.02       | 618.0 ± 8.5                                      | 610.7 ± 90.7*                                   |
| 1.62        | 317.2 ± 7.6                                      | 840.0 ± 28.8*                                   |
| 3.28       | 118.0 ± 8.5                                      | 150.3 ± 9.8*                                    |
| 3.64       | 356.7 ± 58.5                                     | 616.0 ± 63.5*                                   |
| 0.36       | 17.6 ± 5.0                                       | 8.3 ± 1.5                                        |
| 0.42       | 36.8 ± 7.4                                       | 19.7 ± 6.7                                       |
| 0.36       | 134.7 ± 6.2                                      | 21.7 ± 2.5                                       |

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

Table 4
Effect of LDAC on mammalian chromosome aberration in cultured CHO-K1 cells.

| Treatment period (h) | Metabolic Test sample | Aberration frequency<sup>a</sup> | p value<sup>b</sup> |
|----------------------|-----------------------|----------------------------------|---------------------|
| Short-term treatment (3h) Without S9 | Negative control | 1/200 | – |
|                      | Mitomycin C (0.5 µg/mL) | 20/200 | 0.0000* |
|                      | LDAC (0.625 mg/mL) | 1/200 | 0.7358 |
|                      | LDAC (1.25 mg/mL) | 3/200 | 0.1991 |
|                      | LDAC (2.5 mg/mL) | 3/200 | 0.1991 |
|                      | Negative control | 3/200 | – |
|                      | Benzo(a) pyrene (25 µg/mL) | 15/200 | 0.0002* |
|                      | LDAC (1.25 mg/mL) | 3/200 | 0.6472 |
|                      | LDAC (2.5 mg/mL) | 3/200 | 0.6472 |
|                      | LDAC (5 mg/mL) | 3/200 | 0.6472 |
| Long term treatment (18h) Without S9 | Negative control | 3/200 | – |
|                      | Mitomycin C (0.5 µg/mL) | 29/200 | 0.0000* |
|                      | LDAC (0.313 mg/mL) | 4/200 | 0.4335 |
|                      | LDAC (0.625 mg/mL) | 3/200 | 0.6472 |
|                      | LDAC (1.25 mg/mL) | 2/200 | 0.8571 |

<sup>a</sup> The aberration frequency was displayed in the manner of number of cells with chromosome aberration in 200 observed metaphase cells (n=200).

<sup>b</sup> The statistical analysis was performed by Poisson distribution in comparison with negative control. The "*" represents the statistical significance (p < 0.05).

Table 5
Effect of LDAC on percentage of PCE in erythrocytes and micronucleus frequency in PCE.

| Treatment group | PCE% (mean ± S.D, n=5–6) | MN%:PCE (mean ± S.D, n=5–6) |
|----------------|----------------------------|----------------------------|
|                | Female                     | Male                      | Female                     | Male                      |
|                | 48h                         | 72h                        | 48h                         | 72h                        |
| Neg. control (sterile water) | 3.23 ± 0.36           | 3.3 ± 0.14              | 3.67 ± 0.36           | 3.53 ± 0.41              |
|                | 0.17 ± 0.26           | 0.42 ± 0.38              | 0.67 ± 0.68          | 0.50 ± 0.63              |
| Cyclop. (80 mg/kg b.w)   | 3.45 ± 0.33          | 3.3 ± 0.25              | 4.04 ± 0.38          | 3.76 ± 0.21              |
|                | 0.40 ± 0.42          | 0.40 ± 0.42              | 0.40 ± 0.42          | 0.40 ± 0.42              |
| LDAC (700 mg/kg b.w)    | 3.22 ± 0.08          | 3.42 ± 0.28             | 3.52 ± 0.29          | 3.64 ± 0.17              |
|                | 0.30 ± 0.45          | 0.50 ± 0.50              | 0.40 ± 0.42          | 0.50 ± 0.50              |
| LDAC (1400 mg/kg b.w)   | 3.7 ± 0.51           | 3.32 ± 0.18             | 3.72 ± 0.33          | 3.58 ± 0.19              |
|                | 0.40 ± 0.42          | 0.30 ± 0.45              | 0.30 ± 0.45          | 0.20 ± 0.27              |

within the historical data of this laboratory. The number of cells with chromosome aberration in 200 observed metaphase cells in the positive control, mitomycin C (0.5 µg/mL), was 20, 15, and 29 under the three test Schemes 3 h without S9, 3 h with S9 and 18 h without S9, respectively, which was significantly (p < 0.05) increased compared to that of the negative controls. The chromosome aberrations in LDAC treated cells were 1, 3 and 3 at 0.625, 1.25 and 2.5 mg/mL under 3 h without S9 and 3, 3 and 3 at 1.25, 2.5 and 5 mg/mL, respectively under 3 h with S9 metabolic activation. Moreover, the chromosome aberration in 200 observed metaphase cells were 4, 3 and 2 by 0.313, 0.625 and 1.25 mg/mL LDAC, respectively under 18 h without S9 metabolic activation. In summary, data indicate that exposure to LDAC does not significantly induce chromosome aberration in cultured mammalian somatic cells under the test conditions.

3.3. Mammalian micronucleus test

Besides the possible use of LDAC 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 LDAC resulted in chromosome damage
Table 6
Effect of repeated oral dose (90 days) of LDAC on rats: Clinical observation.

| Clinical sign | Male Study period | Control(W/F) | LDAC (mg/kg b.w) | Female Study period | Control(W/F) | LDAC (mg/kg b.w) |
|---------------|------------------|--------------|-----------------|---------------------|--------------|-----------------|
|               |                  | 700          | 1400            | 2800                | 700          | 1400            | 2800            |
| Wound         | Day 27–30        | 1/12         | 0/12            | 0/12                | Day 1–12     | 0/12           | 0/12            | 0/12            |
|               | Day 31–41        | 0/12         | 0/12            | 0/12                | Day 13–18    | 0/12           | 0/12            | 0/12            |
|               | Day 42–43        | 1/12         | 0/12            | 0/12                | Day 19–22    | 0/12           | 0/12            | 0/12            |
|               | Day 44–57        | 0/12         | 0/12            | 0/12                | Day 23–24    | 0/12           | 0/12            | 1/12            |
|               | Day 58–63        | 0/12         | 0/12            | 0/12                | Day 25–36    | 0/12           | 0/12            | 0/12            |
|               | Day 64–75        | 0/12         | 0/12            | 0/12                | Day 37–50    | 0/12           | 0/12            | 0/12            |
|               | Day 76–81        | 0/12         | 0/12            | 0/12                | Day 51–80    | 0/12           | 0/12            | 0/12            |
|               | Day 82–84        | 0/12         | 0/12            | 0/12                | Day 81–92    | 0/12           | 0/12            | 0/12            |
|               | Day 85–92        | 0/12         | 0/12            | 0/12                | 0/12         | 0/12           | 0/12            | 0/12            |
| Hair loss     | Day 27–30        | 0/12         | 0/12            | 0/12                | Total incidence (n/n)^2 | 0/12         | 0/12            | 0/12            |
|               | Day 31–41        | 0/12         | 0/12            | 0/12                | Day 1–12     | 0/12           | 0/12            | 0/12            |
|               | Day 42–43        | 1/12         | 0/12            | 0/12                | Day 13–18    | 0/12           | 0/12            | 0/12            |
|               | Day 44–57        | 0/12         | 0/12            | 0/12                | Day 23–24    | 0/12           | 0/12            | 0/12            |
|               | Day 58–63        | 0/12         | 0/12            | 0/12                | Day 25–36    | 0/12           | 0/12            | 0/12            |
|               | Day 64–75        | 0/12         | 0/12            | 0/12                | Day 37–50    | 0/12           | 0/12            | 0/12            |
|               | Day 76–81        | 1/12         | 0/12            | 0/12                | Day 51–80    | 0/12           | 0/12            | 0/12            |
|               | Day 82–84        | 0/12         | 0/12            | 0/12                | Day 81–92    | 0/12           | 0/12            | 0/12            |
|               | Day 85–92        | 1/12         | 0/12            | 0/12                | Total incidence (n/n)^2 | 0/12         | 0/12            | 0/12            |

^1n/n: Number of animals with observable sign/Number of animals alive.
^2n/n: Total number of animals with observable sign/Total number of animals examined.

Table 7
Effect of repeated oral dose (90 days) of LDAC on rats: Hematological findings.

| Parameters        | MaleLDAC (mg/kg b.w) | FemaleLDAC (mg/kg b.w) |
|-------------------|----------------------|------------------------|
|                   | Control 700 1400 2800| Control 700 1400 2800  |
| WBC (10^3/μL)     | 9.45±2.28 9.34±1.80 8.63±1.56 | 10.25±2.02 |
| RBC (10^6/μL)     | 9.22±0.41 9.33±0.33 9.89±0.35 | 9.12±0.51 |
| HGB (g/dL)        | 16.33±0.19 16.43±0.14 15.99±0.59 | 15.98±0.72 |
| HCT (%)           | 45.08±1.49 45.23±1.10 44.03±1.52 | 44.25±1.95 |
| MCV (fL)          | 48.91±1.81 48.52±1.30 49.05±2.03 | 48.58±2.45 |
| MCH (pg)          | 17.71±0.48 17.62±0.35 17.72±0.54 | 17.53±0.71 |
| MCHC (g/dL)       | 36.19±0.49 36.31±0.38 36.13±0.55 | 36.13±0.52 |
| PLT (10^3/μL)     | 116.6±123.6 113.4±192.1 113.1±109.5 | 1173.5±143.1 |
| NEUT (%)          | 21.72±0.60 25.32±8.43 23.52±8.04 | 19.30±5.33 |
| LYMPH (%)         | 73.18±6.78 69.93±8.73 71.79±8.50 | 76.32±5.37 |
| MONO (%)          | 4.76±1.23 4.41±0.70 4.37±1.03 | 4.00±1.17 |
| EOSIN (%)         | 0.28±0.15 0.30±0.39 0.29±0.16 | 0.32±0.28 |
| BASO (%)          | 0.06±0.07 0.05±0.05 0.03±0.05 | 0.07±0.05 |
| PT (sec)          | 12.12±1.92 12.84±1.74 13.78±2.03 | 13.31±1.91 |
| APTT (sec)        | 16.97±1.12 15.68±2.58 17.13±2.27 | 17.09±0.88 |

*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.

in mice using an in vivo micronucleus test. All test animals were divided into five groups which received negative control (sterile water), positive control (80 mg/kg.b.w cyclophosphamide), or LDAC at dose levels of 700, 1400 or 2800 mg/kg.b.w. After 96 h post-treatment, no mortalities were recorded, and gross necropsy of the animals revealed no macroscopic findings. The mean body weight was analyzed with t-test showed no significant difference between three doses of LDAC and control groups in both genders (Table S3).

The percentage of PCE and micronuclear frequency were analyzed 48 and 72 h post-treatment of control and test sample (Table 5). The percentage of positive control groups at 48 h were 0.60 ± 0.36% in female and 1.02 ± 0.19% in male. The percentage of PCE in positive control was significantly decreased after dosing, which indicates that cyclophosphamide inhibits erythropoiesis. However, the percentage of PCE in LDAC treated groups showed no significant decrease than negative control group, suggest that all the testing doses of LDAC did not affect erythropoiesis. We further examined the micronucleus frequency in 1000 PCE using fluorescence microscope and the data was summarized in Table 5. The micronuclear frequency in 1000 PCE of negative control group at 48 and 72 h were 0.17 ± 0.26 and 0.42 ± 0.38 %, PCE in female, 0.67 ± 0.68 and 0.50 ± 0.63 %, PCE in male, respectively. The micronuclear frequency of 1000 PCE of positive control group at 48 h were 23.80 ± 12.09 in female and 28.80 ± 5.62 %, PCE in male. After statistical analysis with Poisson distribution methods, there was no significant difference between three testing doses of LDAC and negative control group in both genders at 48 and 72 h. Based on
these observations, we conclude that all the testing doses of LDAC does not increase micronucleated PCE in the test condition.

3.4. Acute (14-day) oral toxicity study

Exposure of animals to the tested doses of LDAC (1400, 2800 and 5600 mg/kg b.w) produced neither deaths nor treatment-related signs of toxicity in any of the treatment groups during the study. In addition, no weight loss resulted from the LDAC treatment compared to the control groups in both genders after 1, 4, 8 and 15 days. Data of individual animal body weight and mean body weight are summarized in Table S4. Moreover, there were no abnormal clinical findings from external observations which were attributable to LDAC treatment. However, animals in Group IV showed slight hair loss during the study, which may be caused by the animal fightings which was not related to the LDAC 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 LD50 of LDAC 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.

3.5. Repeated dose (90 days) oral toxicity study

The repeated oral dose (90 days) toxicity study showed that there were no mortalities or ophthalmologic and treatment related signs of toxicity were observed during the study period in any of the treatment groups (Table S5). No statistically significant mean body weight and mean body weight gain in both genders were reported between the test and control groups (Table S6 and S7 respectively). 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 6). However, some clinical signs were observed due to housing behavior (wounds) or individual animal differences (hair loss). In male rats, wound and hair loss
were observed one in 12 rats of vehicle control group. In female rats, wound was observed in 1 in 12 rats of highest LDAC (2800 mg/kg) treated group, and hair loss was found in one in 12 rats of 700 mg/kg LDAC treated group and two in 12 rats of 2800 mg/kg treated group (Table 6).

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 LDAC. Particularly, the hematocrit of the high-dose group (2800 mg/kg) was statistically (p < 0.05) lower than that of vehicle control group (Table 7). Results from individual animal serum chemistry analysis showed some statistically significant differences in both genders as summarized in Table 8. In male rats, the glucose levels in high-dose (2800 mg/kg) treated group was statistically higher than that of control group. The total protein level in the 1400 mg/kg/day LDAC treated group was significantly lower than vehicle control group. In female rats, compared to vehicle control group a significant increase of total cholesterol in 1400 and 2800 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 (2800 mg/kg) treated rats were statistically lower than vehicle control group in both genders (Table S8). 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 liver weight of those male animals in the highest dose (2800 mg/kg) group (Table 9). This difference between control (13.18 ± 1.36g) and high-dose treatment group included the increased weight after 90 days in male (16.01 ± 1.76g). This statistical difference was within normal historical control range and without physiological abnormalities. In females, there was no statistical difference between the vehicle control and LDAC treated groups (Table 9).

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 S9). However, one female animal of vehicle control group was observed to have bone marrow cavity discoloration in the femur, moderate enlargement of spleen and liver. Histopathologically, myeloid hyperplasia of mononuclear cells was noted in the liver, spleen and bone marrow in femur. 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 LDAC exposure (Table S10).

3.6. Reproductive and developmental toxicity assessment

After co-habitation of male and female animals over-night, 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 numbers of animals with impregnation verified in the control and groups given 700, 1400 or 2800 mg/kg b.w. during the study period were 26/29, 25/28, 22/28 and 27/29, respectively. The pregnant animal numbers verified at necropsy in the control and 700, 1400 and 2800 mg/kg LDAC treatment groups were 26, 25, 22 and 27, respectively. The confirmed pregnancy rats were high (80–90%) for all groups. There was no maternal mortality or moribundity found in this study. The maternal body weight was measured in gestation period (G0, G3, G6, G9, G12, G15, G18, G20 and G20 without uterus weight). There was no statistically significant maternal body weight and 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).

Clinical observation was performed once daily during gestation period (G0–G20). Slight to moderate hair loss was observed at various sites in vehicle control, 700 and 1400 mg/kg LDAC treatment groups with the incidence of 2/26, 1/25 and 2/22, respectively Table S11. This clinical sign was considered to be caused by nesting behavior and is commonly observed in pregnant animals and so not related to LDAC administration.
Results of maternal evaluation showed that there were no statistical significant differences between the groups observed in gravid uterus weight, implantation number, corpora lutea number, litter size, live or dead fetal number, male or female fetus number, resorption number, fetal sex ratio (M/F), pre-implantation loss and post-implantation loss (Table S12).

Results from fetal examination showed that there was no statistical significance noted in fetal body weight and body length among the test groups. However, in the gross examination of the fetal appearance, one fetus from 1400 mg/kg LDAC treatment group showed craniarachischisis, exencephaly and open eyelid (Table 10). This finding meet the prevalence with low incidences in normal SD rat population. Fifty percent of each litter were allocated to visceral examination (Table 10). There was no dose response significance noted in the incidence of abnormalities. The observed abnormalities included irregular shape of brain, cerebral ventricular enlargement, asplenia, diaphragmatic hernia, distended renal pelvis, hydroureters and distended ureter. However, the incidences were low and all within historical reference range. Skeletal examination of 50% fetuses of each litter was performed with Alizarin Red S and Alcian Blue staining. LDAC treatment showed no statistically significant incidences or abnormalities in tested fetuses. The fetal skeletal examination suggest that there was no LDAC (700–2800 mg/kg) treatment related abnormalities and LDAC related teratogenic toxicity. 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 [4]. However, the highest oral dose of the present study was 5-fold higher than the previous report.

4. Conclusion

LDAC showed no mutagenic activity in the bacterial reverse mutation test, did not induce micronuclei in mammalian erythrocytes or increase the rates of structural and numerical chromosome aberration of CD mice. The results of acute (14 days) and repeated (90 days) oral toxicity studies of LDAC in rats (LD50 is greater than 5 g/kg b.w and there was no evident toxicity at 2800 mg/kg/day) confirm in part, safety of LDAC for oral consumption. Based on the results of reproductive and developmental toxicity study, there were no observable segment II reproductive and developmental evidences of LDAC. The no observable adverse effect dose level (NOAEL) under the conditions of this study was 2800 mg/kg. Taken together, the present studies demonstrate that LDAC has a very low order of toxicity, which supports the safety of LDAC for human consumption.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxrep.2015.10.007.

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