Evaluation of the Sub-Chronic Toxicity and Teratogenicity of *Antrodia cinnamomea* mycelia

Chun-Chih Huang [1], Mun-Kit Nam [2], Yueh-Ting Tsai [2], Alice Lan [1]*

[1]New Bellus Enterprises Co., LTD., Tainan City 72042, Taiwan
[2]Super Laboratory Inc., Taipei 24890, Taiwan
Corresponding author: alicelan.nb@gmail.com

Abstract: BACKGROUND: *Antrodia cinnamomea* (AC) is a medicinal mushroom used in folk medicine in Taiwan. AC is currently used in the formulation of nutraceuticals and functional foods. The cytotoxic and other effects of AC have been confirmed by various *in vitro* and *in vivo* assays. However, the general public in Taiwan has expressed concerns over the safety of AC consumption, and thus there is a need for more toxicological studies to evaluate its safety. In this study, AC mycelia were evaluated for sub-chronic toxicity and teratogenic effects in Sprague-Dawley (SD) rats. MATERIALS AND METHODS: For the sub-chronic toxicity study, the SD rats were divided into four different groups, control, low-dose AC (2 g/kg Body Weight (BW)/day), mid-dose AC (4 g/kg BW/day) and high-dose AC (6 g/kg BW/day) administered group. The animals were exposed with AC once daily for 90 days. Body weight (BW) analysis, food consumption were monitored at regular intervals. On 90 (th) day of the experiment, the rats were assessed for any ophthalmological abnormalities, and blood sample was collected for clinicochemical and hematological analysis. At the end of the experiment, all the animals were sacrificed for histopathological analysis. For the teratogenicity study, the pregnant rats were exposed with AC (0, 2, 4, and 6 g/kg BW/day) from day 6 of gestation to day 20. On the 20 (th) day of the gestation, the dams were euthanized and the gravid uteri were removed. The gravid uteri and individual fetuses were weighed. The fetuses were evaluated for skeletal malformations. RESULTS: There were no treatment-related effects on mortality or body weight, nor were there any ocular effects or effects on any hematology or clinical chemistry parameters in the sub-chronic toxicity study. No treatment-related abnormalities (gross and microscopic) were present at any dose level in both sub-chronic and teratogenicity studies. CONCLUSIONS: Based on these findings, the highest dose of 6 mg/kg BW/day was considered the no-observed-adverse-effect-level (NOAEL)
[Huang, CC, Nam, MK, Tsau YT, Lan A. **Evaluation of the Sub-Chronic Toxicity and Teratogenicity of Antrodia cinnamomea mycelia.** *Life Sci J* 2014;11(11):1090-1098]. (ISSN:1097-8135). http://www.lifesciencesite.com. 186

Keywords: *Antrodia cinnamomea*; sub-chronic toxicity; teratogenicity

1. Introduction

Antrodia cinnamomea (AC), known as "Niu-Chang-Chih" in Chinese, is a treasured Taiwanese medicinal mushroom. AC fruiting bodies have an orange and yellowish color, and grow in the rotting empty trunks of *Cinnamomum kanehirai*, the only host tree for AC. *C. kanehirai* is an evergreen tree, only native to Taiwan and growing at altitudes between 450m to 1200m (Liao et al. 2010). The traditional uses of this mushroom in folk and aboriginal medicine include relief for headache, diarrhea, abdominal pain, alcohol intoxication, skin itchiness and hypertension. The wild fruiting bodies can take up to a year to grow, and *C. kanehirai* has been listed as grade 1 conservation wood, which significantly limits the supply of wild AC fruiting bodies. These factors have pushed the AC market price to USD$15,000–25,000 per kg (Lu et al. 2013). Mycelia are an attractive alternative to meet the increasing demand for AC fruiting bodies, due to the significantly shorter cultivation time (~2 weeks compared with ~one year for fruiting bodies). More importantly, this could potentially reduce illegal logging and promote *C. kanehirai* conservation. AC contains more than seventy bioactive compounds. and major components include polysaccharides, benzenoids, benzoquinones, succinic and maleic acids, and triterpenoids (Geethangili and Tzeng 2011). Mounting evidence suggests that AC exhibits anti-inflammatory, anti-oxidant, hepatoprotective, vasorelaxative and cytotoxic activities (Chen et al. 2007; Hseu et al. 2005; Hsiao et al. 2003; Lee et al. 2002; Liu et al. 2004; Mau et al. 2004; Rao et al. 2007; Shen et al. 2004; Song and Yen 2002; Wang et al. 2003). AC extracts or isolated bioactive components have demonstrated cytotoxicity to human colon, liver, breast, prostate, lung, leukemia, glioblastoma, and melanoma cancer cell lines (Deng et al. 2009; Hseu et al. 2009; Hseu et al. 2007; Hseu et al. 2002; Hsu et al. 2005; Huang et al. 2012; Kuo et al. 2006; Lee et al. 2007; Nakamura et al. 2004; Rao et al. 2007; Yang et al. 2006; Yeh et al. 2009). Although they are potent in inhibiting the proliferation of cancer cells, such cytotoxicity was not seen in normal cells, such as HBL100breast cells and human umbilical vein endothelial cells (Hseu et

al. 2009; Yang et al. 2006). The current cultivation methods for AC include submerged fermentation, solid-support culture, basswood culture, and dish culture. Culture conditions, such as cultivation methods, temperature, pH, and time, can significantly affect the concentrations of bioactive components. The resulting varied phytochemical compositions can also lead to different results in toxicological studies. To date, there have been few toxicological reports on AC. In a 28-day sub-acute toxicity study, a dose of 2 g/kg BW/day showed minimal effects in sub-acute toxicity, and no genotoxicity in Ames mutagenicity tests, in vitro and in vivo chromosomal aberration tests (Chen et al. 2001). No significant toxicity was seen in a sub-chronic study, in which the rats were gavaged up to 3 g/kg BW/day, and the authors claimed that the non-observed-adverse-effect level (NOAEL) of AC was greater than 3 g/kg BW/day (Chen et al. 2011a). A later study found that AC was not teratogenic up to 500 mg/kg BW/day (Chen et al., 2011). In the present study, SD rats were administered with 2, 4 and 6 g/kg BW/day of AC mycelia to assess potential effects at higher doses.

2. Material and Methods

2.1 Test materials and preparation

Strains of AC were provided by the Research and Development Center (New Bellus Enterprises Co., Ltd, Tainan, Taiwan). Extracts were prepared as previously described (Cheng et al 2014). Briefly, AC was first cultured with Malt Extract Agar (MEA) I broth containing 1.5% agar, 1.5% malt extract, 2% glucose, and 0.1% peptone at 30°C for 7-10 days. AC mycelia were inoculated into MEA I broth and incubated at 30°C with shaking for 8-10 days. After 121°C sterilization for 30 minutes, fermented extracts were centrifuged at 3000 g for 10 minutes and then permeated through a 3000MW ultrafiltration membrane. Finally, concentrated extracts were lyophilized at -40°C for 4 hours and 30°C for 72 hours to prepare the freeze-dried powder. 4-acetylanthroquinonol B served as an index compound for internal control of batch-to-batch differences. The dried Antrodia cinnamomea was redissolved in ethanol (100 mg/mL) and extracted for 60 minutes in a water bath at 28℃. The ethanol extract was passed through a 45 μm filter. The ethanol extract was the separated by a Hitachi HPLC equipped with a UV detector. The Mightysil RP-18 GP column (4.6mm×250mm, 5μm) was used for separation. The gradient elution was consisted of 0.03% phosphoric acid (A) and methanol (B). The gradient profile was as follows: 0-5 min, A:B = 25:75; 5-15 min, A:B = 25:75; 15-28 min, A:B = 0:100; 28-28.1, A:B = 0:100; 28-28.1 min, A:B = 25:75; 28-35 min, A:B = 25:75 with a flow rate of 1.0 mL/min. 4-acetylanthroquinonol B previously isolated by our laboratory served as standards.

2.2 Animals

Sprague-Dawley (SD) rats were purchased from BIOLasco, Taiwan Co., Ltd. (Taipei, Taiwan). Eighty rats (40 male and 40 female) and 96 rats (all female) were used for sub-chronic toxicity and teratogenicity studies, respectively. Ninety-six rats at day 3 of gestation were first acclimatized for three days at 22 ±3°C with 12 hour light/dark cycles. Prior to entering the experiments, the health status of the rats was assessed by veterinarians. The animals were provided with standard diet MFG (Oriental Yeast Co., Ltd, Tokyo, Japan) and sterilized reverse osmosis water ad labium. The study was approved by the Institutional Animal Care and Use Committee (IACUC).

2.3 Study design

For the sub-chronic toxicity study, rats were randomly assigned to four groups (10/sex/group; 80 in total). Groups were assigned to ensure that mean body weight values were comparable among all groups. Control rats were administered with sterilized reverse osmosis water, which was used for dissolving AC powder. Rats in the low-dose, mid-dose, and high-dose groups were administered graded doses of AC (2, 4 and 6 g/kg body weight/day respectively). All rats received a single-dose daily for a period of 90 days. AC solutions were prepared fresh each day using reverse osmosis water at concentrations of 100, 200 and 300 mg/mL. The control group was given sterilized reverse osmosis water. For both control and AC solution, a total of 20 mL/kg BW of solution was administered daily.

For the teratogenicity studies, on day 6 of gestation a number was assigned to each animal and body weights were recorded. The rats were then randomly assigned to four groups (24 rats per group): control, low-dose, mid-dose, and high-dose group. Groups were assigned such that body weight values were comparable among all groups. Doses of 0, 2, 4, and 6 g/kg BW/day were administered to pregnant rats via oral gavage at a dosing volume of 20 mL on gestation days 6-17.

2.4 Sub-chronic toxicity study

2.4.1 Weight, food intake and clinical symptoms

Rats were observed once daily for morbidity and mortality during the study period, and were weighed once a week. Food intake was calculated by adding a fixed amount of feed on the day of weighing and by measuring the remaining feed after one week. In the ophthalmological assessments, visual inspections were used to look for any external abnormalities, while an ophthalmoscope was used to examine the internal structure. All rats were

subjected to these assessments at the beginning of the study and the day before being sacrificed.

2.4.2 Clinical chemistry

Blood was sampled from the heart after carbon dioxide euthanasia. The blood was left at room temperature for coagulation, and then centrifuged to harvest serum. An ADVIA 1800 Chemistry Analyzer was used to examine the following parameters: alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, total protein, total bilirubin, creatinine, blood urea nitrogen (BUN), glucose, cholesterol, triglyceride, phosphorus, calcium, chloride, potassium and sodium.

2.4.3 Hematology

The animals were fasted overnight prior to being sacrificed by carbon dioxide euthanasia. The blood was collected in a tube containing ethylenediaminetetraacetic acid (EDTA). An XE-2100-Hematology-Analyzer (Sysmex) was used to analyze the following hematological parameters: hematocrit, hemoglobin, red blood cell count (RBC), white blood cell count (WBC), platelet counts, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), lymphocyte, neutrophil and monocyte counts. In addition, blood was collected in a tube containing sodium citrate and analyzed with a CA-1500 automated coagulation analyzer (Sysmex) to determine prothrombin time.

2.4.4 Urinalysis

One day prior to being sacrificed, the rats were placed in a metabolic cage for urine collection. An AUTION MAX AX-4030 automatic urine analyzer (ARKRAY Core Laboratory) was used to examine the specific gravity (SG), color, protein, urobilinogen, pH, ketone, bilirubin, glucose, nitrite, and occult blood in urine. Microscopic examination of the sediment after centrifugation of urine included the white blood cell count (WBC), red blood cell count (RBC), epithelial cells (EP), crystals and microorganisms.

2.4.5 Pathology

During the study, deceased animals were necropsied as soon as possible, and tissues were collected for fixing in 10% neutral buffered formalin. After 90 days of dosing, all animals were euthanized by carbon dioxide and subjected to necropsy. Visual inspections of the external, oral, cranial, thoracic and abdominal organs were performed. The following tissues were collected and fixed in 10% neutral buffered formalin: brain, heart, kidneys, liver, spleen, adrenals, testis, ovaries, aorta, bone marrow, duodenum, jejunum, ileum, caecum, colon, rectum, eyes, esophagus, mammary glands, Harderian gland, trachea, lungs, lymph node, pancreas, sciatic nerve, pituitary gland, prostate glands, salivary glands, skin, spinal cord, stomach, skeletal muscle, thymus, thyroid/parathyroid glands, urinary bladder and uterus. Testis were first fixed with modified Davidson's solution for 24 hours and then preserved in 10% neutral buffered formalin. The brain, heart, kidneys, liver, spleen, and adrenal glands of all rats were weighed at necropsy. The percentage of organ weight relative to body weight was calculated as organ weight (g) / body weight (g) x 100%.

2.4.6 Histology

Histopathological examinations were performed on all tissues of the animals (both male and female) in the control and high-dose groups. Specimens from various tissues were fixed in 10% neutral buffered formalin, processed in wax, sectioned at 5 μm and stained with hematoxylin and eosin (HE). If any treatment-related changes were observed in the high-dose group, specimens from the low-dose and mid-dose groups were also processed for the same examinations.

2.5 Teratogenicity study

2.5.1 Maternal findings

The general condition and behavior of all animals were examined daily, and any clinical anomalies or deaths were recorded. Rats with severe illness or signs of spontaneous abortion were euthanized and subjected to necropsy. Body weights were recorded daily from day 6 to day 20 of gestation, and food consumption was calculated daily during the study period. All rats were euthanized by carbon dioxide inhalation on day 20 of gestation. Cesarean section was performed to remove the uterus, ovaries, and fetuses. Fetal body weights were measured. The numbers of corpora lutea, implantations, live fetuses and resorbed fetuses were recorded. In addition, the percentages of pre-implantation loss and post-implantation loss were calculated.

2.5.2 Fetal findings

Each live fetus was examined, and the sex ratio was noted. External examinations were conducted for the following conditions: anencephalia, cerebral edema, encephalocele, incomplete fontanelle closure, hydrocephaly, microcephaly, microphthalmia, anophthalmia, exophthalmos, microtia, anotia, low set ears, agnathia, micrognathia, cleft palate, mandibular cleft, cleft sternum, thoracic cleft, cleft spine, scoliosis, lordosis, kyphosis, hypospadias, imperforate anus, short tail, curved tail, no tail, celoschisis, polymelia, amelia, hemimelia, polydactylia, adactylia, syndactyly, brachydactyly, and ectrodactyly. Half of each litter was processed further for visceral examinations. Briefly, fetuses were fixed with 10% buffered formalin solution, and then the organs in the head, throat, thorax and

abdomen were examined. The other half of the litter was processed for skeletal examinations. The visceral organs were first removed from the thorax and abdomen, and fixed with 95% ethanol for one week. The staining protocol included a 24-hour digestion with 1% potassium hydroxide (KOH), 24-hour staining with Alizarin red in 1% KOH solution, then incubation in 1% KOH solution, followed by sequential clearing and preservation in 0%, 60%, 80% and 95% glycerol solution. The fixed fetal examinations were performed under low-power magnification. Special attention was paid to the size and shape of the cranial bone, as well as ossification in the nasal, frontal, parietal, interparietal, occipital and lower jaw regions. The numbers, sizes, shapes, and degrees of ossification of the following vertebral bones were assessed: cervical; thorax; sacral; and caudal: sternum; and costa. The skeletal abnormalities were determined according to the methods described in Manson and Kang (1994) and Christian (2004).

2.6 Statistical analysis

Group means and standard deviations were calculated for the collected data. In the sub-chronic toxicity study, body weights, food intake, organ weights, hematological and biochemical data were analyzed by one-way analysis of variance (one-way ANOVA), followed by post-hoc Duncan's multiple range testing. Similarly, in the teratogenicity study, the data on the maternal body weights and food consumption, weights of the uterus and fetuses, numbers of fetuses and corpus luteus were analyzed by one-way ANOVA where appropriate, followed by the post-hoc Duncan's multiple range tests. For comparisons on sex-ratio, fetal external, visceral and skeletal examinations, Chi-squared tests were applied. A $p<0.05$ value was considered as the minimum level for significance.

3. Results

3.1 Sub-chronic toxicity study

For quality assessment of the Antrodia cinnamomea mycelia, the major active component of our AC extract was identified as 4-acetylantroquinonol B (2790 ppm (w/w)). No mortality was seen in the control and treatment groups, no clinical signs were reported, and no statistically significant differences in mean body weight were observed in any of the treatment groups compared with the control. Mean body weights for males and females in the treatment groups were not statistically different from those of the controls (Figure 1). At week 2, male rats in the high-dose group had a significantly lower food intake than the controls, and the same was seen at week 8 for the female rats in the same group. Female rats in the low-dose group had significantly higher food intake than the controls (Appendix A. Figure S1 and S2). Ophthalmological assessments did not reveal any significant abnormalities in any of the groups (data not shown). In addition, the relative organ weights did not show any differences between the controls and treatment groups (Appendix A. Table S1).

Hematological examinations revealed an increase in mean eosinophil counts in the male high-dose group. Mean basophil counts were significantly higher in both males and females in the low-dose and high-dose groups (Table 1). Mean prothrombin time was significantly lower in the males of all three treatment groups as compared with the control group. Hemoglobin concentration was significantly lower in females of the low-dose and high-dose groups. Hematocrit was decreased in females of all treatment groups. However, these hematological changes were not considered as having any great toxicological relevance, as they were either not dose-dependent or limited to one sex only.

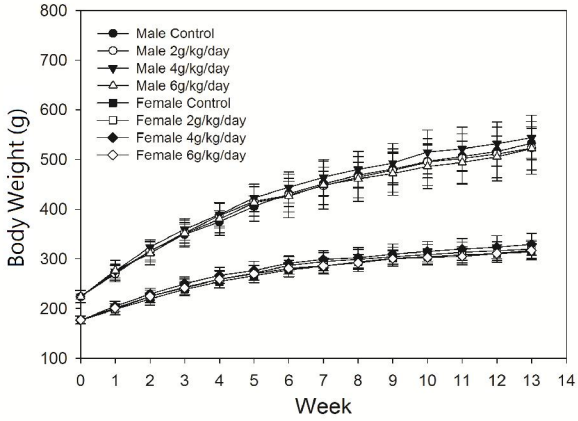

Figure 1. Growth curves for male and female SD rats following 90-day gavage to AC. n=10.

Clinicochemical data are presented in Table 2. Clinical chemistry indicated significantly decreased levels of total protein and creatinine in male high-dose and female mid-dose groups, but these were not dose-dependent, and not seen in females. Increased levels of BUN and phosphorus were seen in the males of the low-dose group.

Significant decreases in ALT were seen in females of the mid-dose and high-dose groups. Decreased globulin levels were observed in females of the low-dose group. Increased levels in creatinine, sodium, and glucose levels were seen in females of some dose groups. However, these differences were not dose-dependent, and limited to one sex only.

Table 1. Hematological findings on Spraque-Dawley rats administered with AC for 90 days.

Parameter	AC (g/kg BW/day)							
	Males				Females			
	Control	Low-dose	Mid-dose	High-dose	Control	Low-dose	Mid-dose	High-dose
	0	2	4	6	0	2	4	6
WBC (10^3/μL)	12.5±3.8	9.9±2.2	11.3±1.7	11.3±1.7	11.6±3.8	9.1±2.3	9.0±2.9	8.5±2.9
RBC (10^6/μL)	9.04±0.27	8.89±0.44	8.92±0.28	8.92±0.28	8.39±0.31	8.11±0.36	8.16±0.32	8.15±0.33
Hemoglobin (g/dL)	16.35±0.39	16.07±0.56	15.97±0.72	15.97±0.72	15.73±0.59	15.15±0.63*	15.20±0.56	14.99±0.60*
Hematocrit (%)	51.8±1.9	51.1±2.1	49.9±1.9	49.9±1.9	50.3±2.3	48.6±1.4*	48.4±1.4*	47.8±1.9*
MCV (fL)	57.3±1.3	57.6±2.7	55.9±1.4	55.9±1.4	59.9±1.9	60.0±1.9	59.4±2.2	58.6±1.4
MCH (pg)	18.1±0.4	18.1±0.8	17.9±0.5	17.9±0.5	18.8±0.5	18.7±0.4	18.6±0.5	18.4±0.4
MCHC (g/dL)	31.6±0.7	31.5±0.6	32.0±0.7	32.0±0.7	31.3±0.7	31.2±0.7	31.4±0.8	31.4±0.6
Platelet (10^3/μL)	747.1±104.3	681.1±111.1	652.3±94.3	652.3±94.3	738.8±81.1	709.2±64.8	743.4±74.5	752.2±81.6
Neutrophil (%)	19.0±3.4	23.3±5.5	22.6±7.5	22.6±7.5	14.1±5.4	14.6±4.7	16.7±6.0	15.6±5.2
Lymphocyte (%)	74.9±3.1	69.0±6.1	70.2±8.2	70.2±8.2	79.9±6.2	79.9±4.6	76.9±6.9	78.1±7.7
Monocyte (%)	4.8±2.3	6.5±2.2	5.5±2.0	5.5±2.0	4.6±1.3	4.3±1.1	4.7±1.3	4.3±2.9
Eosinophil (%)	1.1±0.4	1.0±0.4	1.1±0.3	1.6±0.6*	1.2±0.3	1.0±0.3	1.5±0.7	1.7±0.5
Basophil (%)	0.1±0.1	0.2±0.1*	0.1±0.1	0.2±0.1*	0.1±0.1	0.3±0.1	0.2±0.1	0.4±0.3*
Reticulocyte (%)	1.4±0.6	1.4±0.8	1.0±0.5	1.2±0.6	1.4±0.2	1.5±0.4	1.4±0.4	1.4±0.7
PT (sec.)	17.1±2.8	12.4±1.2*	11.4±0.6*	11.3±0.9*	10.2±0.1	10.3±0.2	10.3±0.2	10.4±0.2

Abbreviations: MCV, Mean corpuscular volume; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; PT, Prothrombin time

Values are means±SD.

*p<0.05 in comparison with control value

Urine and sediment examinations did not reveal any significant differences in any of the parameters (Appendix A. Tables S2 and S3). Histopathological examinations revealed no treatment-related findings (Appendix A. Table S4). The incidences of focal mononuclear cell infiltration of the Harderian gland were one male in the controls and one male in the high-dose group. Very mild focal mononuclear cell infiltration in heart tissue was seen in one male of the control group, and one male and one female of the high-dose group. Only one male in the control group displayed focal mononuclear cell infiltration in kidney tissue. Two males in the high-dose group had focal mononuclear cell filtration in liver tissue. Six males of the control group and four males of the high-dose group had focal mononuclear cell filtration in the prostate glands. These incidences of focal mononuclear cell infiltration were not dose-dependent and/or limited to one sex only.

3.2 Maternal observations in the teratogenicity study

All pregnant rats survived to the end of experiment without any clinical signs or gross abnormalities. Mean weight gains from day 6 to day 20 of gestation for all treatment groups were not statistically different from controls (Figure 2). No significant difference in food consumption from day 6 to day 20 of gestation was observed in any of the treated groups compared with the control. After carbon dioxide euthanasia the whole uterus, including the fetuses, was weighed, and no significant difference was observed between the treated and control groups. There were no significant effects from the treatment with regard to the numbers of implantations and corpus luteus, live births, pre-implantation losses, and post-implantation losses.

No gross defects were observed upon external and visceral examinations of the fetuses (Table 4). The numbers of fetuses with skeletal anomalies in all groups were not statistically different (Table 5). The numbers of fetuses with skeletal anomalies were similar among the control, low- and mid-dose groups. However, compared with the control group, the high-dose group had a significantly lower number of fetuses with skeletal anomalies. Among these anomalies, the incidence of absent sternebrae was significantly lower in all treated groups compared with the control group. The incidence of split sternebrae (bifid) was higher in the high-dose group. The incidence of split sternebrae in all treated groups was similar to that seen in the control group. Only the mid- and high-dose groups had incidences of missing vertebra (0.8% and 4.6%, respectively). The incidence of hemivertebrae only occurred in the high-dose group (3.8%). Similar incidences of excessive ribs were found in all treated groups and the controls. Missing ribs only occurred in the control and the low-dose group, with incidences of 0.8% and 1.4%, respectively. Broken ribs were only found in the control (6.6%), low-dose (4.9%) and mid-dose (11.4%) groups, and the differences were not statistically significant.

Table 2. Clinical chemistry data from SD rats dosed with AC for 90 days.

Parameter	AC (g/kg BW/day)							
	Males				Females			
	Control 0	Low-dose 2	Mid-dose 4	High-dose 6	Control 0	Low-dose 2	Mid-dose 4	High-dose 6
	0	2	4	6	0	2	4	6
AST (U/L)	118.1±23.2	136.4±20.6	116.3±22.1	126.2±31.8	111.2±15.2	102.7±16.7	106.5±20.6	93.6±17.6
ALT (U/L)	35.3±8.5	38.7±7.5	34.7±9.9	45.2±24.2	27.6±3.5	27.4±4.1	23.6±4.6*	21±2.9*
ALP (U/L)	83.7±8.8	88.3±16.0	87.7±20.5	97.6±27.4	49.3±13.1	39.5±12.1	41.9±11.7	40.2±13.2
Total bilirubin (µg/dL)	25.2±10.1	24.4±10.8	27.7±11.9	19.9±5.8	25±9.6	14.5±5.7	23.2±12.0	16.5±10.2
Total protein (g/dL)	7.4±0.3	7.3±0.2	7.1±0.2*	7.1±0.2*	8.2±0.3	7.9±0.3	8.1±0.4	8±0.4
Albumin (g/dL)	4.5±0.1	4.4±0.2	4.4±0.1	4.4±0.1	5.1±0.3	5.1±0.2	5.1±0.4	5±0.3
Globulin (g/dL)	2.8±0.2	2.8±0.2	2.7±0.1	2.7±0.2	3±0.1	2.8±0.1*	3.1±0.2	2.9±0.2
BUN (mg/dL)	12.5±1.3	14.9±1.3*	13.6±2.3	14.1±1.8	15.2±1.1	13.9±1.6	15.5±1.7	14.7±1.5
Creatinine (mg/dL)	0.52±0.06	0.49±0.04	0.48±0.04	0.43±0.03*	0.54±0.03	0.54±0.06	0.59±0.04*	0.57±0.04
Glucose (mg/dL)	159.9±37.1	163.1±26.5	172.4±13.2	167.3±38.3	141.5±21.2	152±20.7	170.4±38.4*	175.8±29.8*
Triglyceride (mg/dL)	61.9±20.3	72.0±24.1	70.8±24.0	65.4±14.8	56.5±11.3	77.8±37.4	73.6±30.2	59±17.0
Cholesterol (mg/dL)	77.1±19.6	72.1±12.6	74.9±14.7	70.7±9.5	104.6±17.8	92.7±21.4	105.3±22.3	102.4±20.5
Sodium (mEq/L)	149.3±1.9	150.3±1.6	149.2±1.3	148.9±2.1	146.9±1.0	148.1±1.1*	148.7±1.2*	148.5±1.1*
Potassium (mEq/L)	6.8±0.8	6.9±1.1	6.9±0.8	7.2±1.2	6.4±0.3	6.3±0.5	6.5±0.5	6.7±0.6
Calcium (mg/dL)	11.9±0.3	11.9±0.4	11.9±0.4	12.0±0.4	12.4±0.3	12.4±0.3	12.6±0.4	12.6±0.4
Chloride (mEq/L)	100.2±1.8	99.8±2.1	100.8±1.7	100.3±1.1	100.5±1.6	100.9±1.6	99.7±0.9	100.1±1.1
Phosphorus (mg/dL)	10.8±0.6	11.7±0.9*	10.4±0.6	11.2±1.3	9.9±1.0	9.1±1.0	9±0.9	9±1.0

Notes: values are means±SD.
*$p<0.05$ in comparison with control value

3.3 Fetal observations in the teratogenicity study

Mean fetal body weight was significantly higher in all treated groups (low, mid, and high) compared with the control group (Table 3). No statistically significant effects were found with regard to the sex-ratio, live births, stillborn births, and the resorption number in any of the treated groups compared with the control (Table 3).

Table 3. Pregnancy and litter data from SD rats administered AC-supplemented diet.

Parameter	AC (g/kg BW/day)			
	Control 0 g/kg	Low dose 2 g/kg	Mid dose 4 g/kg	High dose 6 g/kg
Dams (n)	24	24	24	24
Fetuses (n)	267	308	292	288
Gravid uterine weight (g)	69.3 ±17.6	80.3 ±10.9	76.5 ±12.6	77.0 ±13.9
Mean fetal weight (g)	3.96±0.42	4.03±0.41*	4.13±0.54*	4.17±0.39*
Fetal sex ratio (M/F)	1.04	1.03	0.87	1.04
Corpora lutea (n)	317	327	317	307
No. corpora lutea per dam	13.2±3.1	13.6±2.4	13.2±3.2	12.8±2.4
Implantations (n)	267	308	292	288
No. implantations per dam	11.1±3.2	12.8±1.7	12.2±2.1	12.0±2.1
Viable fetuses (n)	267	308	292	288
Dead fetuses (n)	0	0	0	0
Resorptions (n)	0	0	0	0
Pre-implantation loss (%)[a]	0.14±0.25	0.05±0.07	0.06±0.11	0.05±0.10
Post-implantation loss (%)[b]	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Values are means±SD.
*$p<0.05$ in comparison with control value
[a] Pre-implantation loss (%) = [(no. of corpora lutea – no. of implantation sites)/no. of corpora lutea] x 100.
[b] Post-implantation loss (%) = [(no. of implantation sites – no. of live fetuses)/ no. of implantation sites] x 100.

Table 4. Summary of external and visceral examinations of fetus.

Parameter			AC (g/kg BW/day)			
			Control	Low-dose	Mid-dose	High-dose
			0	2	4	6
External examinations						
	Dams	(n)	24	24	24	24
	Fetuses	(n)	267	308	292	288
	Abnormal fetuses	(n)	0	0	0	0
		(%)	0	0	0	0
Visceral examinations						
	Dams	(n)	24	24	24	24
	Fetuses	(n)	146	165	160	157
	Abnormal fetuses	(n)	0	0	0	0
		(%)	0	0	0	0

Table 4. Summary of external and skeletal examinations of foetuses.

Parameter			AC (g/kg BW/day)			
			Control	Low-dose	Mid-dose	High-dose
			0	2	4	6
Skeletal examinations						
	No. dams	(n)	24	24	24	24
	Dams with skeletal anomalies	(n)	20	21	18	21
		(%)	83.3	87.5	75.0	87.5
Skeletal examinations						
	No. fetuses	(n)	121	143	132	131
	Fetuses with skeletal anomalies	(n)	72	77	62	56*
		(%)	59.5	53.8	47.0	42.7
Skeletal anomalies (n/%)						
	Sternabra	Miss	53/43.8	34/23.8*	22/16.7*	19/14.5*
		Bifid	0	0	0	11/8.4*
	Vertebrate	Bifid	19/15.7	34/23.8	25/18.9	18/13.7
		Miss	0	0	1/0.8	6/4.6*
		Hemivertebra	0	0	0	5/3.8
	Ribs	Excessive	1/0.8	1/0.7	3/2.3	0
		Miss	1/0.8	2/1.4	0	0
		Break	8/6.6	7/4.9	15/11.4	0

*p<0.05 in comparison with control value

4. Discussions

OECD guidelines for toxicity studies state that "unless limited by the physical-chemical nature or biological effects of the test substance, the highest dose level should be chosen with the aim to induce toxicity but not death or severe substance (OECD, 2001)." To minimize the chances of Type II errors and take the daily recommended dose into consideration, the sub-chronic toxicity and teratogenicity were assessed up to a dose of 6 g/kg BW/day. In this study, the doses 2, 4 and 6 g/kg BW/day were evaluated in Spraque-Dawley rats, which were equivalent to 60, 120, and 180 times of the daily recommended dose in humans (2g/60 kg BW/day), respectively.

Our data compares well with other reports on AC toxicity. No significant toxicity was found at 3 g/kg BW/day in a 90-day repeated dose study (Chen et al 2011a), although there were some significant differences in the clinicochemical and hematological parameters used in the current work and this earlier study. However, these results are not considered toxicologically significant, as they were usually seen in only one sex, were not dose-dependent, and were also seen in control animals.

Overall, the AC administration to Spraque-Dawley rats for 90 days at the doses of 2, 4 and 6 g/kg BW/day demonstrated no treatment related effects with regard to mortality or food consumption, or the results of the ophthalmological, clinical chemical, and urinalysis examinations. Some statistically significant changes were observed for the hematological, clinicochemical and histopathological parameters, but these were not dose-dependent and/or were specific to only one sex, and thus are not considered to be treatment related. Histopathological

changes in the Harderian gland, heart, kidney, liver and prostate gland of high-dose rats were seen in only one-sex, or were also observed in the control group, and were of either minimal or slight effects. These results compare well with those reported in Chen et al (2011a), which only observed minimal or slight changes in the heart, kidney and testes.

In the teratogenicity study, we found no effects of AC extracts at 2, 4, and 6 g/kg BW/day doses on the maternal body weight and food consumption. Reduced maternal body weight gain during gestation is the most common indication of some maternal toxicity, which is most likely due to reduced food/water intake upon administration of the test agent (Moriyama et al, 2008). No visceral malformations were observed in any of the treated groups. Incidences of split sternebrae and absent vertebrae were significantly higher in the high-dose group compared with the control. A higher incidence of split sternebrae in the high dose group (500 mg/kg BW/day) was also reported in a previous study (Chen et al., 2011b), although split sternebrae were not observed in the low- and mid-dose groups (2 and 4 g/kg BW/day, respectively) in the current work. Therefore, this skeletal malformation is unlikely to be a consequence of the AC administration. Similarly, absence of vertebra was seen in the control and the low-dose (50 mg/kg) groups in by Chen et al. (2011b). The incidences of absent vertebra lacked a dose-dependent relationship in the current study, and thus it is less likely that they can be attributed to the administration of AC.

The doses tested in toxicity studies were calculated based on the dry weight of AC mycelia, which consists of a mixture of bioactive compounds, and not one single compound. For example, the major bio-active compound identified in the AC extract in this study was 4-acetylantroquinonol B at 2790 ppm (w/w), whereas in another similar study the active components were pyrroledione and ergostatrien-3-ol at 1168 ppm (w/w) and 2750 ppm (w/w), respectively (Chen et al., 2011a). Direct comparison of the doses based on the whole extracts between these two studies may thus be less meaningful. Therefore, this warrants multiple toxicity studies in which the major bioactive components of AC are qualitatively and quantitatively determined, as this would provide a more comprehensive investigation of the toxicities of AC. In addition, pharmacokinetics studies are needed to address the bioavailability of the AC extract. The major active components, such as 4-acetylantroquinonol B, can serve as a bio-marker to preclude the possibility of a lack of toxicity due to no or low bioavailability in future studies.

In conclusion, based on absence of any treatment-related effects in the sub-chronic toxicity and teratogenicity studies, the no-observable adverse effect level (NOAEL) of AC was the highest dose (6 g/kg BW/day).

Supplement data

Supplementary data can be downloaded from: http://tinyurl.com/nrxz2z2

Corresponding Author:
Alice Lan
New Bellus Enterprises Co., Ltd.
Tainan 72042, Taiwan
E-mail: alice.lan@gmail.com

References

1. Chen CC, Lin WH, Chen CN, Hsu SJ, Huang SC, Chen YL. Development of *Antrodia camphorata* mycelium with submerge culture. Fungi Science 2001;16:7–22.
2. Chen JJ, Lin WJ, Liao CH, Shieh PC. Anti-inflammatory benzenoids from *Antrodia camphorata*. Journal of Nature Products 2007;70(6):989–92.
3. Chen TI, Chen CC, Lin TW, Tsai YT, Nam MK. A 90-day subchronic toxicological assessment of *Antrodia cinnamomea* in Sprague-Dawley rats. Food and Chemical Toxicololgy 2011;49(2):429–33.
4. Chen TI, Chen CW, Lin TW, Wang DS, Chen CC. Developmental toxicity assessment of medicinal mushroom *Antrodia cinnamomea* T.T. Chang et W.N. Chou (Higher Basidiomycetes) submerged culture mycelium in rats. International Journals of Medicinal Mushrooms 2011b;13(6):505–11.
5. Christian MS. Test methods for assessing female reproductive and developmental toxicology In: Hayes, AW, ed. Principles and methods of toxicology. Taylor & Francis. Philadelphia, USA. 2004:1641–60.
6. Deng JY, Chen SJ, Jow GM, Hsueh CW, Jeng CJ. Dehydroburicoic acid induces calcium- and calpain-dependent necrosis in human U87MG glioblastomas. Chemical Research in Toxicology 2009;22(11):1817–26.
7. Geethangili M, Tzeng YM. Review of pharmacological effects of *Antrodia camphorata* and its bioactive compounds. Evidence-based Complementary and Alternative Medicine 2011 Jan; 2011:212641.
8. Hseu Y, Yang H, Lai Y, Lin J, Chen G, Chang Y. Induction of apoptosis by *Antrodia camphorata* in induction of apoptosis by *Antrodia camphorata* in human premyelocytic Leukemia HL-60 cells. Nutrition and Cancer 2009;48(2):37–41.
9. Hseu YC, Chang WC, Hseu YT, Lee CY, Yech YJ, Chen PC, et al. Protection of oxidative damage by

aqueous extract from *Antrodia camphorata* mycelia in normal human erythrocytes. Life Sciences 2002;71(4):469–82.
10. Hseu YC, Chen SC, Tsai PC, Chen CS, Lu FJ, Chang NW, et al. Inhibition of cyclooxygenase-2 and induction of apoptosis in estrogen-nonresponsive breast cancer cells by Antrodia camphorata. Food and Chemical Toxicology 2007;45(7):1107–15.
11. Hseu YC, Wu FY, Wu JJ, Chen JY, Chang WH, Lu FJ, et al. Anti-inflammatory potential of *Antrodia camphorata* through inhibition of iNOS, COX-2 and cytokines via the NF-kappaB pathway. International Immunopharmacology 2005;5(13-14):1914–25.
12. Hsiao G, Shen M-Y, Lin K-H, Lan M-H, Wu L-Y, Chou D-S, et al. Antioxidative and hepatoprotective effects of Antrodia camphorata extract. J. Agric. Food Chemistry 2003;51(11):3302–8.
13. Hsu YL, Kuo YC, Kuo PL, Ng LT, Kuo YH, Lin CC. Apoptotic effects of extract from *Antrodia camphorata* fruiting bodies in human hepatocellular carcinoma cell lines. Cancer Letters 2005;221(1):77–89.
14. Huang HC, Liaw CC, Yang HL, Hseu YC, Kuo HT, Tsai YC, et al. Lanostane triterpenoids and sterols from *Antrodia camphorata*. Phytochemistry 2012;84:177–83.
15. Kuo PL, Hsu YL, Cho CY, Ng LT, Kuo YH, Lin CC. Apoptotic effects of *Antrodia cinnamomea* fruiting bodies extract are mediated through calcium and calpain-dependent pathways in Hep 3B cells. Food and Chemical Toxicology 2006;44(8):1316–26.
16. Lee I, Huang R, Chen C, Chen H, Hsu W, Lu M. Antrodia camphorata polysaccharides exhibit anti-hepatitis B virus effects. FEMS Microbiology Letters 2002;209:63–7.
17. Lee TH, Lee CK, Tsou WL, Liu SY, Kuo MT, Wen WC. A new cytotoxic agent from solid-state fermented mycelium of *Antrodia camphorata*. Planta Medica 2007;73(13):1412–5.
18. Liao PC, Kuo DC, Lin CC, Ho KC, Lin TP, Hwang SY. Historical spatial range expansion and a very recent bottleneck of *Cinnamomum kanehirae Hay.* (*Lauraceae*) in Taiwan inferred from nuclear genes. BMC Evolutionary Biology 2010; 10:124.
19. Liu JJ, Huang S, Hsu ML, Chen CC, Lin WS, Lu FJ, et al. Antitumor effects of the partially purified polysaccharides from *Antrodia camphorata* and the mechanism of its action. Toxicology and Applied Pharmacology 2004; 201(2):186–93.
20. Lu MC, El-Shazly M, Wu TY, Du YC, Chang TT, Chen CF, et al. Recent research and development of Antrodia cinnamomea. Pharmacology & Therapeutics 2013;139(2):124–56.
21. Manson JM, Kang YJ. Test methods for assessing female reproductive and developmental toxicology. In: Hayes, AW, ed. Principles and methods of toxicology. Taylor & Francis. Philadelphia, USA. p. 989–1037.
22. Mau JL, Huang PN, Huang SJ, Chen CC. Antioxidant properties of methanolic extracts from two kinds of Antrodia camphorata mycelia. Food Chemistry 2004;86(1):25–31.
23. Nakamura N, Hirakawa A, Gao JJ, Kakuda H, Shiro M, Komatsu Y, et al. Five new maleic and succinic acid derivatives from the mycelium of *Antrodia camphorata* and their cytotoxic effects on LLC tumor cell line. Journal of Natural Products 2004;67(1):46–8.
24. Rao YK, Fang SH, Tzeng YM. Evaluation of the anti-inflammatory and anti-proliferation tumoral cells activities of *Antrodia camphorata*, *Cordyceps sinensis*, and *Cinnamomum osmophloeum* bark extracts. Journal of Ethnopharmacology 2007;114(1):78–85.
25. Shen YC, Chou CJ, Wang YH, Chen CF, Chou YC, Lu MK. Anti-inflammatory activity of the extracts from mycelia of *Antrodia camphorata* cultured with water-soluble fractions from five different Cinnamomum species. FEMS Microbiolgy Letters 2004;231(1):137–43.
26. Song TY, Yen GC. Antioxidant properties of *Antrodia camphorata* in submerged culture. Journal of Agricultural and Food Chemistry 2002;50(11):3322–7.
27. Wang GJ, Tseng HW, Chou CJ, Tsai TH, Chen CT, Lu MK. The vasorelaxation of *Antrodia camphorata* mycelia:involvement of endothelial Ca2+-NO-cGMP pathway. Life Sciences 2003;73(21):2769–83.
28. Yang HL, Chen CS, Chang WH, Lu FJ, Lai YC, Chen CC, et al. Growth inhibition and induction of apoptosis in MCF-7 breast cancer cells by *Antrodia camphorata*. Cancer Letters 2006;231(2):215–27.
29. Yeh CT, Rao YK, Yao CJ, Yeh CF, Li CH, Chuang SE, et al. Cytotoxic triterpenes from *Antrodia camphorata* and their mode of action in HT-29 human colon cancer cells. Cancer Letters; 2009;285(1):73–9.

11/22/2014