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90-day Sub-chronic Oral toxicological assessment of *Antrodia cinnamomea* fruiting body extract in Sprague-Dawley rats

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Abstract

A. cinnamomea is considered to improve physical health and perhaps effectively treat many diseases. In order to ensure that a product prepared from *A. cinnamomea* is safe for long-term consumption, 90-day sub-chronic oral toxicity testing in rats was performed. The test design fully followed the Organization for OECD guideline 408. The test material was an extract of *A. cinnamomea* fruiting bodies cultured by Da-Yi Biotech Corporation, and the dosages tested were 750, 1500 and 3000 mg/kg body weight ; a group administered water served as the control. No rats died during the feeding period. Although two rats died unexpectedly, pathological examination showed sample residue in the lungs, which resulted in asphyxia with respiratory failure. In observations carried out twice a day, no functional or clinical abnormal behavior was noted. Organ weight, body weight, urinalysis, hematologic and biochemical analyses, as well as blood-clotting tests, showed no abnormality associated with *A. cinnamomea* feeding. Pathological examination by visual inspection and histopathologic analysis demonstrated no abnormalities in the 78 surviving rats. The NOAEL of *A. cinnamomea* was determined to be at least 3000 mg/kg; using a safety factor of 100, the ADI was estimated to be 30 mg/kg.

Keywords: A. cinnamomea, OECD guideline 408, NOAEL, ADI

1. Introduction

Antrodia cinnamomea (formerly named *Antrodia camphorata*), also known as "*Niu-chang-chih"*, is a fungus of the family Polyporaceae. *A. cinnamomea* is found at altitudes ranging from 200 to 2000 meters throughout Taiwan, and only grows on the brown heartwood of the inner cavity or a wet surface of *Cinnamomum kanehirae*, an endemic species of native camphor tree in Taiwan. The fruit bodies of *A. cinnamomea* appear in different shapes, include plate-shaped, bell-shaped and flake-shaped bodies, and are of a red-orange color, or light brown on the porous side. Owing to its distinctive aroma and delicate texture, the *C. kanehirae* tree is an excellent hardwood that has long been used for the production of high-end furniture. As it increasingly becomes a very rare and endangered species, the *C. kanehirae* tree is considered Taiwan's national tree, and the Taiwan government has actively preserved and rehabilitated the species in the wild.

A review article by Geethangili and Tzeng in 2011 [1] reported that *A. cinnamomea* has many medical applications and is used widely by members of the general population. It has long been used by the indigenous peoples of Taiwan to treat hangover or fatigue [2], and long-term consumption can help prevent or even treat liver disease, and can treat food poisoning, diarrhea, abdominal pain, hypertension, itchy skin and cancer, and alleviates many pathological conditions [3-5].

In recent years, the number of scientific studies focused on *A. cinnamomea* has dramatically increased, and at least 78 compounds have been identified. The fruiting bodies of *A. cinnamomea* contain 39 terpenoids, of which triterpenoids are the major constituents, accounting for more than 50% of the total compounds, and are also the source of the

bitter taste of *A. cinnamomea*. The other compounds present in the fruiting bodies include benezoids, lignans, benzoquinones, malic and succinic acid derivatives and polysaccharides [6-8]. A recent study showed that wild *A. cinnamomea* has 10- to 30-fold the triterpenoid content of submerged-fermentation *A. cinnamomea* [9, 10]. A study used HPLC-ESI-MS/MS to accurately and rapidly quantify the signature components of the fruiting body of *A. camphorate* [11], and the components were identified as antcins A, B, C, H and K, dehydroeburicoic acid, and 4,7-dimethoxy-5-methyl-1,3-benzodioxole. Most of the individual components and fruiting body extracts have been shown to have the potential to directly prevent liver, breast and cervical cancers, and leukemia in *in vitro* and *in vivo* models, or may be used as an adjuvant therapy [9]. In an induced apoptosis model, the components were demonstrated to prevent alcohol- or chemical-induced liver injury in an animal model [12], as well as protect liver function and exert anti-inflammatory, anti-oxidant and immunomodulatory effects [13]. In pharmacological studies, *A. camphorate* has been shown to be associated with the mechanism of apoptosis, and regulates the activities of key molecules in various signaling pathways, including caspases, Bcl-2 family proteins, NF-κB, Akt, p53 and mitogen-activated protein kinases (MAPK) [1].

At present, study of the safety of *A. camphorate* is limited. Most studies have used submerged fermentation of *A. camphorata* mycelia, and no toxicity was noted. In 2011, a biotech company performed a 90-day sub-chronic toxicological assessment following the OECD guidelines 408, and reported that the no-observed-adverse-effect level (NOAEL) of *A. cinnamomea* was greater than 3000 mg/kg BW/day in rats [14]. Another study used fruiting body extract to carry out 90-day sub-chronic oral toxicity analysis, and reported the NOAEL to be 5000 mg/kg BW/day [15].

However, a news report in 2013 indicated that a biotech company had been commissioned by the Development Center for Biotechnology of the Ministry of Economic Affairs, Taiwan, to perform toxicity testing using doses of 200, 600 and 2000 mg/kg BW/day, which were equivalent to daily oral doses of 12, 36 and 120 g in humans. Their test results showed abnormal cell proliferation, cytoplasmic vacuolation death, and a high toxicity towards the adrenal gland, the damage increasing with increased doses of *A. cinnamomea*.

Currently, methods of *A. cinnamomea* culture can be classified into submerged fermentation, base-wood culture, and dish culture. A range of products prepared using different methods are available on the market. The literature shows that *A. cinnamomea* samples obtained using different cultivation methods contain varied active substances, and different extraction methods may also result in dissimilar activities. Regardless of the preparation method, most products on the market claim similar effects; however, no scientific data have been produced to support the supposed effects of the products. To safeguard public health, a complete toxicological assessment of individual sources of *A. cinnamomea* should be carried out before the effects in terms of health improvement can be clarified.

Da-Yi Biotech Corporation is a biotech company that specializes in culturing *A. cinnamomea* fruiting bodies using *C. kanehirai* as the host wood. The company extracts active natural ingredients from *A. cinnamomea* fruiting bodies that have been grown on the wood for years. To assess the food safety of *A. cinnamomea*, manufacturers need to perform 90-day oral toxicity study based on the regulations announced by the Ministry of Health and Welfare, Taiwan, as described in article MOHW1041302003. The purpose of testing is to determine the toxicity and potential health hazards from repeat doses of various levels of a product during a 90-day test period in a mammalian model, with the aim of obtaining information regarding the safe edible dose. The data obtained in this test can then be used to estimate the acceptable daily intake (ADI) for humans.

2. Materials and methods

Preparation of A. cinnamomea *fruiting body extract*

Samples of *A. cinnamomea* fruiting bodies were provided by Da-Yi Biotech Corporation. 3500 g (wet weight) of *A. cinnamomea* fruiting body were ultrasonically cleaned for 20 minutes, then dried in a 55°C oven for 47 hours. The dried sample was ground and placed into a CO_2 Supercritical Fluid Extraction System (model E-2000); extraction was performed with 2500 ml 95% ethanol at a flow rate of 1 ml/sec, and the process was completed within 6 hours, following which 1500 ml of *A. cinnamomea* fruiting body extract were collected. The extract solution was then concentrated using a vacuum evaporator to a volume of 700 ml, to a final concentration of 5 g/ ml. After diluting with sterile deionized water to 2 g/ml, the sample was then aliquoted and stored in a refrigerator.

Determination of total triterpenoids

The 0.2ml sample was heated to evaporation, 0.2mL new mixed 5% (W/V) vanillin-acetic solution and 1.2 mL perchloric acid were added, mixed and incubated at 70°C for 20 min. The absorbance was

measured at 550 nm against blank using a spectrophotometer (Biorad Model 680). The content of total triterpenoids was expressed as milligram ursolic acid equivalent/gram extract.

Qualitative and quantitative analysis by HPLC

The high performance liquid chromatography (HPLC) analysis using 8 standards was executed by Center for Active Natural Produce Development, NPUST, Dr. Chi-I Chang. The extracts were filtered using a 2.2 μm filter. A 250 × 4.6 mm HyPURITY C18 HPLC column with a Hitachi L-7100 HPLC pump and 7240 UV detection system were used for the analysis under 254 nm UV detection for secondary metabolite profiles. The mobile phase was 30-100% acetonitrile from 0 - 20 min, 100% acetonitrile for next 20 min, then, linear replacement of acetonitrile with 100% methanol for the next 10 min, and finally 100% methanol for next 10 min.

Administration of A. cinnamomea extract to animals

This animal toxicity test employed rats as the model. The protocol of the experiment was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of NPUST. The approval number for this experiment was NPUST-106-006. Eighty 6-week-old (40 male and 40 female) Sprague-Dawley (SD) rats were purchased from BioLASCO Taiwan Co.. The rats were then assigned to one of four groups (for both male and female rats: 4 groups/sex, 10 animals/group) as follows: Group 1, control group fed with sterile water; Groups 2-4, *A. cinnamomea*-fed (Group 2-low dose, 750 mg/kg body weight (BW); Group 3-middle dose, 1500 mg/kg BW; Group 4-high dose, 3000 mg/kg BW). The animals were maintained under temperature 24°C, relative humidity 65%, 12-hour light and darkness cycle condition, with free access to food and water. The animals were housed in cages containing three to four rats. The rats were allowed 7 days in which to recover after transportation, then the rats were trained to be fed by oral gavage needle for 5 days prior to administration of *A. cinnamomea* solution. After training, the rats were administered distilled water or one of three different concentrations of *A. cinnamomea* solution via oral gavage once daily at 10:00 am. The oral gavage needle used in this study was ST-F174 (ψ1.2 mm × L80 mm). The stock *A. cinnamomea* solution of 2 g/ml was diluted using sterile deionized water to concentrations that were equal to 3000, 1500 and 750 mg of fruiting bodies/kg of rat body weight.

Clinical observations: animal behavior, response and survival

All animals were observed in their cages twice daily (10:00 AM and 6:00 PM) in terms of their behavior, response and signs of toxicity. Examinations included observations of bizarre behavior, changes in the level of motor activity, respiratory factors, and skin and fur conditions. Food and water intakes were roughly recorded, and the excretion pattern was also noted.

Ophthalmic examination

Ophthalmic examination was performed by pathology specialist, Dr. Ching-Dong Chang (NPUST, Pingtung, Taiwan). The examination was performed both pre-test and at the end of the experimental period.

Urinalysis

Two days before necropsy examination, urine samples were collected after consecutive 12-hr periods. The samples were analyzed immediately using a Urine Chemistry Analyzer (Clinitek STATUS, Bayer HealthCare) and urine test strips (Multistix 10 SG, Siemens) in Animal Hospital (NPUST, Pingtung, Taiwan).

Blood collection, hematology and analytical instruments

Two days prior to necropsy examination, the rats were fasted for 10 hours before blood samples were collected from the tail vein using a 23G needle. For hematologic analysis, K_2EDTA was used as the anticoagulant, and the analysis was performed using an automated hematology analyzer (Mythic 18; Orphee, Switzerland). For biochemical testing, lithium heparin was used as the anticoagulant, and the analysis was performed using an automated clinical chemistry analyzer (Dri-Chem 3500S; Fujifilm, Japan).The blood and serum tests were performed in the clinical laboratory of the Animal Hospital at NPUST.

Necropsy and histopathologic analysis

This project used a euthanasia chamber approved by the IACUC at NPUST that employed carbon dioxide to euthanize the rats. Necropsy, including the external surface of the body, all orifices, cranial cavities, thoracic cavities, abdominal cavities, liver, kidneys, adrenals, testes, epididymides, uterus, ovaries, thymus, spleen, brain, and heart. Histopathologic examination, including all gross lesions, brain (including cerebrum, cerebellum and medulla/pons), spinal cord (at the cervical, mid-thoracic and lumbar levels), pituitary, thyroid, parathyroid, thymus, esophagus, salivary glands, stomach, small and large intestines including Peyer's patches, liver, pancreas, kidneys, adrenals, spleen, heart, trachea, lungs, aorta, uterus, female mammary gland, prostate, urinary bladder, lymph nodes, tibial

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nerve, bone marrow or fresh bone marrow aspirate, skin and eyes.

Statistical analysis

The data collected in the study was analyzed by one-way ANOVA followed by *Post hoc* analysis with Tukey's methods. *P* values < 0.05 were considered to indicate statistical significance, and are indicated by "*" in the tables. Statistical analysis of the data was analyzed using GraphPad Prism software.

3. Results and Discussion

Total triterpenoids content and composition

The total triterpenoids level of our *A. cinnamomea* fruiting body extract prepared by Da-Yi Biotech was 209.89±17.82 mg ursolic acid equivalent/g extract. The specific compounds in extracts was analyzed by HPLC. Figure 1A revealed the profile of 8 standards, the retention time of each compound was shown. The chromatogram in Figure 1B illustrated the secondary metabolites profile in our extract. The quantitative results indicated the two highest amounts are antcin H (173.26mg/g) and antcin K (136.59mg/g), followed by antcin B (131.25mg/g), DeEA(42.00mg/g), antcin C (37.18 mg/g), DeSA (30.81mg/g), DMMB (17.19mg /g) and antcin A (1.01mg/g).

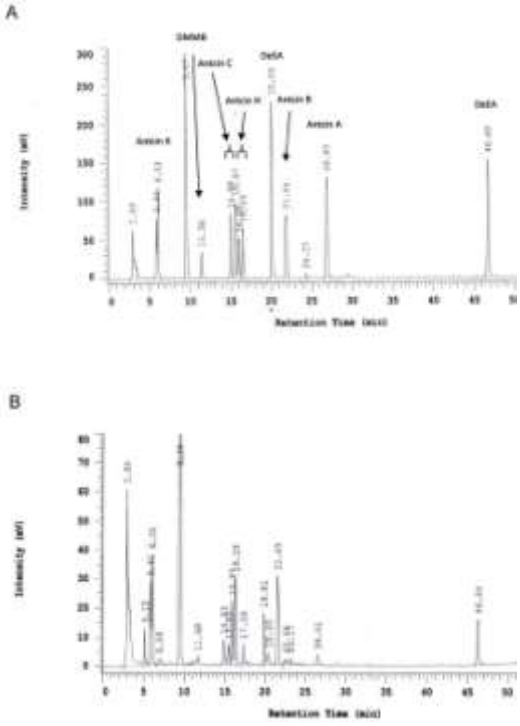

Figure 1. (A) HPLC profiles of eight standards. (B)Chromatogram HPLC profile of *A. cinnamomea* fruiting body extract.

Ophthalmic examination, body weight, water and food intake

The results of the examination indicated that no ophthalmic lesions were found on the peripheral and internal structures of either eye before or after treatment in any animal. The body weight of each rat was measured weekly. Changes in body weight of the male and female rats were calculated. Food consumption was calculated based on the weight of the food dropped at the bottom of the cage subtracted from the weight of the food remaining on top of the cage. Food and drinking water consumptions were analyzed to show the daily consumption of the animals. Based on data published by the Animal Center of the Medical School at National Cheng Kung University, normal rats consume food at a level of 10 g/100g body weight/day and water at a level of 10-12 ml/100g body weight/day. The rats in our experiment consumed food at 5-10 g/100 g BW/day and water at 6-12 ml/100 g BW/day, which were within the normal ranges. The weight increase of the rats was also normal. The data are presented in Tables 1 and 2. One-way analysis of variance (ANOVA) showed that the *P* values of the 90-day percentage of body weight increase of the male and female rats were 0.4917 and 0.7901, respectively. The *P* values of the average daily food consumption per male and female rat were 0.0631 and 0.0072, respectively, and the *P* values of the average daily water consumption per male and female rat were 0.0095 and <0.0001, respectively. Due to continued growth, differences in body size of rats kept in the same cage will affect the food and water intakes of individual rats. In addition, food-dropping and water leakage due to animal activity may all cause some variance in food and water consumption. During the 90-day experiment, some differences between groups were likely due to these factors, rather than caused by *A. cinnamomea* administration.

Table 1. Body weight increase (%), food and water consumption in male rats.

Group/male	90-day body weight increase (%)/per rat	Food consumption (g)/day/per rat	Water consumption (g)/day/per rat
Group 1 (control)	50.17±3.40	24.75±0.75	46.47±3.39
Group 2 (750mg/kg)	52.88±3.13	26.98±3.45	48.36±6.15
Group 3 (1500mg/kg)	51.56±4.32	24.21±3.20	41.46±7.59
Group 4 (3000mg/kg)	52.77±6.17	24.63±0.77	41.53±1.99
ANOVA P value	0.4917	0.0631	0.0095

Table 2. Body weight increase (%), food and water consumption in female rats.

Group/female	90-day body weight increase (%)/per rat	Food consumption (g)/day/per rat	Water consumption (g)/day/per rat
Group 1 (control)	33.62±7.04	16.84±0.89	28.83±2.71
Group 2 (750mg/kg)	35.33±5.14	17.22±0.73	33.74±4.26
Group 3 (1500mg/kg)	33.56±3.67	16.32±0.50	30.40±0.66
Group 4 (3000mg/kg)	33.23±3.51	16.31±0.28	25.90±1.31
ANOVA P value	0.7901	0.0072	<0.0001

Urinalysis

The collected samples were analyzed immediately. The test items included color, specific gravity (SG), protein, urobilinogen, pH, ketones, bilirubin, glucose, nitrite and occult blood. Urine sediment was placed under a microscope in order to count the numbers of red blood cells (RBC), white blood cells (WBC), epithelial cells, and triple phosphate crystals per high power field (HPF), which covered all the items listed in OECD guideline 408. The results were as shown in Table 3, and demonstrated that most of the animals had a normal urine color, with the exception of one male in the control group and one male in the high *A. cinnamomea* dose (3000 mg/kg BW) group, whose urine was of a reddish color. The urine sediments of these two male rats (no. 3 and no. 62) only showed 0-1 red blood cells under the field of view of a microscope at 400× magnification. While the sample of rat no. 3 had negative results in the occult blood test, which ruled out the possibility of urinary tract lesions, the sample of rat no. 62 had strong positive results (+++) in the occult blood test, but almost no red blood cells were seen, which also ruled out the possibility of urinary tract disease. The reddish color of the urine might be due to hemoglobinuria or myoglobinuria, which was likely caused by over-reactive activity or intake of the orange pigment of *A. cinnamomea*. Urine SG values between 1.010 and 1.030 are considered normal, while higher SG values are often seen in normal animals. In the control group, one male and one female rat had abnormal urine SG values, and in the low, middle and high *A. cinnamomea* dose groups, 4, 5 and 4 rats had abnormal urine SG values, respectively (male: 2, 4 and 3; female: 2, 1 and 1), suggesting that *A. cinnamomea* feeding was not correlated with abnormal urine SG. Three rats (two males and one female) in the control group had proteinuria, and in the low, middle and high *A. cinnamomea* dose groups, 6, 6 and 3 rats had proteinuria, respectively (male: 5, 5 and 2; female: 1, 1 and 1), suggesting that *A. cinnamomea* feeding was not correlated with proteinuria. Transient proteinuria is usually benign, and with the exception of rat no. 62 (high *A. cinnamomea* dose) with an occult blood response, the pH values of the remaining rats were all within the normal range, suggesting that renal disease should be excluded. Rat no. 62 had alkaline urine and showed positive responses to ketonuria, bilirubin and occult blood, while biochemical markers of renal function from serum showed that blood urine nitrogen (BUN) and creatinine were within the normal ranges. In addition, pathological examination of the kidneys did not show any abnormality, which ruled out the possibility of uremia or renal dysfunction. It was inferred that the abnormal urinalysis results of rat no. 62 were likely caused by other unknown factors. All three treatment groups had pH values ranging from 5.5-8.5. Although the *A. cinnamomea*-treated groups had fewer lower SG values than the controls, there was no evidence to show that *A. cinnamomea* feeding caused abnormal urine SG. In the ketone test, in the male rats, the control and the low, middle and high *A. cinnamomea* dose groups had 5, 7, 7 and 8 positives, respectively; in the female rats, only the control group had 1 positive test; the *A. cinnamomea*-fed groups results were all negative. A higher ketone level might be due to diabetes or starvation, but all the rats in this study had normal urine glucose and food consumption levels, suggesting that the trace amount of ketones in the male rat urine samples was not caused by *A. cinnamomea* feeding. Abnormal urinary bilirubin was found in 3 males and one female in the control group; both the low and middle *A.*

cinnamomea dose groups had one male with abnormal urinary bilirubin, and two males in the high *A. cinnamomea* dose group had abnormal urinary bilirubin levels. All the female rats in the *A. cinnamomea*-fed groups had normal urinary bilirubin levels. The results indicated that *A. cinnamomea* feeding was not correlated with the urinary bilirubin level. Other urinalysis items, including urobilinogen, urine glucose and nitrite, were all negative and normal. In the blood cell count test of urine sediment, in the control group, there were four males with 0-5 WBC; in the middle *A. cinnamomea* dose group, there were three males with 0-1 or 0-5 WBC; and in the high *A. cinnamomea* dose group, only one male and one female had 0-1 and 0-5 WBC, respectively. The results suggested that the presence of WBC in urine was not associated with *A. cinnamomea* feeding. All the urine samples had a negative nitrite response, indicating no infection in the animals. The control group had 3 males with 0-1 and 0-5 RBC, while the high *A. cinnamomea* dose group only had one male and one female with 0-1 and 0-5 RBC, including the aforementioned rat no. 62. The results suggested that *A. cinnamomea* feeding did not cause an abnormal blood cell count in urine sediment. Three rats in the *A. cinnamomea*-fed groups (two in the middle-dose group, one in the high-dose group) had a positive epithelial cell response in urine sediment. The presence of squamous epithelial cells in urine may be due to contamination of the urine specimen from the urothelium. In most cases, it can be ignored; however, if cells are present in large numbers, this indicates that the specimen was not collected properly. Acidic urine often contains urate, uric acid and calcium oxalate, while alkaline urine often contains phosphates, calcium carbonate, ammonium urate or magnesium ammonium phosphate. In our results, triple phosphate crystals were present in all groups, which are often present in the urine of healthy subjects. In all of the urine samples tested, no pathologic crystals, such as bilirubin, cysteine, cholesterol, tyrosine or leucine crystals, were found. Crystals in urine may change due to urine concentration, temperature and pH, and therefore this test is an often-neglected aspect of urinary sediment analysis.

Hematologic analysis

In the male rats, one-way ANOVA showed that there were significant differences in the mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), WBC and lymphocyte counts between the treatment groups and the control group. The results of Tukey's *post hoc* tests indicated that the low- and middle-dose groups showed significant differences in MCH and MCHC to those of the control group; in addition, all three *A. cinnamomea*-fed groups showed significant differences in WBC and lymphocyte counts in comparison with the control group (Table 4).

In the female rats, there were significant differences in the RBC and mean corpuscular volume (MCV) between the low and high *A. cinnamomea* dose groups (Table 5). MCH refers to the average weight of hemoglobin in the RBC in the sample; MCHC refers to the average concentration of hemoglobin in the RBC contained within the sample. Test results for MCH, MCHC and MCV are used to diagnose types of anemia and their causes: normocytic, macrocytic and microcytic anemia. The results of this experiment showed that in the male rats, there were significant differences in MCH, MCHC, WBC and lymphocyte counts between the *A. cinnamomea*-fed groups and the control group, but all the values were within the normal ranges. In the female rats, no differences were noted between the treatment groups and the control group.

In summary, of the items of hematologic analysis in which a difference was observed, no dose-dependent response was observed. Although some changes were noted, the test values were still within the normal ranges of normal rat hematology [16], suggesting that the changes were independent of *A. cinnamomea* treatment.

Serum biochemistry analysis

The results of one-way ANOVA and *Post hoc* analysis showed that there was a significant difference between the *A. cinnamomea*-treated groups, while the *A. cinnamomea*-treated groups showed no differences from the control group in the male rats (Table 6). In the female rats, there were no differences in total bilirubin between the treatment groups and the control group; only a difference in blood sugar was noted between the low *A. cinnamomea* dose group and the control group (Table 7). The normal ranges of serum biochemistry for rats were based on the literature [16].

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Table 3. Results of Urinalysis

			Group 1 (control)	Group 2 (750mg/kg)	Group 3 (1500mg/kg)	Group 4 (3000mg/kg)
Analysis item		abnormal	Abnormal animal number (male/female)			
Color		white/brown/red/ mucus	1\0	0\0	0\0	1\0
SG (Specific Gravity)		<1.010 or >1.030	1\1	2\2	4\1	3\1
Protein		++	2\1	5\1	5\1	2\1
Urobilinogen		+	0\0	0\0	0\0	0\0
PH		<5.5 or >8.5	3\0	0\0	3\0	1\0
Ketone		+	5\1	7\0	7\0	8\0
Bilirubin		+	3\1	1\0	1\0	2\0
Glucose		+	0\0	0\0	0\0	0\0
Nitrite		+	0\0	0\0	0\0	0\0
Occult blood		+	0\0	0\0	0\0	3\0
Urine sediment/high power field (hpf)	WBC	>1	4\0	0\1	3\0	1\1
	RBC	>1	3\0	0\0	0\0	1\1
	Epithelial cell	+	0\0	1\1	1\0	0\0
	Triple phosphate	+	10\10	10\10	[&]9\10	10\9[#]

[&] rats no. 46 (middle-dose male) died on day 73, final nine rats in total. [#] rats no. 75 (high-dose female) died n day 76, final nine rats in total.

Table 4. Hematological data in male rats.

			Group 1 (control)	Group 2 (750mg/kg)	Group 3 (1500mg/kg)	Group 4 (3000mg/kg)
Item	Unit	Normal range	Male			
Hematocrit ; (Man)	%	36-54	47.9±1.174	47.4±2.716	47.44±5.388	48.25±2.680
Hematocrit ; (Auto)	%	36-54	43.36±2.702	44.25±3.595	44.54±3.183	43.50±3.524
RBC	$\times 10^6/\mu L$	6.76-9.75	7.522±0.559	7.959±0.515	8.052±0.741	7.824±0.806
Hemoglobin	g/dL	11-19.2	17.70±1.909	16.57±1.227	16.61±1.154	17.40±1.601
MCV	fl	48-70	57.72±2.175	55.57±1.838	55.45±2.520	55.76±2.731
MCH	pg	18-23	23.53±1.667	20.83±0.907*	20.67±0.764*	22.29±1.185
MCHC	g/dL	34-38	40.78±2.733	37.48±1.127*	37.3±0.947*	39.98±1.040
WBC	$10^3/\mu L$	6.6-12.6	8.72±2.271	11.81±2.437*	12.44±1.481*	11.67±2.553*
Lymphocytes	$10^3/\mu L$	4.78-9.12	7.256±1.980	10.168±2.328*	10.767±1.509*	9.886±1.584*
Monocytes	/μL	30-180	156.8±113.7	253.9±294.1	205.3±220.4	119.2±112.8
Eosinophils	/μL	10-160	71.9±79.9	112.9±147.5	119.3±146.9	164.2±274.1
Basophils	/μL	0-30	0	0	0	0
PLT	$\times 10^3/\mu L$	638-1177	589±247.5	542.2±224.6	701.7±108.7	675.5±123.4

The data are shown as mean±SD, *p<0.05in comparison with the control group.

Table 5. Hematological data in female rats.

			Group 1 (control)	Group 2 (750mg/kg)	Group 3 (1500mg/kg)	Group 4 (3000mg/kg)
Item	Unit	Normal range	Female			
Hematocrit ; (Man)	%	36-54	41.0±2.461	40.5±4.007	39.40±3.612	38.0±3.52
Hematocrit ;(Auto)	%	36-54	36.76±2.222	37.22±3.678	34.61±2.928	34.47±2.742
RBC	$\times 10^6/\mu L$	6.76-9.75	6.253±0.4492	6.806±1.445	5.917±0.588	5.621±0.4943
Hemoglobin	g/dL	11-19.2	14.53±0.8433	14.97±1.719	14.09±1.137	13.75±1.429
MCV	fl	48-70	58.86±1.760	55.88±7.218	58.63±2.860	61.38±1.868
MCH	pg	18-23	23.26±0.7763	22.46±3.013	23.87±1.047	24.46±1.192
MCHC	g/dL	34-38	39.53±0.6848	40.19±1.748	40.73±0.6093	39.85±1.514
WBC	$10^3/\mu L$	6.6-12.6	8.07±2.971	9.8±2.712	8.61±1.951	8.01±3.418
Lymphocytes	$10^3/\mu L$	4.78-9.12	7.043±2.566	8.423±1.966	8.054±1.844	6.799±3.201
Monocytes	/µL	30-180	240.8±114.2	170.1±236.8	64.10±72.94	244.3±312.8
Eosinophils	/µL	10-160	77.40±69.49	24.50±29.79	23.80±39.92	44.10±63.95
Basophils	/µL	0-30	0	0	0	0
PLT	$\times 10^3/\mu L$	638-1177	647.2±112.6	480.8±260.4	714.3±153.1	528.9±288.9

The data are shown as mean±SD, *p<0.05in comparison with the control group.

Table 6. Serum biochemical analysis in male rats.

			Group 1 (control)	Group 2 (750mg/kg)	Group 3 (1500mg/kg)	Group 4 (3000mg/kg)
Item	Unit	Normal range	Male			
Total serum protein	g/dL	5.6-7.6	6.730±0.3368	7.08±0.5846	6.722±0.2224	6.52±0.4417
Albumin	g/dL	3.8-4.8	3.43±0.3093	3.6±0.5375	3.567±0.3041	3.59±0.3107
Globulin	g/dL	1.8-2.5	3.3±0.4216	3.48±0.5224	3.156±0.2007	2.93±0.2163
AST	U/L	45.7-80.8	91.6±53.27	79.3±17.82	77.44±38.58	74.6±21.46
ALT	U/L	17.5-30.2	31.3±10.03	35.2±5.037	37±22.85	34.2±9.727
ALP	U/L	86-247	569.7±247.3	486.4±142	522.4±117.3	503.2±229.6
T. Bilirubin	mg/dL	0.2-0.5	0.26±0.06992	0.333±0.1118	0.3889±0.1537	0.3±0.09428
Cholesterol	mg/dL	40-130	68.5±12.6	71.78±22.35	74.78±13.21	69.6±16.6
Triglyceride	mg/dL	16-175	103.9±60.74	163.3±86.89	129.2±76.29	85.4±48.19
Glucose	mg/dL	50-160	176.5±39.13	170.7±38.7	206±44.87	170.7±27.92
BUN	mg/dL	15-21	18.17±2.123	18.99±2.878	18.53±3.173	17.89±1.501
Creatinine	mg/dL	0.2-0.8	0.22±0.1033	0.29±0.09944	0.2±0.07071	0.24±0.1075
Ip	mg/dL	3.11-11	5.89±1.339	6.45±1.083	6.289±0.9688	6.910±1.051
Ca^{2+}	mg/dL	5.3-13	8.8±1.036	9.32±0.6647	8.144±0.7178	8.65±0.6604
Na^+	mEq/L	140-150	144±2.494	146.6±3.596	145.4±3.812	141±2.625
K^+	mEq/L	4.3-5.6	8.910±13.39	5.270±0.4296	4.978±0.4994	5.18±0.4104
Cl^-	mEq/L	95-115	102.7±3.368	104.5±5.297	104.1±2.571	101.6±2.459

The data are shown as mean±SD, *p<0.05in comparison with the control group.

Table 7. Serum biochemical analysis in female rats.

			Group 1 (control)	Group 2 (750mg/kg)	Group 3 (1500mg/kg)	Group 4 (3000mg/kg)
Item	Unit	Normal range	Female			
Total serum protein	g/dL	5.6-7.6	7.630±0.3057	7.730±0.5945	7.89±0.404	7.844±0.4333
Albumin	g/dL	3.8-4.8	4.29±0.6045	4.490±0.5174	4.42±0.4733	4.756±0.3779
Globulin	g/dL	1.8-2.5	3.34±0.6653	3.24±0.5835	3.47±0.5870	3.089±0.4595
AST	U/L	45.7-80.8	102.8±67.13	121.6±102.4	61.8±16.61	76.22±19.25
ALT	U/L	17.5-30.2	45.9±27.46	38±18.94	28±7.165	31.67±5.788
ALP	U/L	86-247	275.3±105.8	370±150.8	305.6±147	377.8±133.8
T. Bilirubin	mg/dL	0.2-0.5	0.4±0.1333	0.28±0.1033	0.47±0.1418	0.3778±0.1856
Cholesterol	mg/dL	40-130	84±11.94	94.4±17.29	85±26.10	80.11±19.90
Triglyceride	mg/dL	16-175	105.1±60.72	96.8±64.67	77.40±30.81	79.67±53.66
Glucose	mg/dL	50-160	189.9±33.7	159.1±22.03*	161.0±22.66	179.9±12.94
BUN	mg/dL	15-21	19.10±3.483	19.16±2.762	21.07±2.840	19.21±2.827
Creatinine	mg/dL	0.2-0.8	0.23±0.06749	0.27±0.09487	0.24±0.09661	0.1889±0.09280
Ip	mg/dL	3.11-11	4.83±1.261	5.33±0.7394	5.89±0.9927	5.963±0.8245
Ca^{2+}	mg/dL	5.3-13	9.67±0.5165	9.1±0.8055	9.17±0.6273	8.967±0.8646
Na^{+}	mEq/L	140-150	143.3±2.983	141.9±4.306	145.4±5.337	145.4±4.126
K^{+}	mEq/L	4.3-5.6	4.44±0.5562	5.05±1.244	4.89±0.5065	5.038±9.7009
Cl^{-}	mEq/L	95-115	102±3.333	99.6±3.471	103.6±8.884	102.8±3.898

The data are shown as mean±SD, *p<0.05in comparison with the control group.

Blood clotting

Clotting time (CT) was estimated using the slide method, and the prothrombin time (PT) and activated partial thromboplastin time (APTT) were obtained using a coagulometer (Coag Dx Analyzer; IDEXX, MA, USA). One-way ANOVA showed that there were no differences (all *P* values > 0.05) in CT, PT or APTT between the treatment and control groups in both the male and female rats. The values, presented as mean ± SD, are shown in Table 8 (male rats) and Table 9 (female rats).

Necropsy and organ weights

At the end of the oral feeding experiment, the rats were necropsied. The mean ± SD of the organ weights in each group are listed in Table 10 (male rats) and Table 11(female rats). One-way AVOVA showed no differences between the treatment and control groups.

Pathological examination by visual inspection

During the necropsies, the veterinary students were supervised and supported by animal specialist/veterinary pathologist Dr. Ching-Dong Chang to identify any lesions or other pathological conditions. No pathological changes were seen in any of the rats.

Histopathologic analyses

Organ samples were weighed and fixed in 10% neutral buffered formalin. Tissue samples were then transferred into an automated tissue processor for the fixation, dehydration and infiltration process. The parafilm tissue blocks were cut using a microtome at thicknesses of 2-4 μm, following which the sections were placed onto slides, put into an automated stainer for hematoxylin and eosin (H&E) staining, then mounted. The specimen sections were subsequently examined by a pathologist. Pathological examination identified feeding sample residue in the lungs, confirming that the deaths of rat no. 46 (middle-dose male) and rat no. 75 (high-dose female) on day 73 and day 76 were due to a feeding accident causing sample inhalation into the lungs.

Table 8. Blood clotting test data in male rats.

		Group 1 (control)	Group 2 (750mg/kg)	Group 3 (1500mg/kg)	Group 4 (3000mg/kg)	ANOVA p value
Item (sec)	Normal range (sec)	Male				
Clotting time	60-300	117.9±89.4	122.10±93.2	60.70±30.6	111.60±84.1	0.0746
PT	No reference data	23.56±4.09	23.9±7.43	24.70±4.71	26.90±4.89	0.2238
APTT	No reference data	47.50±5.50	42.70±8.73	42.80±5.9	43.50±6.08	0.3975

The data are shown as mean±SD.

Table 9. Blood clotting test data in female rats.

		Group 1 (control)	Group 2 (750mg/kg)	Group 3 (1500mg/kg)	Group 4 (3000mg/kg)	ANOVA p value
Item (sec)	Normal range (sec)	Female				
Clotting time	60-300	103.50±93.20	78.70±58.20	80.10±64.10	114.40±80.30	0.2745
PT	No reference data	26.10±3.38	23.50±2.32	25.70±6.41	24.10±4.84	0.1351
APTT	No reference data	46.40±5.42	42.70±9.19	44.70±5.79	44.90±7.63	0.5829

Table 10. Organ weight of male rats.

Group/male	liver	kidney and adrenal gland	testes and epididymis	thymus	spleen	cerebrum, cerebellum and pons	heart
Group 1 (control)	22.56±2.71	4.53±0.66	6.16±0.92	0.40±0.23	0.928±0.1	4.32±6.56	2.03±0.27
Group 2 (750mg/kg)	26.11±4.02	4.94±0.63	5.78±0.71	0.46±0.13	1.05±0.12	2.14±0.19	2.09±0.29
Group 3 (1500mg/kg)	24.65±3.4	4.78±0.67	6.172±0.79	0.44±0.19	0.93±0.22	2.07±0.24	1.96±0.3190
Group 4 (3000mg/kg)	25.33±3.10	4.79±0.69	6.03±0.86	0.43±0.18	0.98±0.16	2.24±0.13	1.995±0.29
ANOVA p value	0.119	0.571	0.686	0.929	0.225	0.362	0.753

The data are shown as mean±SD.

Table 11. Organ weight of female rats.

Group/female	liver	kidney and adrenal gland	uterus and overies	thymus	spleen	cerebrum, cerebellum and pons	heart
Group 1 (control)	14.35±1.47	3.09±0.62	2.72±1.49	0.28±0.16	0.67±0.13	2.00±0.09	1.29±0.21
Group 2 (750mg/kg)	13.91±1.05	2.99±0.57	2.27±1.26	0.30±0.20	0.70±0.14	1.945±0.1440	1.28±0.09
Group 3 (1500mg/kg)	14.08±1.40	3.12±0.60	2.78±1.80	0.31±0.16	0.61±0.11	1.92±0.17	1.20±0.13
Group 4 (3000mg/kg)	14.99±1.23	3.02±0.46	2.31±1.73	0.32±0.09	0.65±0.20	2.000±0.06	1.27±0.18
ANOVA p value	0.280	0.954	0.835	0.976	0.610	0.327	0.508

The data are shown as mean±SD.

4. Conclusions

The data from this experiment demonstrated that the extracted solution of *A. cinnamomea* fruiting bodies obtained from Da-Yi Biotech Corporation did not cause adverse effects or toxicity in the organs of rats administered with the doses tested. The NOAEL was at least 3000 mg/kg, and using a safety factor of 100, the ADI was estimated to be 30 mg/kg.

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