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Repeated Dose 90-Day Oral Toxicity Study of an *Antrodia cinnamomea* Product via a Novel Process in Sprague Dawley Rats

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Abstract. *Antrodia cinnamomea* as a medicinal mushroom exhibited neuroprotective, hepatoprotective, anti-inflammatory, antioxidant, or even antineoplastic activities. However, wood-cultured *A. cinnamomea* products cost too high to afford, and some people in Taiwan still had a concern for the safety of *A. cinnamomea*. Hence, the purpose of the study was conducted to evaluate repeated dose 90-day oral toxicity of a high-quality and low-price *A. cinnamomea* product via a novel process, spraying solid-state-cultivated *A. cinnamomea* extracts on wood-cultivated *A. cinnamomea* powder, in Sprague Dawley rats. Three dose levels were conducted in the study, namely 1000, 2000, and 4000 mg/kg BW. Results showed that no clinical abnormality of the rats was observed, and all rats gained weights normally without any significant difference. No treatment-related histopathological changes were observed, but significantly different and dose-independent responses were revealed for leukocyte esterase in male urine, chloride in male serum, and female liver weight. The significant changes of chloride in male serum, hemoglobin and mean corpuscular hemoglobin in male blood, and relative weight of adrenal glands were within normal ranges. In conclusion, the repeated dose 90-day oral toxicity study showed no treatment-related adverse effect in the Sprague Dawley rats after administration of the *A. cinnamomea* product via the novel process.

INTRODUCTION

Antrodia cinnamomea, usually growing on the inner rotting trunk of *Cinnamomum kanehirai* Hayata endemic to Taiwan, had more than 97 bioactive compounds identified and structurally elucidated [1-6], and these compounds exhibited neuroprotective, hepatoprotective, anti-inflammatory, antioxidant, or even antineoplastic activities [2,7]. However, in acute toxicity studies, diarrhea was found on the day when administration of 15000 mg/kg body weight (BW) of *A. cinnamomea* concentrate in Sprague Dawley rats [8]. On the other hand, Kunming mice's activities were not affected when administration of 20000–22900 mg/kg BW of *A. cinnamomea* suspension [9,10]. Additionally, in 2013, it was reported that *A. cinnamomea* overdoses could harm the kidneys of rats [11], and some people in Taiwan still had a concern for the safety of *A. cinnamomea*. Thereafter, the Ministry of Health and Welfare of the Republic of China made a public announcement that *A. cinnamomea* as food ingredients should have certified documents about detailed manufacturing processes, specifications, and 90-day oral toxicity studies due to the safety issue in 2015 [12]. Ninety-day oral toxicity studies can provide information on major toxic effects, target organs, possibilities of accumulation, and an estimate of a no-observed-adverse-effect level of exposure [13].

In the past, *A. cinnamomea* only come from the wild, and the high-grade fruiting bodies cost about US$15–44/g[14,15]. Nowadays, *A. cinnamomea* could be artificially cultivated on the trunk of *C. kanehirai* Hayata, on solid media, or in liquid media [16]. The fresh *A. cinnamomea* artificially cultivated on the trunk of *C. kanehirai* Hayata cost the highest, ranging from US$14.2–29.3/g. The price of the solid-state-cultivated *A. cinnamomea* was from US$1.0–4.8/g, and the liquid-state-fermented *A. cinnamomea* could be bought for US$0.6/g. However, the highest cost of the wild or wood-cultivated *A. cinnamomea* limited the desire in some persons in spite of better effectiveness. It was why a novel process was developed in the study to spray solid-state-cultivated *A. cinnamomea* extract on wood-

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cultivated *A. cinnamomea* powder to make a high quality and low price product. For powder of liquid-state-fermented mycelia [17,18], powder of solid-state-cultivated mycelia [19,20], extract of wood-cultivated fruiting bodies [21], extract of wood-cultivated fruiting bodies mixed with powder of solid-state-cultivated mycelia [22] of *A. cinnamomea*, no treatment-related observable adverse effects were found in 90-day oral toxicity studies, but there was not any study reported about the safety of the *A. cinnamomea* product via the novel process. Hence, the purpose was to evaluate the 90-day oral toxicity of the *A. cinnamomea* product via the novel process.

MATERIALS AND METHODS

Mycelia Source, Medium, and Culture Condition

Mycelia of *Antrodia cinnamomea* supplied by the Greenrays International Co., Ltd. (Chiayi, Taiwan) were grown in solid-state condition and on stout camphor (*Cinnamomum kanehirai* Hayata) logs. In solid-state-cultured condition, the mycelia were cultured using mushroom grow bags. Each bag contained corn cob, rice bran, wheat bran, and corn powder in the ratio 15.8:2.6:2.6:1 (w/w), adding 0.1% magnesium sulfate ($MgSO_4$) and 0.1% zinc sulfate ($ZnSO_4$), with water content of 53.94%. After sterilizing by autoclaving at 120°C for 2 hours, the bag was cooled down to 20 °C and then inoculated with the mycelia. After cultivation at 25 °C for 3 months, the grow bag was removed and reverse osmosis water was sprayed to induce primordia. The fruiting bodies were selected and harvested after 6 months, and the harvested mycelia with substrates were dried with air to water content of 5 % and then crushed. In wood-cultured condition, each stout camphor log inoculated with the mycelia was placed in a growth chamber equipped with a Supa Fine HF-096 cool mist humidifier (Shivn Feng Enterprise Co., Ltd., New Taipei City, Taiwan) running at 7–9 AM and 8–9 PM to achieve average humidity of 85 % and at 25 °C in a dark environment. The fruiting bodies on stout camphor logs were selected and harvested after 6 months. The solid-state-cultured and wood-cultured fruiting bodies were dried and powdered into 100–150 meshes.

Extraction and Concentration

Six hundred kilograms powder of mycelia with substrates was extracted 3 different extraction solvents. First, the powder was extracted with 6,000 kg of 55% ethanol at 50 °C for 18 hours. Second, the filter residue was extracted with 6,000 kg of reverse osmosis water at 90 °C for 18 hours. Finally, the second filter residue was extracted with 6,000 kg of reverse osmosis water under high pressure at 120 °C for 18 hours. These three filtrates were then concentrated under vacuum of 680 ± 20 mmHg at 52 ± 2 °C, respectively [23]. The three concentrates had 975 kg in total.

Spray Granulation

The three concentrates were used for spray granulation with 56 kg of the powdered fruiting bodies as starter cores in reversed order using a fluid-bed spray granulator YS-FDG-600 (Inora Pharmaceutical Machinery Co., Ltd., Taichung City, Taiwan). The inlet air temperature was 40 °C during the mixing and spraying phases and was raised to 70 ± 2 °C during the drying phase. The liquid pump speed and the fan speed were 35 rpm and 40 rpm, respectively. After granulation, total 164 kg of *A. cinnamomea* granules were obtained. The granules thereafter powdered into 100 meshes again.

Animal and Experiment Design

Eighty 5-week-old Sprague Dawley rats were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan) and were tested in Super Laboratory Co., Ltd. (New Taipei City, Taiwan). Two rats of the same sex per cage were kept under a 12-h light/dark cycle at 22 ± 3 °C and relative humidity of 55 ± 15 %. The rats were given *ad libitum* access to a normal chow diet MFG (Oriental Yeast Co., Ltd, Tokyo, Japan) and sterilized RO water. After 1-week acclimation and quarantine period, the animals were randomly assigned to a control group or one of three treatment groups. Every group had 10 male and 10 female rats. The control group was only administered 10 mL/kg body weight (BW) of RO water. Three treatment groups, i.e. AC1000, AC2000, and AC4000 groups, were set according to the three dose levels of the *A. cinnamomea* granules suspended in RO water. The dose levels used in the study were 1000, 2000, and 4000

mg/kg BW of the *A. cinnamomea* suspension, respectively. Each rat was administered a volume of 10 mL/kg BW of the *A. cinnamomea* suspension via oral gavage every day for 90 days.

Body Weight, Food Consumption, and Ophthalmological Examination

The body weight of each rat was measured once a week while the food consumption was recorded. All rats' eyes were examined using an ophthalmoscope prior to the administration of the *A. cinnamomea* suspension and at the end of the study.

Urinalysis

At the ninetieth day, the rats were moved to metabolic cages for 16 h to collect urine. A portion of the urine was analyzed using a urine analyzer PocketChem UA PU-4010 (ARKRAY, Inc., Kyoto, Japan) to examine specific gravity, pH value, glucose, ketone, protein, bilirubin, urobilinogen, nitrite, occult blood, and leukocyte esterase. The color and clarity of the urine were examined with the naked eye. Another portion of the urine after centrifuged was observed for red blood cell, white blood cell, epithelial cell, urinary crystal, and microorganism in its sediment by using Nikon Optiphot-2 microscope (Nikon Corporation, Tokyo, Japan).

Hematology and Serum Biochemical Assay

At the end of study, the rats after overnight fasting were anesthetized with CO_2. Blood sample was taken from the heart, and one part of the blood sample was collected in a blood collection tube with EDTA or sodium citrate. After homogeneous mixing, the sample in the tube with EDTA was examined using an automated hematology analyzer XT-1800iV (Sysmex Corporation, Kakogawa, Japan) for red blood cell count (RBC), hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), hemoglobin, hematocrit, reticulocyte, white blood cell count (WBC), neutrophil, eosinophil, basophil, monocyte, lymphocyte, platelet count. The sample in the tube with sodium citrate was tested using an automated blood coagulation analyzer CA-1500 (Sysmex Corporation, Kakogawa, Japan) for prothrombin time.

Another part of the blood sample was held at room temperature for clotting. After coagulation, the serum of the centrifuged sample was analyzed using an automatic chemistry analyzer Hitachi 7070 (Hitachi, Ltd., Tokyo, Japan) for aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyltranspeptidase (GGT), total bilirubin, total protein, albumin, globulin, creatinine, blood urea nitrogen (BUN), glucose, cholesterol, triglyceride, calcium, phosphorus, sodium, chloride, and potassium.

Histopathology

At the end of study, the rats received humane euthanasia by CO_2. The brain, heart, liver, spleen, kidneys, adrenal glands, testes, or ovaries were excised and weighed after removal of visceral fat. Then, the ratio of organ-to-body weight (relative organ weight) was calculated [24]. Eyes, lungs, forestomach, glandular stomach, pancreas, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, spinal cord, sternal marrow, femur bone, aorta, tongue, trachea, esophagus, skeletal muscle, skin, pituitary gland, thyroid glands, Harderian glands, salivary glands, thymi, mammary glands, prostate gland, coagulation glands, mandibular lymph nodes, mesenteric lymph nodes, optic nerve, sciatic nerve, epididymides, seminal vesicles, uterus, and vagina were also excised. The testes were fixed in modified Davidson's solution for 24 hours and were thereafter preserved in 10 % neutral buffered formalin. The other tissues and organs were fixed and preserved in 10 % neutral buffered formalin. The tissues and organs of the control group and the high dose group were selected and were embedded into paraffin blocks. The paraffin-embedded specimens were cut into slices between 4 and 5 μm in thickness using a semi-motorized rotary microtome Leica RM 2145 (Leica Biosystems Nussloch GmbH, Nussloch, Germany) and these slices were stained with hematoxylin and eosin (H&E). The stained specimens were observed by using Nikon Optiphot-2 microscope (Nikon Corporation, Tokyo, Japan). Lesion severity scored from 1 to 5, and minimal severity (< 1 %) was denoted by 1 while massive lesion (76–100 %) was represented by 5 [25].

Statistical Analysis

The following statistical analyses were conducted using SAS Studio 3.4 (SAS Institute Inc., Cary, NC, USA), and a significance level of 0.05 was chosen in the study. The body weight and daily food consumption were analyzed for repeated measures analysis using a mixed model. The results of urinalysis and the histopathological counts of the tissues and organs were compared for the chi-square test. The one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test was performed for the results of hematological analyses and serum biochemical assays, and absolute and relative weights of the organs. For the histopathological lesion severity scores of the tissues and organs, the independent t test was conducted.

RESULTS

Body Weight, Food Consumption, and Ophthalmological Examination

No clinical abnormality of the rats was observed, and all rats were alive after administration of the *A. cinnamomea* suspension at three dose levels for 90 days. Although the daily food consumption of the male Sprague Dawley rats either in the AC1000 group (20.6 ± 1.4 g, $P = 0.025$) or in the AC2000 group (21.2 ± 1.2 g, $P = 0.001$) was significantly higher than that in the control group (19.5 ± 1.7 g) at the 13th week after 90-day treatment, the rats gained weights normally without any significant difference (Figure 1).

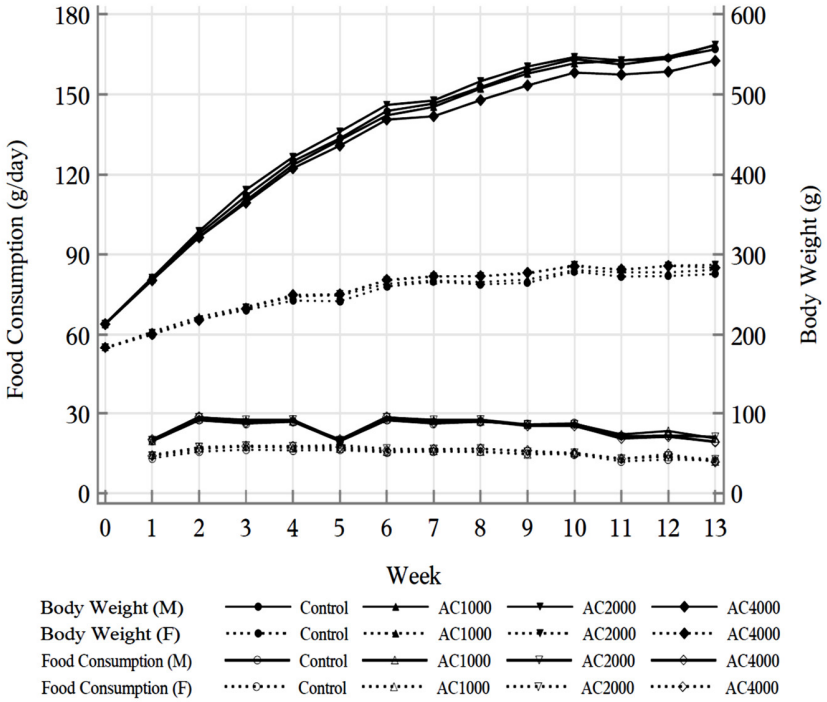

FIGURE 1. Body weight and weekly changes of daily food consumption of the Sprague Dawley rats given the *A. cinnamomea* suspension once a day during 90-day treatment

Urinalysis

The rats were detected negatively for glucose and bilirubin in the urines, and 0–1 red blood cell, 0–1 epithelial cell per high power field and no urinary crystal were found in the sediments. The color, clarity, specific gravity, pH value, ketone, and occult blood in urines and white blood cell in the sediments varied, but they were not significantly different between the control group and the treatment groups in both sexes. In addition, there were no protein and urobilinogen in the female rats' urines, and there were not significantly different for protein and urobilinogen examination in the

male rats' urines. After administration of the *A. cinnamomea* suspension once a day for 90 days, however, more female rats had positive nitrite in the AC4000 group than in the control group, and more male rats had 1+ reading of leukocyte esterase in the AC2000 group than in the control group (Table 1).

TABLE 1. Urinalysis in the Sprague Dawley rats after administration of the *A. cinnamomea* suspension once a day for 90 days

	Male					Female				
Item	**Control**	**AC1000**	**AC2000**	**AC4000**	***P***	**Control**	**AC1000**	**AC2000**	**AC4000**	***P***
Color					0.414					0.759
Yellow	7	5	8	8		3	3	4	5	
Pale yellow	3	5	2	2		7	7	6	5	
Clarity					0.592					0.502
Clear	2	3	1	1		7	9	9	9	
Light turbid	8	7	9	9		3	1	1	1	
Specific gravity					0.436					0.740
<1.01	1	4	2	2		2	4	4	3	
1.01-1.06	9	6	8	8		8	6	6	7	
pH value					0.634					0.270
<6.7	2	1	2	1		10	9	8	7	
6.7-7.3	5	6	2	4		0	1	2	3	
7.4-8.2	3	3	6	5		0	0	0	0	
Glucose					NA					NA
Negative	10	10	10	10		10	10	10	10	
Ketone					0.744					0.380
Negative	8	7	7	7		9	10	10	10	
Trace	2	2	3	3		1	0	0	0	
1+	0	1	0	0		0	0	0	0	
Protein					0.795					NA
Negative	5	4	2	3		10	10	10	10	
Trace	3	4	3	2		0	0	0	0	
1+	2	2	4	4		0	0	0	0	
2+	0	0	1	1		0	0	0	0	
Bilirubin					NA					NA
Negative	10	10	10	10		10	10	10	10	
Urobilinogen					0.380					NA
Negative	10	10	9	10		10	10	10	10	
1+	0	0	1	0		0	0	0	0	
Nitrite					0.725					0.011
Negative	7	7	7	5		9	10	7	4	
Positive	3	3	3	5		1	0	3	6	
Occult blood					0.291					0.412
Negative	10	9	7	10		9	9	10	10	
Trace	0	1	2	0		1	0	0	0	
1+	0	0	1	0		0	1	0	0	
Leukocyte esterase					0.020					0.412
Negative	8	5	1	1		10	9	10	9	
Trace	0	4	4	6		0	0	0	1	
1+	1	0	4	2		0	1	0	0	
2+	1	1	1	1		0	0	0	0	
Red blood cell					NA					NA
0–1/HPF	10	10	10	10		10	10	10	10	
White blood cell					0.121					0.473
0–1/HPF	5	8	5	9		8	8	9	10	
2–5/HPF	5	2	5	1		2	2	1	0	
Epithelial cell					NA					NA
0–1/HPF	10	10	10	10		10	10	10	10	
Urinary crystal					NA					NA
None found	10	10	10	10		10	10	10	10	

Hematology and Serum Biochemical Assay

The RBC, MCV, MCHC, hematocrit, reticulocyte, WBC, neutrophil, eosinophil, basophil, monocyte, lymphocyte, platelet count, prothrombin time in the bloods were not significantly different between the control group and the treatment groups in both sexes after administration of the *A. cinnamomea* suspension once a day for 90 days. The MCH (17.56±0.83pg) and hemoglobin (15.99±0.75 g/dL) of the male rats in the AC4000 group were significantly lower than those in the control group (18.48±1.00 pg and 17.01±0.84 g/dL, respectively), but no significant difference was found in the female rats (Table 2).

TABLE 2. Hematological analysis in the Sprague Dawley rats after administration of the *A. cinnamomea* suspension once a day for 90 days

	Male				Female			
Item	**Control**	**AC1000**	**AC2000**	**AC4000**	**Control**	**AC1000**	**AC2000**	**AC4000**
RBC (10^6/μL)	9.21±0.50	9.39±0.50	9.06±0.35	9.13±0.61	8.54±0.41	8.39±0.31	8.44±0.28	8.30±0.65
MCV (fL)	58.42±5.09	56.89±3.40	56.18±3.50	56.64±4.26	61.08±2.08	60.70±2.61	60.49±1.99	61.28±3.43
MCH (pg)	18.48±1.00	17.78±0.45	17.89±0.73	17.56±0.83*	18.79±0.58	18.70±0.27	18.52±0.46	18.82±0.59
MCHC (g/dL)	31.75±1.51	31.31±1.53	31.88±1.26	31.09±1.17	30.75±0.83	30.85±1.01	30.64±0.53	30.77±1.24
Hemoglobin (g/dL)	17.01±0.84	16.67±0.86	16.20±0.50	15.99±0.75*	16.03±0.79	15.67±0.62	15.64±0.69	15.63±0.91
Hematocrit (%)	53.68±4.37	53.32±3.05	50.84±1.86	51.52±3.39	52.13±2.26	50.82±2.45	51.07±2.53	50.87±3.55
Reticulocyte (%)	2.34±1.32	1.99±0.43	1.37±1.01	1.82±0.51	2.52±0.76	2.62±0.79	2.77±0.67	2.39±0.61
WBC (10^3/μL)	11.61±2.89	10.20±1.30	12.13±2.67	12.53±1.46	8.57±2.17	8.31±1.83	8.16±2.30	8.16±2.08
Neutrophil (%)	20.49±4.76	19.59±7.58	17.99±5.37	15.13±4.45	12.19±5.85	11.98±2.80	10.91±3.06	11.32±2.79
Eosinophil (%)	1.46±0.66	1.30±0.46	1.27±0.59	1.58±0.34	1.12±0.36	1.25±0.50	1.42±0.70	1.26±0.53
Basophil (%)	0.15±0.07	0.14±0.07	0.19±0.06	0.17±0.05	0.16±0.10	0.20±0.13	0.25±0.08	0.23±0.12
Monocyte (%)	4.44±2.11	4.20±1.45	4.22±1.28	3.35±1.76	2.53±1.28	3.13±2.28	2.65±1.26	2.14±0.98
Lymphocyte (%)	73.46±6.36	74.77±8.74	76.33±6.49	79.77±6.11	84.00±6.29	83.44±4.19	84.77±3.85	85.05±3.36
Platelet count (10^3/μL)	883.4±145.4	952.2±162.2	840.7±171.4	918.7±145.8	836.2±75.3	851.7±118.6	803.0±112.2	856.1±85.0
Prothrombin time (s)	15.05±2.08	13.91±2.21	14.25±1.96	13.38±1.72	9.93±0.16	9.72±0.23	9.78±0.23	9.72±0.23

The AST, ALT, ALP, GGT, total bilirubin, total protein, albumin, globulin, creatinine, BUN, glucose, cholesterol, triglyceride, calcium, phosphorus, sodium, and potassium in the serums were not significantly different among the groups. There was significantly higher chloride level (102.2±1.3meq/L) in the AC2000 group than in the control group (100.2±2.3 meq/L) in the male rats, but the chloride levels were not significantly different between the AC4000 group and the control group in either male or female rats (Table 3).

TABLE 3. Serum biochemical assays in the Sprague Dawley rats after administration of the *A. cinnamomea* suspension once a day for 90 days

	Male				Female			
Item	**Control**	**AC1000**	**AC2000**	**AC4000**	**Control**	**AC1000**	**AC2000**	**AC4000**
AST (U/L)	141.8±64.1	114.3±27.1	112.0±40.2	111.8±23.2	118.1±45.1	116.8±45.0	110.4±33.6	112.6±49.1
ALT (U/L)	76.8±54.1	55.0±26.6	47.7±16.6	55.1±18.9	42.1±21.6	40.7±20.3	41.6±18.0	47.2±33.5
ALP (U/L)	82.30±17.42	84.10±20.17	77.70±12.88	91.70±16.79	45.60±8.40	47.60±14.83	49.60±15.60	38.10±5.99
GGT (U/L)	0.47±0.37	0.13±0.20	0.52±0.63	0.44±0.40	0.59±0.31	0.39±0.36	0.33±0.32	0.78±0.42
Total Bilirubin (μg/dL)	20.12±8.71	13.08±6.74	19.67±8.36	19.05±7.38	33.64±15.25	34.85±16.69	37.38±10.19	29.92±15.24
Total Protein (g/dL)	7.07±0.38	7.03±0.33	6.96±0.28	6.99±0.29	7.49±0.59	7.62±0.52	7.70±0.75	7.73±0.48
Albumin (g/dL)	4.36±0.22	4.39±0.20	4.33±0.15	4.36±0.13	4.76±0.42	4.94±0.36	4.97±0.45	4.98±0.35
Globulin (g/dL)	2.71±0.21	2.64±0.17	2.63±0.18	2.63±0.19	2.73±0.22	2.68±0.22	2.73±0.32	2.75±0.18
Creatinine (mg/dL)	0.53±0.06	0.53±0.06	0.47±0.06	0.49±0.04	0.54±0.05	0.54±0.08	0.54±0.08	0.54±0.07
BUN (mg/dL)	16.01±2.25	15.43±1.98	15.33±1.92	14.10±1.92	16.33±1.68	14.52±1.91	16.91±2.15	15.19±2.64
Glucose (mg/dL)	260.2±95.6	278.2±52.2	230.3±50.2	286.2±30.2	153.6±53.9	189.5±64.1	170.3±72.1	197.7±55.9
Cholesterol (mg/dL)	58.60±15.81	64.30±16.99	62.20±13.12	45.80±10.00	82.10±15.81	70.90±19.62	84.80±18.68	73.10±10.00
Triglyceride (mg/dL)	84.50±57.30	79.50±30.57	65.90±15.05	61.40±20.13	72.90±25.90	64.40±25.82	52.80±8.85	56.30±22.86
Calcium (mg/dL)	11.91±0.75	12.29±0.39	12.00±0.54	12.07±0.19	12.08±0.43	12.37±0.65	12.26±0.71	12.43±0.41
Phosphorus (mg/dL)	11.33±1.92	11.26±1.26	10.95±1.01	11.25±1.55	10.23±1.44	9.53±0.98	10.48±1.18	10.31±1.66
Sodium (meq/L)	149.6±1.2	150.6±1.4	150.8±1.6	150.1±0.6	147.2±2.2	147.1±1.6	145.9±2.9	146.6±2.0
Chloride (meq/L)	100.2±2.3	100.4±1.6	102.2±1.3*	100.9±1.1	101.9±2.7	101.7±2.7	101.0±1.9	100.7±1.8
Potassium (meq/L)	6.91±1.20	7.04±0.96	6.96±1.08	6.73±0.97	7.43±1.24	7.49±1.23	7.75±1.39	7.54±1.31

Histopathology

The weights of brains, hearts, spleens, and kidneys were not significantly different between the control group and the treatment groups in both sexes. Neither the weight of the male's testes nor the weight of the female's ovaries was significantly different. But, the weight of the female rat's liver in the AC2000 group (8.19 ± 0.72 g) and the weight of the female rat's adrenal glands in the AC4000 group (0.07 ± 0.01 g) were found to be significantly higher than those in the control group (7.32 ± 0.66 g and 0.06 ± 0.01 g, respectively). Additionally, the organ-to-body weight ratios of the male's and female's adrenal glands in the AC4000 group were significantly higher than those in the control group (Table 4).

TABLE 4. The weights of the organs in the Sprague Dawley rats after administration of the *A. cinnamomea* suspension once a day for 90 days

	Male				Female			
Item	**Control**	**AC1000**	**AC2000**	**AC4000**	**Control**	**AC1000**	**AC2000**	**AC4000**
Brain (g)	2.14 (0.41)	2.19 (0.42)	2.20 (0.43)	2.08 (0.42)	1.95 (0.77)	2.02 (0.79)	1.99 (0.74)	1.92 (0.75)
Heart (g)	1.76 (0.34)	1.76 (0.33)	1.77 (0.34)	1.78 (0.36)	0.94 (0.37)	1.01 (0.40)	0.96 (0.36)	0.91 (0.36)
Liver (g)	15.86 (3.03)	15.89 (3.03)	15.79 (3.02)	15.41 (3.07)	7.32 (2.89)	7.88 (3.09)	8.19* (3.05)	8.01 (3.14)
Spleen (g)	0.78 (0.15)	0.80 (0.15)	0.79 (0.15)	0.72 (0.14)	0.47 (0.18)	0.49 (0.19)	0.50 (0.18)	0.50 (0.19)
Kidneys (g)	3.87 (0.74)	3.86 (0.74)	3.83 (0.73)	3.75 (0.75)	1.90 (0.75)	2.00 (0.79)	2.06 (0.76)	2.06 (0.81)
Adrenal glands (g)	0.06 (0.01)	0.05 (0.01)	0.06 (0.01)	0.07 (0.01)†	0.06 (0.02)	0.07 (0.03)	0.07 (0.02)	0.07* (0.03)†
Testes (g)	3.49 (0.67)	3.64 (0.69)	3.44 (0.66)	3.43 (0.69)	NA	NA	NA	NA
Ovaries (g)	NA	NA	NA	NA	0.08 (0.03)	0.09 (0.04)	0.08 (0.03)	0.09 (0.04)

In spite of higher absolute and relative weights of the female rat's adrenal glands, the lesion count in the AC4000 group was not significantly different from that in the control group (Table 5), and the lesion severity scores were not significantly different, either (Table 6). The similar results were found in the male's adrenal glands (Table 5 and Table 6). In the study, little lesions were also found for heart, liver, kidneys, lungs, glandular stomach, pancreas, rectum, and Harderian glands in both sexes, ovaries in the female rats, and forestomach, prostate gland, and epididymides in the male rats, but these were not significantly different (Table 6). The forebrain, midbrain, cerebellum, pons, spleen, duodenum, jejunum, ileum, caecum, colon, urinary bladder, spinal cord, sternal marrow, femur bone, aorta, tongue, trachea, esophagus, skeletal muscle, skin, salivary glands, thymi, prostate gland, mandibular lymph nodes, and mesenteric lymph nodes were all normal. In addition, the male rats' testes, coagulation glands, and seminal vesicles, and the female rats' mammary glands, uteri, and vaginas were also normal.

TABLE 5. The histopathological counts of the tissues and organs in the Sprague Dawley rats after administration of the *A. cinnamomea* suspension once a day for 90 days

	Male			Female		
Item	**Control**	**AC4000**	***P***	**Control**	**AC4000**	***P***
Heart	7/3	6/4	0.639	6/4	3/7	0.178
Liver	10/0	9/1	0.305	9/1	10/0	0.305
Kidneys	9/1	6/4	0.121	5/5	5/5	>0.999
Adrenal glands	6/4	9/1	0.121	2/8	4/6	0.329
Ovaries	NA	NA	NA	0/10	1/9	0.305
Eyes	0/10	1/9	0.305	0/10	0/10	NA
Lungs	8/2	10/0	0.136	8/2	9/1	0.531
Forestomach	0/10	2/8	0.136	0/10	0/10	NA
Glandular stomach	0/10	1/9	0.305	2/8	0/10	0.136
Pancreas	8/2	10/0	0.136	3/7	6/4	0.178
Rectum	2/8	1/9	0.531	3/7	2/8	0.606
Pituitary gland	0/10	1/9	0.305	2/8	2/8	>0.999
Thyroid glands	3/7	2/8	0.606	0/10	2/8	0.136
Harderian glands	0/10	2/8	0.136	3/7	0/10	0.060
Prostate gland	6/4	5/5	0.653	NA	NA	NA
Optic nerve	2/8	1/9	0.531	0/10	0/10	NA
Sciatic nerve	1/9	0/10	0.305	0/10	0/10	NA
Epididymides	1/9	1/9	>0.999	NA	NA	NA

TABLE 6. The histopathological lesion severity scores of the tissues and organs in the Sprague Dawley rats after administration of the *A. cinnamomea* suspension once a day for 90 days

	Male			Female		
Item	**Control**	**AC4000**	***P***	**Control**	**AC4000**	***P***
Heart	0.5±0.4	0.4±0.3	0.382	0.4±0.3	0.2±0.2	0.145
Liver	0.6±0.2	0.5±0.3	0.391	0.4±0.3	0.4±0.1	0.487
Kidneys	0.3±0.2	0.2±0.2	0.247	0.2±0.2	0.1±0.1	0.382
Adrenal glands	0.4±0.4	0.8±0.4	0.051	0.1±0.2	0.2±0.3	0.355
Ovaries	NA	NA	NA	0.0±0.0	0.2±0.6	0.343
Lungs	0.4±0.3	0.5±0.2	0.388	0.2±0.1	0.3±0.1	0.441
Forestomach	0.0±0.0	0.2±0.6	0.343	0.0±0.0	0.0±0.0	NA
Glandular stomach	0.0±0.0	0.1±0.3	0.343	0.2±0.4	0.0±0.0	0.168
Pancreas	0.3±0.3	0.5±0.4	0.250	0.1±0.1	0.1±0.2	0.145
Rectum	0.3±0.5	0.1±0.3	0.458	0.2±0.5	0.1±0.2	0.559
Harderian glands	0.0±0.0	0.4±0.8	0.168	0.3±0.5	0.0±0.0	0.081
Prostate gland	1.1±1.1	0.8±0.9	0.517	NA	NA	NA
Epididymides	0.1±0.3	0.1±0.3	>0.999	NA	NA	NA

DISCUSSION

The study revealed that no treatment-related histopathological changes were observed after administration of the *A. cinnamomea* suspension. According to control data of previous reports, the lesions of heart, kidneys, lungs, pancreas, and prostate gland spontaneously occurred after administration of distilled water for 13 weeks [26], and the lesions of liver, adrenal glands, forestomach, glandular stomach, Harderian glands, ovaries, and epididymides also naturally happened in SD rats [9,19,27,28]. Although the occurrence of the lesions was higher in the study, there was no significant difference between the control group and the AC4000 group (Table 5).

It was noted that the organ-to-body weight ratios of adrenal glands in the AC4000 group were significantly different from those in the control group (Table 4), but they were within the normal range [24,29]. Additionally, although the livers of the female SD rats in the AC2000 group were significantly enlarged, the livers of the male SD rats in the AC2000 group were smaller than those in the control group. The result was not a dose-dependent response, so the enlarged livers might not be caused by administration of the *A. cinnamomea* suspension. The similar results were also observed in the serum biochemical assays and in the hematological analysis. The level of chloride in the AC2000 group of the male SD rats was significantly higher than that in the control group, but the result was not a dose-dependent response and was within the normal range [29-32]. The levels of MCH and hemoglobin in the AC4000 group of the male SD rats were significantly decreased, but they were both within the normal range [29-32].

On top of that, leukocyte esterase in the male SD rats' urine and nitrite in the female SD rats' urine were significantly different among the control group and the treatment groups (Table 1). The positive leukocyte esterase test result could be considered to be pyuria [33]. Nevertheless, more male SD rats in the AC2000 group had the positive results (Table 1), and positive results of leukocyte esterase in male SD rats' urine were also found in other control SD rats [31], so it might not be resulted from administration of the *A. cinnamomea* suspension. For the increased urinary nitrite in the female SD rats, it might be due to urinary tract infections [34] or inflammatory bowel disease [35]. However, the inflammation of any related organ was not significantly different between the control group and the AC4000 group (Table 4, Table 5, and Table 6), and the clarity of the female SD rats' urine in the treatment groups was most clear (Table 1). Neither urinary tract infections nor inflammatory bowel disease would happen after administration of the *A. cinnamomea* suspension. Hence, more studies would be needed to figure out the reason why the urinary nitrite increased in the female SD rats.

CONCLUSION

In summary, the *A. cinnamomea*product via the novel process would be safe. The repeated dose 90-day oral toxicity study showed no treatment-related adverse effect in the Sprague Dawley rats after administration of the *A. cinnamomea* product, and the no-observed-adverse-effect level of exposure was at 4000 mg/kg BW.

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