90-day Sub-chronic Oral Toxicity Analysis of Antrodia cinnamomea (“Niu-chang-chih”) Fruit Body Extract in Rats

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Abstract

Antrodia cinnamomea is considered to improve physical health and traditionally used treat many diseases. In order to ensure that a fruit body extract prepared from A. cinnamomea is safe for long-term consumption, 90-day sub-chronic oral toxicity testing in rats was performed. The test design followed the Organization for Economic Co-operation and Development (OECD) Guideline 408. The dosages tested in this experiment were 1250, 2500 and 5000 mg/kg body weight of the animal for 90 consecutive days and a group administered reverse osmosis water was used as the controls. No rats died during the feeding period in this experiment. The groups administered A. cinnamomea extract showed no significant differences to the controls in terms of body weight increase, food and water consumption, pre- and post-test ophthalmic examinations, organ to body weight ratios, blood clotting tests, pathological examination by visual inspection and histopathologic analysis. Urinalysis, hematologic analysis and biochemical analysis demonstrated no treatment-related pathological changes. According to the above experiments and examinations, the no-observed-adverse-effect level (NOAEL) of our A. cinnamomea was determined and the acceptable daily intake (ADI) was estimated.

Keywords: Antrodia cinnamomea; fruit body; sub-chronic; OECD; NOAEL

1. Introduction

Antrodia cinnamomea (formerly named Antrodia camphorata) also known as “Niu-chang-chih”, is a fungus that only grows on the brown heartwood of the inner cavity or wet surface of Cinnamomum kanehirae, an endemic species of native camphor tree in Taiwan. The fungus has a plate-shaped or flake-shaped appearance, with a red-orange color on its porous side. It has long been used by the indigenous peoples of Taiwan to treat hangover or fatigue [1]. Many medical usages of A. cinnamomea have been widely circulated by members of the public, including the beliefs that long-term consumption can help prevent or even
treat liver diseases, that it can be used for treatment of food poisoning, diarrhea, abdominal pain, hypertension, itchy skin, and cancer, and for alleviation of many pathological conditions [2-4]. In recent years, the number of scientific studies on *A. cinnamomea* have dramatically increased, and at least 78 compounds have been identified. The fruiting bodies of *A. cinnamomea* contain 39 known terpenoids, in which triterpenoids are the major constituents, accounting for more than 50% of the total compounds. The other compounds present in the fruiting bodies include benzoids, lignans, benzoquinones, malic and succinic acid derivatives and polysaccharides [5, 6]. Recent study showed that wild *A. cinnamomea* has 10- to 30-fold the triterpenoids content than submerged-fermentation *A. cinnamomea* [7, 8]. 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. Regardless of the preparation method, most products claim similar effects; however, no scientific data have been produced to support the claimed effects of the products.

In biological and pharmacological activity studies, crude extracts of mycelia and fruiting bodies of *A. cinnamomea* were both shown to have potent activity against cancer cell proliferation and to induce apoptosis in several human cancer cell lines. *A. cinnamomea* extract also exhibits activity against tumor growth in mice models. The compounds in *A. cinnamomea* are thought to correlate with the activities of caspases, Bcl-2 family proteins, NF-kB, Akt, p53 and mitogen-activated protein kinases (MAPK), which are important molecules in the mechanisms that regulate anti-tumor effects [5]. Aqueous, methanolic or ethyl acetate extracts of *A. camphorata* have all been shown to have anti-inflammatory, immunomodulatory, anti-oxidant and hepatoprotective effects in *in vitro* studies. Previous studies concluded that the contents of active substances vary according cultivation conditions. In addition, the active substances obtained using different extraction methods are also significantly different. Of the active substances derived using different methods, triterpenoids are the most important due to their biological efficacy [9, 10]. However, biological analyses of these triterpenoids have mainly been based on *in vitro* cell culture models, very few having been performed in animal experiments.

Filtrate of fermented mycelium from *A. cinnamomea* was tested previously in terms of the 90-day subchronic toxicity in Sprague-Dawley rats, and clinical data were collected. The results of the study indicated that the no-observed-adverse-effect level (NOAEL) of *A. cinnamomea* was greater than 3000 mg/kg BW/day in Sprague–Dawley rats [11]; however, no similar tests have been performed using fruiting bodies of *A. cinnamomea*.

We extract active natural ingredients from *A. cinnamomea* fruiting bodies that have been grown on the wood for 2.5-3 years. In order to ensure the safety and efficacy of their products, the quality assurance and quality control processes include the commissioning of academic institutions to perform identification of constituents and safety tests. The project carried out repeated-dose 90-day oral toxicity study in rats, following the OECD Guidelines for the Testing of Chemicals (OECD408).

Therefore, in this 90-day oral toxicity study, we followed the regulations of OECD408 and aimed to obtain an accurate No-observed-adverse-effect level (NOAEL) of the *A. cinnamomea* product. The data obtained in this test can then be used to estimate the acceptable daily intake (ADI) for humans.

### 2. Materials and Methods

**Fruit body culture and extract preparation**

*A. cinnamomea*, a Taiwan strain BCRC 35396 was purchased from the Culture Collection and Research Center (Hsinchu, Taiwan) and inoculated into Malt Extract Agar, then subjected to 28-days culture at 25°C to obtain the mycelia. The cultured mycelia cut into small pieces and then incubated in liquid Malt Extract broth under 25°C for 28-days. The wood of *Cinnamomum kanehirai* is autoclaved, transfer to a cleanroom at class10000 level for 24 h. The liquid cultured *A. cinnamomea* is smeared and inoculated onto the autoclaved wood segments, put in a plastic bag for one year culture with temperature of 25°C and 50% moisture. After one year, remove the bag, the wood maintained in a water-contained black box for additional culture to obtain the fruit body. Dried fruit bodies were smashed and immersed in 75% ethanol for 24 h then filtered through Whatman No. 1 filter paper to separate the liquid part and insoluble part. The extract was subjected to rotary evaporator (R-2000V1, Panchun Scientific Corp., Taiwan) to remove ethanol and a concentrated *A. cinnamomea* ethanolic extracts was obtained. The cultured fruit body sample was identified by Food Industry Research and Development Institute (Hsinchu, Taiwan), based on the morphology, microstructure and DNA sequence of *rDNA ITS1-5.8S-ITS2* region and LSU *rDNA D1-D3.*

**Qualitative and quantitative analysis of fruit body extract by using LC/MS/MS**

A series of various concentrations of 8 standard stock solutions were prepared and kindly provided
by Dr. Chi-I Chang (NPUST, Pingtung, Taiwan). Chromatographic separation was performed on a Phenomenex C18 100A column (150x2.1mm I.D., 5μm, Kinetex), qualitative LC-MS analysis was performed using an Thermo TSQ system. Sample diluted in methanol, the mobile phase A consisted of 0.2% formic acid aqueous solution, acetonitrile as mobile phase B, and a gradient elution program was set as follows: mobile phase B, 35-55% (0-7 min), 55-65% (7-10 min), 65-100% (10-19 min), 100-65% (19-21 min), 65-55% (31-25 min), 55-35% (25-30 min), the flow rate was set 0.4 mL/ min. The condition of negative mode and positive mode, as well as data process was described in the literature [12].

Animal grouping and care

This animal toxicity test used 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-104-052. Eighty 6-week-old (40 male and 40 female) Sprague-Dawley (SD) rats were purchased from BioLASCO Taiwan Co. Ltd. The rats were assigned to one of four groups (for both male and female rats: 4 groups/sex, 10 animals/group) as follows: Group 1 as the control group, Groups 2-4 as A. cinnamomea – treated groups (Group 2–low dose: 1250 mg/kg body weight; Group 3–middle dose: 2500 mg/kg; Group 4–high dose: 5000 mg/kg). Concentrated extracted was diluted in reverse osmosis water daily before administration. The animals were maintained under laboratory conditions (temperature 24°C, relative humidity 65%, 12-hour light and darkness cycle), with free access to food and water. The details of the animal care protocol were as stated in the Laboratory Animal Care and Use Guide, 3rd edition, published by the Taiwan Society of Laboratory Animal Science.

Ophthalmic examination

Ophthalmic examination was performed by Dr. Ching-Dong Chang (NPUST, Pingtung, Taiwan), a pathology specialist. The examination was performed on all animals both pre-test and at the termination of the experimental period. The pathology specialist used an ophthalmoscope (HEINE, Germany) to observe the fundus and other eye structures.

Clinical observations: animal behavior, response and survival

All animals were observed in their cages twice daily (7:00 am and 7:00 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, skin and fur condition. Food and water intake were also recorded, and the excretion pattern was also noted. The body weight of each rat was measured weekly.

Urinalysis

Two days before necropsy examination, urine samples were collected from each rat 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, blood chemistry an, blood clotting

Two days before necropsy, the rats were fasted for 10 hours before blood samples were collected. For hematologic analysis, K₂EDTA was used as the anticoagulant, and the analysis was performed using an automated hematolyser analyzer (Mythic 18; Orphee, Switzerland). For biochemical tests, lithium heparin was used as the anticoagulant, and the analysis was performed using an automated clinical chemistry analyzer (Dri-Chem 3500iS; Fujifilm, Japan). 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). All tests were performed in Animal Hospital (NPUST, Pingtung, Taiwan).

Pathological examination by visual inspection and histopathologic analyses

During the necropsies, veterinary students were supervised and supported by animal specialist veterinary pathologist to identify any lesions or other pathological conditions. 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/infiltration process. The paraffin tissue blocks were then cut using a microtome at thicknesses of 2-4 μm. The sections were then placed onto slides, put into an automated stainer for hematoxylin and eosin (H&E) staining, then mounted. All histopathological evaluations were carried out in a double-blind manner by a professional pathologist from the Animal Disease Diagnostic Center (NPUST, Pingtung, Taiwan).

Statistical analyses

The data are presented as the mean ± SD, and differences between groups were calculated by one-way ANOVA. Post hoc analysis with Tukey’s
methods was used for validation of differences between a treatment group and the control group. P values < 0.05 were considered to indicate statistical significance, and are indicated by “*” in the tables. Statistical analysis of the data collected in the study were analyzed using SPSS.

3. Results

Composition

The specific compounds in our extract was analyzed by LC/MS/MS, 7 known compounds and 1 unknown were identified and demonstrated in Figure 1. The chromatogram showed retention times of each component: Antcin A, 17.30 min; Antcin B, 14.84 min; unknown, 14.76 min; Antcin H, 11.61 min; Antcin K, 2.13 min; dehydroeburicoic acid (DeEA), 22.96 min; dehydrosulphurenic acid (DSA), 13.59 min; 1,4-dimethoxy-2,3-methylenedioxy-5-methylbenzene (DMB), 8.21 min. The quantitative analytical results indicated the highest amounts of these compounds of our concentrated extracts are antcin B (263.9 mg/ml) and DeEA (247.0 mg/ml), followed by DSA (142.2 mg/ml), antcin H (137.8 mg/ml), Antcin A (41.1 mg/ml) and Antcin K(24.9 mg/ml). The signal of DMB is below the limit of quantification.

Figure 1: LC/MS/MS profiling of ethanolic extracts of A. cinnamomea fruiting bodies.

Clinical response and eye examination

All clinical observations did not reveal any treatment-related adverse effects. At the end of the 90-day test period, all animals survived. Physical and behavioral observation did not reveal any different response in all groups before and after dosing. The results of our study showed that the body weight increase, water and food consumption were all within the normal ranges. The results were as shown in Tables 1 (male) and Table 2 (female). One-way analysis of variance (ANOVA) showed no differences between the A. cinnamomea-treated groups and the control group. Eye examination results showed that no ophthalmic lesions were found on the peripheral and internal structures of either eye before or after treatment in each animal (data not shown).

<table>
<thead>
<tr>
<th>Group/male</th>
<th>90-day body weight increase (%)/animal</th>
<th>Food consumption (g)/day/animal</th>
<th>Water consumption (g)/day/animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Control)</td>
<td>65.65 ± 3.90</td>
<td>24.00 ± 8.87</td>
<td>43.77 ± 15.25</td>
</tr>
<tr>
<td>Group 2 (1250 mg/kg)</td>
<td>65.86 ± 2.65</td>
<td>24.66 ± 9.57</td>
<td>48.27 ± 20.79</td>
</tr>
<tr>
<td>Group 3 (2500 mg/kg)</td>
<td>65.95 ± 4.83</td>
<td>24.05 ± 8.94</td>
<td>43.85 ± 18.25</td>
</tr>
<tr>
<td>Group 4 (5000 mg/kg)</td>
<td>61.91 ± 3.11</td>
<td>22.51 ± 7.46</td>
<td>39.33 ± 12.76</td>
</tr>
</tbody>
</table>

Table 2. Body weight increase, food and water consumption in the groups of male rats.

Urine analysis

The test items covered all the items listed in OECD Guideline 408. The results were as shown in Table 3, demonstrating that the animals in the treatment groups fed with A. cinnamomea solution had results similar to those in the control group.

Table 3. Urine analysis results in the groups of male rats.

<table>
<thead>
<tr>
<th>Group/male</th>
<th>90-day body weight increase (%)/animal</th>
<th>Food consumption (g)/day/animal</th>
<th>Water consumption (g)/day/animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Control)</td>
<td>48.61 ± 2.17</td>
<td>21.06 ± 3.27</td>
<td>38.67 ± 5.4</td>
</tr>
<tr>
<td>Group 2 (1250 mg/kg)</td>
<td>47.73 ± 2.45</td>
<td>20.70 ± 3.04</td>
<td>34.52 ± 5</td>
</tr>
<tr>
<td>Group 3 (2500 mg/kg)</td>
<td>49.54 ± 2.87</td>
<td>20.16 ± 3.25</td>
<td>34.81 ± 5.44</td>
</tr>
<tr>
<td>Group 4 (5000 mg/kg)</td>
<td>46.71 ± 3.74</td>
<td>19.64 ± 3.49</td>
<td>35.28 ± 6.97</td>
</tr>
</tbody>
</table>
Table 3. Results of urinalysis in the groups of male and female rats.

<table>
<thead>
<tr>
<th>Analysis item</th>
<th>Abnormal</th>
<th>Group 1 (Control)</th>
<th>Group 2 (1250 mg/kg)</th>
<th>Group 3 (2500 mg/kg)</th>
<th>Group 4 (5000 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>White/brown/red/mucus</td>
<td>0/0</td>
<td>0/1</td>
<td>1/2</td>
<td>4/2</td>
</tr>
<tr>
<td>Specific gravity (SG)</td>
<td>&lt;1.010 or &gt;1.030</td>
<td>3/4</td>
<td>0/1</td>
<td>1/2</td>
<td>4/2</td>
</tr>
<tr>
<td>Protein</td>
<td>++Positive</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>Urobilinogen</td>
<td>+ Positive</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>pH</td>
<td>&lt;5.5 or &gt;8.5</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>Ketone</td>
<td>+ Positive</td>
<td>5/0</td>
<td>7/0</td>
<td>6/0</td>
<td>6/0</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>+ Positive</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>Glucose</td>
<td>+ Positive</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>Nitrite</td>
<td>+ Positive</td>
<td>1/0</td>
<td>1/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>Urine sediment / high power field (hpf)</td>
<td>WBC &gt;1 Positive</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>RBC &gt;1 Positive</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>Epithelial cells + Positive</td>
<td>1/2</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>Triple phosphate + Positive</td>
<td>6/1</td>
<td>6/7</td>
<td>6/4</td>
<td>3/5</td>
</tr>
</tbody>
</table>

**Hematology**

Statistical analysis indicated that hemoglobin, Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) and were significantly different between the group administered the lowest dose of A. cinnamomea (Group 2) and the control group in the male rats, while only MCH was significantly different between the group administered the lowest dose (Group 2) and the control group in the female rats. Details of the hematologic test results are shown in Table 4 (male) and Table 5 (female).

**Serum biochemistry analysis**

The data showed that there were 7 test items in the male rats and 2 test items in the female rats for which significant differences were observed between the treatment and control groups. In Tables 6 and 7, the values of each test item in the individual groups are listed.

**Blood clotting**

One-way ANOVA showed that there were no differences in CT, PT and APTT between the treatment and control groups in both the male and female rats. In the male rats, the p values of CT, PT and APTT were 0.322, 0.597 and 0.337, respectively; in the female rats, the p values of CT, PT and APTT were 0.620, 0.521 and 0.720, respectively. The values presented as mean ± SD are shown in Table 8 (male) and Table 9 (female).

**Necropsy and organ weights**

At the end of the oral feeding experiment, the rats were necropsied. The organ to body weight ratios in each group were as listed in Table 10 (male) and Table 11 (female). One-way AVOVA showed no differences between the treatment and control groups. Necropsy showed that one female (#14) rat in control group had an enlarged thyroid on the right side. No other pathological change was seen in the other rats.

**Histopathologic analyses**

According to OECD Guideline 408, histopathologic examination should include 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 nerve, bone marrow or fresh bone marrow aspirate, skin, and eyes. The specimen sections of 80 rats were examined by a pathologist, who confirmed that the thyroid of the #14 female rat in control group was infected with *Streptococcus*, which caused the enlargement. The infection was then identified to be correlated with a puncture wound from a skin injury, and was not associated with the present experiment. No any treatment-related changes were observed for all examined tissues.

Table 4. Hematologic data in different groups of male rats.

<table>
<thead>
<tr>
<th>Item</th>
<th>Unit</th>
<th>Normal range</th>
<th>Group 1 (Control)</th>
<th>Group 2 (1250 mg/kg)</th>
<th>Group 3 (2500 mg/kg)</th>
<th>Group 4 (5000 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit; PCV (Man)</td>
<td>%</td>
<td>36-54</td>
<td>43.80 ± 1.69</td>
<td>43.40 ± 3.17</td>
<td>45.70 ± 1.49</td>
<td>44.30 ± 2.26</td>
</tr>
<tr>
<td>Hematocrit; PCV (Auto)</td>
<td>%</td>
<td>36-54</td>
<td>46.06 ± 2.01</td>
<td>44.78 ± 2.59</td>
<td>46.46 ± 1.54</td>
<td>45.68 ± 1.06</td>
</tr>
<tr>
<td>RBC</td>
<td>×10⁶/μL</td>
<td>6.76-9.75</td>
<td>7.478 ± 0.33</td>
<td>7.45 ± 0.30</td>
<td>7.65 ± 0.33</td>
<td>7.69 ± 0.20</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>g/dL</td>
<td>11-19.2</td>
<td>17.28 ± 0.87</td>
<td>16.21 ± 1.10*</td>
<td>16.95 ± 0.56</td>
<td>16.56 ± 0.40</td>
</tr>
<tr>
<td>MCV</td>
<td>fl</td>
<td>48-70</td>
<td>61.62 ± 1.95</td>
<td>60.08 ± 1.74</td>
<td>60.78 ± 2.29</td>
<td>59.43 ± 1.66</td>
</tr>
<tr>
<td>MCH</td>
<td>pg</td>
<td>18-23</td>
<td>23.12 ± 0.93</td>
<td>21.74 ± 0.72*</td>
<td>22.18 ± 0.97</td>
<td>21.54 ± 0.53</td>
</tr>
<tr>
<td>MCHC</td>
<td>g/dL</td>
<td>34-38</td>
<td>37.51 ± 0.72</td>
<td>36.19 ± 0.76*</td>
<td>36.490.66</td>
<td>36.25 ± 0.49</td>
</tr>
<tr>
<td>WBC</td>
<td>10³/μL</td>
<td>6.6-12.6</td>
<td>9.89 ± 3.18</td>
<td>11.15 ± 2.57</td>
<td>10.3 ± 1.53</td>
<td>9.81 ± 2.05</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>10³/μL</td>
<td>4.78-9.12</td>
<td>8.60 ± 2.89</td>
<td>9.83 ± 2.66</td>
<td>7.65 ± 2.77</td>
<td>7.60 ± 2.50</td>
</tr>
<tr>
<td>Monocytes</td>
<td>/μL</td>
<td>30-180</td>
<td>131.80 ± 236.94</td>
<td>178.90 ± 165.55</td>
<td>125.40 ± 165.30</td>
<td>201.10 ± 192.22</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>/μL</td>
<td>10-160</td>
<td>77.40 ± 60.27</td>
<td>89.30 ± 111.15</td>
<td>182.20 ± 113.78</td>
<td>158.60 ± 102.69</td>
</tr>
<tr>
<td>Basophils</td>
<td>/μL</td>
<td>0-30</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>PLT</td>
<td>×10³/μL</td>
<td>638-1177</td>
<td>533.70 ± 232.74</td>
<td>602.80</td>
<td>649.60 ± 186.41</td>
<td>571.10135.76</td>
</tr>
</tbody>
</table>

The data are shown as mean ± SD, *p*<0.05 in comparison with the control group.
Table 5. Hematologic data in different groups of female rats.

<table>
<thead>
<tr>
<th>Item</th>
<th>Unit</th>
<th>Normal range</th>
<th>Group 1 (Control)</th>
<th>Group 2 (1250 mg/kg)</th>
<th>Group 3 (2500 mg/kg)</th>
<th>Group 4 (5000 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit; PCV (Man)</td>
<td>%</td>
<td>36-54</td>
<td>41.00 ± 2.98</td>
<td>40.70 ± 1.42</td>
<td>41.10 ± 3.03</td>
<td>40.30 ± 1.95</td>
</tr>
<tr>
<td>Hematocrit; PCV (Auto)</td>
<td>%</td>
<td>36-54</td>
<td>43.11 ± 3.52</td>
<td>42.44 ± 1.46</td>
<td>42.85 ± 1.81</td>
<td>42.04 ± 1.63</td>
</tr>
<tr>
<td>RBC</td>
<td>×10^6/μL</td>
<td>6.76-9.75</td>
<td>6.69 ± 0.573</td>
<td>6.78 ± 0.25</td>
<td>6.70 ± 0.32</td>
<td>6.66 ± 0.36</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>g/dL</td>
<td>11-19.2</td>
<td>16.03 ± 1.38</td>
<td>15.54 ± 0.63</td>
<td>15.57 ± 0.88</td>
<td>15.78 ± 0.60</td>
</tr>
<tr>
<td>MCV</td>
<td>fl</td>
<td>48-70</td>
<td>64.45 ± 2.59</td>
<td>62.62 ± 2.00</td>
<td>64.02 ± 1.03</td>
<td>63.17 ± 1.87</td>
</tr>
<tr>
<td>MCH</td>
<td>pg</td>
<td>18-23</td>
<td>23.98 ± 1.25</td>
<td>22.92 ± 0.49*</td>
<td>23.25 ± 0.57</td>
<td>23.71 ± 0.58</td>
</tr>
<tr>
<td>MCHC</td>
<td>g/dL</td>
<td>34-38</td>
<td>37.21 ± 1.22</td>
<td>36.62 ± 0.75</td>
<td>36.32 ± 0.53</td>
<td>37.54 ± 0.78</td>
</tr>
<tr>
<td>WBC</td>
<td>10^9/μL</td>
<td>6.6-12.6</td>
<td>6.74 ± 1.86</td>
<td>6.10 ± 2.38</td>
<td>8.96 ± 2.62</td>
<td>7.90 ± 2.44</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>10^9/μL</td>
<td>4.78-9.12</td>
<td>5.42 ± 1.96</td>
<td>5.21 ± 2.04</td>
<td>7.45 ± 2.43</td>
<td>6.64 ± 1.86</td>
</tr>
<tr>
<td>Monocytes</td>
<td>/μL</td>
<td>30-180</td>
<td>104.30 ± 57.54</td>
<td>121.30 ± 69.11</td>
<td>130.40 ± 157.24</td>
<td>106.20 ± 108.81</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>/μL</td>
<td>10-160</td>
<td>83.60 ± 75.36</td>
<td>54.60 ± 31.36</td>
<td>162.70 ± 143.06</td>
<td>106.20 ± 108.81</td>
</tr>
<tr>
<td>Basophils</td>
<td>/μL</td>
<td>0-30</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>9.70 ± 29.10</td>
</tr>
<tr>
<td>PLT</td>
<td>x10^3/μL</td>
<td>638-1177</td>
<td>501.60 ± 151.22</td>
<td>556.00 ± 157.87</td>
<td>463.40 ± 222.96</td>
<td>454.70 ± 72.11</td>
</tr>
</tbody>
</table>

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

Table 6. Serum biochemistry data in the different groups of male rats.

<table>
<thead>
<tr>
<th>Item</th>
<th>Unit</th>
<th>Normal range</th>
<th>Group 1 (Control)</th>
<th>Group 2 (1250 mg/kg)</th>
<th>Group 3 (2500 mg/kg)</th>
<th>Group 4 (5000 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total serum protein (TSP)</td>
<td>g/dL</td>
<td>5.6-7.6</td>
<td>6.66 ± 0.46</td>
<td>7.27 ± 0.46*</td>
<td>6.95 ± 0.24</td>
<td>6.86 ± 0.43</td>
</tr>
<tr>
<td>Albumin</td>
<td>g/dL</td>
<td>3.8-4.8</td>
<td>4.07 ± 0.35</td>
<td>3.85 ± 0.40</td>
<td>3.85 ± 0.34</td>
<td>3.81 ± 0.38</td>
</tr>
<tr>
<td>Globulin</td>
<td>g/dL</td>
<td>1.8-2.5</td>
<td>2.59 ± 0.55</td>
<td>3.42 ± 0.32*</td>
<td>3.10 ± 0.49</td>
<td>3.05 ± 0.37</td>
</tr>
<tr>
<td>AST</td>
<td>U/L</td>
<td>45.7-80.8</td>
<td>70.20 ± 4.73</td>
<td>72.71 ± 10.71</td>
<td>75.30 ± 9.09</td>
<td>67.50 ± 17.53</td>
</tr>
<tr>
<td>ALT</td>
<td>U/L</td>
<td>17.5-30.2</td>
<td>28.40 ± 2.80</td>
<td>26.40 ± 4.17</td>
<td>22.80 ± 4.61</td>
<td>19.70 ± 6.04*</td>
</tr>
<tr>
<td>ALP</td>
<td>U/L</td>
<td>86-247</td>
<td>357.00 ± 98.38</td>
<td>384.80 ± 126.64</td>
<td>236.50 ± 30.43</td>
<td>247.40 ± 87.15</td>
</tr>
<tr>
<td>T. Bilirubin</td>
<td>mg/dL</td>
<td>0.2-0.5</td>
<td>0.13 ± 0.07</td>
<td>0.13 ± 0.05</td>
<td>0.10 ± 0</td>
<td>0.14 ± 0.07</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>mg/dL</td>
<td>40-130</td>
<td>66.10 ± 14.39</td>
<td>80.50 ± 18.22</td>
<td>72.60 ± 20.26</td>
<td>58.10 ± 7.78</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>mg/dL</td>
<td>16-175</td>
<td>114.80 ± 38.82</td>
<td>82.01 ± 31.26</td>
<td>61.70 ± 23.65*</td>
<td>54.10 ± 11.70*</td>
</tr>
</tbody>
</table>
The data are shown as mean ± SD, *p<0.05 in comparison with the control group.

<table>
<thead>
<tr>
<th>Item</th>
<th>Unit</th>
<th>Group 1 (Control)</th>
<th>Group 2 (1250 mg/kg)</th>
<th>Group 3 (2500 mg/kg)</th>
<th>Group 4 (5000 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>mg/dL</td>
<td>50-160</td>
<td>203.70 ± 49.46</td>
<td>189.10 ± 60.57</td>
<td>178.30 ± 32.28</td>
</tr>
<tr>
<td>BUN</td>
<td>mg/dL</td>
<td>15-21</td>
<td>16.70 ± 1.30</td>
<td>16.21 ± 3.36</td>
<td>15.43 ± 1.91</td>
</tr>
<tr>
<td>Creatinine</td>
<td>mg/dL</td>
<td>0.2-0.8</td>
<td>0.27 ± 0.05</td>
<td>0.29 ± 0.12</td>
<td>0.24 ± 0.11</td>
</tr>
<tr>
<td>Ip</td>
<td>mg/dL</td>
<td>3.11-11</td>
<td>6.24 ± 0.74</td>
<td>7.47 ± 1.17</td>
<td>8.36 ± 1.48*</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>mg/dL</td>
<td>5.3-13</td>
<td>9.82 ± 0.49</td>
<td>10.56 ± 0.86</td>
<td>9.93 ± 0.64</td>
</tr>
<tr>
<td>Na⁺</td>
<td>meq/L</td>
<td>140-150</td>
<td>140.10 ± 3.14</td>
<td>141.50 ± 3.24</td>
<td>139.6 ± 1.84</td>
</tr>
<tr>
<td>K⁺</td>
<td>meq/L</td>
<td>4.3-5.6</td>
<td>3.69 ± 0.40</td>
<td>3.99 ± 0.38</td>
<td>4.00 ± 0.22</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>meq/L</td>
<td>95-115</td>
<td>91.2 ± 4.30</td>
<td>97.00 ± 3.65*</td>
<td>91.70 ± 2.11</td>
</tr>
</tbody>
</table>

The data are shown as mean ± SD, *p<0.05 in comparison with the control group.
Table 8. Blood clotting test data in male rats.

<table>
<thead>
<tr>
<th>Item (unit: sec)</th>
<th>Normal range (sec)</th>
<th>Group 1 (Control)</th>
<th>Group 2 (1250 mg/kg)</th>
<th>Group 3 (2500 mg/kg)</th>
<th>Group 4 (5000 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clotting time</td>
<td>60-300</td>
<td>117.9 ± 89.4</td>
<td>122.10 ± 93.2</td>
<td>60.70 ± 30.6</td>
<td>111.60 ± 84.10</td>
</tr>
<tr>
<td>PT</td>
<td>No reference data</td>
<td>23.56 ± 4.09</td>
<td>23.9 ± 7.43</td>
<td>24.70 ± 4.71</td>
<td>26.90 ± 4.89</td>
</tr>
<tr>
<td>APTT</td>
<td>No reference data</td>
<td>47.50 ± 5.50</td>
<td>42.70 ± 8.73</td>
<td>42.80 ± 5.9</td>
<td>43.50 ± 6.08</td>
</tr>
</tbody>
</table>

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


<table>
<thead>
<tr>
<th>Item (unit: sec)</th>
<th>Normal range (sec)</th>
<th>Group 1 (Control)</th>
<th>Group 2 (1250 mg/kg)</th>
<th>Group 3 (2500 mg/kg)</th>
<th>Group 4 (5000 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clotting time</td>
<td>60-300</td>
<td>103.50 ± 93.2</td>
<td>78.70 ± 58.20</td>
<td>80.10 ± 64.10</td>
<td>114.40 ± 80.3</td>
</tr>
<tr>
<td>PT</td>
<td>No reference data</td>
<td>26.10 ± 3.38</td>
<td>23.50 ± 2.32</td>
<td>25.70 ± 6.41</td>
<td>24.10 ± 4.84</td>
</tr>
<tr>
<td>APTT</td>
<td>No reference data</td>
<td>46.40 ± 5.42</td>
<td>42.70 ± 9.19</td>
<td>44.70 ± 5.79</td>
<td>44.90 ± 7.63</td>
</tr>
</tbody>
</table>

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

Table 10. Ratios of organs:body weight in male rats.

<table>
<thead>
<tr>
<th>Male rats</th>
<th>Liver</th>
<th>Kidney and adrenal gland</th>
<th>Testes and epididymis</th>
<th>Thymus</th>
<th>Spleen</th>
<th>Cerebrum, cerebellum and pons</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Control)</td>
<td>16.58 ± 2.17</td>
<td>4.06 ± 0.81</td>
<td>6.45 ± 1.60</td>
<td>0.34 ± 0.14</td>
<td>0.77 ± 0.11</td>
<td>2.16 ± 0.13</td>
<td>1.74 ± 0.17</td>
</tr>
<tr>
<td>Group 2 (1250 mg/kg)</td>
<td>12.81 ± 4.21</td>
<td>3.27 ± 1.31</td>
<td>6.82 ± 2.47</td>
<td>0.30 ± 0.09</td>
<td>0.61 ± 0.10</td>
<td>2.10 ± 0.17</td>
<td>1.35 ± 0.34</td>
</tr>
<tr>
<td>Group 3 (2500 mg/kg)</td>
<td>10.56 ± 2.47</td>
<td>2.62 ± 0.63</td>
<td>9.04 ± 4.35</td>
<td>0.25 ± 0.05</td>
<td>0.56 ± 0.10</td>
<td>2.01 ± 0.07</td>
<td>1.30 ± 0.29</td>
</tr>
<tr>
<td>Group 4 (5000 mg/kg)</td>
<td>14.91 ± 3.66</td>
<td>3.65 ± 1.09</td>
<td>6.66 ± 2.25</td>
<td>0.29 ± 0.12</td>
<td>0.73 ± 0.13</td>
<td>2.21 ± 0.17</td>
<td>1.79 ± 0.37</td>
</tr>
</tbody>
</table>

The data are shown as mean ± SD, *p<0.05 in comparison with the control group.
In this study, as the amounts were low, we therefore excluded the possibility of pathological proteinuria. The increased urine protein in these three rats was likely benign and transient proteinuria.

Regarding on the urine examination, all three rats in the control group had a weak positive response. The presence of squamous epithelial cells in the male rat urine samples was not caused by A. cinnamomea feeding. Nitrite was found in the urine samples of one rat in the control group and one male rat in Group 2, the group administered the lowest dose of A. cinnamomea. As the amounts were low, and only a marginal positive response was found, it was thought that this was not associated with treatment. As bacteria can convert nitrate to nitrite, the presence of nitrite in the urine is usually a sign of bacterial infection. We therefore checked whether urinary tract infection was present in these rats; however, no urine white blood cells (WBC) were detected, and therefore the possibility of the nitrite positive response having been caused by urinary tract infection was excluded.


<table>
<thead>
<tr>
<th>Female rats</th>
<th>Liver (g)</th>
<th>Kidney and adrenal gland (g)</th>
<th>Uterus and ovaries (g)</th>
<th>Thymus (g)</th>
<th>Spleen (g)</th>
<th>Cerebrum, cerebellum and pons (g)</th>
<th>Heart (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Control)</td>
<td>9.72 ± 1.11</td>
<td>2.4 ± 0.31</td>
<td>1.34 ± 0.44</td>
<td>0.27 ± 0.05</td>
<td>0.54 ± 0.07</td>
<td>1.99 ± 0.07</td>
<td>1.18 ± 0.17</td>
</tr>
<tr>
<td>Group 2 (1250 mg/kg)</td>
<td>14.02 ± 4.36</td>
<td>3.43 ± 1.17</td>
<td>1.17 ± 0.46</td>
<td>0.29 ± 0.11</td>
<td>0.68 ± 0.17</td>
<td>2.15 ± 0.17</td>
<td>1.73 ± 0.43</td>
</tr>
<tr>
<td>Group 3 (2500 mg/kg)</td>
<td>14.90 ± 3.56</td>
<td>3.68 ± 1.05</td>
<td>1.44 ± 0.14</td>
<td>0.32 ± 0.11</td>
<td>0.74 ± 0.13</td>
<td>2.24 ± 0.14</td>
<td>1.76 ± 0.45</td>
</tr>
<tr>
<td>Group 4 (5000 mg/kg)</td>
<td>10.4 ± 2.57</td>
<td>2.64 ± 0.62</td>
<td>1.48 ± 0.45</td>
<td>0.30 ± 0.07</td>
<td>0.58 ± 0.11</td>
<td>1.95 ± 0.30</td>
<td>1.29 ± 0.33</td>
</tr>
</tbody>
</table>

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

4. Discussion

The current study is the first article provided a complete 90-days subchronic oral toxicity examination using fruit body extracts of A. cinnamomea in rat model, which fully under the guideline of OECD 408. No abnormality of ophthalmoscopic results, no difference on body weight increased, food and water consumption, clotting time, ratios of organs/body weight in all groups of both gender.

Triterpenoids are considered to be the most critical bioactive compounds [8]. The contents of our fruit bodies extracts included several well-known index compounds of various antcin, DeEA and DSA. Critically, the level of individual compound of our fruit body extract was much higher than previous literature [13, 14], it most likely because we had been established a best state on standard operation process of A. cinnamomea fruit body. The potential of biomedical activities of our fruit body extracts will be tested subsequently.

Regarding on the urine examination, all three treatment groups showed clear urine with a light yellow color, with pH values ranging from 5.5-8.5. Urine SG values between 1.010 and 1.030 are considered normal. The numbers of animals with abnormal urine SG values in the groups fed with A. cinnamomea were lower than the number in the control group, suggesting that A. cinnamomea feeding did not cause abnormal urine SG. Three male rats had urine protein levels, while no occult blood was found, and the pH values were all within the normal range. In addition, serum renal function tests showed all items of the three rats to be normal, and therefore we excluded the possibility of pathological proteinuria. The increased urine protein in these three rats was likely benign and transient proteinuria.

In a ketone test, there were no differences in the number of male rats with abnormal results. Only trace amounts of ketones were found in all groups of male rats, and the results were negative in all female rats in all groups. 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. Nitrite was found in the urine samples of one rat in the control group and one male rat in Group 2, the group administered the lowest dose of A. cinnamomea. As the amounts were low, and only a marginal positive response was found, it was thought that this was not associated with treatment. As bacteria can convert nitrate to nitrite, the presence of nitrite in the urine is usually a sign of bacterial infection. We therefore checked whether urinary tract infection was present in these rats; however, no urine white blood cells (WBC) were detected, and therefore the possibility of the nitrite positive response having been caused by urinary tract infection was excluded. The remaining urine test items, including urobilinogen, bilirubin, glucose, and occult blood, were all normal in all groups. Three rats in the control group had a weak positive epithelial cell response in urine sediment, while all rats in the A. cinnamomea-treated groups were negative. 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 often a neglected aspect of urinary sediment analysis [15, 16].

About hematology tests, 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. The 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 no differences in RBC and MCV. All the animals had a MCH in the range of 21.5-24.0 pg/cell, and the MCHC ranged from 36.2-37.5 g/dL. The hemoglobin values of the male rats ranged from 14.3-18.6. In summary, of the items of hematologic analysis in which a difference was observed, few changes were seen in the group administered the lowest dose of A. cinnamomea. No dose-dependent response was observed. Although some changes were noted, the test values were still within the normal ranges of normal rat hematology, suggesting that A. cinnamomea does not affect blood indices [17].

For the serum biochemical analysis, in the male rats, the total serum protein (TSP) of Group 2 was significantly higher than that of the controls, though the value was still within the normal range. Globulin in Group 2 was also significantly higher than in the controls, and the albumin/globulin ratios of the animals in Group 2 ranged from 0.93-1.37, which were lower than the normal range of 1.5-2.33. In the study by Ziaias et al., in 2009 [18], the albumin/globulin ratio was reported to be reduced with increasing age in rats. The albumin/globulin ratio was 0.88 ± 0.03 when our rats were 60-days-old, but by the time the rats were necropsied, they were 20-weeks-old, and therefore the decrease in the albumin/globulin ratio should be considered to be a normal phenomenon due to aging. Aminotransferase (ALT) in Group 4 was significantly lower than that in the controls. Abnormal increased ALT may be a sign of liver damage. Although the ALT value of Group 4 was significantly different from the control group, it was decreased, and was still within the normal range. Triglyceride in Group 4 was significantly decreased in comparison with the controls, but was still within the normal range. A change in triglyceride requires reference to the values of total cholesterol, LDL cholesterol and HDL cholesterol for the diagnosis of hyperlipidemia. The total cholesterol was normal in the rats of all groups, suggesting that the possibility of hyperlipidemia could be ruled out. The inorganic phosphorus (Ip) values of Group 3 and Group 4 were significantly higher than that of the control group, but were still within the normal range. Potassium (K⁺) and chloride (Cl⁻) were significantly high in Group 4 as compared with the controls, but were all within the normal ranges. Electrolytes are important for normal bodily functions, as the electrolyte balance affects blood osmotic pressure, and individual ions also have specific functions. The levels of sodium and chloride ions in the blood are correlated with the amount of sodium chloride uptake. Potassium ions are involved in nerve conduction and muscle contraction. Calcium ions are associated with bone, parathyroid and kidney functions. Many factors affect the level of phosphate in the blood, and abnormal blood phosphorus requires other tests to confirm the diagnosis.

In the female rats, the ALT value in Group 2 was significantly lower than that in the controls, while the ALT levels of Group 3 and Group 4 were both normal. In clinical application, only increased AST and ALT values require further follow-up, as higher AST and ALT are considered to indicate liver inflammation. The blood urine nitrogen (BUN) level in Group 4 was significantly higher than in the controls, although it was within the normal range. It should be noted that increased BUN may indicate increased release of protein metabolites, which can be a sign of renal dysfunction, but cannot indicate the degree of abnormality. For evaluation of renal function, creatinine is a better indicator. In our experiment, no abnormal creatinine levels were seen [19, 20].

Taken together, the data from this experiment demonstrated that the extracted solution of A. cinnamomea fruiting bodies does not cause adverse effects or toxicity in the organs of rats administered with the doses tested. The NOAEL was 5000 mg/kg, and using a safety factor of 100, the ADI was estimated to be 50 mg/kg.

Acknowledgement

We appreciate the assistance of OxBiosci for English editing.

Conflict of interest

The authors declare that there is no conflict of interest involved in this study.

References


Figure Legends

Figure 1. LC/MS/MS profiling of ethanolic extracts of A. cinnamomea fructing bodies.