Acute Oral Toxicity ($LD_{50}$) of Crude Exopolysaccharide from *Rhodotorula minuta* BIOTECH 2178 in ICR Mice

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Abstract

Acute toxicity study on lyophilized exopolysaccharide (EPS) produced from *Rhodotorula minuta* BIOTECH 2178 EPS was evaluated using laboratory mice. In this study, 20 male ICR mice were divided into four (4) groups: Group I received 0.5 ml saline solution; Group II was treated with 50mg EPS/kg body weight; Group III was treated with 225mg EPS/kg body weight; and Group IV was treated with 400 mg EPS/kg body weight. Blood samples were collected via orbital sinus for hematological analysis. Body weight, feed and water consumption were recorded daily. Liver and kidney were collected for histopathological analysis after 15 days administration of EPS. Blood chemistry analysis showed that mice fed with crude EPS had comparable BUN, creatinine and ALT values with that of the control. Liver histopathological analysis of the control and treated group showed normal hepatic architecture with intact hepatic cells, nucleus, sinusoidal spaces and a central vein. Histopathological analysis of the kidney showed normal renal structure of cortex, indicating a normal architecture of renal glomeruli, proximal convoluted tubule and distal convoluted tubules. Moreover, the tubules showed a relatively regular distinct lumen of both control and treated groups. Thus, crude EPS from R. minuta BIOTECH 2178 is neither hepatotoxic nor nephrotoxic and likely to be safe for human consumption. Moreover, its safety property lays a good foundation for application of EPS in food and pharmaceutical industries.

Keywords: Histopathological analysis; Creatinine; Hepatotoxic; Nephrotoxic

Introduction

One of the major roles of food scientist in developing novel products is to ensure their safety for human consumption. Basically, toxicological tests establish a margin of safety before it reaches to the consumer. Helferich and Winter (2001) noted that microbial safety would remain as a major health concern in human wherein chronic consumption of bioactive metabolites or compounds produced by several microbes from our diet has a direct relationship in the development and progression of several diseases. Toxicological test used *in vitro* methods and animal models have been widely accepted by the research community.

Toxicological studies on the use of exopolysaccharides (EPS) are very minimal since most studies used bacterial EPSs coming from lactic acid bacteria (LAB), which
are “generally recognized as safe” (GRAS). The major EPS produced by yeast are beta-Glucans, a group of highly conserved carbohydrate biopolymers that form a fibrous structural extracellular matrix. Animal studies revealed that these compounds showed no adverse or toxic effects after acute and sub-chronic oral administration in laboratory animals (Babicek et al., 2006 and Turmina et al., 2012). There are actually a few pre-clinical safety evaluations that have been performed on beta-glucans derived from *S. cerevisiae* and *Candida albicans* (Feletti et al., 1992 as cited in Turmina et al., 2012). However, based on their studies, the effect of beta-glucan can vary significantly depending on the method of preparation. Fungal EPS from *Phellinus baumii* were found non-toxic and instead, showed a remedial role in liver functions when applied in diabetic (STZ-induced) rats (Hwang et al., 2004).

*Rhodotorula* species are generally considered safe for humans. However, in the last 20 decades, these species have emerged as opportunistic pathogens that have the ability to colonize and infect both immunocompetent and immunocompromised patient (De Almeida et al., 2008). Wirth and Goldani (2012) studied the pathogenesis of human mycosis caused by *Rhodotorula* species using laboratory mice. Result showed that the most infected organs were the lungs, kidney, spleen, and liver, which experienced a severe degree of infection.

Studies on the acute and sub-chronic intake of exopolysaccharide *in vivo* using animal models that assess toxicological effects are scarce. To the authors’ knowledge, there are no reports assessing the toxicity of EPS from *R. minuta* BIOTECH 2178. In the work presented here, toxicological examination of crude EPS from *R. minuta* BIOTECH 2178 using acute oral toxicity test (LD50) were assessed by examining various biochemical and histopathological parameters that may cause metabolic alterations in the vital organs (kidney and liver) of ICR mice. The protocol of this study was approved by the Institutional Animal Care and Use Committee (IUCAC), College of Veterinary Medicine, University of the Philippines Los Baños.

The dose that was administered to the mice in this study ranged between 50 – 400 mg EPS/kg bodyweight of ICR mice. This was based on several review that studied the hypoglycemic effect of EPS from fungi wherein the investigators suggested a suitable dose range of 100-300mg EPS/kg BW because beyond this range negative signs were observed in the animals such as diarrhea and extreme weight loss (Kim et al., 2001; Lo et al., 2004 and Benwahhoud et al., 2001). Hwang et al. (2005) used 200mg EPS from *P. baumii* kg BW of rats that is considered as the most suitable dose to have an anti-diabetic effect in streptozotocin (STZ)-induced diabetic rats.

**Materials and Methods**

**Microorganisms, media and growth conditions**

Pure cultures of *Rhodotorula minuta* BIOTECH 2178 was isolated from fresh water Laguna Lake, Philippines and was stored in the Philippine National Collection of Microorganisms located at the National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños (UPLB). The yeast strains were maintained on malt yeast agar (MYA) slants and stored at 4°C. The basal medium for EPS production mainly contained (g/L): yeast extract, 18.75; xylose, 6.25; (NH4)2SO4, 2.5; KH2PO4, 1; MgSO4.7H2O, 0.5; NaCl, 0.1 and CaCl2.2H2O, 0.1. The initial pH was adjusted to 5.5, and the medium was sterilized at 121°C for 15 minutes. All chemicals that were used in this study were of analytical reagent grade.

Prior to inoculation, yeast cells were counted using Neubauer hemacytometer. Then, yeast cells (5%v/v) were aseptically inoculated into the Erlenmeyer flask (250
mL) containing 50 mL basal medium and incubated in a rotary shaker (180rpm) at room temperature (Ramirez, et al., 2015; Ghada et al., 2012)

Isolation of crude exopolysaccharide by *R. minuta* BIOTECH2178

After four days fermentation, the culture broth was centrifuged at 5000 rpm for 5 minutes at 5°C to separate the yeast cells. The exopolysaccharide was precipitated in the cell free supernatant with cold 96% at 1:2 (v/v) ethanol:supernatant ratio then stored at 4°C for 24 hours. The precipitated EPS was separated by centrifugation at 5000 rpm for 15 minutes at 12°C. The supernatant was discarded and the precipitate was immediately stored in the freezer. Frozen EPS was lyophilized (Leybold-Heracus, Germany) until a constant weight was observed (Ramirez, et al., 2015). A precision analytical balance was used to verify the quantity of EPS obtained (grams EPS per liter of culture medium).

Test Material Preparation

The optimized crude EPS from *Rhodotorula minuta* BIOTECH 2178 was used as a test material for acute toxicity studies. EPS was prepared in a sterile isotonic saline solution at a concentration of 5g NaCl/L.

Care of Experimental Animals

Adult male ICR mice were obtained from the animal house of Food and Drug Administration (FDA), Muntinlupa City, Philippines. Upon arrival of the mice in the laboratory, they were acclimatized for a minimum of seven (7) days, and ensured to be 5–6 weeks of age and weighed between 20-26 g at the initiation of the treatment. They were initially housed as groups of five (5) animals per cage in polycarbonate cages. The temperature inside the experimental room was kept at 22°C ±2°C and the relative humidity was maintained at 40–70% (with an aim of 50–60%). Additional environmental controls involved the use of artificial lighting (12 h light/12 h dark) and microbiological environmental monitoring. Sterilized litter (wood chips) was inspected daily and changed three (3) times a week. Animals received sterilized standard pellets diets and sterilized drinking water (Wilkins or Absolute) ad libitum. All animals received proper care and the experimental protocols were in compliance with institutional guidelines for the use of laboratory animals.

Experimental Design

The mice were randomly selected and divided into four equal groups (five mice each group) and treated as follows: group I was the control given 0.5 ml saline solution; group II was treated with 50mg EPS/kg body weight; group III was treated with 225mg EPS/kg body weight; and group IV was treated with 400 mg EPS/kg body weight. The doses were set on the basis of the recently recorded body weight of each individual animal. Then it was suspended in sterile saline solution with a constant volume of 0.5ml per mice. They were individually caged and treated for 14 successive days by oral gavage.

Physical and Physiological Parameters

Safety assessment was based on the physical and physiological observations for toxicological evaluation. Signs of acute toxicity were observed daily in EPS-treated group during the 14-day experimentation period. The study was based on the following procedures by Morgan et al. (1989) as cited in Reginio (2013): (a) animals were observed undisturbed in their cages; (b) animals were removed from their cages and given some examination, and (c) animals were observed after being returned to their cages. Physiological parameters included the body weights, food and water intakes,
Table 1. General appearance and behavioral observations of control and treated groups of male ICR mice.

<table>
<thead>
<tr>
<th>Observations</th>
<th>Control 6h</th>
<th>Control 14h</th>
<th>Treated 6h</th>
<th>Treated 14h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin and fur</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Eyes</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Mucus Membrane</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Salivation</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Lethargy</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Sleep</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Stool</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Coma</td>
<td>N.O.</td>
<td>N.O.</td>
<td>N.O.</td>
<td>N.O.</td>
</tr>
<tr>
<td>Tremors</td>
<td>N.O.</td>
<td>N.O.</td>
<td>N.O.</td>
<td>N.O.</td>
</tr>
</tbody>
</table>

N.O. = Not observed

gastrointestinal signs such as inappetence, occurrence of diarrhea or constipation, dehydration, bloody feces and emaciation, over-all physical characteristics of mice particularly the hair coat and the nostril, and behavioral signs of irritability or inactivity.

Biochemical Analyses

Biochemical tests were determined before oral administration of EPS and at the end of the administration period in all treated animals. Blood samples (placed in heparinized tubes) were withdrawn in the orbital sinus from each mouse. Blood plasma was separated by centrifugation 3000 x g for 10 minutes and immediately processed for biochemical parameters: alanine transaminase (ALT), Blood Urea Nitrogen (BUN), and creatinine levels.

Histopathological Analyses

Liver and kidney organs were removed from all experimental animals following euthanasia and fixed in 10% formaldehyde. After 48 hours, the liver organs were sliced in its desired section while the kidney was sliced into half and transferred in fresh formaldehyde. These were sectioned using a microtome and stained with Hematoxylin-Eosin (H.E.).

Statistical Analysis

Data on weekly body weights, food consumption, biochemical and hematological parameters were calculated by a one-way analysis of variance (ANOVA) and F-values were presented only if $p < 0.05$. Post-hoc analysis was performed using the Tukey’s test when a significant dose-effect was observed.

Results and Discussion

The optimized crude EPS from \textit{R. minuta} BIOTECH 2178 was used to feed the ICR mice for toxicity test. The treatment groups were fed with crude EPS at varying concentrations dissolved in 0.5% saline solution while the control group was fed with saline solution only. When studying oral toxicity test, the most convenient and commonly used method is through the use of gastric gavage to ensure that the test material is taken by the animal. The absorption might be slow, but this method costs less and painless to the mice (Jothy et al., 2011). The behavioral patterns of the mice were observed after six hours and followed by 14 hours after administration of crude EPS in control and treated groups (Table1). Results showed that the mice did not display any significant changes in behavior having normal food intake and water consumption. No toxic symptom and mortality for the control and treated groups 14 hours
Mean body weight of male ICR mice given with daily doses of crude EPS by oral gavage for 14 days (n=5 mice per group).

Mean average (±SD) of food and water intake of male and female mice fed with crude EPS from *R. minuta*

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>Mean Food Intake</th>
<th>Mean Water Intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.35±0.79</td>
<td>5.9±2.19</td>
</tr>
<tr>
<td>T1 - 50 mgEPS/Kg BW</td>
<td>3.50±1.13</td>
<td>5.66±2.02</td>
</tr>
<tr>
<td>T2 - 225 mgEPS/Kg BW</td>
<td>3.65±0.97</td>
<td>5.79±2.05</td>
</tr>
<tr>
<td>T3 - 400mg EPS/Kg BW</td>
<td>3.69±1.01</td>
<td>6.01±2.10</td>
</tr>
</tbody>
</table>

Each value is a mean of five mice taken for 14 days. There was no significant difference on food and water intake between control and crude EPS fed groups (p<0.05).

Mean Body weight

The 14 days administration effect of *R. minuta* EPS on the body weight gain in male ICR mice are presented in Figure 1. As shown in the graph, the weight of the male mice are within the normal weight range of the mice which is 20 gm to 30 gm. It also exhibited a normal increment in body weight without a drastic change between the control and treated groups.

Mean Food Consumption and Water Intake

In general, the administration of crude yeast EPS even at higher dose did not significantly affect the mean food and water intake in both sexes of treatment group and were comparable to that of the control group (Table 2), indicating no signs of inappetence. Its erratic nature is considered normal since there are days that these mice may have taken more feeds or water which resulted in less intake the following day.

Dehydration, diarrhea or constipation,
Table 3. Mean creatinine, BUN, and ALT of the negative control and EPS-treated male and female ICR mice.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>CREATININE (mg/dL)</th>
<th>BUN (mg/dL)</th>
<th>ALT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 day</td>
<td>14 days</td>
<td>0 day</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.20±0.01</td>
<td>0.21±0.01</td>
<td>12.87±0.06</td>
</tr>
<tr>
<td>T1</td>
<td>0.18±0.01</td>
<td>0.19±0.00</td>
<td>12.71±0.39</td>
</tr>
<tr>
<td>T2</td>
<td>0.19±0.01</td>
<td>0.20±0.01</td>
<td>12.92±0.08</td>
</tr>
<tr>
<td>T3</td>
<td>0.19±0.01</td>
<td>0.19±0.01</td>
<td>12.89±0.04</td>
</tr>
</tbody>
</table>

BUN – Blood urea nitrogen; ALT – L-alanine-2-oxoglutarate aminotransferase.

Each value is a mean average of five mice. No significant difference between control and R. minuta crude EPS-fed groups (p>0.05)

bloody stool were not observed. Its stool were frequent, non-sticky, dark and pelleted indicating that there were no signs of gastrointestinal disturbances during the 14 days administration of EPS in treated groups. Throughout the study, the overall physical appearances of the EPS treated group were also comparable with the control groups showing active but not irritable, bright clear eyes and shiny hair coat.

Blood Chemistry (Creatinine, Serum BUN and ALT Levels)

Kidney is a paired organ whose functions include removing waste products from the blood and regulating the amount of fluid in the body. A disturbance in one of the fundamental mechanisms of the body’s self-regulatory control systems can lead to renal failure. Estimation of the renal excretion of the waste products such as creatinine (CRT) and Blood Urea nitrogen (BUN) provides a useful tool on the health status of the kidney. An elevated CRT and BUN in the blood (which is above 1 mg/dL and 21mg/dL, respectively) indicate biochemical damage of the kidney.

Creatinine is a breakdown product of creatinine phosphate released from the skeletal system filtered by the glomerulus in the kidney (Kahn et al., 2010). It is generally used to estimate how well the kidney are filtering blood and usually used as a screening test for early kidney impairment. As shown in Table 3, the creatinine levels for male group treated with crude EPS showed no significant difference on the negative control. Moreover, all mean creatinine levels are within the normal range taken before (0 day) and after (14 days) administration of EPS.

Serum blood urea nitrogen (BUN) test revealed how well the liver and kidney are working. The liver produces ammonia after the breakdown of protein needed in the body and combined with other elements to form urea, a chemical waste product. The urea travels from the liver to the kidney through the bloodstream and filtered freely in the glomerulus which is excreted in the body through the urine (Harris and Crabb, 2010). An elevated urea nitrogen suggest that the kidney or liver is not working properly (Niedert and Dorner, 2004). The BUN values obtained from day 0 to day 14 in EPS-treated group were insignificantly different from that of the control group, as shown in Table 3.

Hepatic enzymes such as alanine aminotransferases (ALT) are a reliable marker of hepatocellular injury or necrosis. An elevated ALT in the blood is due to the metabolic changes acted by the liver, such as administration of toxin, cirrhosis, hepatitis and liver cancer (Chalasani et al., 1997). Results showed no significant difference observed in ALT levels of the treatment male and female group from day 0 to day 14. Furthermore, all ALT values in both control and treatment groups were within the normal range (25-76IU/L).
Histopathological Examination of the Liver

The liver is a large, complex organ that is well designed for various metabolic functions playing a key role in detoxification, regulation and maintaining homeostasis (Hwang et al., 2007). Macroscopic examination of liver organs of the animals treated with crude EPS from *Rhodotorula minuta* BIOTECH 2178 showed no changes in color compared to the control. Figure 2 show the microscopic structure of the male hepatocyte, respectively. The control and treated group showed normal hepatic architecture with hepatic cells, nucleus, sinusoidal spaces and a central vein.

Histopathological Examination of the Kidney

The main role of the kidney is the elimination of toxins or waste products from the plasma and maintains homeostasis of essential cellular biomolecules (Hwang et al., 2007). The histopathological examinations of kidney did not detect any severe alterations in male mice from both control and crude-EPS treated groups (Figure 3). The kidneys showed normal renal structure of cortex, which showed a normal architecture of renal glomeruli, proximal convoluted tubule and distal convoluted tubules. Moreover, the tubules showed a relatively regular distinct

Table 4. Quantitative analysis of the cellular damage in liver and kidney organ observed in control and EPS-treated ICR mice.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>LIVER</th>
<th>KIDNEY</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>1.8±0.22</td>
<td>1.0±0.00</td>
</tr>
<tr>
<td>T1</td>
<td>1.6±0.55</td>
<td>1.1±0.22</td>
</tr>
<tr>
<td>T2</td>
<td>1.6±0.67</td>
<td>1.2±0.27</td>
</tr>
<tr>
<td>T3</td>
<td>1.7±0.45</td>
<td>1.0±0.00</td>
</tr>
</tbody>
</table>

Grading for statistical analysis: No lesions -1.0; very mild – 1.5; mild – 2.0; mild to moderate – 2.5; moderate – 3.0; moderate to severe - 3.5; severe – 4.0. No significant difference between control and R. minuta crude EPS-fed groups (p>0.05)
lumen in both control and treated groups. The control and treated ICR mice were rated quantitatively according to the damage of the liver and kidney. Based on the results, there is no significant difference between the negative control and treated male and female ICR mice in terms of the quantitative rating of the hepatocyte and nephrocyte (Table 4). The rating of the liver and kidney ranges from 1-1.5 (absence of lesion to very mild fibroblasts proliferation on the cortico-medullary junction) in the control and treated mice. Fibroblasts are considered mesenchymal cells that display a spindle-shaped morphology and originally identified as the cells being responsible for wound contraction which eventually turned into a scar (Strutz and Muller 2006; Majno et al., 1971). Mild fibroblast proliferation in this study are not directly associated with the treatment of EPS since it was also seen in the control group. Probably the mice might have previously infected and was healed in the process.

Conclusion and Recommendation

This study demonstrated that the intake of freeze-dried exopolysaccharide powder did not produce signs of toxicity in mice, regardless of gender. The administration of EPS by gavage at the concentration of 400 mg/kg b.w./day showed no alteration in the hematological and histopathological analysis of male ICR mice after 14 days administration. Thus, the EPS produced by Rhodoturula minuta BIOTECH 2178 is likely to be safe for human consumption. Future studies will be directed towards the analysis of pharmaceutical properties of the compound and its mechanism of action involved.

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