EVALUATION OF POLYSACCHARIDE EXTRACT FROM *GANODERMA LUCIDUM* (CENDAWAN SENDUK) ON BALB/C 3T3 NRU CYTOTOXICITY AND ORAL ACUTE TOXICOLOGY UP-AND-DOWN PROCEDURE (UDP) – LIMIT TEST

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ABSTRACT

The animal tests are mandatory for the evaluation of toxicity in order to determine the effective dose for new nutraceutical or medicinal product. In this study, the polysaccharide extract from Ganoderma lucidum (Cendawan Senduk) was evaluated for the safety and efficacy of the extract using Balb/c 3T3 NRU Cytotoxicity and acute oral toxicity UDP – Limit Test. This method is one of the most used in toxicology test to reduce the number of animals. The median inhibition concentration (IC50) of the polysaccharide extract was obtained at 21,075.33 µg/mL ± 1,779.21 µg/mL. Hence, the UDP starting dose for the acute oral systemic toxicity study Up-and-Down Procedure limit test is 2000 mg/kg. Acute toxicity study conducted revealed that the administration of polysaccharide extract (up to a dose of 2000 mg/kg) of G.lucidum (Cendawan senduk) did not produce any significant changes in behavior of the animals. No death was observed up to the dose of 2000 mg/kg body weight. The rats were physically active. These effects were observed during the experimental period (14 days). The results showed that in single dose the polysaccharide extract had no adverse effect, indicating that the medium lethal dose (LD_{50}) could be greater than 2000 mg/kg body weight in rats. No toxic symptoms were observed up to dose of 2 g/kg body weight. All animals behaved normally. No neurological or behavioral effects could be noted. No mortality was found up to 14 days study.

INTRODUCTION

Nowadays, there are increasing consumption of natural product and natural chemical from plant and fungi as nutraceutical product either used for human health or disease management. Therefore, the regulatory and safety aspect of nutraceutical product especially fungal origin is important to be studied. *Ganoderma lucidum* being treasured for its medicinal value for more than 1000 years and known to be safely used in traditional herbal medicine (TCM) and pharmaceutical industry as well as food consumption. However, the *ganoderma* strain used in this study was a local isolates and artificial cultivated using a complex medium of spent yeast and brown sugar by submerged cultivation. The growth of *ganoderma* sp. is greatly influence by the growth medium/substrates and condition of cultivation. Therefore, the safety is vitally needed to be studied for the range of doses that could be used subsequently, and also to study the possible clinical signs (adverse effect) elicited by the substances. Cendawan senduk, a local *ganoderma* strain was traditionally used as self medication by

indigenous communities in Malaysia as herbal remedy based on their ethnomycological knowledge for natural-healing, alternative medicine and improvement of body strength. But it is not ensure that this strain is safe without any compromising of health effect.

Herbal remedies are often believed to be safe, harmless, free of side effect and less damaging to the human body compared to synthetic drug (Pakehh and Chanda, 2006 and Lopes et al. 2000). Therefore, there is a need for thorough scientific safety evaluation of this medicinal mushroom. The evaluation of the toxic action of the mushroom extract is indispensable in order to know the effective dose and to be considered a safe treatment. The method for determining the in vitro cytotoxicity of test substances using neutral red uptake (NRU) is to determine the starting doses for in vivo acute oral systemic toxicity tests for the up-and-down procedure that may reduce the number of animals required. The acute oral toxicity Up-and-Down Procedure (UDP) – Limit Test – is a sequential test which uses a maximum of five animals. Animals are dosed in sequential manner with the next animal receiving the same dose only if the first animal survives the limit dose.

MATERIALS AND METHODS

Extraction of polysaccharide

The strain of *Ganoderma lucidum,* (Cendawan Senduk, strain KUM 61076) was grown in 2L STL Reactor for 4 days at 26^{0}C at 160rpm. The mycelium were separated using ultracentrifuge (Beckman Coulter, Germany) at 10,000 rpm for 10 min. The mycelium was dried at 50±2^{o}C for a constant weight. The dried mycelia extract were extracted with hot water extract (1:40 w/v) for 30 minutes. Then, it was filtered and the precipitated was overnight with addition of double volume of 95% ethanol. After centrifuged and filtered, the polysaccharide extracts were freeze dried for 5 days.

T3 Neutral Red Uptake (NRU) Cytotoxicity Assay

A murine fibroblast cell line, BALB/c 3T3 cells, clone A31, was obtained from American Type Culture Collection (ATCC), Manassas, USA (Catalog no. CCL-163). The Balb/c 3T3 cells are maintained in DMEM (Thermo Scientific, buffered with sodium bicarbonate) supplemented with calf serum (Sigma), Penicillin and Streptomycin (PAA Lab). The procedure of BALB/c 3T3 NRU cytotoxicity test was carried out as recommended by ICCVAM (NIH Publication No. 07-4519, 2006), The cell line was incubated for 24 hours for formation of monolayers after cells were removed from flask by trypsinization. The cells were then seeded into 96-well tissue culture microtiter plates with NR medium (50 g/mL NR in DMEM Medium) and incubated for approximately 3 hours. The NR medium was then removed and the cells were again carefully rinsed with PBS. NR Desorb solution (1% Glacial acetic acid and 50% ethanol) was added to all wells and the plate was rapidly shaken on a microtiter plate shaker for 10 minutes to 30 minutes. Optical density was measured at 570 nm ± 10 nm (OD570) in a microplate spectrophotometer. The IC50 values were calculated using Hill function analysis using GraphPad Prism® version 5.04 for Windows software.

Acute toxicity

Acute toxicity was assessed with five female sprague dawley rats, by oral administration of a selected dose as recommendation by BALB/c 3T3 NRU cytotoxicity test. This method of acute toxicity UDP-limit test was conducted according to the Organization of Economic Cooperation and Development (OECD) Guidelines (OECD,2010). The adverse effects occurring within a short time after oral administration of a single dose of a substance was determined according to Chan and Hayes (1994). A starting dose of 2000 mg/kg was selected based on recommendation from the BALB/c 3T3 NRU cytotoxicity test. The freeze dried polysaccharide extract was freshly prepared prior to dosing. Special attention was given during the first 4 hours and periodically during 48 hours post-dosing. Daily observation was carried out for 14 days. The number of animals killed for each of the doses was noted and the LD50 calculated by the Up and Down method. The female sprague dawley rats (152-218 g, 2 months old) were divided into two groups (n=10) (control group and treated group), Each group contained five animals. The animals were kept under observation for mortality and symptoms for a period of 24 h and then sacrificed by cervical dislocation. Liver, spleen, stomach, lungs and kidneys were dissected out and assessed for gross pathology. Histopathological examination was carried out after histochemical processing and staining with hematoxylin and eosin.

RESULTS AND DISSCUSSION

The Balb/c 3T3 NRU cytotoxicity test was determined the median inhibition concentration (IC50) of the polysaccharide extract at 21,075.33 µg/mL ± 1,779.21 µg/mL. The regression formula calculates the estimated value of LD50 to be 4,289.9 mg/kg. Therefore, the UDP starting dose for the acute oral systemic toxicity study Up-and-Down Procedure limit test is 2000 mg/kg. The average and S.D IC50 value from triplicate NRU assay was determined 21,075.33 µg/mL ± 1,779.21 µg/mL.

The toxic effect of polysaccharide of extract from Cendawan Senduk on the appearance and the general behavioral pattern of rats were also observed at first 6 h and followed by 14 h after the administration. Results shows that the animals in both control group and treated groups were normal and did not display any significant changes in behavior, skin effects, breathing, impairment in food intake and water consumption and postural abnormalities. No toxic symptoms or mortality were observed in animals, at the administration of 2000 mg/kg of polysaccharide extract of Cendawan Senduk (Table 1). Throughout the 14-day observation period of oral acute toxicology test (UDP-limit test), all animals appeared active and healthy. All animals gained body weight over the 14-day observation period for both groups. Individual body and organ weights of control group and treated group are summarized in Tables 2. There were significant values ($p<0.05$) between the organ and body weights of treated group.

Macroscopic examination of the organs of the animals treated with extract shows no changes in color compared to control. Autopsy at the end of the experiment period revealed no apparent changes in the liver, kidney, lungs, heart and spleen organs from both control and treated mice in the histopathology analysis. The microscopic structures of the organs depicted through shows unnoticeable differences between the control and test groups. The microscopic examination revealed that all the organs from the extract treated mice does not show any alteration in cell structure or any

unfavorable effects when viewed under the light microscope using multiple magnification power. The structure or coordination of cells in extract treated organs more or less similar compared to the control organs.

Table 1. Mortality of Sprague Dawley Rats treated with polysaccharides extracts of Cendawan Senduk.

Observation Day	Control groups*	Treated groups*
Day 0	0/5	0/5
Day 7	0/5	0/5
Day 14	0/5	0/5

*Number of dead rats/ number of rats used

Table 2. Summary of body and organ weights of animal group (**Mean ± S.E. (n=5)**

Organ/Body	**Treated Group (g)**	**Control Group (g)**
Heart	0.370 ± 0.01	0.375 ± 0.12
Liver	2.687 ± 0.07	2.601 ± 0.05
Spleen	0.200 ± 0.00	0.188 ± 0.01
Kidneys	0.711 ± 0.01	0.713 ± 0.01
Stomach	0.300 ± 0.01	0.304 ± 0.01
Body Weight (g)	210.0 ± 4.6	211.6 ± 9.6

Organ body index = (organ weight × 100)/body weight; polysaccharides extract of Cendawan senduk was administered to rats at a dose of 2000 mg/kg; values are mean ± SD (n = 3) at 5% level of significance (* = $P < 0.05$).

CONCLUSION

In conclusion, the results of the present study clearly show that polysaccharide extract at up to 2000 mg/kg body weight/day for 14 days did not cause either mortality or toxicity rat. Therefore, no observed adverse effect level for polysaccharide extract derived from our results was 2000 mg/kg body weight/day thus establishing its safety in use.

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