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Preclinical toxicological evaluations of the sclerotium of *Lignosus rhinocerus* (Cooke), the Tiger Milk mushroom

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ABSTRACT

Ethnopharmacological relevance: Lignosus rhinocerus (Tiger Milk mushroom) is distributed in South China, Thailand, Malaysia, Indonesia, Philippines and Papua New Guinea. In Malaysia, it is the most popular medicinal mushroom used by the indigenous communities to relieve fever, cough, asthma, cancer, food poisoning and as a general tonic. In China, this mushroom is an expensive traditional medicine used to treat liver cancer, chronic hepatitis and gastric ulcers. The sclerotium of the mushroom is the part with medicinal value. This rare mushroom has recently been successfully cultivated making it possible to be fully exploited for its medicinal and functional benefits. The present study was carried out to evaluate the chronic toxicity of the sclerotial powder of *Lignosus rhinocerus* cultivar (termed TM02), its anti-fertility and teratogenic effects as well as genotoxicity.

Materials and methods: Sprague Dawley rats (10 rats/group/sex) were fed orally with 250, 500 and 1000 mg/kg of sclerotial powder of TM02. The sclerotial powder was orally administered once daily and consecutively for 180 days. At the completion of the oral feeding period, analysis of hematological and clinical biochemical parameters, urine profiles, organ weight as well as histopathological analysis were carried out. The effect of the sclerotial powder on fertility and its possible teratogenicity were examined by feeding rats orally with 100 mg/kg sclerotial powder consecutively for 7–8 weeks. Genotoxicity was evaluated by Ames test using *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and *Escherichia coli* WP2 uvrA.

Results: The results showed that oral administration of the sclerotial powder of the *Lignosus rhinocerus* cultivar at daily dose of up to 1000 mg/kg for 180 days had no adverse effect on the general clinical observations, body weight, hematology, clinical biochemistry, urinalysis, absolute organ weight as well as relative organ weight, nor induced histological changes in the organs. Oral administration of 100 mg/kg sclerotial powder of the *Lignosus rhinocerus* for 7–8 weeks did not affect the fertility of the rats nor induce teratogenic effect on their offspring. *Lignosus rhinocerus* sclerotial powder up to 5000 μg/plate in the presence and absence of metabolic activation did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used.

Conclusion: Our results showed that the no-observed-adverse-effect level (NOAEL) dose of the sclerotial powder of *Lignosus rhinocerus* in 180-day chronic toxicity study is more than 1000 mg/kg. Oral feeding of the sclerotial powder at 100 mg/kg did not induce adverse effect on rats' fertility nor causing teratogenic effect on their offspring. In the reverse mutation Ames test, the sclerotial powder at all tested concentration did not show any genotoxicity.

1. Introduction

Lignosus rhinocerus (Synonym *Lignosus rhinocerotis* (Cooke) Ryvarden), commonly known as Tiger Milk mushroom, belongs to the Polyporaceae family. Its geographical distribution is only in the tropical rainforest in the region of South China, Thailand, Malaysia, Indonesia, Philippines and Papua New Guinea (Tan et al., 2012). In Malaysia, it is also known as '*Cendawan susu rimau*' and is the most popular medicinal mushroom used by the indigenous communities of Peninsular Malaysia to relieve fever, cough, asthma, cancer, food poisoning and as a general tonic (Lee et al., 2009). In China, the sclerotium of the mushroom is an expensive traditional medicine used for treatment of liver cancer, chronic hepatitis and gastric ulcers (Wong and Cheung, 2008).

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Phylogenetic analysis indicated that the mushroom is closely related to *Ganoderma lucidum* and *Trametes versicolor,* the two most popular medicinal mushrooms used in Asia (Tan et al., 2010).

The sclerotium of *Lignosus rhinocerus* has been demonstrated to exhibit anti-proliferative activity. Lai et al. (2008) demonstrated that sclerotial polysaccharides from *Polyporus rhinocerus* (synonym of *Lignosus rhinocerus*) exhibited antiproliferative effects on several kinds of leukemic cell lines. Wong et al. (2009) reported that the hot water extract of *Polyporus rhinocerus* exhibited immunomodulatory effect by stimulating human innate immune cells. Our studies demonstrated that the cold water extract of *Lignosus rhinocerus* sclerotium exhibited direct cytotoxicity on human breast carcinoma (MCF-7) and human lung carcinoma (A549) cell lines (Lee et al., 2012a). It has also been demonstrated that the sclerotial extracts of *Lignosus rhinocerus* exhibited anti-acute inflammatory activity in an animal model study (Lee et al., 2012b). Gao et al. (2009) showed that the non-digestible carbohydrates may function as novel prebiotics. Recently, aqueous sclerotial extract of *Lignosus rhinocerus* was reported to contain neuroactive compounds that stimulated neurite outgrowth in PC-12 cell line (Eik et al., 2012).

In view of its wide ethno-botanical usages as well as proven *in vitro* anti-proliferative and anti-inflammatory activities, *Lignosus rhinocerus* sclerotium may be used as a health supplement. It is therefore necessary to carry out in depth safety evaluation of the sclerotium. Earlier studies to evaluate the subacute toxicity of the sclerotial powder of *Lignosus rhinocerus* cultivar (termed TM02) demonstrated its no-observed-adverse-effect level (NOAEL) was higher than 1000 mg/kg in a 28 days animal studies using rats. In the present study, we carried out a 180 day chronic toxicity study of the sclerotial powder, as well as evaluating its possible anti-fertility and teratogenic effects and genetoxicity.

2. Materials and methods

2.1. Preparation of Lignosus rhinocerus sclerotial powder

Sclerotial powder of the *Lignosus rhinocerus* cultivar TM02 was provided by Ligno Biotech Sdn. Bhd. (Selangor, Malaysia). The fungus was identified by internal transcribed spacer (ITS) regions of the ribosomal DNA (Tan et al., 2010). The sclerotial powder was freeze-dried and milled into powder using 0.2 mm sieve. The powder is light brown, dry fluffy powder with milk like taste.

2.2. Animals

Sprague Dawley (SD) rats aged 5 weeks old (male and female) were supplied by Chenur Supplier (Selangor, Malaysia). The animals were kept under standard conditions (temperature at 22 ± 2 °C, 12 h light, 12 h dark), and given water *ad libitum*. Animals were used after 14 days of acclimatization. Experimental protocols reported in this study were approved by Institutional Animal Care and Use Committee, University of Malaya (UM IACUC-Ethics reference no. PM/16/11/2010/0812/FSY (R)).

2.3. Chronic toxicity study

The chronic toxicity study was carried out in compliance with the guidelines from the Organization for Economic Cooperation and Development (OECD) (2009). Ten male (7 week old) and ten female (7 week old) Sprague Dawley (SD) rats were used for each treatment group. The *Lignosus rhinocerus* sclerotial powder was suspended in distilled water as vehicle. The animals were divided into eight groups (four male and four female groups) of 10 each as follow: Group 1 male and female (control group) received distilled water only throughout the entire test period (180 days). Animals in Group 2–4 (10 rats/group/sex) daily received 1000, 500 and 250 mg/kg sclerotial powder of *Lignosus rhinocerus* (cultivar TM02) orally, respectively. The highest dose of 1000 mg/kg was chosen based on the results of the 28-day subacute toxicity studies (Lee et al., 2011). The 500 and 250 mg/kg were chosen to demonstrate if there is any dose related response.

2.3.1. Blood analysis

At the end of 180 days, rats were fasted for 18 h. The rats were then anaesthetised with ketamine (45 mg/kg) and xylazine (4.5 mg/kg). Blood samples were withdrawn using cardiac puncture. Hematological examinations and clinical biochemistry were performed using Advia 2120 Hematology System (Siemen, Germany) and Advia 2400 Chemistry System (Siemen, Germany), respectively. The parameters for hematological examination include red blood cell (RBC) count, hemoglobin concentration, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, white blood cell (WBC) count, neutrophil count, lymphocyte count, monocyte count, eosinophil count, basinophil count, atypical lymphocyte count, prothrombin time and activated partial thromboplastin time (APTT). Biochemical tests included glucose, urea, creatinine, calcium, inorganic phosphate, uric acid, sodium, potassium, chloride, total cholesterol, total protein, albumin, globulin, albumin globulin ratio, total bilirubin, alkaline phosphatase, serum glutamic oxaloacetic transaminase (SGOT or AST), serum glutamic pyruvic transaminase (SGPT or ALT) and gamma-glutamyl transpeptidase (GTT).

2.3.2. Urinalysis

Urine was collected over 18 h using metabolic cages, on the last day of oral feeding (day 180th). Appearance, colour and volume of urine were recorded. Specific gravity, pH, total protein, and glucose, ketone, occult blood, urobilinogen and bilirubin were analysed using SD UroColor™ and Urometer 720™ (Standard Diagnostics, INC, Korea). White blood cell, red blood cell and epithelial cells were observed using microscope (Olympus CH, America).

2.3.3. Harvesting of the organs and histopathological analysis

After blood collection, the following organs were weighed to calculate weights relative to the final body weight: the adrenals, brain, epididymis, heart, lungs, kidneys, liver, ovaries, spleen, testes, and uterus. Organ weight relative to body weight was calculated as follows:

$$\text{Organ weight relative to body weight (\%)} = \frac{\text{Organ weight (g)}}{\text{Body weight (g)}} \times 100\%$$

In addition to these organs, aorta, caecum, cervix, coagulating gland, colon, duodenum, eyes, Harderian gland, ileum, jejunum, lacrimal gland (exorbital), lymph nodes, oesophagus, pancreas, prostate, rectum, salivary gland (parotid, submaxillary and sublingual), seminal vesicle, skeletal muscle, skin, stomach, thymus, trachea, urinary bladder and vagina were removed and preserved in 10% buffered formalin. The tissues were dehydrated by serial ethanol solution, cleared with xylene, paraffin embedded, sectioned and stained with hematoxylin and eosin. Light microscopic examinations of multiple tissue sections from each organ were performed.

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2.4. Anti-fertility and teratogenicity studies

The method to study anti-fertility and teratogenicity effects were modified from Oliveira et al. (1991) and Tabach et al. (2009). The rats were divided into two groups; each group consists of five female rats and two male rats. Group 1 (control group) was orally given 10 mL/kg distilled water daily while Group 2 (test group) was orally fed with 100 mg/kg sclerotial powder of *Lignosus rhinocerus* cultivar TM02 daily. After 28 days (without interrupting the treatment), the five female rats were left to mate with the two male rats from the same treatment group for 10 days. After the mating period, male rats were separated from the female rats, and the female rats were continually fed with the sclerotial powder until delivery. The female rats gave birth between 7 and 8 weeks of treatment.

The main parameters used to assess the anti-fertility effect and possible teratogenic effect on the offspring were: number of female rats with delivery, number of offspring/delivery, any external signs of malformation of the litter, weight gain/loss, day of eyes opened, righting reflex and ambulation of the litter (Oliveira et al., 1991 and Tabach et al., 2009). Seven pups per litter were randomly selected for analysis of righting reflex and ambulation. Body weight per litter was recorded on day 1, 7, 14 and 21. The righting reflex was evaluated on days 1, 3 and 7 by placing the animals on their backs and recording the time needed to return to the right posture. The ambulation was recorded on days 8 and 13 by placing each animal on a surface divided in 9 squares of 10 cm each and recording the number of squares crossed in 2 min.

2.5. Assessment of genotoxicity of the sclerotial powder of Lignosus rhinocerus (Ames test)

The genetoxicity of the sclerotial powder of the *Lignosus rhinocerus* cultivar TM02 or its potential to induce gene mutations was assessed using the plate incorporation test and pre-incubation test. The Ames test study was carried out by BSL Bioservice (City, Germany) according to internationally accepted guidelines: Organization for Economic Cooperation and Development (OECD) (1997), United States Environmental Protection Agency (EPA) (1998) and Commission Regulation (EC) (2008). The bacterial strains tested included: *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and *Escherichia coli* WP2 uvrA. Each assay was conducted with and without metabolic activation of a mammalian microsomal fraction S9 mix. The concentrations, including the controls were tested in triplicate at 3.16, 10.0, 31.6, 100, 1000, 2500 and 5000 μg/plate.

2.6. Statistical evaluation

All data were expressed as mean ± standard deviation. Data for chronic toxicity study was analyzed using one-way analysis of variance (ANOVA). Statistical differences between the means of control and treatment groups were determined using Dunnett's *t* (two-sided) test. In case of variance heterogeneity, Dunnett T3 test were used. The homogeneity of variances was calculated using Levene statistics. Results for anti-fertility effect and teratogenic effect on the offspring were analyzed using Independent Samples *T*-test (SPSS v14). Results were considered significant at $p < 0.05$.

3. Results

3.1. Chronic toxicity study

3.1.1. Body weight and general clinical observations

Oral administration of the sclerotial powder of *Lignosus rhinocerus* (cultivar TM02) at all the doses tested did not produce any treatment related abnormality in the rats, and there were no death observed. Figs. 1 and 2 show the body weight of male and female rats treated with various doses of the sclerotial powder, and that of the control group for 180 days (26 weeks). The net body weight gain of the all the treated groups were not significantly different from the control animals (data not shown, $p > 0.05$).

3.1.2. Blood analysis

3.1.2.1. Hematological examinations. Tables 1 and 2 show the results of the hematological examinations of the blood samples from the treated and control groups, for male and female rats, respectively, after the 180 days treatment. There were no significant differences in the values of RBC, hemoglobin, PCV, MCV, MCH, MCHC, platelet count, WBC, neutrophil, lymphocyte, monocyte, eosinophil, basinophil, atypical lymphocyte between the treated groups and the control group ($p > 0.05$).

3.1.2.2. Clinical biochemistry. The results of clinical biochemistry are shown in Tables 3 and 4, respectively, for male and female rats. Generally, there were no significant differences ($p > 0.05$) in the levels of serum glucose, urea, creatinine, calcium, inorganic phosphate, uric acid, sodium, potassium, chloride, total cholesterol, total protein, albumin, globulin, albumin globulin ratio, total bilirubin, alkaline phosphatase, SGOT, SPGT and GGT between the treated groups and the control group, except for the following:

- Sodium levels in the female group treated with 500 mg/kg (147.30 ± 1.89 mmol/L) and 250 mg/kg (146.30 ± 1.70 mmol/L)

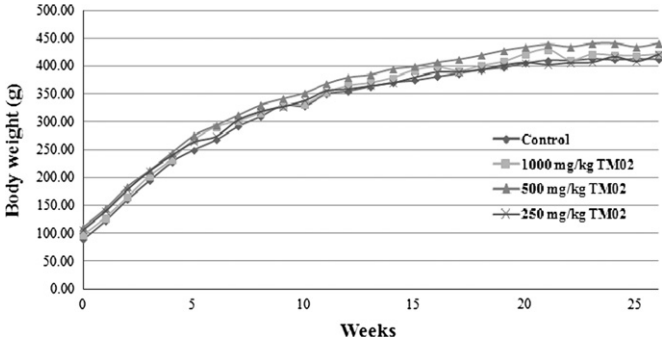

Fig. 1. Body weight of male rats treated with 1000, 500 and 250 mg/kg sclerotial powder of *Lignosus rhinocerus* (TM02) and the control group for 26 weeks (180 days). Body weight is shown as mean ± SD ($n = 10$).

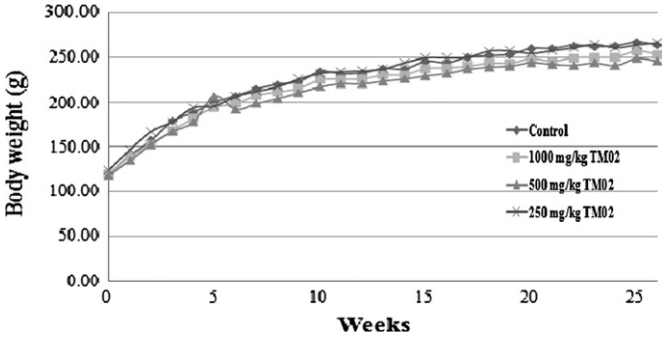

Fig. 2. Body weight of female rats treated with 1000, 500 and 250 mg/kg sclerotial powder of *Lignosus rhinocerus* (TM02) and the control group for 26 weeks (180 days). Body weight is shown as mean ± SD ($n = 10$).

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Table 1
Hematological parameters of male rats treated with various doses of sclerotial powder of *Lignosus rhinocerus* (TM02) for 180 days.

Treatment (mg/kg)	Control ($n=10$)	1000 mg/kg sclerotial powder ($n=10$)	500 mg/kg sclerotial powder ($n=10$)	250 mg/kg sclerotial powder ($n=10$)
RBC ($\times 10^{12}$/L)	9.45 ± 0.56	9.00 ± 0.45	9.02 ± 0.34	9.07 ± 0.43
Hemoglobin (g/dL)	15.53 ± 0.71	15.13 ± 1.00	14.95 ± 0.45	15.01 ± 0.77
PCV (%)	46.10 ± 2.60	45.90 ± 2.42	44.60 ± 1.07	44.70 ± 2.00
MCV (fL)	48.90 ± 2.47	51.00 ± 1.89	49.40 ± 1.71	49.40 ± 2.37
MCH (pg)	16.50 ± 0.71	16.80 ± 0.42	16.70 ± 0.67	16.60 ± 0.70
MCHC (g/dL)	33.80 ± 0.63	33.00 ± 1.05	33.40 ± 0.70	33.60 ± 0.84
Platelet count ($\times 10^{9}$/L)	1047.30 ± 140.63	985.70 ± 205.09	1188.50 ± 170.68	1129.20 ± 206.65
WBC ($\times 10^{9}$/L)	8.42 ± 2.39	11.01 ± 3.46	9.21 ± 1.92	11.16 ± 2.74
Neutrophil (%)	39.20 ± 8.55	35.50 ± 8.02	31.30 ± 4.76	32.60 ± 7.04
Lymphocyte (%)	52.70 ± 7.76	55.20 ± 9.16	55.70 ± 3.53	55.70 ± 6.52
Monocyte (%)	7.80 ± 3.33	8.90 ± 2.42	12.60 ± 4.50	11.40 ± 2.07
Eosinophil (%)	0.30 ± 0.67	0.40 ± 0.84	0.40 ± 0.97	0.30 ± 0.95
Basinophil (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Atypical lymphocyte (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Prothrombin time (s)	9.39 ± 0.47	8.95 ± 0.51	9.18 ± 0.74	9.40 ± 0.49
APTT (s)	19.80 ± 1.69	18.70 ± 0.95	19.50 ± 1.35	18.40 ± 1.43

Values are mean ± SD ($n=10$/group). There was no significant difference between control and treatment groups ($p > 0.05$).

Table 2
Hematological parameters of female rats treated with various doses of sclerotial powder of *Lignosus rhinocerus* (TM02) for 180 days.

Treatment (mg/kg)	Control ($n=10$)	1000 mg/kg sclerotial powder ($n=10$)	500 mg/kg sclerotial powder ($n=10$)	250 mg/kg sclerotial powder ($n=10$)
RBC ($\times 10^{12}$/L)	8.27 ± 0.76	8.05 ± 0.48	8.16 ± 0.41	8.12 ± 0.37
Hemoglobin (g/dL)	14.64 ± 0.94	14.60 ± 0.62	14.69 ± 0.50	14.80 ± 0.86
PCV (%)	45.30 ± 3.06	44.60 ± 1.26	44.40 ± 1.51	44.40 ± 2.01
MCV (fL)	55.30 ± 4.81	55.60 ± 3.24	54.70 ± 3.23	55.10 ± 2.56
MCH (pg)	17.80 ± 1.03	18.30 ± 0.48	18.10 ± 0.74	18.30 ± 1.06
MCHC (g/dL)	32.20 ± 1.23	32.90 ± 1.10	33.10 ± 0.99	33.10 ± 0.57
Platelet count ($\times 10^{9}$/L)	953.80 ± 143.67	1029.70 ± 188.36	1035.50 ± 144.66	992.40 ± 190.33
WBC ($\times 10^{9}$/L)	7.45 ± 2.90	7.95 ± 1.94	6.98 ± 1.47	7.77 ± 2.86
Neutrophil (%)	29.40 ± 5.76	24.40 ± 7.59	23.50 ± 4.74	27.30 ± 10.32
Lymphocyte (%)	65.70 ± 7.47	70.20 ± 5.71	71.70 ± 6.57	67.80 ± 9.59
Monocyte (%)	4.10 ± 2.73	4.70 ± 3.20	4.80 ± 2.94	4.40 ± 2.01
Eosinophil (%)	0.60 ± 0.84	0.70 ± 1.06	0.80 ± 1.03	0.50 ± 0.85
Basinophil (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Atypical lymphocyte (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Blood samples collected from female rats was insufficient for the determination of prothrombin time and APTT.
Values are mean ± SD ($n=10$/group). There was no significant difference between control and treatment groups ($p > 0.05$).

Table 3
Clinical biochemistry parameters of male rats treated with various doses of sclerotial powder of *Lignosus rhinocerus* (TM02) for 180 days.

Treatment (mg/kg)	Contro ($n=10$)	1000 mg/kg sclerotial powder ($n=10$)	500 mg/kg sclerotial powder ($n=10$)	250 mg/kg sclerotial powder ($n=10$)
Glucose (mmol/L)	8.29 ± 0.58	8.23 ± 0.90	8.42 ± 1.16	8.10 ± 1.13
Urea (mmol/L)	6.77 ± 0.90	6.92 ± 1.21	6.25 ± 0.94	7.10 ± 1.05
Creatinine (μmol/L)	40.60 ± 10.28	44.00 ± 9.68	36.30 ± 4.35	38.40 ± 6.65
Calcium (mmol/L)	2.47 ± 0.04	2.45 ± 0.09	2.45 ± 0.04	2.54 ± 0.08
Inorganic pohosphate (mmol/L)	2.26 ± 0.36	2.44 ± 0.31	2.24 ± 0.25	2.33 ± 0.16
Uric acid (mmol/L)	0.08 ± 0.05	0.09 ± 0.04	0.06 ± 0.01	0.07 ± 0.01
Sodium (mmol/L)	144.30 ± 3.53	144.90 ± 1.37	143.20 ± 0.79	143.00 ± 2.05
Potassium (mmol/L)	4.56 ± 0.69	4.57 ± 0.40	4.50 ± 0.20	4.53 ± 0.25
Chloride (mmol/L)	104.10 ± 1.52	103.60 ± 2.22	103.10 ± 1.45	105.70 ± 3.20
Total cholesterol (mmol/L)	1.47 ± 0.12	1.45 ± 0.14	1.52 ± 0.27	1.30 ± 0.23
Total protein (g/L)	77.20 ± 4.49	73.80 ± 4.94	74.60 ± 3.98	78.10 ± 4.56
Albumin (g/L)	35.80 ± 1.99	33.50 ± 3.21	35.90 ± 1.29	36.10 ± 2.38
Globulin (g/L)	41.00 ± 3.27	40.30 ± 3.77	38.70 ± 3.43	42.00 ± 4.00
A/G ratio	0.88 ± 0.06	0.82 ± 0.11	0.93 ± 0.08	0.87 ± 0.11
Total bilirubin (μmol/L)	3.10 ± 0.32	3.30 ± 0.48	3.10 ± 0.32	3.20 ± 0.42
Alkaline phosphatase (IU/L)	105.40 ± 20.51	79.00 ± 34.79	107.30 ± 28.56	113.40 ± 28.41
SGOT (AST) (IU/L)	162.70 ± 27.11	187.80 ± 32.55	172.10 ± 38.21	191.30 ± 31.88
SGPT (ALT) (IU/L)	59.70 ± 11.51	57.10 ± 9.73	52.10 ± 7.45	60.10 ± 13.36
GGT (IU/L)	1.90 ± 2.28	0.00 ± 0.00	0.20 ± 0.63	0.40 ± 0.97

Values are mean ± SD ($n=10$/group). There was no significant difference between control and treatment groups ($p > 0.05$).

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Table 4
Clinical biochemistry parameters of female rats treated with various doses of sclerotial powder of *Lignosus rhinocerus* (TM02) for 180 days.

Treatment (mg/kg)	Control ($n=10$)	1000 mg/kg sclerotial powder ($n=10$)	500 mg/kg sclerotial powder ($n=10$)	250 mg/kg sclerotial powder ($n=10$)
Glucose (mmol/L)	8.29 ± 1.18	8.59 ± 0.84	8.58 ± 1.51	8.65 ± 1.00
Urea (mmol/L)	7.90 ± 1.61	7.53 ± 1.06	7.71 ± 0.68	7.65 ± 1.45
Creatinine (μmol/L)	47.80 ± 5.59	48.10 ± 610	42.40 ± 6.35	46.70 ± 8.06
Calcium (mmol/L)	2.67 ± 0.10	2.58 ± 0.11	2.63 ± 0.12	2.69 ± 0.09
Inorganic pohosphate (mmol/L)	1.79 ± 0.33	1.91 ± 0.64	2.02 ± 0.41	1.95 ± 0.24
Uric acid (mmol/L)	0.08 ± 0.02	0.09 ± 0.05	0.06 ± 0.01	0.06 ± 0.02
Sodium (mmol/L)	144.00 ± 1.94	144.70 ± 2.54	147.30 ± 1.89*	146.30 ± 1.70*
Potassium (mmol/L)	4.10 ± 0.31	4.36 ± 0.66	4.11 ± 0.25	4.18 ± 0.36
Chloride (mmol/L)	105.00 ± 1.94	102.70 ± 2.26	107.50 ± 2.01*	106.50 ± 2.27
Total cholesterol (mmol/L)	2.09 ± 0.46	2.12 ± 0.36	1.85 ± 0.32	2.44 ± 0.53
Total protein (g/L)	84.50 ± 5.28	80.50 ± 6.92	81.10 ± 4.98	84.30 ± 5.87
Albumin (g/L)	44.40 ± 4.25	41.10 ± 4.77	41.20 ± 3.46	43.60 ± 4.03
Globulin (g/L)	40.10 ± 3.73	39.40 ± 3.44	39.90 ± 3.51	40.70 ± 3.62
A/G ratio	1.13 ± 0.14	1.06 ± 0.13	1.03 ± 0.13	1.08 ± 0.11
Total bilirubin (μmol/L)	4.00 ± 0.00	3.50 ± 0.53	4.00 ± 0.00	3.70 ± 0.48
Alkaline phosphatase (IU/L)	56.10 ± 28.75	68.70 ± 21.96	58.40 ± 19.24	71.80 ± 20.47
SGOT (AST) (IU/L)	156.20 ± 33.03	152.90 ± 30.53	148.70 ± 39.06	150.90 ± 18.39
SGPT (ALT) (IU/L)	52.10 ± 25.02	43.90 ± 12.12	53.20 ± 20.78	48.30 ± 14.59
GGT (IU/L)	0.10 ± 0.32	0.00 ± 0.00	0.20 ± 0.42	0.00 ± 0.00

Values are mean ± SD ($n=10$/dose).

* Significant difference from control (ANOVA, Dunnett's t (two-sided) test, $p<0.05$).

Table 5
Organ weight relative to body weight of male and female rats treated with various doses of sclerotial powder of *Lignosus rhinocerus* (TM02) for 180 days.

Treatment (mg/kg)	Control ($n=10$)		1000 mg/kg sclerotial powder ($n=10$)		500 mg/kg sclerotial powder ($n=10$)		250 mg/kg sclerotial powder ($n=10$)	
	Male	Female	Male	Female	Male	Female	Male	Female
Heart	0.32 ± 0.06	0.35 ± 0.03	0.32 ± 0.04	0.36 ± 0.04	0.31 ± 0.05	0.35 ± 0.01	0.34 ± 0.04	0.35 ± 0.02
Left lung	0.17 ± 0.03	0.24 ± 0.06	0.18 ± 0.04	0.23 ± 0.05	0.18 ± 0.03	0.22 ± 0.03	0.18 ± 0.03	0.21 ± 0.02
Right lung	0.36 ± 0.09	0.48 ± 0.17	0.35 ± 0.05	0.45 ± 0.08	0.34 ± 0.05	0.44 ± 0.06	0.34 ± 0.05	0.41 ± 0.07
Liver	2.57 ± 0.31	2.73 ± 0.25	2.52 ± 0.19	2.61 ± 0.20	2.70 ± 0.23	2.62 ± 0.21	2.55 ± 0.33	2.78 ± 0.40
Left kidney	0.32 ± 0.02	0.32 ± 0.03	0.33 ± 0.03	0.32 ± 0.04	0.31 ± 0.03	0.32 ± 0.03	0.33 ± 0.04	0.33 ± 0.04
Right kidney	0.32 ± 0.02	0.33 ± 0.03	0.34 ± 0.04	0.32 ± 0.04	0.32 ± 0.02	0.34 ± 0.03	0.33 ± 0.04	0.34 ± 0.04
Left adrenal	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Right adrenal	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Spleen	0.15 ± 0.04	0.18 ± 0.03	0.16 ± 0.03	0.20 ± 0.04	0.16 ± 0.02	0.20 ± 0.03	0.17 ± 0.02	0.20 ± 0.02
Brain	0.47 ± 0.06	0.73 ± 0.08	0.48 ± 0.06	0.75 ± 0.07	0.46 ± 0.06	0.78 ± 0.07	0.48 ± 0.03	0.74 ± 0.08
Left testes/ovary	0.35 ± 0.06	0.02 ± 0.00	0.38 ± 0.07	0.02 ± 0.00	0.30 ± 0.14	0.02 ± 0.00	0.35 ± 0.07	0.02 ± 0.01
Right testes/ovary	0.35 ± 0.06	0.02 ± 0.01	0.38 ± 0.07	0.02 ± 0.00	0.31 ± 0.10	0.02 ± 0.00	0.35 ± 0.06	0.02 ± 0.01
Left epididymis/uterus	0.17 ± 0.03	0.24 ± 0.06	0.17 ± 0.04	0.25 ± 0.07	0.15 ± 0.02	0.26 ± 0.09	0.16 ± 0.02	0.23 ± 0.04
Right epididymis	0.18 ± 0.04	–	0.16 ± 0.03	–	0.16 ± 0.03	–	0.16 ± 0.02	–

Values are percentage mean ± SD ($n=10$/dose). There was no significant difference between control and treatment groups of rats at each sex ($p>0.05$).

sclerotial powder of TM02 were slightly higher than the control group (144.00 ± 1.94 mmol/L, $p<0.05$);

- Chloride level in the female group treated with 500 mg/kg sclerotial powder of TM02 (107.50 ± 2.01 mmol/L) was slightly higher than the level in control group (105.00 ± 1.94 mmol/L, $p<0.05$).

3.1.3. Urinalysis

No significant changes in appearance, colour, volume, specific gravity, pH, total protein, glucose, ketone, occult blood, urobilinogen, bilirubilin, white blood cell, red blood cell and epithelial cells were noted in all treated groups as compared to the control group of both sexes (data not shown).

3.1.4. Absolute and relative organ weight

There were no significant changes in the absolute (data not shown) and relative organ weights (Table 5) in all male and female rats orally treated with the sclerotial powder at various doses as compared to the control group.

3.1.5. Histopathological examinations

There were no macroscopic abnormality of organs observed in the control and treated rats. As such, histopathological examinations were only performed on organs from animals in the control and highest-dose treated group of both sexes (1000 mg/kg sclerotial powder). After 180 days treatment with the *Lignosus rhinocerus* sclerotial powder, there was no alteration worthy of note in the microscopic examinations of the organs in all treated and control rats.

3.2. Assessment of the anti-fertility and teratogenic effects of the sclerotial powder of Lignosus rhinocerus

Our results showed that oral administration of 100 mg/kg sclerotial powder of *Lignosus rhinocerus* for 7–8 weeks did not significantly ($p>0.05$) alter the number of pregnant female rats, number of pups per litter, day of eyes opened of the litter, weight of pups and righting reflex as well as the ambulation of the litter (Tables 6 and 7). Besides, there was no external sign of malformation of the litter.

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3.3. Assessment of the genotoxicity of the sclerotial powder of *Lignosus rhinocerus* using Ames test

The potential of the sclerotial powder of *Lignosus rhinocerus* to induce gene mutations was assessed using the plate incorporation test and pre-incubation test. The results showed that there were no toxic effects in any of the five tester strains used, and up to the highest dose group (5000 μg/plate), evaluated with and without metabolic activation. There were no biologically relevant increases in revertant colony numbers of any of the five tester strains following treatment with the sclerotial powder at any concentration level, either in the presence or absence of metabolic activation. On the other hand, the reference mutagens induced a distinct increase of revertant colonies indicating the validity of the experiments.

4. Discussion

Changes in body weight have been used as an indicator of adverse effects of drugs and chemicals (Hilaly et al., 2004; Mukinda and Eagles, 2010). In the present studies, the similar growth pattern as shown by body weight (Figs. 1 and 2) and body weight gain (data was not shown) for the treated and the control groups indicated that oral administration of sclerotial powder of *Lignosus rhinocerus* (TM02) at a daily dose of up to 1000 mg/kg and for 180 days had no adverse effect on the growth of the rats.

It has been established that the highest overall concordance of toxicity in animals with humans is hematological parameters (Olson et al., 2000). Our studies show that there was no significant difference between the hematological parameters of rats fed with sclerotial powder of *Lignosus rhinocerus* for 180 days and that of the control groups, indicating that the sclerotia of the mushroom had no adverse toxic effect as assessed by hematological examinations. This is further supported by clinical biochemistry studies, which showed that the 180 days' treatment with the sclerotial powder up to 1000 mg/kg did not affect the renal functions (urea, creatinine and uric acid levels), hepatic functions (total protein, albumin, globulin, albumin globulin ratio, total bilirubilin, alkaline phosphatase, SGOT, SGPT and GGT), serum electrolytes (calcium, inorganic phosphate, sodium, potassium, chloride) as well as glucose and total cholesterol levels. Even though the sodium levels of the 250 and 500 mg/kg female treated groups and the chloride levels in the 500 mg/kg female treated group were slightly higher ($p < 0.05$) than the control group (144.00 ± 1.94 mmol/L), the values are within the reference ranges established by Sharp and La Regina (1998). It is also noted that the differences observed were not dose dependent. Therefore, these very minor variations are unlikely to be of any toxicological significance.

Organ weight changes have long been accepted as a sensitive indicator of chemically induced changes to organs (Michael et al., 2007). Our present study show that there were no significant alterations in the absolute and relative organ weights as a result of the sclerotial powder treatment ($p > 0.05$), indicating that consumption of the sclerotial powder for the extended period (180 days) did not induce organ changes. This conclusion was supported by histological examinations of the organs, which showed that oral feeding of up to 1000 mg/kg of sclerotial powder of *Lignosus rhinocerus* (TM02) did not induce histopathological changes to all the internal organs examined.

The recommended daily consumption of the sclerotial powder of *Lignosus rhinocerus* as neutraceutical is approximately 5–10 mg/kg, whereas for cancer patients, up to 100 mg/kg of sclerotial powder is recommended per day (above figures provided by supplier of the sclerotial powder, Ligno Biotech Sdn. Bhd., Selangor, Malaysia). As such, in the anti-fertility and teratogenicity studies a dose 100 mg/kg was used. Our results show that oral administration of 100 mg/kg sclerotial powder of *Lignosus rhinocerus* (TM02) for 7–8 weeks did not cause any adverse effect to the fertility of the rats, nor did the treatment induce teratogenic effect on their offspring. Genetoxicity studies using Ames test also demonstrated that the sclerotial powder of *Lignosus rhinocerus* did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used.

Table 6
Assessments of the anti-fertility and teratogenic effects of consumption of *Lignosus rhinocerus* sclerotial powder in rats.

Treatment	Number of pregnant female rats	Number of pups per litter (mean ± SD)	Day of eyes opened of the litter (mean ± SD)	External signs of malformation of the litter
Control	4/5	8.33 ± 1.15	16.38 ± 0.50	No
100 mg/kg TM02	5/5	9.00 ± 1.87	16.64 ± 0.74	No

The values for each parameter are expressed as mean ± SD (except number of pregnant female rats). All parameters of the treated group (100 mg/kg sclerotial powder) were not significantly different from the control group ($p > 0.05$).

5. Conclusions

Our results showed that there are no treatment-related chronic toxicity in rats of both sexes following the long term (180-days) oral administration of 250, 500 and 1000 mg/kg of *Lignosus rhinocerus* (TM02) sclerotial powder, as shown by the clinical observations, body weight gain, hematological analysis, clinical biochemistry, urinalysis, absolute organ weight as well as relative organ weight, and histological examinations of the organs. Thus, the no-observed-adverse-effect level (NOAEL) dose of the sclerotial powder of *Lignosus rhinocerus* (TM02) in our 180-day chronic toxicity study is more than 1000 mg/kg. The sclerotial powder also did not cause adverse effect on fertility nor teratogenic effect on the offspring of the treated rats. The bacterial reverse mutation assay also shown that the sclerotial powder was not mutagenic.

Table 7
Assessments of the possible teratogenic effects of consumption of *Lignosus rhinocerus* sclerotial powder in rats.

Treatment	Weight of pups (g)				Righting reflex (s)			Ambulation	
	Day 1	Day 7	Day 14	Day 21	Day 1	Day 3	Day 7	Day 8	Day13
Control	6.46 ± 0.74	13.59 ± 1.45	24.52 ± 2.62	37.11 ± 5.00	12.00 ± 7.71	8.62 ± 7.97	1.90 ± 2.61	2.19 ± 1.63	3.43 ± 2.04
100 mg/kg sclerotial powder	6.14 ± 0.67	12.30 ± 3.29	22.90 ± 4.22	34.63 ± 7.27	12.17 ± 7.05	10.24 ± 9.28	1.60 ± 1.01	1.40 ± 1.63	3.40 ± 2.25

Weight of pups was recorded on day 1, 7, 14 and 21. Righting reflex was evaluated on day 1, 3 and 7. Ambulation was assessed on day 8 and 13. The values for each parameter are expressed as mean ± SD. All parameters for the offspring of the treated group were not significantly different from the control group ($p > 0.05$).

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