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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.

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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 $22 \pm 2\,^{\circ}$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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