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Original Article

TOXICOLOGICAL EVALUATION OF *LEPISTA NUDA* (BULL. EX FR.) COOKE MYCELIUM PRODUCED BY AN *IN VITRO* CULTURE METHODOLOGY

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ABSTRACT

Objective: Considering the interest in *L. nuda* as a source of ingredients for the development of functional food and nutraceuticals has increased, the objective of this study was to evaluate its general toxicity and possible genotoxic effects in rats to assess its safety.

Methods: This study evaluated the safety of *L. nuda* mycelium by using genotoxicity assays (reverse mutation, chromosomal aberration, and micronuclei tests) and a short-term toxicity test.

Results: Our results have indicated that *L. nuda* mycelium did not significantly increase the number of revertant colonies and chromosomal aberration in both *in vitro* assays. Furthermore, it did not induce any increase in micronuclei formation in mouse bone marrow.

Conclusion: In summary, no mutagenic effects and no evidence of systemic toxicity were found in this safety assessment, and the use of *L. nuda* mycelia is safe at a dose of 3 g/kg body weight in S-D rats. Using a safety factor of 100, the calculated acceptable daily intake in humans is 30 mg/kg body weight/d.

Keywords: *Lepista nuda* mycelium, Oral toxicity, Genotoxicity, Fermentation.

INTRODUCTION

Mushrooms have been a part of regular diets for their nutritional and medicinal values since ancient times. They are low in fat content and high in minerals, vitamins, fibers, proteins and polysaccharides [1]. Recently, there has been an increased consumption of mushrooms due to a significant number of studies conducted that focused on isolating and identifying compounds with promising therapeutic properties from various species of fungi [2]. These mushrooms possess bioactive compounds such as phenolics, tocopherols, ascorbic acid, carotenoids and ergothioneine (EGT) [3]which are identified as ingredients for the development of functional foods or medicinal products. Moreover, such bioactive substances are not only available in the form of fruiting bodies, but also as mycelial biomass that may be beneficial for human health [4].

Lepista nuda (Bull. ex Fr.) Cooke (Tricholomataceae) is an edible woodland mushroom that grows in Asia, Africa, America, Australia and Europe [5]. It is valued internationally for its lilac color, delicate flavor, special aroma and good post harvest conservation, all of which lead to its consumption and even its cultivation [6]. Recent studies indicate that *L. nuda* has a wide range of pharmacological functions, including anti-tumor [7], immune-modulatory [8], anti-oxidant [9], anti-microbial [10], anti-diabetic and anti-hyperlipidemic [11] properties. The fruiting body of *L. nuda* contains a number of secondary metabolites, including sterols, triterpenoids [12], ceramides [13] and EGT [14], which are known to possess various beneficial health effects.

In general, the production of a fruiting body is a time-consuming and labor-intensive process. From an ecological point of view, utilizing *in vitro* culture minimizes the overexploitation of endangered, rare or valuable species, representing a promissory methodology that prioritizes sustainable conservation and rational use of biodiversity. The use of submerged cultivation of edible and medicinal mushrooms has started to attract attention around the world and is viewed as a promising alternative for the production of important metabolites [15-17]. Therefore, it seems highly desirable to produce *L. nuda* via submerged fermentation in a batch stirred tank bioreactor with controlled culture parameters for faster production of mycelia biomass and metabolites, which would be useful for further development on an industrial scale.

Considering the interest in *L. nuda* as a source of ingredients for the development of functional food and nutraceuticals has increased, the objective of this study was to evaluate its general toxicity and possible genotoxic effects in rats to assess its safety. To the best of our knowledge, there have been no toxicological or genotoxicological studies on the safety of *L. nuda* mycelium. Hence, this study will evaluate the safety of *L. nuda* in subchronic studies and genotoxicity assays.

MATERIALS AND METHODS

Sample preparation

L. nuda strain NO. 8620 purchased from German Collections of Microorganisms and Cell Cultures (DSMZ) biological resource center (Braunschweig, Germany) was cultured on potato dextrose agar at 24 °C for 14 d. The agar block (1 cm^3) containing mycelia was then transferred to a 2-L Erlenmeyer flask with 1l broth (composed of soybean powder, glucose, maltose syrup, peptone, yeast extract, $MgSO_4$ and KH_2PO_4, adjusted to pH 4.5 with 1N HCl) and was cultured at 24 °C for 12 don a rotary shaker (120 rpm) for seed culturing prior to its scale-up production step. The scale-up of the fermentation process was performed using the same media in the 200-L fermenter agitated at 60 rpm with an aeration rate of 0.375 vvm at 24 °C for 14 d. At the end of the cultivation, *L. nuda* mycelia were lyophilized, reduced to a fine dried powder using a 60 mesh and stored in a desiccator at room temperature. The dry sample was then analyzed by high performance liquid chromatography (HPLC) to evaluate the active EGT content in the mycelia based on its dry weight. HPLC analysis of EGT was executed according to the previous study [14], and the retention time of EGT was approximately ~9 min at a flow rate of 1.0 ml/min with a scanning UV wavelength at 254 nm. Proximate analysis and heavy metals determination were carried out using the standard procedures [18].

Animals and husbandry

Seven-week-old male and female ICR mice and Sprague-Dawley (S-D) rats were purchased from Bio LASCO Taiwan Co., Ltd (I-Lan, Taiwan). The studies were conducted in compliance with Good Laboratory Practice (21 CFR Part 58), conformed to the guidelines set forth by the National Institutes of Health (NIH) for the care and

use of laboratory animals and approved by the Institutional Animal Care and Use Committee (Approval no. 130110-13 and 130111-12 for *in vivo* erythrocyte micronucleus, acute and subchronic tests, respectively). Animals were kept in polypropylene cages under an ambient temperature of 21±2 °C and 55±20 % relative humidity with a 12 h light/dark cycle throughout the test period. Water and food (Rodent Lab Diet 5010; PMI® Nutrition International, LLC, Indiana, USA) was available *ad libitum*. All animals had their health status evaluated, quarantined and acclimatized for a week before any test.

Ames test (bacterial reverse mutation test)

The protocol was conducted according to Organization for Economic Cooperation and Development (OECD) Test No. 471[19]: Bacterial reverse mutation test. Five *Salmonella typhimurium* strains of histidine auxotrophs TA98, TA100, TA102, TA1535 and TA1537 (MoltoxInc, USA), were used to evaluate the genotoxic potential of *L. nuda* mycelium in the presence and absence of S9 metabolic activation [20]. 0.05 ml of each testing sample (*L. nuda* powder, positive control, and negative control separately), 2 ml top agar containing 0.5 mM histidine/biotin solution, and 0.1 ml bacterial culture liquid (37±1 °C cultured overnight) were mixed thoroughly with and without S9 activation and then poured onto the surface of minimal glucose agar plates. Afterwards, the plates were incubated invertedly at 37±1 °C in the dark for 48-72 h prior to revertant colonies counting.

***In vitro* chromosome aberration test**

The test was performed according to OECD Guideline Test No. 473 [21]: *In vitro* mammalian chromosome aberration test. Chinese hamster ovary cells (CHO-K1; BCRC 60006) seeded at a density of 3-4×10^5 cells/well in 6 cm dishes were exposed to dimethyl sulfoxide, positive controls (mitomycin C (0.5 µg/ml) and benzo (a) pyrene (25 µg/ml))or *L. nuda* mycelium (1.25, 2.5 or 5 mg/ml) for 3 -24 h, with and without S9-mix metabolic activation. At the end of the treatment, cells were treated with 0.1 µg/ml demecolcine solution (Sigma-Aldrich, MO, USA), swelled by 0.075 M KCl and fixed with methanol/acetic acid solution (3:1, v/v). The cells were then spread on glass slides and stained with Giemsa. Structural chromosome aberration such as chromosome-type break (csb), chromosome-type exchange (cse), chromatid-type breakage (ctb) and chromatid-type exchange (cte) were observed and recorded.

***In vivo* erythrocyte micronucleus test**

This test protocol is based on OECD Guideline Test No. 474 [22]: Mammalian erythrocyte micronucleus test. Each testing group (positive control, negative control, low, mid and high dose group) contained five females and five male ICR mice. Negative controls were administrated with sterilized water, and positive controls were given an intraperitoneal injection of 8 ml/kg body weight cyclophosphamide monohydrate. The low, mid and high dose groups were orally treated with a single dose of *L. nuda* mycelium at 1250, 2500 and 5000 mg/kg, respectively. Peripheral blood (2-3 ml) was collected from the tail vein at 48 h and 72 h after dosing, which was then applied to acridine orange-coated slides in the dark for 2~3 h under room temperature and was examined using a fluorescent microscope. The percentage of polychromatic erythrocytes (PCEs) in 1000 erythrocytes and the incident of micro nucleated polychromatic erythrocytes (MNPCEs) in 2000 PCEs were calculated for each animal collected at 24 and 72 h time points.

4-w subchronic toxicity test

This test was conducted in accordance to OECD Guideline Test No. 407 [23]: Repeated dose 28-d oral toxicity study in rodents. Eighty healthy adult S - Drats were randomly divided into four groups according to their body weight (10 males and 10 females per group). Our earlier study on acute oral toxicity indicated that acute oral LD_{50} of *L. nuda* was greater than 12 g/kg in S-D rats (data not shown). Hence, the following three doses were selected for the study: 1 g/kg/d (Low dose), 2 g/kg/d (Mid dose) and 3 g/kg/d (High dose). Distilled water was served as the control. Body weight, food consumption, and water intake (the difference between the food given and their remnants the next day) was recorded weekly. Mortality and morbidity were observed twice a day. At the end of the stipulated experiment, all overnight fasted rats (with water still supplied *ad libitum*) were sacrificed and the toxicological and histopathological changes were evaluated.

Toxicological evaluation

Urine and blood samples were collected from the rats before they were sacrificed. The 12 to 16 h urine specimens were collected using metabolic cages and were evaluated by using urinalysis equipment (Bayer Clinitek-Status, Siemens, USA) for the following parameters: pH, specific gravity, protein, glucose, ketone, bilirubin, urobilinogen, occulted blood, leukocytes and nitrite. Urinary sediments examination was further evaluated by using a microscopic equipment (Model-E200, Nikon, Japan) to observe the presence of red blood cells, white blood cells, epithelial cells, casts, crystals and microbes. The blood samples were collected via the abdominal aortic method and were transferred into non-anticoagulated-containing tubes for serum biochemistry analysis and sodium citrate-containing tubes for haematological analysis. Serum biochemistry and haematological profiles were obtained using a biochemistry analysis equipment (Model-7080, Hitachi, Japan) and haematology analyzers (Sysmex Xt-1800i and Sysmex CA-500, Sysmex Corporation, Japan), respectively. For the serum biochemistry analysis, amylase, albumin, alkaline phosphatase (ALP), total bilirubin (T-BIL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl-transferase (γ-GT), total protein (TP), creatinine (CRE), blood urea nitrogen (BUN), cholesterol (CHO), triglycerides (TG), creatine kinase (CK), chloride (Cl), sodium (Na), potassium (K), glucose (GLU), calcium (Ca) and phosphorus (P) were examined. The haematological parameters including red blood cell (RBC), white blood cell (WBC), platelet, haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), neutrophils, eosinophils, basophils, monocytes, lymphocytes, activated partial thrombo plastin time (APTT) and prothrombin time (PT) were measured.

Pathology and histopathology

Gross necropsies were performed in order to analyze the external and internal surfaces of the body. Organs such as heart, liver, spleen, kidney (paired), adrenal gland (paired), thymus, epididymis (paired), testes (paired), ovary (paired), and uterus were weighted and then fixed in 10 % formalin neutral buffer. The tissues obtained from these fixed organs were then stained with haematoxylin-eosin (H&E) and were viewed using optical microscope (Model-E200, Nikon, Japan).

Statistical analysis

All values in this study were presented as mean±standard deviation (SD). Poisson distribution was used for the statistical evaluation of the number of cells in the chromosome aberration test and the frequency of micronucleus in the erythrocyte micronucleus test. Values were compared with the control group by Dunnett's t-test in Ames and 4-w subchronic toxicity tests. In the chromosome aberration test and micronucleus test, the Cochran-Armitage trend test (C-A test) was conducted in order to identify any dose-response that may be significantly different between the testing group and the negative control group. A P value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

L. nude has been widely consumed for a long period of time, especially in Europe. They are valued as highly flavorsome foods and are also considered as a rich source of bioactive compounds such as EGT. Despite its widespread use, no scientific studies have been undertaken to determine its toxicity. The nutrient contents of the mushrooms are influenced by many factors, such as the types of substrate on which they were cultivated [24]. For this reason, submerged fermentation proved to be a promising and reproducible alternative as it avoids the overexploitation of endangered, rare or valuable species, and offers advantages of efficient production of mushroom mycelium and metabolites in a faster and more easily controlled process [25]. Since the chemical composition, nutritional values and medicinal values of fruiting bodies may differ between

the natural environment and submerged cultured mycelia, the safe dose range of this mushroom obtained from submerged fermentation should be analyzed. Hence, this is the first report on the evaluation of the toxicity of submerged cultured *L. nuda* mycelium, which was conducted in parallel with the OECD guidelines to assess its safety as a source for the development of medicines, nutraceuticals or functional foods.

The proximate composition of freeze-dried *L. nuda* mycelia is shown in table 1. *L. nuda* mycelium was composed of 11.85 % moisture, 4.65 % ash, 32.8 % crude protein, 9.65 % crude fat and 41.05 % carbohydrate, which were completely distinct from the values of the fruiting body reported earlier [5] (93.77 %, 1.15%, 3.70%, 0.11 % and 1.55 %, respectively). The moisture content of the mycelia exhibited lower values than the fruiting body.

This finding supports previous research indicating that fresh fruiting bodies contained about 90 % moisture and 10 % dry matter, and dried mushrooms contained approximately 90 % dry matter and 10 % moisture [26]. The greater ash content showed that *L. nuda* mycelium is higher in mineral content. Furthermore, the relatively higher contents of protein, carbohydrate, and low fat in *L. nuda* mycelium provide a proof of it being a better candidate than the fruiting body as an ideal functional food for human consumption.

Table 1: Proximate analysis (g/100g) of freeze-dried *L. nuda* mycelium

Proximate composition	**Freeze-dried *L. nuda***		
Moisture	11.85	±	5.73
Ash	4.65	±	0.78
Crude Protein	32.80	±	3.96
Crude Fat	9.65	±	2.47
Carbohydrate	41.05	±	7.99
Energy	382	±	38
Average±SD, n=2			

EGT has been known as an excellent antioxidant that has a protective effect on the organs of rats against lipid peroxidation [27]. Unfortunately, this unique naturally occurring potent antioxidant cannot be made in human cells and therefore is only available from diet through a specific ergothioneine transporter [28]. Moreover, food processing tends to destroy many active compounds [29], but in this study, a proportion of the EGT remained. 60 ppm EGT in *L. nuda* mycelium was confirmed and quantified by HPLC as shown in fig. 1. This suggests that there is a potential in developing ergothioneine-rich *L. nuda* mycelium nutritional supplement as an ingredient in functional foods or medicinal products intended to prevent and treat chronic inflammatory diseases.

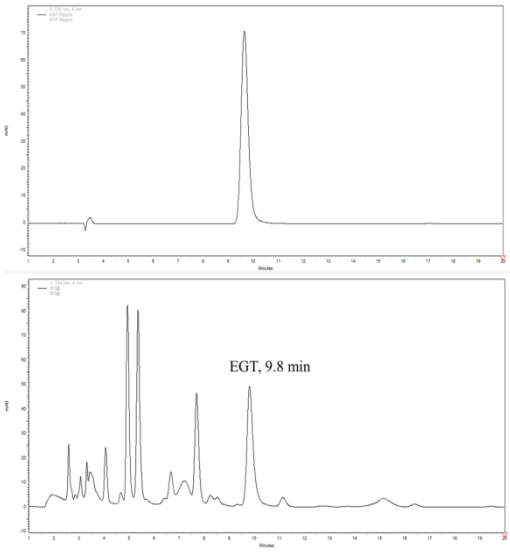

Fig. 1: HPLC chromatograms of standard EGT sample (top; 50 ppm EGT) and extracted *L. nuda* mycelium (bottom; 60 ppm EGT) from 200-L bioreactor (UV detection at 254 nm). Retention time of EGT is 8.9 min

When a functional food contains novel ingredients, its safety for consumption as well as its acceptability becomes particularly important to investigate. Earlier studies have raised concerns about possible contamination of *L. nuda* fruiting bodies with heavy metals such as arsenic, lead, cadmium and mercury [30]. In this study, *L. nuda* mycelia were analyzed for the presence of arsenic, lead, cadmium and mercury. The mean values and range of heavy metals were all within the daily intake levels (data not shown). The overall result from proximate and metal analyses showed *L. nuda* mycelia being a more suitable candidate than its fruiting bodies as an excellent food for good health.

In vitro methods with high throughput screening capacity and good predictability such as the Ames test, the chromosomal aberration test and the micronucleus test have a high false-positive rate for predicting *in vivo* genotoxicity and carcinogenicity [31]. Hence, the bacterial reverse mutation test, chromosome aberration test and micronucleus test were conducted to evaluate the safety of *L. nuda* mycelia consumption for the general public. Ames test has been used worldwide for the geno toxicity of a compound [32]. In our study, all *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 exposed to different concentrations of *L. nuda* mycelia with the presence and absence of S9-Mix metabolic activators did not show a two-fold increase in revertant numbers over the negative control (table 2). The validity of the assay was observed by the marked increase in the number of revertant colonies in the positive control ($P<0.05$). These results of Ames test indicated that *L. nuda* mycelia displayed no mutagenicity.

Similarly, *in vitro* chromosomal aberration test did not show any significant alterations up to a dose of 5 mg/culture when compared to the negative control in the presence and absence of S9-Mixture (table 3). The validity of the assay is supported as the negative controls induced less than 3 % of cells with chromosomal aberrations, and positive controls induced a significant increase of aberrations ($P<0.05$). The number of cells with chromosome aberration recorded in 200 metaphase cells under 3 h treatment in the absence of metabolic activation was 5, 2, and 1 at 5, 2.5 and 1.25 mg per culture respectively, with 6 in vehicle control group and 22 in the positive control group. Likewise, longer treatment or metabolic activation of *L. nuda* mycelia did not interfere with the mitotic process or cell cycle progression. In summary, *L. nuda* mycelia up to 5 mg/ml were found to be non-clastogenic to CHO-K1 cell line in both the presence and absence of metabolic activation.

L. nuda mycelia were further investigated by using the *in vivo* erythrocyte micronucleus test in ICR mice to test whether it resulted in chromosomal damage. The results of ICR mice bone marrow erythrocyte micronucleus assay are shown in Tables 4 and 5. A reduced ratio of polychromatic erythrocytes to total erythrocytes and an increased frequency of micro nuleated polychromatic erythrocytes were found in the positive control group treated with cyclophosphamide ($P<0.05$). The proportion of PCEs and the incidence of MNPCEs in *L. nuda* mycelia treatment groups (1.25, 2.5 and 5 g/kg body weight) were not significantly different from the negative control groups ($P>0.05$). These results were thus consistent with those of the *in vitro* mutagenicity test, providing that *L. nuda* mycelia are not mutagenic at the tested dosage range.

Table 2: Bacterial reverse mutation test of *L. nuda* mycelia using *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537

Dose (mg/plate)	Number of revertants/plate (without S9 activation)														
	TA98			TA100			TA102			TA1535			TA1537		
Negative Control[a]	54.3	±	10.3	181.7	±	9.1	306.7	±	17.0	9.7	±	4.0	12.0	±	2.6
Positive Control[b]	197.7	±	25.2*	729.3	±	89.7*	3392.0	±	334.1*	426.7	±	26.3*	251.0	±	26.8*
0.625	38.0	±	5.0	196.3	±	8.0	291.3	±	17.2	16.3	±	2.9	15.0	±	5.3
0.313	35.7	±	4.0	183.7	±	8.4	297.3	±	24.2	17.3	±	4.0	12.0	±	2.0
0.156	34.7	±	4.2	189.0	±	9.5	287.3	±	14.6	9.7	±	0.6	10.7	±	2.9
0.078	31.3	±	3.5	188.3	±	13.4	309.7	±	11.0	12.0	±	3.6	13.0	±	3.0
0.039	37.0	±	5.2	181.0	±	16.5	299.7	±	7.5	10.7	±	3.1	14.0	±	1.7
	Number of revertants/plate (with S9 activation)														
	TA98			TA100			TA102			TA1535			TA1537		
Negative Control[a]	36.3	±	8.4	182.3	±	8.4	321.7	±	9.6	12.0	±	2.6	11.3	±	4.5
Positive Control[c]	918.3	±	126.1*	833.3	±	30.0*	956.7	±	88.2*	264.5	±	6.4*	601.7	±	82.0*
0.625	51.0	±	2.6	186.3	±	3.1	390.7	±	11.2	20.4	±	2.6	14.7	±	4.2
0.313	45.0	±	1.0	186.0	±	3.5	391.3	±	20.4	16.3	±	3.8	11.0	±	2.6
0.156	43.0	±	6.2	191.0	±	8.9	405.7	±	9.0	15.3	±	5.7	12.7	±	3.1
0.078	41.3	±	2.5	181.7	±	13.4	383.7	±	17.9	14.7	±	1.5	10.3	±	3.2
0.039	41.0	±	1.0	180.3	±	7.4	384.0	±	6.6	14.7	±	0.6	11.3	±	3.1

Data were expressed as mean±SD (n=3), [a]Sterilized water, [b]TA98: 2-nitrofluorene, 1μg/plate; TA100: Sodium azide, 1μg/plate; TA102: Mitomycin C, 0.2μg/plate; TA1535: Sodium azide, 1μg/plate; TA1537: 9-aminoacridine, 50 μg/plate, [c]TA98: 2-aminoanthracene, 1μg/plate; TA100: Benzo[a]pyrene, 1μg/plate; TA102: 2-aminoanthracene, 5μg/plate; TA1535: 2-aminoanthracene, 5μg/plate; TA1537: 2-aminoanthracene, 5 μg/plate, *A two-fold or greater increase in revertant numbers over the negative control.

Table 3: Chromosome aberration test of *L. nuda* mycelia using CHO-K1 cells

Dose	S9-Mixture	ctb	cte	csb	cse	other	AF[d]	Total number of aberrations
3 h treatment								
Negative control[a]	-	5	0	0	1	0	5/200	6
Positive control[b]	-	13	5	3	1	0	21/200*	22
5 mg/ml	-	3	1	1	0	0	5/200	5
2.5 mg/ml	-	1	0	1	0	0	2/200	2
1.25 mg/ml	-	0	0	0	0	1	1/200	1
Negative control[a]	+	4	0	0	0	0	1/200	4
Positive control[c]	+	12	8	1	2	1	23/200*	24
5 mg/ml	+	3	1	0	0	0	4/200	4
2.5 mg/ml	+	2	0	0	0	0	2/200	2
1.25 mg/ml	+	1	0	0	1	1	3/200	3
18h treatment								
Negative control[a]	-	1	0	1	0	0	2/200	2
5 mg/ml	-	5	0	0	0	0	5/200	5
2.5 mg/ml	-	2	0	0	1	0	3/200	3
1.25 mg/ml	-	0	0	1	0	0	1/200	1

[a]Culture medium. [b]MitomycinC (0.5 μg/ml)., [c]Benzo(a)pyrene (25 μg/ml)., [d]Aberration frequency (AF): Number of cells with chromosome aberration in 200 metaphase cells (n/200). ctb: chromatid breakage; cte: chromatid exchange; csb: chromosome breakage; cse: chromosome exchange; other: other abnormalities, *Significantly different when compared to the negative control, P<0.05.

Table 4: Percentage of PCEs in erythrocytes of male and female mice at 48 h and 72 h after *L. nuda* mycelia treatment

Dose (mg/kg)	PCE (%)											
	Male						Female					
	48 h			72 h			48 h			72 h		
Negative control	3.92	±	0.27	3.70	±	0.07	3.80	±	0.07	3.78	±	0.04
Positive control (80 mg/kg)	1.40	±	0.07	-			1.38	±	0.24	-		
1.25	3.86	±	0.21	3.86	±	0.05	3.94	±	0.23	3.74	±	0.05
2.5	3.92	±	0.16	3.78	±	0.08	3.84	±	0.13	3.78	±	0.08
5	3.92	±	0.19	3.76	±	0.05	3.88	±	0.22	3.82	±	0.08

Data are expressed as the percentage of polychromatic erythrocytes (PCEs) in 1000 erythrocytes (n=5)

Table 5: Micronucleus frequency in PCEs of male and female mice at 48 h and 72 h after *L. nuda* mycelia treatment

Dose (g/kg)	MNPCE (%)											
	Male						Female					
	48 h			72 h			48 h			72 h		
Negative control	0.50	±	0.35	0.30	±	0.27	0.30	±	0.27	0.40	±	0.65
Positive control (80 mg/kg)	13.40	±	3.49*	-			12.10	±	2.97*	-		
1.25	0.40	±	0.22	0.50	±	0.35	0.40	±	0.22	0.40	±	0.42
2.5	0.50	±	0.35	0.50	±	0.35	0.20	±	0.27	0.20	±	0.27
5	0.40	±	0.22	0.40	±	0.55	0.50	±	0.35	0.50	±	0.00

Data are expressed as the incident of micronuleated polychromatic erythrocytes in 2000 PCEs (n=5), *$p < 0.05$: Significantly different when compared to negative control via Poisson distribution.

Usually an LD_{50} between 5 and 15g/kg of any substance is considered non-toxic [33]. From our previous study, *L. nuda* mycelia could be characterized as non-toxic because its LD_{50} was found to be greater than 12 g/kg in S-D rats (data not shown). However, this LD_{50} value does not reflect enough information to categorize the toxic property of the compound nor does it suggests the mechanism of action of the compound. In order to select the appropriate starting dose that is safe for human consumption, the information on the degree of toxicity after repeated exposure to it over a long period of time is therefore required. Regarding the long term effects of *L. nuda* mycelia in S-D rats, no toxicity, morbidity nor mortality was noted in both the male and female rats that were given three dosages (1, 2 and 3 g/kg/body weight) during the 4-w testing period (n=10). No significant changes were found in body weight (P>0.05) (fig. 2) or food consumption (P>0.05) (fig. 3), regardless of sex or treatment. All haematology measures (WBC, RBC, HGB, HCT, MCV, MCH, MCHC, platelet, neutrophil, lymphocyte, monocyte, eosinophil, basophil, PT and APTT) in treated rats revealed no significant difference (P>0.05) between the treatment group and the control group in both male and female rats (table 6). Serum biochemistry profiles are shown in table 7.

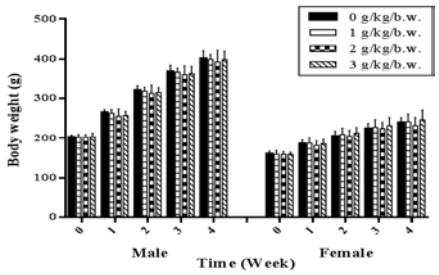

Fig. 2: Effects of *L. nuda* mycelia on body weight in male and female S-D rats during the 4-w safety assessment. Data were expressed as mean±SD (n=10)

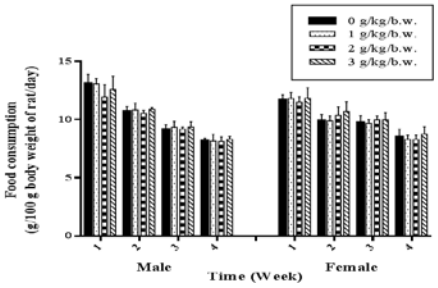

Fig. 3: Effects of *L. nuda* mycelia on food intake in male and female S-D rats during the 4-w safety assessment. Data were expressed as mean±SD (n=5 cages, two animals per cage)

Most serum biochemistry parameters in treated rats were not significantly different from the negative control group, with the exception of variations in AST, TP, ALP, P, Na and Cl values of male rats and in CK measures of female rats. AST levels were significantly decreased (P<0.05) (mid-dose group: 97.97±14.20 U/l) while TP (low-dose group: 5.82±0.15 g/dl), ALP (high-dose group: 668.93±163.19 U/l) and P (high-dose group: 8.48±0.37 mg/dl) concentrations were significantly increased (P<0.05) compared to the control male rats. However, these parameters were not dose-dependent and therefore were not considered to be a treatment-related effect. Additionally, all values were in the range of normal values [34]. Significant increases (P<0.05) in serum electrolytes such as sodium and chloride were noted in all dosage groups, but these values remained within the normal range [35]. Furthermore, female rats demonstrated a significant elevation in creatine kinase levels (low-dose group: 274.58±153.56 U/l) when compared to control animals. However, such alteration was not dose-related and should be assumed as toxicologically irrelevant as it was within normal physiological ranges [36].

Table 6: Effects of *L. nuda* mycelia on haematological parameters in male and female S-D rats during the 4-w safety assessment

Parameters	Control (Distilled water)			*L. nuda* mycelia (g/kg/d)							
				1 g/kg/d			2 g/kg/d			3 g/kg/d		
Male												
WBC (10^3 µl)	9.030	±	1.690	8.867	±	2.587	8.917	±	1.460	9.553	±	1.307
RBC (10^6 µl)	8.028	±	0.478	7.985	±	0.308	8.128	±	0.292	7.921	±	0.327
HGB (g/dl)	15.87	±	0.57	15.66	±	0.32	15.95	±	0.34	15.64	±	0.53
HCT (%)	44.09	±	1.60	43.37	±	0.89	44.17	±	1.09	43.98	±	1.68
MCV (fl)	55.04	±	2.94	54.38	±	2.27	54.39	±	1.96	55.54	±	1.34
MCH (pg)	19.80	±	0.88	19.64	±	0.71	19.65	±	0.67	19.76	±	0.48
MCHC (g/dl)	36.00	±	0.40	36.11	±	0.48	36.11	±	0.26	35.56	±	0.57
Platelet (10^3 µl)	1152.7	±	127.6	1212.2	±	109.4	1186.4	±	121.6	1186.6	±	118.2
Neutrophil (%)	17.99	±	4.95	16.24	±	3.82	14.07	±	2.98	18.90	±	5.31
Lymphocyte (%)	78.02	±	4.69	79.31	±	4.03	81.68	±	3.02	76.95	±	5.72
Monocyte (%)	3.74	±	0.82	4.25	±	0.75	4.04	±	0.45	3.94	±	1.07
Eosinophil (%)	0.18	±	0.12	0.12	±	0.09	0.14	±	0.08	0.15	±	0.14
Basophil (%)	0.07	±	0.05	0.08	±	0.06	0.07	±	0.05	0.06	±	0.05
PT (sec)	12.84	±	1.27	12.94	±	0.83	13.37	±	1.31	12.67	±	2.13
APTT (sec)	17.33	±	1.36	17.42	±	0.77	17.36	±	1.20	17.26	±	1.49
Female												
WBC (10^3 µl)	6.832	±	1.828	5.831	±	1.295	5.931	±	2.502	7.517	±	2.335
RBC (10^6 µl)	7.654	±	0.340	7.575	±	0.314	7.743	±	0.264	7.612	±	0.283
HGB (g/dl)	15.16	±	0.44	15.10	±	0.61	15.26	±	0.52	15.08	±	0.57
HCT (%)	41.03	±	0.89	40.78	±	1.74	41.47	±	1.33	40.93	±	1.14
MCV (fl)	53.64	±	1.81	53.89	±	2.23	53.59	±	2.00	53.81	±	1.38
MCH (pg)	19.82	±	0.46	19.96	±	0.74	19.71	±	0.71	19.81	±	0.61
MCHC (g/dl)	36.94	±	0.45	37.04	±	0.50	36.80	±	0.32	36.83	±	0.59
Platelet (10^3 µl)	1070.2	±	161.3	1068.2	±	40.3	1067.7	±	112.0	1106.3	±	150.5
Neutrophil (%)	13.18	±	4.61	14.86	±	3.27	12.29	±	1.66	15.99	±	10.15
Lymphocyte (%)	83.94	±	5.16	82.34	±	3.51	85.05	±	2.00	81.15	±	10.39
Monocyte (%)	2.53	±	0.89	2.58	±	0.69	2.34	±	0.76	2.37	±	0.46
Eosinophil (%)	0.30	±	0.14	0.21	±	0.14	0.24	±	0.12	0.43	±	0.18
Basophil (%)	0.05	±	0.08	0.01	±	0.03	0.08	±	0.10	0.06	±	0.07
PT (sec)	9.54	±	0.28	9.43	±	0.15	9.30	±	0.15	9.65	±	0.35
APTT (sec)	14.37	±	0.88	14.55	±	1.27	14.19	±	0.47	14.27	±	0.92

Data were expressed as mean±SD (n=10), White blood cell (WBC), red blood cell (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), prothrombin time (PT) and activated partial thromboplastin time (APTT).

Table 7: Effects of *L. nuda* mycelia on serum biochemistry parameters in male and female S-D rats during the 4-w safety assessment

Parameters	Control (Distilled water)	*L. nuda* mycelia (g/kg/d)		
		1 g/kg/d	2 g/kg/d	3 g/kg/d
Male				
AST (U/l)	123.55 ± 19.28	105.81 ± 18.19	97.97 ± 14.20*	109.34 ± 22
ALT (U/l)	30.88 ± 8.64	30.93 ± 5.29	29.26 ± 3.27	33.03 ± 5.90
GLU (mg/dl)	168.86 ± 32.94	150.62 ± 13.01	165.71 ± 18.38	160.12 ± 28.84
TP (g/dl)	5.56 ± 0.18	5.82 ± 0.15*	5.64 ± 0.20	5.62 ± 0.21
Albumin (g/dl)	4.09 ± 0.11	4.22 ± 0.11	4.16 ± 0.13	4.07 ± 0.22
T-BIL (mg/dl)	<0.05	<0.04	<0.06	<0.04
BUN (mg/dl)	12.58 ± 1.95	13.35 ± 1.77	12.10 ± 1.52	11.62 ± 1.15
CRE (mg/dl)	0.32 ± 0.04	0.34 ± 0.05	0.31 ± 0.03	0.32 ± 0.04
γ-GT (U/l)	<2.0	<2.0	<2.0	<2.0
ALP (U/l)	512.85 ± 87.17	607.07 ± 125.80	615.63 ± 91.05	668.93 ± 163.19*
CHO (mg/dl)	50.07 ± 7.59	51.04 ± 7.54	46.30 ± 6.68	46.72 ± 12.80
TG (mg/dl)	33.11 ± 9.71	26.83. ± 6.93	29.46 ± 9.11	26.60 ± 10.04
Ca (mg/dl)	9.91 ± 0.24	9.99 ± 0.30	10.12 ± 0.14	9.81 ± 0.20
P (mg/dl)	8.09 ± 0.23	8.39 ± 0.41	8.34 ± 0.37	8.48 ± 0.37*
CK (U/l)	676.26 ± 301.71	547.51 ± 244.85	461.83 ± 181.23	498.95 ± 238.96
Amylase (U/l)	1328.6 ± 222.0	1388.9 ± 202.6	1345.2 ± 188.8	1269.9 ± 243.5
Na (mmol/l)	146.17 ± 1.53	147.89 ± 1.38*	148.49 ± 0.71*	149.25 ± 1.23*
K (mmol/l)	4.118 ± 0.303	4.180 ± 0.216	4.205 ± 0.144	4.206 ± 0.200
Cl (mmol/l)	106.68 ± 1.46	109.03 ± 1.25*	109.93 ± 1.54*	109.66 ± 1.80*
Female				
AST (U/l)	68.22 ± 10.17	78.05 ± 11.62	72.60 ± 9.12	67.82 ± 10.96
ALT (U/l)	21.89 ± 2.83	23.43 ± 4.05	22.70 ± 2.14	20.99 ± 2.59
GLU (mg/dl)	157.31 ± 33.17	158.97 ± 24.36	157.42 ± 27.26	154.42 ± 27.59
TP (g/dl)	5.58 ± 0.21	5.76 ± 0.33	5.46 ± 0.30	5.63 ± 0.26
Albumin (g/dl)	4.30 ± 0.18	4.43 ± 0.29	4.19 ± 0.34	4.33 ± 0.28
T-BIL(mg/dl)	<0.04	<0.04	<0.05	<0.04
BUN (mg/dl)	13.91 ± 1.00	14.10 ± 2.55	13.76 ± 1.67	12.81 ± 1.62
CRE (mg/dl)	0.32 ± 0.04	0.36 ± 0.05	0.34 ± 0.05	0.32 ± 0.06
γ-GT (U/l)	<2.0	<2.0	<2.0	<2.0
ALP (U/l)	340.48 ± 96.47	351.38 ± 89.08	321.68 ± 61.10	345.88 ± 81.68
CHO (mg/dl)	65.72 ± 17.87	63.43 ± 12.52	69.48 ± 12.38	59.13 ± 7.40
TG (mg/dl)	19.89 ± 5.70	19.08 ± 8.04	21.73 ± 8.88	22.15 ± 8.41
Ca (mg/dl)	9.77 ± 0.24	9.93 ± 0.25	9.77 ± 0.18	10.00 ± 0.29
P (mg/dl)	6.92 ± 0.65	6.78 ± 0.72	6.86 ± 0.50	7.12 ± 0.69
CK (U/l)	162.93 ± 54.08	274.58 ± 153.56*	241.08 ± 79.15	191.40 ± 87.57
Amylase (U/l)	716.1 ± 90.7	841.9 ± 285.4	812.3 ± 171.3	755.3 ± 122.4
Na (mmol/l)	144.13 ± 1.36	143.88 ± 1.27	143.67 ± 1.02	144.46 ± 1.80
K (mmol/l)	3.796 ± 0.163	3.987 ± 0.240	3.922 ± 0.406	4.013 ± 0.333
Cl (mmol/l)	109.67 ± 1.32	109.60 ± 1.67	108.57 ± 1.70	109.28 ± 1.63

Data were expressed as mean±SD (n=10), Aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose (GLU), total protein (TP), total bilirubin (T-BIL), blood urea nitrogen (BUN), creatinine (CRE), gamma-glutamyltransferase (γ-GT), alkaline phosphatase (ALP), cholesterol (CHO), triglycerides (TG), calcium (Ca), phosphorus (P), creatine kinase (CK), sodium (Na), potassium (K), and chloride (Cl), * Statistically significant ($p<0.05$) when compared to the control group.

There were no abnormal urinalysis (table 8) and no abnormal relative organ weight findings (table 9) in any of the *L. nuda* mycelia treated groups when compared to either sex of the control group. Upon histopathological examination of the control and high dose groups, no treatment-related toxicological significances were observed (data not shown). Summing up the results, the NOAEL (no-observable-adverse-effect-level) of *L. nuda* mycelia is 3 g/kg/d, which could be used as a reference for possible exposure levels of its consumption.

Table 8: Urinalysis values of male and female SD rats with *L. nuda* mycelia for 4 w

Parameters	Control (Distill water)	*L. nuda* mycelia (g/kg/d)		
		1 g/kg/d	2 g/kg/d	3 g/kg/d
Male				
Volume (ml)	15.05 ± 7.32	14.40 ± 6.43	22.00 ± 11.03	21.15 ± 4.16
Specific Gravity	1.0170 ± 0.0059	1.0135 ± 0.0041	1.0178 ± 0.0051	1.0170 ± 0.0026
pH	7.00 ± 0.00	7.00 ± 0.00	6.90 ± 0.21	6.95 ± 0.16
Urobilinogen (EU/dl)	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00
Female				
Volume (ml)	15.05 ± 7.32	14.40 ± 6.43	14.40 ± 7.86	16.40 ± 6.89
Specific Gravity	1.0150 ± 0.0041	1.0165 ± 0.0041	1.0170 ± 0.0035	1.0144 ± 0.0017
pH	7.05 ± 0.16	7.10 ± 0.21	7.25 ± 0.54	6.95 ± 0.16
Urobilinogen (EU/dl)	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00

Data were expressed as mean±SD (n=10)

Table 9: Effects of *L. nuda* mycelia on relative organ weight of male and female SD rats during the 4-w safety assessment

Relative weight (%)	Control (Distilled water)			*L. nuda* mycelia (g/kg/d)							
				1 g/kg/d			2 g/kg/d			3 g/kg/d		
Male												
Adrenal gland (Paired)	0.05597	±	0.00540	0.05664	±	0.00701	0.05901	±	0.00735	0.05663	±	0.00594
Thymus	0.656	±	0.091	0.566	±	0.115	0.577	±	0.118	0.625	±	0.095
Heart	1.268	±	0.092	1.239	±	0.091	1.203	±	0.107	1.243	±	0.093
Kidney (Paired)	3.048	±	0.219	3.054	±	0.290	3.028	±	0.261	3.053	±	0.289
Liver	11.738	±	0.956	11.774	±	0.762	11.031	±	1.171	11.670	±	1.213
Spleen	0.714	±	0.119	0.716	±	0.141	0.708	±	0.072	0.721	±	0.091
Testis (Paired)	3.166	±	0.184	3.180	±	0.417	3.236	±	0.296	3.184	±	0.241
Epididymis (Paired)	0.929	±	0.095	0.937	±	0.127	0.946	±	0.102	0.937	±	0.056
Female												
Adrenal gland (Paired)	0.06602	±	0.01083	0.07203	±	0.00993	0.06669	±	0.01039	0.06986	±	0.01560
Ovary and fallopian tube	0.13446	±	0.02095	0.14608	±	0.02262	0.13085	±	0.02296	0.13506	±	0.02151
Heart	0.857	±	0.070	0.879	±	0.092	0.833	±	0.062	0.866	±	0.070
Kidney (Paired)	1.787	±	0.103	1.881	±	0.225	1.808	±	0.214	1.909	±	0.215
Liver	7.229	±	0.629	7.510	±	0.920	7.098	±	0.838	7.461	±	0.988
Uterus and cervical	0.540	±	0.293	0.443	±	0.136	0.409	±	0.097	0.393	±	0.100
Thymus	0.545	±	0.067	0.538	±	0.113	0.469	±	0.077	0.498	±	0.065
Spleen	0.555	±	0.075	0.535	±	0.085	0.487	±	0.072	0.538	±	0.086

Data were expressed as mean±SD (n=10).

CONCLUSION

In summary, no mutagenic effects and no evidence of systemic toxicity were found in this safety assessment, which suggest that the use of *L. nuda* mycelia is safe at a dose of 3 g/kg body weight in S-D rats. Using a safety factor of 100, the calculated acceptable daily intake in humans is 30 mg/kg body weight/d. Additional research on *L. nuda* mycelia, including a longer term animal study and a year-long human clinical trial, is suggested to further support the safety of its consumption.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest

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