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Safety assessment of the standardized aqueous extract from solid-state cultured *Xylaria nigripes* (Wuling Shen) in rats

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Abstract

Background: *Xylaria nigripes* (Koltz.) Cooke, also known as Wuling Shen, is a high-value medicinal mushroom. It is a herbal medicine traditionally used for treating insomnia, trauma and depression. However, its toxicity has never been systematically evaluated. This study aimed to evaluate the safety of a standardized aqueous extract (XNE), an ingredient of commercial products, prepared from solid-state cultured *X. nigripes* in rats.

Methods: A 90-day subchronic toxicity study was conducted by oral administration of XNE at daily doses of 20, 1000 and 2000 mg/kg body weight to Sprague-Dawley rats of both sexes, and the control group was given distilled water (vehicle). All animals were checked daily for general behavior, body weight changes and signs of toxicity. At the end of the treatment period, hematological analysis, biochemical analysis and histopathological examination of organs were conducted.

Results: At tested concentrations, oral XNE administration caused no treatment-induced adverse effects on general health, body weight gain, relative organ weights, and hematological and biochemical parameters. Histopathological results also showed no significant structural changes in organs even in high-dose XNE-treated animals.

Conclusion: This study suggests that treatment with XNE for 90 days does not produce significant toxicity, even up to 100 fold (2000 mg/kg body weight/day) of the recommended daily intakes. Therefore, the use of XNE as herbal medicines is considered to be relatively safe.

Keywords: *Xylaria nigripes*, Mushroom, Toxicity, Hematology, Histology, Rat

Introduction

Xylaria nigripes (Koltz.) Cooke, also known as Wuling Shen, is a high-value medicinal mushroom from the family of Xylariaceae. It is found growing in wilds around the abandoned termite nests. Traditionally, it is used for treating insomnia, trauma, and as a diuretic and tonic for weak nerve [1], as well as for relieving depression [2, 3]. Previous studies have shown that *X. nigripes* extracts possessed antioxidant [4, 5], immunomodulatory [6] and hepatoprotective [7] activities.

Clinical studies showed that Wuling capsules prepared from liquid cultured mycelia of *X. nigripes* were effective in inducing sleepiness and maintaining good sleep in insomnia patients [8–10], and caused no adverse effect to the body [10]. Furthermore, they were demonstrated to reduce spatial memory impairment [11], and alleviate depression and anxiety [12–14]. Wuling capsules also showed effectiveness in treating post-stroke depression [15], and co-morbid depression in patients with epilepsy [16]. They can reduce inflammation and oxidative stress in patients with Type 2 diabetic patients [14].

Mushrooms and mushroom-derived products have been used for prevention and control of diseases since ancient times. As polysaccharides are recognized to be

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the most potent bioactive compounds of mushrooms, polysaccharide-rich fungi and plants have been employed for centuries by cultures around the world for their dietary and therapeutic benefits [17]. Fruiting bodies of *X. nigripes* were shown to contain ergostarien-3β-ol and ergosterol peroxide [18], agroclavine, xylanigripones A-C, 8,9-didehydro-10-hydroxy-6,8-dimethylergolin and (6S)-agroclavine N-oxide [19]. Although these herbal products are generally perceived as safe or free from toxic effects, scientific validation aims to ensure their safety remain essential. To-date, despite numerous studies have reported on the medicinal uses of *X. nigripes*, there is no toxicity information available on products derived from solid-state cultured *X. nigripes*. Hence, this study aimed to conduct a subchronic toxicity study to evaluate the safety profile of a commercially prepared extract of cultivated *X. nigripes* in rats.

Materials and methods

Mushroom materials

The *X. nigripes* materials were produced by solid-state culture system. The authenticity of *X. nigripes* species was confirmed by Prof. Airong Song, Qingdao Agricultural University (Shandong, China), and its culture specimen (no. KJ-XN-07-1) was deposited at Kang Jian Biotech Corp., Ltd. (Nantou County, Taiwan), whereas its ribosomal RNA/internal transcribed spacer sequences were deposited in the National Center for Biotechnology Information GenBank database (no. KJ627786).

Preparation of *X. nigripes* standardized extract

The commercial *X. nigripes* extract composing of fungal mycelium and fruiting bodies (Kang Jian Biotech Corp., Ltd.) was prepared by boiling water at 95 °C for 2 h. The decoction was filtered and concentrated under vacuum to produce a thick concentrated extract, which was subjected to spray-drying to obtain the dried powder, followed by passing through a 60-mesh sieve to obtain a final powdered product (XNE); this standardized extract contained about 5.5% of water soluble β-linked polysaccharides with molecular weight greater than 10 kDa, and composing of 86.6% glucose, 6.8% mannose and 6.6% galactose.

Animals

Three-week-old male and female Sprague-Dawley rats were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan), they were maintained under standard laboratory conditions (12 h light/dark cycle, a temperature of 22 ± 2 °C and a relative humidity of 55 ± 5%) with free access to standard pellet food (Oriental Yeast Co., Ltd., Tokyo, Japan) and water. Ethical approval for use of animals was obtained from the Institutional Animal Ethical Committee, with the protocol approval number 105–59. The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, MD, USA, 1996).

Experimental design

The subchronic toxicity study was carried out according to the protocol described by the OECD guideline 408 for testing chemicals [20]. Rats were allowed to acclimatize to the laboratory conditions and the gavage procedures for 15 days. Healthy animals were then selected, weighed and randomly divided into four groups, namely control group, low-, medium- and high-dose groups, with each group contained 20 animals (10 males and 10 females). Rats of the treatment groups were intragastrically (orally) administered with XNE at 20 mg/kg/day (recommended intake), 1000 mg/kg/day (50 times the recommended intake) and 2000 mg/kg/day (100 times the recommended intake) daily for 90 days, and the dosing volume was 5 mL/kg body weight; the control group received the same volume of distilled water (vehicle) for the same duration.

Visual observations for mortality, behavioral patterns, physical appearance and symptoms of illness for all animals were performed throughout the experimental period. Food intake was recorded daily, while body weights of the animal were measured every 3 days. The dose received by each animal was calculated based on the individual animal body weight, and adjusted according to the subsequent changes in body weight.

At the end of the experimental period, all rats were euthanized with carbon dioxide after overnight starvation (about 15 h), blood and organ samples were then collected for hematological and biochemical measurements, and histopathological examination.

Relative organ weight

Following blood collection, liver, kidney, heart, lungs, spleen, stomach, testicles, ovary, brain, and pancreas of all animals were carefully dissected free of connective tissue and fat, and then weighed and observed for abnormalities. The relative organ weight of each animal was calculated as follows: Relative organ weight = Absolute organ weight (g)/Body weight of the animal on sacrifice day (g) × 100.

Hematology and serum biochemistry

For the hematological investigation, whole blood was collected in ethylenediamine-tetraacetic acid (EDTA) tubes and processed immediately without any delay. The parameters measured were white blood cells (WBC), red blood cells (RBC), haemoglobin, lymphocytes, monocytes, haematocrit, mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration

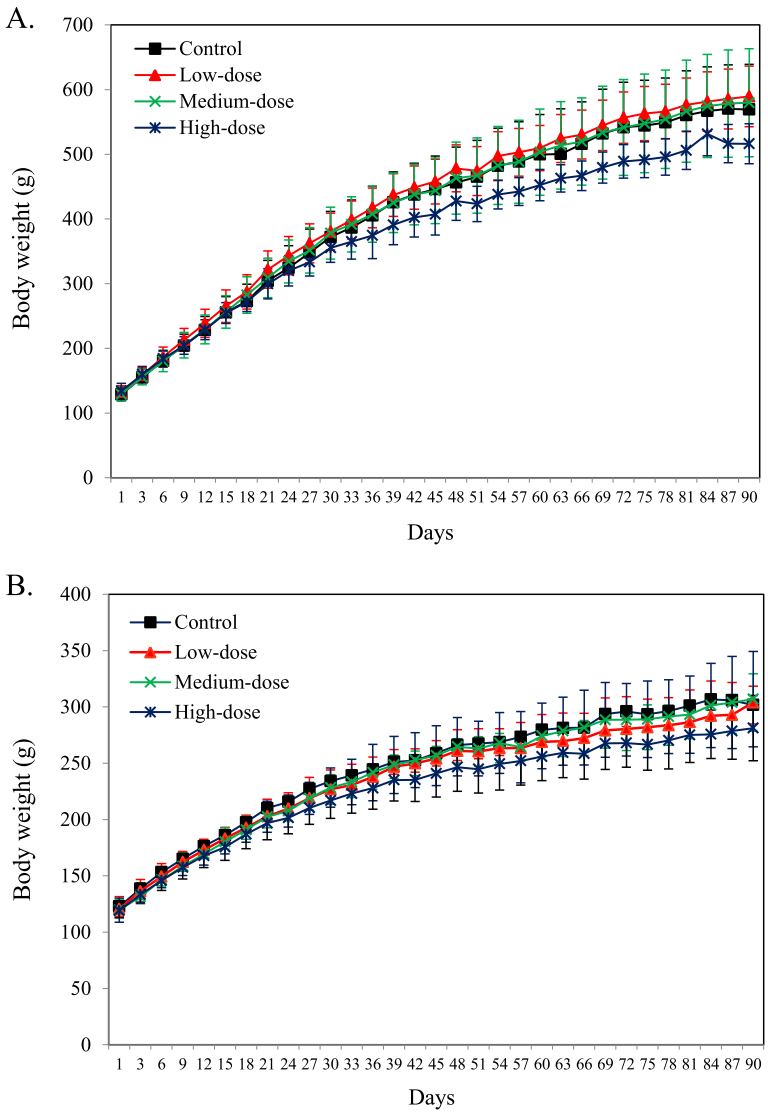

Fig. 1 Changes of body weight of male and female rats during the 90-day experimental period. **a**. Male rats; **b**. Female rats

(MCHC), mean corpuscular volume (MCV), platelet count, neturophils, basophils, eosinophils, prothrombin time and activated partial thromboplastin time (APTT) using a hematology analyzer MEK-6318 K (Nihon Kohden Corp., Tokyo, Japan).

For biochemical assay, blood without anticoagulant were centrifuged at 3000×g at 5 °C for 15 min. Serum samples were then collected for measuring biochemical parameters comprising aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine, total protein, albumin, total bilirubin and glucose (GLU), as well as serum electrolytes such as sodium (Na), potassium (K), calcium (Ca), chloride (Cl) and phosphorous (P) using an automated biochemistry analyzer (Cobas Integra® 400 plus, Roche, Basel, Switzerland).

Histopathological studies

All organ and tissue samples were fixed in 10% neutral buffered formalin, dehydrated in graded ethanol, cleared in xylene, embedded in paraffin, and then sectioned at about 3–5 μm thickness, followed by staining with hematoxylin-eosin (H & E) dye. The microscopic features of the organs of male and female XNE-treated rats were compared with that of the control group.

The following tissues were examined microscopically: adrenal gland, brain, esophagus, bone femur (including marrow), cervix, heart, small intestine

(duodenum, jejunum, and ileum), large intestine (cecum, colon, and rectum), kidney, liver, lung, lymph nodes (mesenteric), ovary, pancreas, pituitary gland, parathyroid gland, prostate gland, sciatic nerve, spinal cord, spleen, stomach, testicles, thymus, thyroid gland, trachea, urinary bladder and uterus.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Statistical significances between control and treated groups were determined by one-way analysis of variance (ANOVA), followed by post-hoc Duncan's multiple range tests. Difference was considered significant when P-value was < 0.05.

Results

Body weight changes and food intake

Results showed that there were no significant differences in mean body weights between animals receiving different XNE treatments and the control (Fig. 1). Furthermore, animals of both sexes appear to increase in body weight normally during the 90-day repeated oral administration of different XNE doses. No difference was also noted in food intake between the different treatment groups (data no shown).

Survival and clinical observations

There was no mortality in any group throughout the study. Daily and weekly detailed clinical and functional observations of the animals did not reveal any toxicologically relevant abnormalities, such as weakness and reduced activity, and other adverse clinical manifestations; these observations indicate that the 90-day repeated oral administration of XNE caused no observable toxic symptoms in animals of both sexes.

Relative organ weights

Compared with the control group, XNE showed no effect on the value of relative organ weights of rats in both sexes, and there was also no significant difference in the relative organ weights between animals receiving different doses of XNE (Table 1).

Haematological parameters

Compared with the control group, all doses of XNE caused no significant effect on any of the haematological indices tested in both male and female animals (Table 2), however, it was noted that female rats receiving XNE appear to have a higher count of leukocytes (WBC) than the control group.

Biochemical parameters

Among the various biochemical parameters, AST and ALT enzymes are excellent markers in evaluation of hepatocellular injury, and changes in bilirubin and urea levels are good indicators of hepatic and renal conditions, respectively [21]. In this study, the results show that there was no difference in serum biochemical indices between XNE-treated and the control

Table 1 Effects of XNE on relative organ weights of male and female rats at the end of the 90-day safety assessments

	Relative organ weight (%)[a]			
	Control	Low	Medium	High
Male				
Heart	0.31 ± 0.03	0.32 ± 0.04	0.32 ± 0.04	0.33 ± 0.02
Liver	3.29 ± 0.42	3.19 ± 0.17	3.17 ± 0.20	3.17 ± 0.40
Kidney	0.71 ± 0.06	0.68 ± 0.05	0.72 ± 0.05	0.75 ± 0.05
Adrenal glands	0.012 ± 0.002	0.011 ± 0.002	0.011 ± 0.002	0.011 ± 0.001
Testicles	0.60 ± 0.06	0.61 ± 0.06	0.58 ± 0.10	0.67 ± 0.04
Brain	0.40 ± 0.04	0.39 ± 0.05	0.40 ± 0.05	0.44 ± 0.03
Female				
Heart	0.35 ± 0.03	0.34 ± 0.04	0.33 ± 0.04	0.36 ± 0.05
Liver	3.22 ± 0.16	2.96 ± 0.36	2.83 ± 0.29	2.90 ± 0.35
Kidney	0.78 ± 0.05	0.73 ± 0.07	1.01 ± 1.03	0.73 ± 0.08
Adrenal glands	0.022 ± 0.004	0.022 ± 0.003	0.023 ± 0.003	0.023 ± 0.008
Ovary	0.028 ± 0.004	0.030 ± 0.005	0.029 ± 0.003	0.035 ± 0.011
Brain	0.70 ± 0.05	0.70 ± 0.08	0.68 ± 0.09	0.74 ± 0.08

Key: Data are expressed as mean ± SD ($n = 10$)
[a]Relative organ weight (%) = Organ weight / Body weight × 100

Table 2 Effects of XNE on haematological parameters in male and female rats during the 90-day safety assessments

Parameters	Control	Low	Medium	High
Male				
WBC (10^3/μL)	12.38 ± 3.16	12.65 ± 4.86	14.01 ± 3.77	12.22 ± 3.05
RBC (10^6/μL)	10.41 ± 0.58	10.10 ± 0.69	9.81 ± 0.67	10.18 ± 0.57
Haemoglobin (g/dL)	17.84 ± 1.05	17.35 ± 0.84	17.17 ± 1.09	17.64 ± 0.73
Hematocrit (%)	61.50 ± 3.51	59.62 ± 3.22	58.08 ± 3.76	60.30 ± 3.78
MCV (fL)	59.14 ± 2.41	59.09 ± 1.55	59.23 ± 1.47	59.23 ± 1.14
MCH (pg)	17.16 ± 0.88	17.20 ± 0.50	17.52 ± 0.67	17.35 ± 0.47
MCHC (g/dL)	29.00 ± 0.64	29.14 ± 0.85	29.57 ± 0.47	29.28 ± 0.81
Platelet (μL)	1512 ± 259	1443 ± 257	1422 ± 143	1439 ± 243
Neutrophils (%)	14.35 ± 4.14	12.36 ± 2.64	12.01 ± 4.21	13.04 ± 3.22
Lymphocyte (%)	79.40 ± 7.19	81.98 ± 3.74	83.03 ± 5.99	80.98 ± 4.88
Monocyte (%)	5.10 ± 3.42	4.36 ± 3.21	3.63 ± 3.18	4.95 ± 3.81
Eosinophil (%)	0.98 ± 0.35	1.05 ± 0.45	1.14 ± 0.20	0.80 ± 0.36
Basophil (%)	0.16 ± 0.07	0.25 ± 0.21	0.19 ± 0.13	0.23 ± 0.14
PT (sec)	11.15 ± 1.76	10.59 ± 0.78	10.82 ± 0.81	11.20 ± 0.86
APTT (sec)	42.38 ± 10.46	44.26 ± 6.34	40.33 ± 7.09	41.62 ± 4.90
Female				
WBC (10^3/μL)	7.91 ± 2.33	8.72 ± 2.49	11.08 ± 3.04	9.77 ± 2.06
RBC (10^6/μL)	9.31 ± 0.51	8.60 ± 0.61	9.04 ± 0.53	8.91 ± 0.70
Haemoglobin (g/dL)	16.92 ± 1.14	15.87 ± 0.86	16.56 ± 0.72	16.23 ± 1.16
Hematocrit (%)	56.36 ± 3.66	52.41 ± 3.39	54.14 ± 2.38	53.21 ± 4.17
MCV (fL)	60.50 ± 1.89	60.97 ± 1.52	59.94 ± 2.07	59.74 ± 2.10
MCH (pg)	18.17 ± 0.58	18.48 ± 0.71	18.34 ± 0.63	18.24 ± 0.55
MCHC (g/dL)	30.02 ± 0.58	30.30 ± 0.64	30.59 ± 0.71	30.54 ± 0.57
Platelet (μL)	1188 ± 249	1071 ± 395	1115 ± 182	1051 ± 300
Neutrophils (%)	9.27 ± 2.50	9.68 ± 2.60	9.08 ± 3.07	8.58 ± 2.24
Lymphocyte (%)	86.27 ± 5.38	85.26 ± 3.81	86.18 ± 5.69	87.03 ± 3.26
Monocyte (%)	3.56 ± 3.78	4.25 ± 3.14	3.67 ± 3.52	3.39 ± 2.91
Eosinophil (%)	0.76 ± 0.37	0.63 ± 0.29	0.89 ± 0.60	0.82 ± 0.67
Basophil (%)	0.14 ± 0.10	0.18 ± 0.14	0.18 ± 0.10	0.18 ± 0.11
PT (sec)	9.51 ± 0.27	9.38 ± 0.27	9.84 ± 0.60	10.11 ± 0.89
APTT (sec)	42.19 ± 5.71	38.05 ± 8.42	37.61 ± 5.93	36.54 ± 3.58

Key: Data are expressed as mean ± SD ($n = 10$). *WBC* White blood cells, *RBC* Red blood cells, *MCV* Mean corpuscular volume, *MCH* Mean corpuscular haemoglobin, *MCHC* Mean corpuscular haemoglobin concentration, *PT* Prothrombin time, *APTT* Activated partial thromboplastin time

animals in both sexes. Compared to the control groups, the kidney and liver biochemical indices showed no significant differences between animals receiving repeated oral administration of 20, 1000 and 2000 mg/kg BW of XNE (Table 3). Furthermore, the results also revealed no statistically significant differences in electrolyte concentrations in rats of both sexes receiving different treatments; however, the potassium levels in XNE-treated animals appear to be lower than the control group.

Histopathological examination

Pathological examination revealed no observable lesions in any of the excised organs in either male or female rats receiving repeated different doses of XNE (Table 4). However, certain animals in the control and XNE-treated groups appear to have slight abnormal histological appearance in the organ anatomy, for instance: the hearts of the control and high-dose XNE-treated male rats show a slight focal mononuclear cell infiltration (Fig. 2), with an occurrence rate of 1/10 and 1/10,

Table 3 Effects of XNE on serum biochemical parameters in male and female rats during the 90-day safety assessments

Parameters	Control	Low	Medium	High
Male				
AST (U/L)	108.3 ± 28.8	120.2 ± 24.9	112.9 ± 15.0	115.9 ± 23.9
ALT (U/L)	49.20 ± 20.81	56.30 ± 15.59	46.2 ± 8.93	51.40 ± 7.92
ALP (U/L)	110.5 ± 25.6	119.4 ± 30.4	105.4 ± 16.6	106.0 ± 18.8
Total bilirubin (mg/dL)	0.45 ± 0.08	0.48 ± 0.11	0.49 ± 0.11	0.41 ± 0.07
Total protein (g/dL)	6.50 ± 0.36	6.36 ± 0.21	6.41 ± 0.21	6.30 ± 0.24
Albumin (g/dL)	3.60 ± 0.21	3.57 ± 0.12	3.54 ± 0.16	3.52 ± 0.18
BUN (mg/dL)	12.18 ± 2.95	10.55 ± 2.29	13.11 ± 1.96	12.68 ± 2.09
Creatinine (mg/dL)	0.62 ± 0.09	0.60 ± 0.08	0.61 ± 0.05	0.64 ± 0.10
Glucose (mg/dl)	300.9 ± 107.7	354.8 ± 109.2	253.4 ± 59.3	259.9 ± 83.1
Sodium (mmol/L)	142.1 ± 2.8	141.5 ± 2.0	141.3 ± 1.9	141.3 ± 1.5
Potassium (mmol/L)	9.67 ± 5.48	9.61 ± 1.85	9.66 ± 2.71	9.62 ± 2.23
Calcium (mmol/L)	12.25 ± 0.35	12.16 ± 0.58	11.79 ± 0.57	11.91 ± 0.62
Chloride (mmol/L)	94.30 ± 2.36	95.10 ± 1.52	94.50 ± 1.27	94.00 ± 1.05
Phosphorus (mmol/L)	14.32 ± 4.14	14.16 ± 3.87	14.16 ± 4.31	14.86 ± 4.34
Female				
AST (U/L)	157.9 ± 51.3	167.9 ± 207.2	137.4 ± 60.1	122.7 ± 19.3
ALT (U/L)	67.70 ± 27.31	62.10 ± 49.36	53.00 ± 33.21	47.20 ± 13.13
ALP (U/L)	60.00 ± 18.07	67.40 ± 24.26	57.3 ± 18.39	49.90 ± 12.4
Total bilirubin (mg/dL)	0.52 ± 0.13	0.45 ± 0.11	0.54 ± 0.13	0.45 ± 0.13
Total protein (g/dL)	6.97 ± 0.27	6.83 ± 0.36	6.97 ± 0.19	7.01 ± 0.50
Albumin (g/dL)	4.13 ± 0.17	4.15 ± 0.17	4.18 ± 0.18	4.20 ± 0.31
BUN (mg/dL)	12.21 ± 2.01	13.26 ± 3.00	14.72 ± 2.58	13.85 ± 1.39
Creatinine (mg/dL)	0.71 ± 0.12	0.64 ± 0.10	0.65 ± 0.08	0.66 ± 0.09
Glucose (mg/dl)	230.5 ± 165.3	275.1 ± 121.2	190.2 ± 87.8	222.5 ± 124.0
Sodium (mmol/L)	137.7 ± 4.4	139.3 ± 1.4	138.7 ± 1.8	139.9 ± 1.9
Potassium (mmol/L)	13.50 ± 5.48	8.76 ± 2.85	10.94 ± 3.03	8.66 ± 2.05
Calcium (mmol/L)	12.59 ± 0.74	12.07 ± 0.93	12.05 ± 0.52	12.24 ± 0.45
Chloride (mmol/L)	96.60 ± 1.17	95.40 ± 3.17	96.60 ± 1.35	95.40 ± 1.78
Phosphorus (mmol/L)	17.25 ± 4.37	13.43 ± 6.06	13.84 ± 4.63	12.89 ± 4.69

Key: Data are expressed as mean ± SD ($n = 10$). *AST* Aspartate aminotransferase, *ALT* Alanine aminotransferase, *ALP* Alkaline phosphatase, *BUN* Blood urea nitrogen

respectively. Prostate observation found that both the control and high-dose XNE-treated groups have an incidence rate of 5/10 exhibiting slight mononuclear inflammatory cell infiltrate. Besides, several individual animals were also found to have pinworm infestation in the rectum, with an incidence rate of 3/10 and 1/10 in the control and high-dose XNE-treated male and female rats, respectively; however, there was no correlation between the disease and the incidence rate. In addition, the kidneys of female animals at this dose level were also observed to have focal and mild tubular infarct (an incidence rate of 2/10), and they were found unrelated to the XNE treatment.

Discussion

Herbal medicines and health foods derived from either mycelia or fruiting bodies of *X. nigripes* have become increasingly popular in the healthcare market in China. The present results showed that administration of XNE at doses 50 and 100 folds higher than the recommended dosage orally for 90 days exhibit no significant effect on experimental parameters. Compared with the control group, there was no significant difference in weight gains between the different treatments. It has been reported that the body weight changes may reflect the general health status of animals [22]. In this study, the body weight gain in all XNE-treated animals was normal, even

Table 4 Summary on the incidence and pathological changes in organs of rats exposed to XNE by gavage for 90 days

Organ	Lesions	Group			
		Control		High dose	
		Male	Female	Male	Female
Adrenal gland		–	–	–	–
Brain					
Fore		–	–	–	–
Middle		–	–	–	–
Cerebellum		–	–	–	–
Stem		–	–	–	–
Bone femur		–	–	–	–
Bone marrow		–	–	–	–
Cervix		N	–	N	–
Esophagus		–	–	–	–
Heart	Infiltration, mononuclear cell, focal, slight[a]	2 (1/10)[b]	–	2 (1/10)	–
Intestine, small					
Duodenum		–	–	–	–
Jejunum		–	–	–	–
Ileum		–	–	–	–
Intestine, large					
Caecum		–	–	–	–
Colon		–	–	–	–
Rectum	Pinworm infestation, slight	–	2 (3/10)	2 (1/10)	–
Kidney	Infarct, tubule, focal, slight	–	–	–	2 (2/10)
Liver		–	–	–	–
Lung		–	–	–	–
Lymph node, mesenteric		–	–	–	–
Ovary		N	–	N	–
Pancreas		–	–	–	–
Pituitary gland		–	–	–	–
Parathyroid gland		–	–	–	–
Prostate gland	Infiltration, mononuclear cell, focal, slight	2 (5/10)	N	2 (5/10)	N
Sciatic nerve		–	–	–	–
Spinal cord					
Cervical		–	–	–	–
Lumbar		–	–	–	–
Thoracic		–	–	–	–
Spleen		–	–	–	–
Stomach		–	–	–	–
Testicles		–	N	–	N
Thymus		–	–	–	–
Thyroid gland		–	–	–	–
Trachea		–	–	–	–
Urinary bladder		–	–	–	–
Uterus		N	–	N	–

Key: Control, Distilled water; High dose, 2000 mg/kg; N, no tissue available. -, No effect; [a]Degree of lesions was graded from one to five depending on severity: 1 = minimal (< 1%); 2 = slight (1–25%); 3 = moderate (26–50%); 4 = moderate/severe (51–75%); 5 = severe/high (76–100%); [b]Incidence: Affected rats/Total examined rats ($n = 10$)

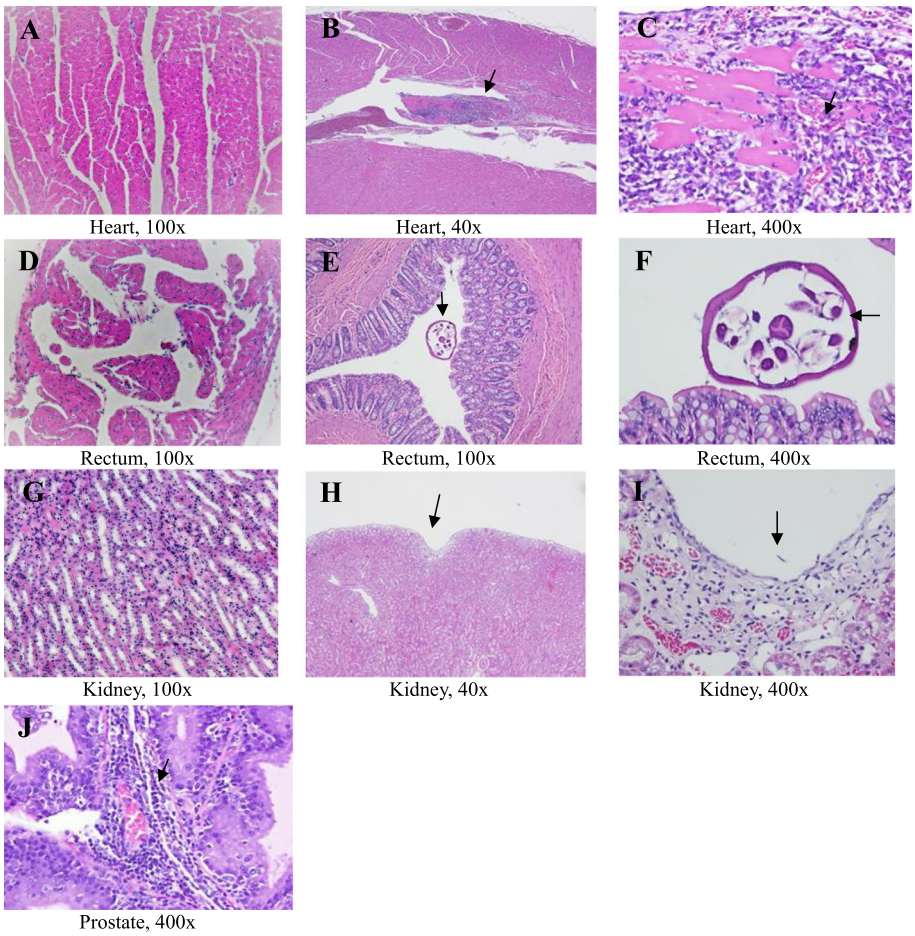

Fig. 2 Non-specific histopathological alterations in organs of rats receiving XNE for 90 days. Heart (**a**, 100x), rectum (**d**, 100x) and kidney (**g**, 100x) of the control animals, non-specific lesions of focal mononuclear cell infiltration in the heart (**b**, 40x and **c**, 400x; control male rat no. XMC4–1), pinworm infestation in the rectum (**e**, 100x and **f**, 400x; control female rat no. XFC4–3), focal tubular infarct in the kidney (**h**, 40x and **i**, 400x; high dose XNE-treated female rat no. XFH1–3) and slight inflammation in the prostate (**j**, 400x, control male rat no. XMC3–1) were found in few control and XNE-treated animals as indicated by arrow. H&E stain

at a dose of up to 100 folds higher than the recommended dosage, no adverse effect was noted on the growth rate; this suggests that the different dosages of XNE did not affect the normal body metabolism of animals, and are not harmful to their growth.

Increased organ weight (either absolute or relative) has been considered as a sensitive indicator of organ toxicity by known toxicants [23]. Compared with the control group, an insignificant difference in the weight of the excised vital organs (e.g. liver, kidney, heart, brain, spleen and lungs) indicates that XNE on prolonged use or intake did not affect the normal functions of organs. As there was no reduction in body and relative organ weights in all XNE-treated rats, hence it can be assumed that the extract is not toxic to these organs.

Assessments of hematological parameters can be used to determine the extent of deleterious effect of extracts on the blood [24]. Compared to the control animals, no significant effects on RBC, MCV, and haemoglobin values were noted on XNE-treated rats; this suggests that the erythropoiesis, morphology or osmotic fragility of RBC are not affected by XNE. Similarly, the insignificant changes in neutrophils, lymphocytes, and monocytes suggest that all tested doses of XNE do not affect the intact state of the immune system, and cause injury to the tissues. These hematological results further justified the safety potential of XNE.

ALT, AST and ALP are sensitive markers for assessing the liver function or liver injury [25]. Elevated activities of these enzymes are associated with liver or heart damage [26, 27]. In this study, oral administration of XNE at dosage up to 2000 mg/kg for 90 consecutive days had no adverse effect on serum biochemistry of rats in both sexes, and serum AST and ALT levels of XNE-treated

animals were within the normal ranges; this suggests that XNE is not hepatotoxic, and has no deleterious effect on the heart.

The serum total protein and albumin levels provide a useful indication of nutritional status, and functions of liver and kidney [24, 28], whereas serum urea and creatinine levels reflect the likelihood of renal problems or dysfunction [29, 30]. Compared with the control group, an insignificant difference in serum level of these parameters was observed in prolonged XNE-treated animals, this further corroborates the fact that XNE does not cause liver damage and affect normal renal function.

Electrolytes (Na, K and Cl) play a prominent role in the gas exchange and the intercompartmental water balance, and they are often used to assess the renal function [31]. Increase or decrease in the levels of these electrolytes within the serum can be a consequence of the hypo- or hyper-functioning of the kidney. In this study, no significant change in Na, K and Cl levels was noted between XNE-treated and the control animals, suggesting a normal functioning status of the kidney.

From the results of histological examination, there was no treatment related alteration and abnormalities observed in the cell structure of organs. Although a few histopathological changes were observed in the sections of the heart, kidney and prostate, these changes are not treatment related as they are also observed in the control rats. In addition to this observation, as the administration of XNE at concentration 2000 mg/kg body weight (100 folds more than the recommended dosage) caused no mortality or any adverse clinical signs, and led to changes in the organ weights, hematological and biochemical parameters of rats in both sexes; these results indicate that the lethal dose (LD_{50}) of XNE is greater than 2000 mg/kg, it is considered to have low toxicity and hence is relatively safe for consumption.

Conclusion

The oral administration of XNE up to a dose of 2000 mg/kg exhibited no clinical signs or toxic effects in animals with regard to behavioral patterns, body and relative organ weights, haematological, biochemical and histopathological parameters in rats for a period of 90-day treatment. These results conclude that XNE has a high margin of safety in rats with NOAEL of up to 2000 mg/kg/day. This finding also suggests that XNE is relatively safe for use as herbal medicines and health foods.

Abbreviations

XNE: Aqueous extract from *X. nigripes*; WBC: White blood cells; RBC: Red blood cells; MCHC: Mean corpuscular haemoglobin concentration; MCV: Mean corpuscular volume; APTT: Activated partial thromboplastin time (APTT); AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen; NOAEL: No observed adverse effect level

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Authors' contributions

MNL initiated and coordinated the study, and reviewed the data. HCH performed the study, analyzed the data and prepared the draft manuscript. LTN analyzed and interpreted the data, revised and edited the manuscript. All authors have read and approved the manuscript.

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Availability of data and materials

The dataset supporting the conclusions of this article is included within the article.

Declarations

Ethics approval and consent to participate

The study protocol and experiments were approved by the Animal Ethics Committee of National Ilan University, Ilan County, Taiwan.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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