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Sub-acute toxicity of the standardised extract of *Boesenbergia rotunda* in rats

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ABSTRACT: An *in vitro* study of Thai medicinal plants revealed anti-SARS-CoV-2 activity of ethanolic extract of *Boesenbergia rotunda* and its bioactive component of panduratin A. The present study aims to evaluate the safety of the extract after 28 consecutive days of oral doses according to OECD GLP 407. The 28-day repeated oral doses were performed in both male and female Wistar rats. Three doses of 150, 300 and 600 mg/kg/day were assigned as low, medium and high doses, respectively. During the 28-day administration period, there was no evidence of morbidity, mortality, or neurological toxicity for both gender in all doses. Weight and food and water intake in all doses were similar to the control group. The hematological and clinical biochemistry parameters were within normal ranges of Wistar rats. The oestrous cycle of female animals of all groups was normal and the vaginal cytology investigation showed no abnormal cellular types. The statistically significant difference of the weight of organs showed no-test item-related effects and histopathological examination revealed no remarkable lesion. The no observed adverse effect level (NOAEL) of the ethanolic extract of *B. rotunda* after 28 consecutive days was considered to be 600 mg/kg body weight per day. These findings are useful information for developing *B. rotunda* extract as a potential phytopharmaceutical product for future clinical investigation.

KEYWORDS: *B. rotunda*, fingerroot, subacute toxicity, OECD GLP 407, anti-SARS-CoV-2

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by the severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) that has rapidly spread across the world, affecting millions of individuals worldwide. As the number of confirmed COVID-19 cases continues to rise, it is critical to develop effective and economical therapeutic agents for the prevention or treatment of COVID-19. Numerous repurposed medications including lopinavir-ritonavir, ribavirin, interferon, and hydroxychloroquine have been tested to immediately help prevent morbidity, mortality, and virus spread [1]. However, the efficacy of these drugs was unclear and several drugs showed some toxicities [2–4]. For example, the use of hydroxychloroquine is associated with cardiovascular adverse reactions in COVID-19 patients [5]. Drug-related problems such as diarrhea were reported with the use of lopinavir-ritonavir [2]. Remdesivir, the first antiviral drug approved by the European Medicines Agency and Food and Drug Administration for SARS-CoV-2 infection, is available in the injection form, limiting the use of this drug outside hospital setting [6]. Remdesivir reduces the recovery time and the risk of complications but fails to decrease the mortality rate [7]. Molnupiravir and nirmatrelvir/ritonavir are current oral treatment approved for SARS-CoV-2 infection [8]. The use of molnupiravir in pregnant women is not recommended due to its mutagenic effects. Traditional medicines were also investigated for their activities against SARS-CoV-2; however, the *in vivo* information on safety was required for appropriate dose extrapolation to human [9, 10].

Boesenbergia rotunda (L.) Mansf. (Family: *Zingiberaceae*) also known as *B. pandurata*, *Kaempferia pandurata*, and fingerroot, is mostly consumed as a dietary ingredient and used in traditional medicine in Southeast Asia. Alkaloids, flavonoids, essential oil, and phenolic compounds are phytochemical components found in *B. rotunda* [11]. The extract from rhizomes of *B. rotunda* showed diverse biological activities including anti-inflammatory, antioxidant, anti-bacterial, anti-herpes viral, and hepatoprotective activities [12–17]. Our recent *in vitro* high-content screening of Thai medicinal plants for anti-SARS-CoV-2 activities indicated *B. rotunda* as one of the good candidates. The extract of *B. rotunda* showed IC_{50} to SARS-CoV-2 at 3.62 ng/ml, and its bioactive components of panduratin A exhibited IC_{50} at 0.81 nM [18]. In addition to

in vitro anti-SARS-CoV-2 activity, one main important factor to decide the fate of drug development is toxicity. Acute and sub-acute toxicity studies in animals are required for the determination of human dosing and the registration of the investigational new drugs in humans [19].

Although plant extracts possess many interesting *in vitro* pharmacological activities, these extracts could contain components that can be harmful in *in vivo* models. It is necessary to investigate the potential hazardous characteristics of natural product extracts and their components before human use [20]. The previous study in healthy male and female rats over a 15-day period reported no evidence of toxicity from the consumption of high doses of the ethanolic extract of *B. rotunda* rhizome at 2 and 5 g/kg/day [12]. Oral administration of the ethanolic extract of *B. rotunda* at 60, 120, and 240 mg/kg in a sub-chronic toxicity test in male rats for 60 days was found to be safe [21]. The oral consumption of pinostrobin and pinocembrin from *B. rotunda* at 100 mg/kg for 7 days showed no toxicity and no genotoxicity to male Wistar rats [22]. Dose selection of *B. rotunda* extract was calculated based on our preliminary efficacy study in SARS-CoV-2 infected golden Syrian hamsters with effective dose range of 100–1000 mg/kg/day. Nevertheless, previous studies of toxicity of *B. rotunda* extract never reported the percentage of major bioactive ingredient, panduratin A, and focused only on blood biochemistry, hematological toxicity and liver pathology. Therefore, the present study aimed to determine the repeated dose toxicity of standardised extract of *B. rotunda* with the known levels of panduratin A in male and female rats over a 28-day period according to OECD GLP 407. The reported parameters were general clinical observation, neurological examinations, stage of oestrous cycle, hematological analysis, clinical chemistry analysis and histopathological analysis of liver, kidneys, heart, lung, spleen, thyroid and parathyroid glands, stomach, and small and large intestines. The information obtained from this study will be useful for phytopharmaceutical product development of *B. rotunda* extract against SARS-CoV-2 infection in the near future.

MATERIALS AND METHODS

Preparation of standardised ethanolic extract of *B. rotunda*

B. rotunda rhizomes were purchased from contract farming in Ratchaburi province, Thailand. Plant identification and comparison to the depository plant materials was performed by the Excellent Center for Drug Discovery (ECDD), Faculty of Science, Mahidol University. The ethanolic extraction method used to prepare a crude extract of *B. rotunda* was as follows: 2.5 kg of *B. rotunda* rhizomes were ground into fine powder, dried, then percolated with 6 l of 95% ethanol for 4 times during a 7-day period at room temperature. After the removal of solvent, the level of panduratin A in the final product with the appearance of a dark brown viscous liquid was evaluated using liquid chromatography-mass spectrometry. The standardised extract of *B. rotunda* contains panduratin A 5.68% w/w. The *B. rotunda* extract was stored in the custodian room at 4 °C until used [18].

Experimental animals

Sixty Wistar rats (30 females and 30 males) obtained from the National Laboratory Animal Center, Mahidol University, Thailand, were used in the 28-day repeated oral toxicity study. The rats aged 8 weeks weighed between 228–247 g in males and 178–192 g in females were used. The animals were housed in plastic cages filled with corn cob at 22±3 °C with a relative humidity of 30–70% and a 12-hour light-dark cycle. The standard diet (082: Perfect Companion Group, Thailand) and reverse osmosis water were provided *ad libitum*. All animals were acclimatised to laboratory conditions for at least 5 days before the experiment was initiated.

Ethics statement

Guidelines for the care and use of laboratory animals (Institute of Laboratory Animal Resources, NIH publication number #85-23, revised 2011) were strictly followed throughout these *in vivo* toxicity studies, and were also referred to in the Organisation for Economic Co-operation and Development (OECD) Guideline for Testing of Chemicals 407 [23]. The study was approved by National Laboratory Animal Center Animal Care and Use Committee, Mahidol University, Thailand, NLAC-ACUC No. RA2021-19 on April 30, 2021.

Repeated dose 28-day oral toxicity study

The animals were weighed and randomly distributed into each group. The mean weight difference between each group was not more than 20%. The *B. rotunda* extract was calculated, weighed, and dissolved using 1% w/v carboxymethylcellulose (CMC) to three dose levels of 150, 300, and 600 mg/kg body weight. Sixty rats were randomised into four treatment groups and two control groups, each with 10 animals (5 males and 5 females). Group 1 (as control), 2, 3, and 4 received 150, 300, and 600 mg/kg/day of the *B. rotunda* extract, respectively. Group 5 and 6, designated as recovery groups, received 1% w/v CMC and 600 mg/kg/day of the *B. rotunda* extract for 4 weeks followed by the observation of exacerbation and/or reversibility of possible adverse effects for another 14 days. The dosing preparation of *B. rotunda* extract was prepared immediately before the administration every day. The oral administration was performed by passing the needle into the esophagus in a straight line to the stomach once a day for a period of 28 days.

Clinical observation and health examination

The daily observation focused on changes in general clinical signs, at a similar time and in a standard area. These observations were made outside the cage. Individual body weights were recorded once during the acclimatisation period and once a week until the day of necropsy. Feed and drinking water consumption were measured once during the acclimatisation period and daily after the first date of dosing. The clinical signs of toxicity, including health examinations such as changes in skin, fur/coat, eyes and mucous membrane, occurrence of secretions and excretions, autonomic activity (lacrimation, piloerection, pupil size and respiratory pattern), changes in gait, posture and response to handing, presence of clonic and tonic movements, stereotype/bizarre behavior (excessive grooming, repetitive cycling, self-mutilation and walking backwards) and neurological examinations (auditory, visual, proprioception, fore-limb and hind-limb grip strength test and motor activity assessment) were examined once a week.

Clinical pathology

On the day of necropsy, all animals were scheduled for overnight fasting for 15–18 h prior to blood collection. Blood samples were collected from the posterior vena cava. Whole blood samples were separated into 2 tubes for hematological and clinical biochemistry tests. Hematological parameters including red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT), white blood cell count (WBC), neutrophil (NEUT), lymphocyte (LYMPH), monocyte (MONO), eosinophil (EO) and basophil (BASO) were analysed using an automated analyser (IDEXX Procyte DXTM, USA). Clinical biochemistry parameters including sodium (Na), potassium (K), chloride (Cl), glucose (SGLU3), cholesterol (CHO2l), triglyceride (TRIGL), uric acid (UA2), blood urea nitrogen (U-BUN), creatinine (CREA2), total protein (TP2), albumin (ALB2), globulin (GLO), high-density lipoprotein (HDLC4), low-density lipoprotein (LDLC3), alanine amino transferase (ALTL), aspartate amino transferase (ASTL) and alkaline phosphatase (ALP2S) were performed on serum obtained after centrifugation of total blood (without anticoagulant) using Cobas C311 automated blood analyzer (Roche, Switzerland).

Anatomical pathology

The animals were euthanised using carbon dioxide inhalation for pathological examinations of organs. At the time of necropsy, the stage of the oestrous cycle (metestrus, diestrus, and proestrus) of all female animals was determined by taking vaginal smears. These stages were defined by the absence, presence, and proportion of 4 basic cell types (leucocytes, nucleated epithelial, cornified, and non-nucleated epithelial) as well as the cell density and arrangement. The following organs (liver, kidney, heart, adrenal gland, brain, testes, prostate glands, epididymis, ovaries, and oviduct, uterus, spleen, thymus, thyroid, and parathyroid glands, and pituitary gland) were trimmed, weighed (all the paired organs were weighed separately) and preserved in 10% v/v neutral buffered formalin in plastic bags. The weights of these organs were converted to relative organ weights (organ-to-body weight ratios). For histopathological analysis, the selected organs (liver, kidneys, heart, lung, spleen, thyroid and parathyroid glands, stomach, small intestine, and large intestine) were trimmed and preserved in 10% v/v neutral buffer formalin for tissue slide preparations. Slides of tissue specimens were made with the paraffin section technique and microsections of 4–5 mum thickness were stained with hematoxylin and eosin. The histopathological examinations were individual diagnosed and immediately recorded in Microsoft Excel by the pathologist. The diagnostic terms and glossary were based on the International Harmonization of Nomenclature and Diagnostic Criteria, which was developed by Societies of Toxicologic Pathology from Europe, Great Britain, Japan, and North America. Lesion scoring was classified into 5 levels, which were assigned as absent, minimal, mild, moderate, and severe numerical correlates of 0, +1, +2, +3, and +4 were generally applied semi-quantitatively in direct proportion to the number of foci or the area of lesions.

Statistical analysis

The quantitative results of the treatment group were expressed as an average ± standard deviation. All statistical analyses were done with the commercial programme SPSS® Statistic software version 18.0.0. The significance level was considered at 0.05 levels, $p < 0.05$. The obtained data were statistically analysed by Kolmogorov-Smirnova and Levene's test for normality and homogeneity of variances. For parametric statistics, homogenous data was compared between the vehicle control group and each treatment group by a two-sided Dunnett test. Heterogeneous data were compared between the vehicle control group and each treatment group by the two-sided Dunnett's T3 test, using ANOVA analysis. For non-parametric statistics, the data were compared between the vehicle control group and each treatment group by Mann Whitney U test. Quantitative results of the recovery group were expressed as an average ± standard deviation. The obtained data were statistically analysed using Levene's test for equality of variances and the T-test for equality of averages, which were compared between

the vehicle control-recovery group and the high dose-recovery group.

RESULTS

Effects of the standardised ethanolic extract of *B. rotunda* on clinical observation and health examination

There was no death or clinical sign of toxicity in rats during the 28-day administration period. However, salivation in the high dose group was found after administration and returned to normal within 15 min. There was no statistically significant difference in body weight when compared with the control group at 0.05 levels (Fig. 1). Although the averages of feed and drinking water consumption of all animals showed statistically significant differences in some groups (Figs. S1 and S2), the differences were not related to the animal body weight and had no effect on animal health.

The neurological examination of auditory, visual, proprioception, and motor activity assessment revealed no abnormal neurological sign. The 30-second assessment of animal motor activity revealed no statistically significant difference as compared to the control group (Fig. S3). The fore-limb grip strength in female rats at low dose and hind-limb grip strength tests in males at high dose-recovery group were statistically different from their control groups (Fig. 2). However, the results were not related to doses and had no effect on animal health.

Effects of the standardised ethanolic extract of *B. rotunda* on hematological parameters

There is no statistical difference among control and low-medium-high doses of *B. rotunda* extract, except for white blood cells. In both sexes, the average WBC in the low, medium, and high dose groups was significantly higher than in the control group. For male animals, the average number of monocytes in the medium and high dose groups were significantly higher than in the control group. For female animals, the average of basophil in the low dose group was significantly lower than that in the control group. The statistically different results of hematological parameters were within the normal ranges of rats (Table 1).

Effects of the standardised ethanolic extract of *B. rotunda* on clinical biochemistry parameters

Most of blood chemistry parameters showed no statistically significant difference among control, low-medium-high doses of *B. rotunda* extract (Table 2). Some statistically significant differences in the results of clinical biochemistry parameters were within the normal ranges of rats and had no dose related effect. The average of Na^+ levels in the low dose group in male animals was significantly higher than that in the control group. In the medium dose group, the average of TRIGL was significantly higher than that in the control group, and the averages of ASTL and Cl levels were significantly lower than those in the control group. In the high dose group, the averages of TRIGL and Na^+ were significantly higher than those in the control group. For female animals, the average of SGLU3 in the low dose group was significantly higher than that in the control group. In the medium dose group, the averages of K^+ and Cl^- were significantly lower than those in the control group.

Effects of the standardised ethanolic extract of *B. rotunda* on anatomical pathology

There was no dose-related effect in the statistically significant difference in animal organ weight results (Table 3). Some statistically significant differences of organ weight were found among different groups as follow. The average heart weight in the medium dose group was significantly lower than that in the control group. The average spleen weight in the high dose-recovery group was significantly lower than that in the control group, and the average right epididymis weight was significantly higher than that in the control group. Cytological evaluation results in three stages of the oestrous cycle of this study revealed no abnormal cellular types (Table S1).

Effects of the standardised ethanolic extract of *B. rotunda* on necropsy examination and histopathology evaluation

There is a minimal histopathologic change among control and low-medium-high doses of *B. rotunda* extract. Only some animals had minor changes as follows: a female animal in the medium dose group had a hepatodiaphragmatic nodule. The histopathological examination of kidneys found one male animal in the high dose group with minimal focal basophilic tubule in the right kidney and one male animal in the high dose-recovery group with minimal basophilic and dilation tubule in the left kidney. There was no remarkable lesion in all organs of female animals in any group.

DISCUSSION

The present study assessed the sub-acute oral toxicity of the *B. rotunda* extract in Wistar rats after repeated oral administrations over a 28-day period in accordance with the OECD's guideline for chemical testing 407 to define NOAEL and target organs [23]. Clinical observation and health examination are two of the most critical observations in identifying the toxicity effects of the test compounds. Both male and female rats receiving standardised extract of *B. rotunda* at doses of 150, 300, and 600 mg/kg body weight showed no clinical symptoms of toxicity, morbidity, or mortality. The physical properties of the skin, fur, eyes, mucous

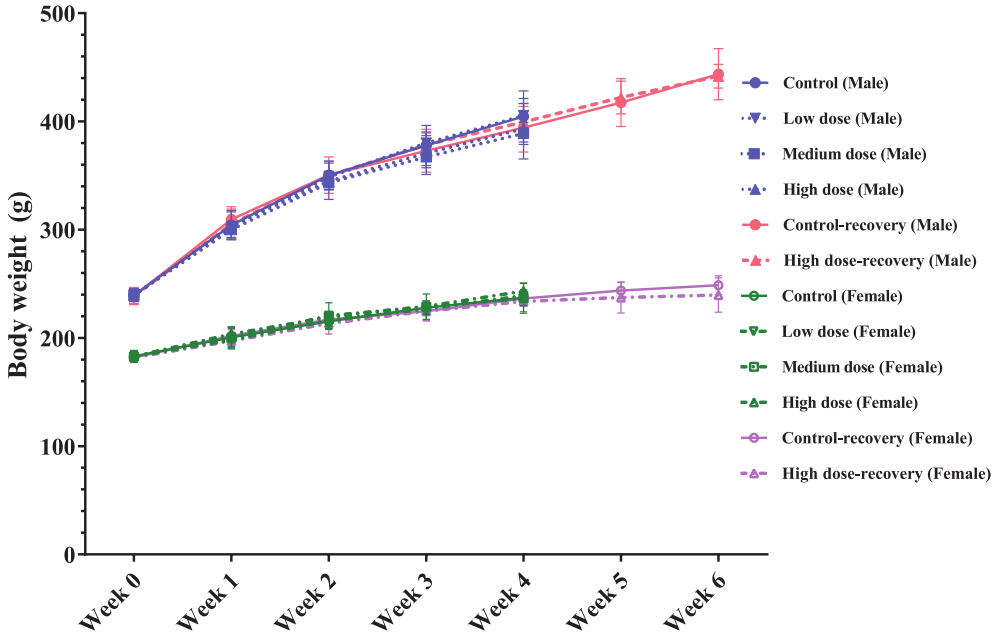

Fig. 1 Effects of the *B. rotunda* extract on averages of animal body weights. Averages of animal body weights in male and female rats. Data are shown as average ± standard deviation ($n = 10$ per group).

Table 1 Effect of the *B. rotunda* extract on hematological parameters.

Parameter	Control	Dose level			Recovery group	
		Low	Medium	High	Control	High dose
Male						
RBC (M/µl)	9.42±0.31	9.51±0.34	9.71±0.34	9.77±0.34	9.60±0.41	9.89±0.34
HGB (g/dl)	17.5±0.43	17.6±0.58	18.0±0.78	18.1±0.31	17.6±0.40	17.7±0.31
HCT (%)	54.0±1.68	54.9±2.26	55.7±1.82	55.6±1.15	54.0±2.00	54.5±0.95
MCV (fl)	57.3±1.42	57.7±0.45	57.3±0.78	56.9±1.12	56.3±1.31	55.1±1.17
MCH (pg)	18.6±0.45	18.5±0.23	18.5±0.17	18.5±0.41	18.4±0.65	17.9±0.38
MCHC (g/dl)	32.4±0.21	32.1±0.38	32.3±0.59	32.5±0.28	32.7±0.70	32.4±0.19
PLT (K/µl)	764±129.98	736±80.63	789±55.65	740±66.34	673±81.25	698±46.72
WBC (K/µl)	7.50±0.53	9.72±0.82*	10.38±1.23*	10.88±1.06*	6.68±0.44	10.63±1.62**
NEUT (%)	7.7±6.44	6.2±4.29	2.1±2.30	5.8±4.38	11.3±1.51	7.4±4.00
LYMPH (%)	86.6±6.46	88.2±4.15	90.6±2.88	86.5±4.01	82.2±2.41	86.6±4.24
MONO (%)	4.9±0.88	4.9±0.61	6.7±1.12*	7.0±1.25*	5.4±1.10	5.1±0.44
EO (%)	0.6±0.15	0.5±0.17	0.5±0.11	0.6±0.11	0.8±0.23	0.7±0.19
BASO (%)	0.2±0.25	0.1±0.09	0.0±0.09	0.1±0.11	0.3±0.11	0.3±0.21
Female						
RBC (M/µl)	9.29±0.29	9.35±0.48	9.13±0.20	9.02±0.42	9.50±0.13	9.39±0.45
HGB (g/dl)	17.3±0.54	17.5±0.82	17.0±0.49	17.0±0.62	17.7±0.29	18.0±0.72
HCT (%)	53.0±1.98	54.0±2.78	52.4±1.68	52.2±2.20	53.9±1.16	55.2±2.18
MCV (fl)	57.1±1.00	57.7±0.94	57.3±0.89	57.9±0.48	56.8±0.86	58.8±1.07**
MCH (pg)	18.7±0.15	18.7±0.34	18.6±0.25	18.9±0.26	18.6±0.29	19.1±0.36
MCHC (g/dl)	32.7±0.38	32.4±0.22	32.5±0.13	32.5±0.41	32.8±0.53	32.6±0.26
PLT (K/µl)	811±47.00	757±203.04	773±61.95	657±56.02*	805±126.88	848±284.47
WBC (K/µl)	5.57±0.81	7.65±0.95*	8.56±1.10*	8.78±1.04*	5.88±1.42	7.30±0.65
NEUT (%)	5.9±3.47	4.4±3.20	3.9±2.66	5.9±1.77	6.9±0.91	5.9±0.68
LYMPH (%)	87.8±4.13	90.4±3.58	89.7±1.87	87.6±3.05	87.6±1.19	88.6±1.74
MONO (%)	5.5±1.28	4.9±1.15	6.0±0.85	6.0±1.39	4.9±1.09	4.8±0.89
EO (%)	0.6±0.15	0.4±0.11	0.4±0.15	0.4±0.16	0.6±0.24	0.5±0.25
BASO (%)	0.2±0.09	0.0±0.00*	0.1±0.10	0.2±0.15	0.1±0.10	0.1±0.07

Values are average ± standard deviation ($n = 10$, per group for male and female rats). * $p < 0.05$ levels of control group. ** $p < 0.05$ levels of control-recovery group. Abbreviations: red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT), white blood cell count (WBC), neutrophil (NEUT), lymphocyte (LYMPH), monocyte (MONO), eosinophil (EO) and basophil (BASO).

Table 2 Effect of the *B. rotunda* extract on clinical biochemistry.

Parameter	Control	Dose level			Recovery group	
		Low	Medium	High	Control	High dose
Male						
SGLU3 (mg/dl)	427.5±36.93	370.3±42.58	426.0±57.33	388.1±53.97	397.2±36.25	397.0±45.93
U-BUN (mg/dl)	22.5±2.98	23.0±2.70	23.2±0.87	24.7±2.60	20.0±2.53	20.0±2.52
CREA2 (mg/dl)	0.42±0.03	0.40±0.01	0.38±0.02	0.40±0.03	0.40±0.03	0.40±0.02
UA2 (mg/dl)	7.2±1.04	6.4±0.83	7.4±1.38	6.8±1.26	6.9±0.70	7.4±0.78
CHO2I (mg/dl)	76.3±13.42	81.7±14.00	77.0±11.80	78.4±10.37	79.6±5.24	76.6±10.62
TRIGL (mg/dl)	86.2±9.99	89.0±20.08	157.0±41.40*	154.9±46.66*	106.7±17.63	105.3±31.17
LDLC3 (mg/dl)	9.0±4.02	11.1±4.55	9.7±3.59	9.7±2.98	11.0±2.05	9.3±3.06
ASTL (U/l)	78.9±7.60	81.4±6.82	67.9±1.90*	70.9±6.88	114.1±77.50	110.0±31.69
ALTL (U/l)	49.3±9.00	60.7±9.59	39.8±3.11	47.1±8.45	88.4±87.15	92.8±40.21
ALP2S (U/l)	118±3.81	113±13.18	108±13.28	103±10.80	103±13.54	106±12.12
TP2 (g/dl)	7.22±0.26	7.33±0.35	7.20±0.24	7.20±0.11	6.99±0.16	7.04±0.14
ALB2 (g/dl)	5.16±0.18	5.19±0.21	5.09±0.16	5.10±0.09	5.02±0.14	5.03±0.08
HDLC4 (mg/dl)	61.3±9.96	63.0±9.30	57.4±8.41	56.4±8.35	60.0±7.05	57.4±8.31
Na (mmol/l)	148±0.45	150±1.14*	148±0.89	150±1.10*	147±2.07	147±2.07
K (mmol/l)	9.39±0.57	9.45±0.37	9.00±0.63	9.31±0.76	10.32±1.54	10.64±1.59
Cl (mmol/l)	102.4±0.80	102.0±0.98	99.7±1.77*	100.7±1.59	102.1±1.63	100.5±0.72
GLO (mg/dl)	2.06±0.16	2.14±0.15	2.11±0.10	2.11±0.08	1.97±0.07	2.01±0.12
Female						
SGLU3 (mg/dl)	262.9±52.64	344.9±54.69*	293.6±44.32	318.4±42.97	220.6±108.75	265.5±138.82
U-BUN (mg/dl)	23.1±3.35	22.1±1.59	22.7±0.88	22.9±2.02	20.5±1.55	18.7±2.84
CREA2 (mg/dl)	0.42±0.02	0.42±0.03	0.40±0.02	0.40±0.02	0.47±0.04	0.46±0.02
UA2 (mg/dl)	4.9±0.33	5.5±0.82	5.1±0.50	5.1±1.10	4.7±0.79	5.1±0.87
CHO2I (mg/dl)	104.9±6.88	103.6±8.55	100.5±12.61	96.6±14.12	96.5±11.95	94.7±23.74
TRIGL (mg/dl)	85.9±23.89	95.5±34.36	102.6±9.30	108.3±51.50	69.8±7.51	74.2±13.06
LDLC3 (mg/dl)	11.4±2.74	10.4±2.32	10.6±3.46	10.5±2.76	9.8±2.33	11.3±5.32
ASTL (U/l)	73.3±5.80	72.7±2.21	77.0±11.16	71.9±6.61	83.1±5.41	85.3±9.43
ALTL (U/l)	43.3±5.66	45.8±5.69	53.9±17.94	43.8±7.06	47.2±4.28	42.9±6.08
ALP2S (U/l)	68±8.60	65±8.61	69±14.88	60±12.46	56±1.64	54±8.02
TP2 (g/dl)	7.08±0.26	7.42±0.29	7.06±0.28	7.15±0.33	7.11±0.29	7.05±0.32
ALB2 (g/dl)	5.22±0.21	5.47±0.19	5.18±0.24	5.18±0.23	5.31±0.22	5.19±0.17
HDLC4 (mg/dl)	83.6±3.54	85.0±7.13	81.7±8.84	77.7±9.08	78.8±9.46	76.3±17.96
Na (mmol/l)	147±0.71	148±1.34	147±0.89	149±1.14	147±1.58	147±2.95
K (mmol/l)	10.11±0.90	10.51±0.62	8.13±0.57*	9.22±1.00	10.96±0.79	11.76±1.14
Cl (mmol/l)	105.2±0.83	104.2±1.16	102.0±1.40*	103.8±1.39	104.3±2.04	105.4±1.14
GLO (mg/dl)	1.86±0.11	1.95±0.15	1.88±0.13	1.97±0.13	1.80±0.10	1.87±0.20

Values are average ± standard deviation ($n = 10$, per group for male and female rats). * $p < 0.05$ levels of control group. ** $p < 0.05$ levels of control-recovery group. Abbreviations: sodium (Na), potassium (K), chloride (Cl), glucose (SGLU3), cholesterol (CHO2l), triglyceride (TRIGL), uric acid (UA2), blood urea nitrogen (U-BUN), creatinine (CREA2), total protein (TP2), albumin (ALB2), globulin (GLO), high-density lipoprotein (HDLC4), low-density lipoprotein (LDLC3), alanine amino transferase (ALTL), aspartate amino transferase (ASTL) and alkaline phosphatase (ALP2S).

membrane, secretions, excretions, and autonomic activity were all determined to be normal. Salivation was observed in the high dose group after treatment and it recovered to normal within 15 min. *Zingiber officinale* or ginger, another plant in the zingiberaceae family, also increased salivation in rats and cattles [24, 25]. The peak of salivation was observed at 7 minutes after an injection with the ethanolic extract of *Z. officinale* [24]. The bioactive component of ginger, 6-gingerol, was also reported to increase saliva flow in humans [26]. Although the rhizome of *B. rotunda* is less pungent, it is possible to contain pungent compounds that trigger salivation.

Alteration in body weight during toxicological testing can be one of the markers of the adverse effects of the test material [27]. Food and water consumption is critical to physiological status of animals; therefore, the reduction of these parameters could indicate potential problems in physical status [28]. Throughout the investigation, the body weights of all animals increased without any significant difference. In some groups, there was a statistically significant difference in feed and drinking water consumption. Nonetheless, the increase or decrease was transient. The findings were unrelated to the doses of *B. rotunda* extract and had no effect on animal's health. These data demonstrate that the *B. rotunda* extract has no deleterious effect on the growth of animals. No neurological abnormality was observed in any of the animals. The difference in grip strength is not related to dose and no adverse event on animal's health were observed. The loss of grip strength could be a sign of muscle wasting or

Table 3 Effect of the *B. rotunda* extract on animal organ weight (g) per 100 g body weight.

Organ	Control	Dose level			Recovery group	
		Low	Medium	High	Control	High dose
Male						
Liver	3.1647±0.20	3.1699±0.22	3.2294±0.18	3.4229±0.11	2.9512±0.22	2.9489±0.14
Kidney R	0.2976±0.01	0.3021±0.01	0.3062±0.02	0.3091±0.01	0.3012±0.02	0.3006±0.02
Kidney L	0.2885±0.01	0.2980±0.01	0.2941±0.01	0.2917±0.01	0.2849±0.01	0.2850±0.01
Heart	0.3263±0.01	0.3284±0.01	0.3043±0.01*	0.3102±0.01	0.3023±0.01	0.3001±0.01
Spleen	0.2062±0.02	0.2003±0.02	0.2071±0.02	0.2010±0.01	0.2062±0.01	0.1879±0.00**
Brain	0.5251±0.03	0.5269±0.03	0.5425±0.03	0.5299±0.03	0.4982±0.03	0.5020±0.02
Adrenal R	0.0107±0.00	0.0118±0.00	0.0111±0.00	0.0123±0.00	0.0091±0.00	0.0092±0.00
Adrenal L	0.0118±0.00	0.0113±0.00	0.0124±0.00	0.0125±0.00	0.0103±0.00	0.0108±0.00
Testis R	0.4471±0.02	0.4586±0.03	0.4738±0.03	0.4896±0.04	0.4438±0.02	0.4388±0.02
Testis L	0.4509±0.02	0.4637±0.03	0.4788±0.03	0.4883±0.05	0.4405±0.03	0.4497±0.02
Epididymis Rt.	0.1275±0.00	0.1305±0.01	0.1328±0.01	0.1305±0.01	0.1298±0.00	0.1337±0.00**
Epididymis Lt.	0.1277±0.00	0.1291±0.01	0.1336±0.01	0.1302±0.01	0.1273±0.01	0.1328±0.01
Prostate Gland	0.1056±0.01	0.0944±0.01	0.1061±0.02	0.1007±0.01	0.0979±0.01	0.1047±0.01
Thymus	0.1025±0.01	0.1007±0.02	0.0954±0.01	0.0994±0.01	0.0873±0.01	0.0998±0.01
Thyroid and parathyroid glands R	0.0020±0.00	0.0019±0.00	0.0021±0.00	0.0023±0.00	0.0014±0.00	0.0016±0.00
Thyroid and parathyroid glands L	0.0021±0.00	0.0021±0.00	0.0021±0.00	0.0021±0.00	0.0017±0.00	0.0018±0.00
Pituitary gland	0.0031±0.00	0.0023±0.00	0.0028±0.00	0.0029±0.00	0.0025±0.00	0.0024±0.00
Female						
Liver	3.0140±0.16	3.0855±0.10	3.1797±0.13	3.1165±0.26	2.8470±0.25	2.9474±0.18
Kidney R	0.3177±0.02	0.3218±0.01	0.3159±0.01	0.3160±0.01	0.3077±0.02	0.3162±0.01
Kidney L	0.3061±0.01	0.3160±0.01	0.3188±0.02	0.2963±0.01	0.2790±0.01	0.2911±0.01
Heart	0.3623±0.02	0.3640±0.02	0.3546±0.01	0.3431±0.02	0.3368±0.03	0.3489±0.02
Spleen	0.2527±0.03	0.2659±0.04	0.2606±0.03	0.2601±0.03	0.2385±0.01	0.2591±0.02
Brain	0.8288±0.04	0.8578±0.03	0.8405±0.05	0.8202±0.01	0.7882±0.05	0.8378±0.04
Adrenal R	0.0216±0.00	0.0199±0.00	0.0228±0.00	0.0212±0.00	0.0214±0.00	0.0209±0.00
Adrenal L	0.0237±0.00	0.0222±0.00	0.0235±0.00	0.0229±0.00	0.0223±0.00	0.0217±0.00
Ovaries and oviduct R	0.0303±0.01	0.0296±0.00	0.0318±0.00	0.0323±0.00	0.0285±0.00	0.0310±0.00
Ovaries and oviduct L	0.0300±0.00	0.0312±0.00	0.0298±0.00	0.0324±0.01	0.0309±0.01	0.0310±0.00
Uterus	0.1756±0.05	0.1840±0.04	0.2261±0.07	0.1379±0.02	0.1672±0.04	0.1535±0.02
Thymus	0.1509±0.01	0.1480±0.02	0.1360±0.01	0.1492±0.02	0.1293±0.02	0.1250±0.01
Thyroid and parathyroid glands R	0.0030±0.00	0.0028±0.00	0.0026±0.00	0.0035±0.00	0.0017±0.00	0.0022±0.00
Thyroid and parathyroid glands L	0.0032±0.00	0.0031±0.00	0.0028±0.00	0.0036±0.00	0.0025±0.00	0.0029±0.00
Pituitary gland	0.0051±0.00	0.0047±0.00	0.0046±0.00	0.0049±0.00	0.0055±0.00	0.0052±0.00

Values are average ± standard deviation ($n = 10$, per group for male and female rats). * $p < 0.05$ levels of control group. ** $p < 0.05$ levels of control-recovery group.

motor neurotoxicity. The change of body weight or peripheral neuropathy can affect grip strength [29]. In the present study, the low dose of *B. rotunda* extract increased fore-limb grip strength plus the difference is not related to dose. The decrease of grip strength in male animals in the high dose-recovery group occurred during the recovery period, in which no extract had been given for one or two weeks. Because no adverse event on animal's health were observed, our results pointed out that this extract does not cause neurotoxicity.

Clinical pathology including hematological and clinical biochemistry testings are necessary in order to determine the toxicity induced by treatment [30]. The average counts of WBC in plasma were markedly higher in the low, medium, and high dose groups than those in the control group in both sexes. The *B. rotunda* extract showed an immunostimulant effect in tilapia [31]. It is possible that the *B. rotunda* extract could have the immunostimulant effect in mammals. The plasma levels of monocytes significantly increased in the medium and high dose groups in male rats; however, there was no change in the levels of neutrophils, eosinophils, basophils, and lymphocytes. Further studies on the effect of *B. rotunda* extract on WBC from the spleen to determine cell polarization and signaling pathways could yield a better understanding of this immunostimulant effect on monocytes.

Clinical biochemistry helps determine the toxicity in liver and kidneys [23]. To assess liver impairment, the enzymatic activity of liver function was determined. This included serum total protein, albumin, aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase activities. The results indicated that none of the doses used in this study resulted in any significant change in the serum levels of liver markers. Blood urea nitrogen and creatinine levels, markers to assess renal function, were similar between the treated and the control group. Clinical biochemistry analysis indicated that *B. rotunda* extract does not impair liver or kidney function. A previous study in rats showed hepatoprotective effect of panduratin A, one of

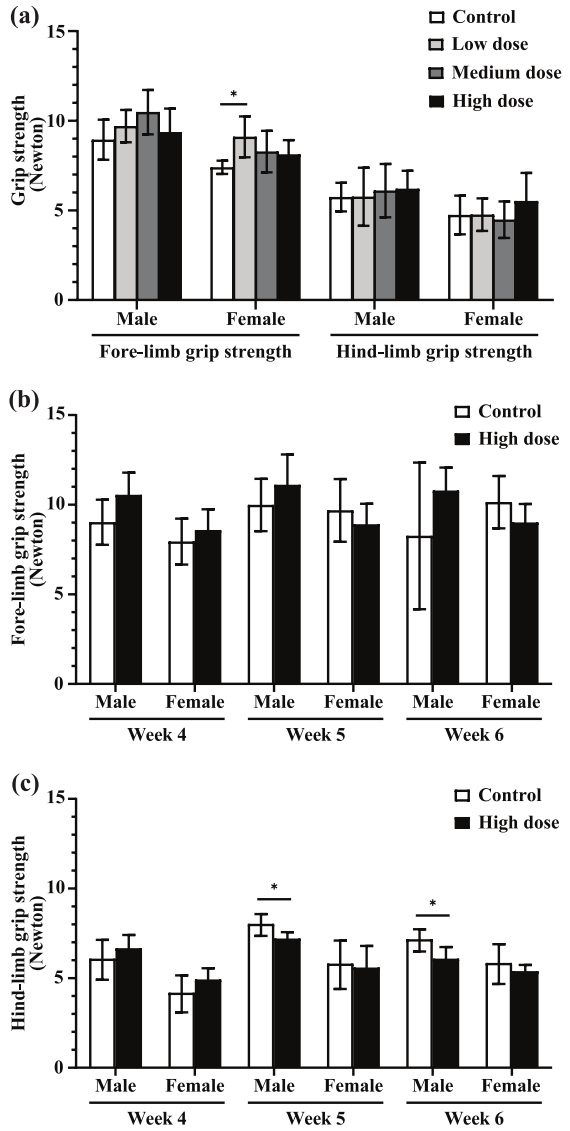

Fig. 2 Effects of the *B. rotunda* extract on fore-limb grip and hind-limb grip strength. (a) Fore-limb grip and hind-limb grip strength at week 4. (b) Fore-limb grip strength of recovery group at week 4, 5, and 6. (c) Hind-limb grip strength of recovery group at week 4, 5, and 6. Values are average ± standard deviation ($n = 10$). * $p < 0.05$ levels compared to the control group.

the main active components in the *B. rotunda* extract in thioacetamide-induced cirrhosis [32]. Pinocembrin, another main active component in the *B. rotunda* extract, did not alter the activities of cytochrome P450 and phase II xenobiotic-metabolising enzymes in rat liver [33]. The lack of hepatotoxicity and nephrotoxicity in the present study suggest the safety for subacute uses; however, the effect of *B. rotunda* extract on phase I and phase II xenobiotic-metabolising enzymes in human should be further investigated.

Anatomical pathology parameters were considered as a basic test in safety assessment of the test material [23]. The difference of organ weight in the *B. rotunda* extract-treated group was not related to doses and macroscopic and microscopic evaluation revealed no abnormality, indicating no organ toxicity. The hepatodiaphramatic nodule in one rat is likely a background lesion found in Wistar rats [34]. The basophilic and dilation tubule could occur spontaneously in Wistar rats [35]. These findings are considered to be an individual incidence and show no relationship with the test compound. No significant difference in severity grading median between the control group and the high dose group in both main group and recovery period group, indicated no delay occurrence or exacerbation of toxic effects [19]. No microscopic abnormality in lung tissues indicates the safety of *B. rotunda* extract. There was no difference in the weight of female reproductive organs, no disturbance of the synchronisation of the oestrous cycle, and no abnormal cell types from vaginal smears, indicating that *B. rotunda* extract had minimal endocrine effect.

The present findings about no notable toxicity are consistent with two previous studies using high doses of the *B. rotunda* extract at 2 and 5 g/kg/day for 15 days [12] and 60, 120, and 240 mg/kg for 60 days [21]. Pinostrobin and pinocembrin, major compounds in the *B. rotunda* extract, were found to have no adverse effect or behavioral abnormality when a single dose of 500 mg/kg or 100 mg/kg for 7 days was given [22]. In the present study, *B. rotunda* extract contained 5.68% w/w of panduratin A. The maximal test dose of the standardised *B. rotunda* extract was 600 mg/kg body weight; therefore, the maximal level of panduratin A was 34.08 mg/kg body weight. The limitation of the study is the water solubility of the *B. rotunda* extract due to the lipophilicity of components including panduratin A, pinostrobin, pinocembrin and volatile oils. High lipophilicity of plant extracts indicates poor aqueous solubility, which might lead to the poor oral bioavailability.

CONCLUSION

There were no signs of toxicity regarding clinical observation and health examination, hematological and clinical biochemistry testing, necropsy examination, and histopathology evaluation in sub-acute oral toxicity tests. It can be summarised that the *B. rotunda* extract had minimal toxicity in rats. Based on a 28-day repeated dose toxicity study, Wistar rats can tolerate the *B. rotunda* extract up to the dose of 600 mg/kg per day under experimental condition. *In vitro* activity against SARS-CoV-2 virus reported previously and no remarkable toxicity after daily consumption of *B. rotunda* extract for 28 days suggests the potential use for treating SARS-CoV-2 viral infection in *in vivo* models.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2024.057.

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Appendix A. Supplementary data

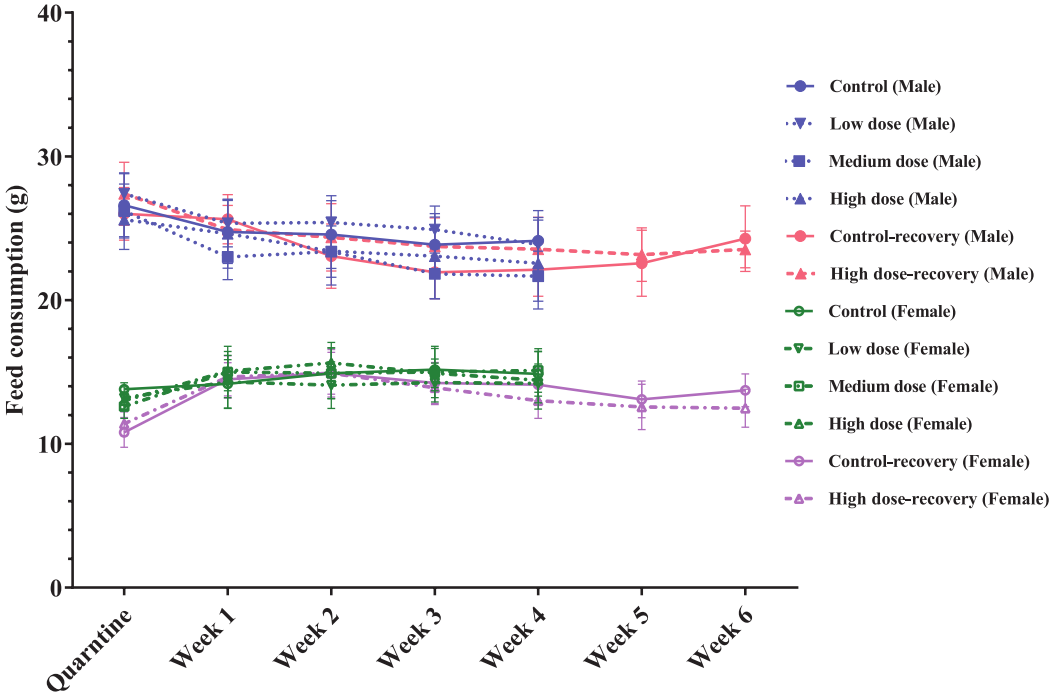

Fig. S1 Effect of the *B. rotunda* extract on average of animal feed consumption in male and female rats. Data are shown as average ± standard deviation ($n = 10$ per group).

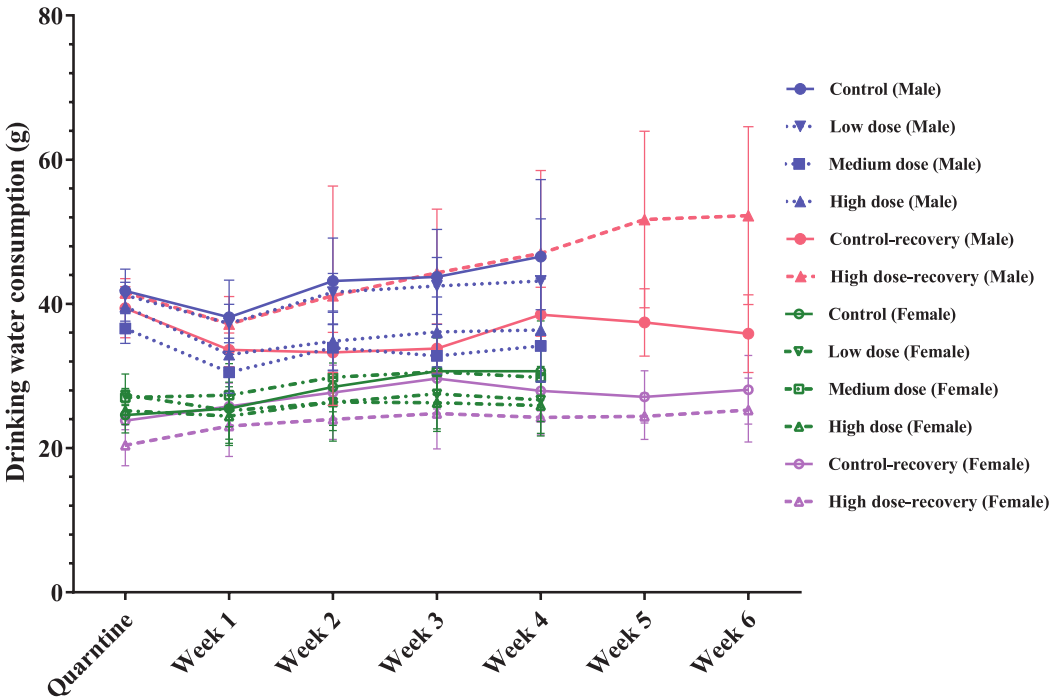

Fig. S2 Effect of the *B. rotunda* extract on average of drinking water consumption in male and female rats. Data are shown as average ± standard deviation ($n = 10$ per group).

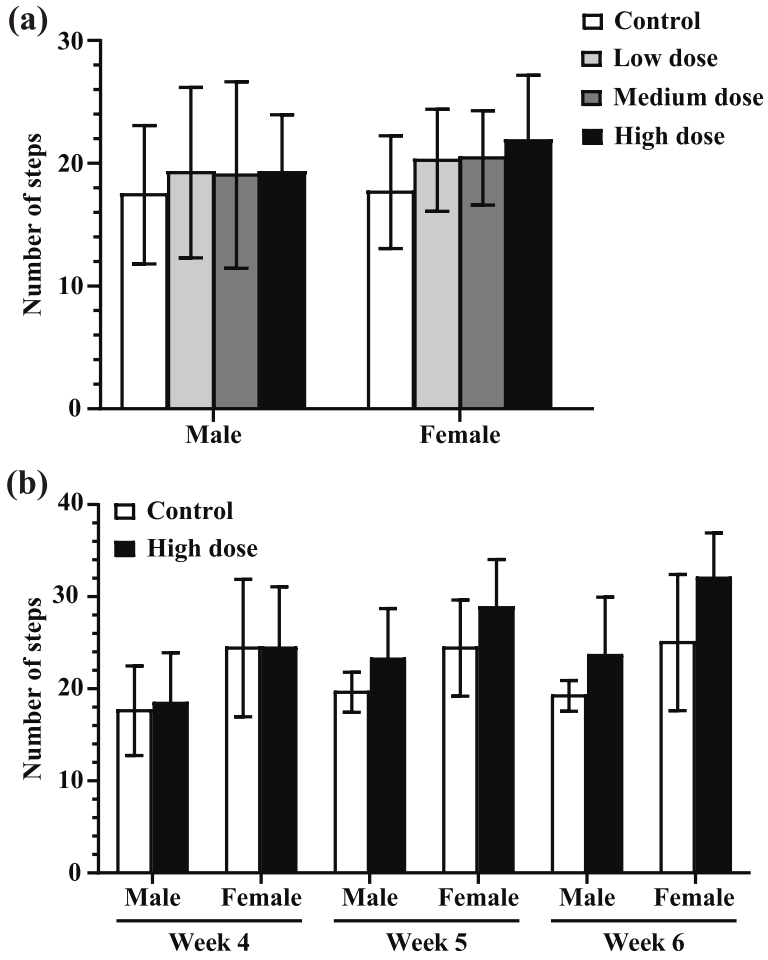

Fig. S3 Animal motor activity assessment (Number of steps) (a) at week 4 (b) recovery group at week 4, 5, and 6. Values are average ± standard deviation ($n = 10$, per group for male and female rats).

Table S1 Effect of the *B. rotunda* extract on vaginal cytology test result.

Vaginal cytology test	Control	Dose level			Recovery group	
		Low	Medium	High	Control	High dose
Stage						
Metestrus	0/5	2/5	0/5	0/5	0/5	1/5
Diestrus	5/5	3/5	0/5	5/5	1/5	4/5
Proestrus	0/5	0/5	5/5	0/5	4/5	0/5