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Assessment of Prenatal Developmental Toxicity Study of Bitter Melon Seed Extract from Supercritical Carbon Dioxide (scCO2) Extraction in Wistar Rats

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Abstract

Bitter melon (*Momordica charantia*) seed extracts exert hypoglycemic effects on animals and humans, but their safety and toxicological effects are still controversial in pregnant women. This study aims to assess the prenatal developmental toxicity of a bitter melon seed extract prepared from supercritical carbon dioxide ($scCO_2$) extraction in Wistar rats based on the Organization for Economic Cooperation and Development (OECD) guideline No. 414. The extract was administered orally from gestation day (GD) 5 to 19, through gavage, to 25 mated female rats per group at the dose levels of 0, 250, 500, and 1000 mg/kg body weight (BW)/day, and all rats were sacrificed on GD 20. There were no mortality, morbidity, and obvious clinical signs of toxicity observed in the control and treatment groups. The results of body weight, weight gain, reproductive parameters (e.g., live/dead foetuses, sex ratio, anogenital distance), fetal examination (i.e., external and visceral observations), organ weight, and thyroid hormone analysis (i.e., tri-iodothyronine (T3), thyroxine (T4), thyroid stimulating hormone (TSH)) in all treatment groups were comparable with those of the control group. The changes in the mean body weight at GD 20, carcass weight in the middle and high groups, and the number of unossified xiphisternum in the high group were considered treatment related but non-adverse. Moreover, gross pathological examination did not find any abnormal lesions in vital organs and thyroid gland. Accordingly, the no observed adverse effect level (NOAEL) of the bitter melon seed extract for maternal and developmental toxicity was determined to be 1000 mg/kg BW.

Introduction

The global prevalence of diabetes continues to aggravate annually, and 80% of adults with diabetes around the globe live in low-income and middle-income regions [1]. Diabetes and its derived complications (e.g., cardiovascular and kidney diseases) impose heavy economic burdens on the healthcare systems, and the global cost is projected to reach USD 2.1 trillion by 2030 [2]. Apart from conventional medical care, complementary and alternative medicines have recently provided an alternate route to help patients with diabetes to manage blood sugar levels [3].

Bitter melon is a popular ethnomedical plant for treating diabetes in traditional medicine [4]. Pharmacological studies have unveiled the hypoglycemic effect of bitter melon fruit, seed, and leave extracts in animals or humans [5–9]. Some active ingredients isolated from bitter melon seeds (e.g., charatin, momordin, polypeptide-p, polypeptide-k, mcIRBP-19), may play a similar role with insulin and have potential to be developed into anti-diabetic supplement [10–12]. Certain insulin-like peptides have demonstrated the effect of glycemic control on patients with diabetes, while bitter melon seeds are not commonly recognized as a dietary ingredient in most countries due to a lack of history of use and possible adverse effects (e.g., hemolytic anemia, stomach pain, coma, abortion, antifertility) [13–16]. The cytotoxicity, immunotoxicity, acute toxicity, developmental toxicity, and reproductive toxicity for the bitter melon seed extracts were observed in cell or animal models [17–24]. The organic extracts of bitter melon seeds led to histological alterations in the prostate and testes in rodents [20–22]. Uche-Nwachi *et al.* reported that a water extract of whole bitter melon incurred teratogenic effects in rats [23]. Farooq Khan *et*

al. reveled that a water extract of bitter melon seeds induced developmental anomalies in zebrafish embryos [24]. Nevertheless, to the best of our knowledge, the developmental toxicity of bitter melon seed extracts in rodents in compliance with the OECD test guidelines are unavailable in scientific reports.

In this study, we attempted to investigate the prenatal developmental oral toxicity of a bitter melon seed extract from $scCO_2$ extraction in Wistar rats based on the OECD guideline No. 414. We believe that our efforts may help to identify the safety concern of bitter melon seeds in pregnant women.

Materials And Methods

Preparation of the bitter melon seed extract

230 kg of bitter melon seeds were collected and washed with running and distilled water. The seeds were dehydrated by food dehydrators at 40°C and grounded with a blender. The dried bitter melon seeds were extracted with supercritical carbon dioxide (pressure: 26-27 MPa; temperature 50-53℃). Subsequently, the extract was evaporated by freeze drying for 3 days.

Housing and acclimatization

Throughout the experimental period, female Wistar rats (*Rattus norvegicus*) rats were housed individually except during the mating period. During acclimatization period, male rats were housed in groups (2 rats/cage). Rats were housed in a group of two rats/cage (one male plus one female). Mated female rats were housed individually. Enrichment material (wooden chew block) was provided to all rats. A total of 100 male and 120 female rats were obtained from the Jai Research Foundation and acclimated in the experimental room (temperature: 19-22℃; relative humidity: 55-66%; light cycle: 12 h light/dark) for a period of 7 days before the cohabitation.

The rats were fed *ad libitum* with a standard rodent pellete diet (Certified Teklad Global 14% Protein Rodent Diet, Batch No 2014SC-010621MA, procured from Envigo, Inc., USA) with an unlimited supply of drinking water in polypropylene bottles (capacity 250 mL). Drinking water was filtered through a reverse osmosis (RO) water filtration system.

Procedure for cohabitation and allocation

After the acclimatization period, female rats were cohabitated with untreated male rats (1:1) until the evidence of copulation. Detection of mating was confirmed by the evidence of a copulatory plug in the vagina or by a vaginal lavage for sperm. After confirmation of mating, the female rat was returned to an individual cage (assigned to a group), and the day was designated as day 0 of gestation (GD 0). Mated females were assigned to dose groups by stratified randomization based on GD 0 body weights. Mating of rats was conducted until the requisite number of mated females (25 females/group) were obtained.

Dose Administration

Different doses of the test article [control: 0 mg/kg BW; low: 250 mg/kg BW; middle: 500 mg/kg BW; high: 1000 mg/kg BW] prepared in RO water were administered to mated female rats once daily through gavage from the 5th day of gestation (GD 5) to the 19th day of gestation (GD19) at approximately the same time each day in the morning. The dose-volume for the administration was 10 mL/kg body weight. The doses were adjusted according to the most recently recorded body weight. Gavage was performed with a stainless-steel cannula attached to a syringe.

Body Weight

Body weights for the mated female rats were recorded individually on days 0, 3, 5, 8, 11, 14, 17, and 20 of gestation.

Thyroid Hormone Analysis

Serum thyroid hormones were analyzed from all surviving female rats at the terminal sacrifice. T3 and T4 were analyzed with the liquid chromatography tandem-mass spectrometry (LC-MS/MS) technique and TSH was analyzed by the ELISA method.

Necropsy

On day 20 of gestation, all surviving female rats were weighed, euthanised by carbon dioxide asphyxiation, dissected and examined macroscopically. The non-gravid uteri were further examined by staining with 5% ammonium sulphide to confirm non-pregnant status. Ovaries and gravid uteri, including the cervix, were removed, and examined immediately.

Organ weight and histopathology

At the time of terminal sacrifice, the thyroid gland was collected and weighed (post-fixation) from all female rats and preserved for histopathology. Detailed histological examination of the thyroid gland was performed in all female rats.

Foetus allocation

Allocation of foetuses for the skeletal and soft tissue evaluation was performed by selecting alternate live foetus. Each litter's first live fetus was allocated to skeletal, and the second live foetus was allocated to soft tissue evaluation.

Foetus observations

Definitions of the foetal findings. Malformation: major abnormal structural change considered detrimental to the animal (may also be lethal). Variation: minor abnormal structural change considered to have little or no detrimental effect on the animal; may be transient and may occur relatively frequently in the control population.

External evaluation

All foetuses (100%) were examined externally. External foetal sex (as determined by gross examination) was compared with internal (gonadal) sex in all foetuses (examined for both skeletal and soft tissue malformations).

Soft tissue evaluation

Approximately 50% of the foetuses in each litter were subjected to the detailed visceral evaluation by microdissection. The procedure for evaluation of soft tissue was performed with the Staple's technique.

Skeletal evaluation

The remaining approximately 50% of the foetuses in each litter were subjected to the detailed skeletal evaluation after processing and staining with alizarin red. Foetuses were preserved in isopropyl alcohol and glycerin solution. The skeletal evaluation was performed with the Staples and Schnell method.

Good laboratory practice

The study was undertaken in compliance with the guidelines of the "Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International" and "Guidelines for Laboratory Animals Facility" issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. The project proposal for the experimentation was approved by the "Institutional Animal Ethics Committee (IAEC)", JRF.

Statistical analysis

Data were processed to get mean values and standard deviations (S.D.) with significance between the control and experimental groups. Non-pregnant female rats and female rats with complete resorption were excluded from statistical analysis appropriately.

Kolmogorov-Smirnov test or Shapiro–Wilk test were used to normality check and decide parametric or non-parametric data. Based on the outcome of homogeneity of variance (F-test, Bartlett's test, Levene's test, or Brown–Forsythe test), the significance of parametric data [body weight, body weight change/gain, food consumption, carcass weight, uterine weights, organ weight, male sex ratio/male percent, pre-and postnatal loss (%), live foetus (%), and resorptions (%), ano-genital distance (normalized)] was determined by Student's t-test, Satterthwaite method, or analysis of variance (ANOVA) with Dunnett's test. The significance of non-parametric data [rreproductive performancee, foetal observations, litter size, number of corpora lutea, number of implants, number of live foetus, number of dead foetus, number of pre-and postnatal losses, number of resorptions] was decided by Kruskal–Wallis test with Dunnett's test, Mann-Whitney U test, or Chi-square test. The significance was considered $p < 0.05$.

Results

No mortality, morbidity, and obvious clinical signs of toxicity observed in the control and experimental groups. There were 23, 25, 23, and 22 female rats got pregnant in the control, low, middle, and high dose groups, respectively, and 23, 24, 22, and 22 females in the corresponding groups had live fetuses (Table 1).

Effect of the extract on body weight and food consumption

Table 2 shows the results of body weights and body weight gains of all groups. The mean body weights at GD 20 in all experimental groups were comparable with the result of the control group. The mean body weight gain of the pregnant female rats at 250 mg/kg BW was comparable with that of the control group, whereas the body weight gains in the middle and high dose groups at GD 5-8 were significantly depressed in comparison with the change of the control group. Food consumption of all groups during the treatment period was consistent with the lower body weight gains (data not shown). The food consumption changes in the middle and high dose groups at GD 5-20 were decreased by 13.6% and 16.7%, respectively, relative to the control group.

Effect of the extract on fetuses

Prenatal observation did not indicate any serious abnormality. The mean numbers of corpora lutea, implants, pre-implantation loss, total resorption, live and dead fetuses, fetal males and females, and post-implantation loss in all treated groups were comparable with those of the control groups (Table 3). In addition, there were no significant findings in uterus weight, litter weight, and anogential distance in all groups. The carcass weights in the middle and high dose groups were decreased by approximately 6% relative to the result control group, which was considered a significant change.

Table 4 reveals the results of the external, visceral, and skeletal examinations for each litter. A total of 255, 269, 256, and 249 foetuses were examined in the control, low, middle, and high dose groups, respectively. The % litter and % fetal data of runt were comparable for the treatment groups with the control group. A significant decrease in the % litter effected with haemetoma was observed in foetuses of the high dose group. A total of 124, 128, 120, and 119 foetuses were examined in the control, low, middle, and high dose groups, respectively. There were no treatment-related visceral anomalies observed in foetuses in all experimental groups. Some spontaneous findings in dilated ureter (nine foetuses from the control and five foetuses from the middle group) and convoluted ureter (four foetuses in control group) were observed. Moreover, the incidences of skeletal malformations were not observed in all experimental groups, while there was a significant decrease in the number of foetuses with unossified xiphisternum in the middle group.

Effect of the extract on structural abnormalities of the dams

External and internal gross examination of terminally sacrificed female rats did not reveal any obvious pathological lesions in vital organs although congenital lesions, such as ectopic thymic tissue and ultimobranchial cyst, were recorded in a few female rats across the groups (data not shown). The

absolute weights of the experimental groups were comparable with the result of the control group (Table 5), and the results of thyroid hormone analysis and histopathological assessment corresponded with the negative findings (Table 6, Figure S1). In accordance with the study results, the NOAEL of the extract of bitter melon seeds extracted by $scCO_2$ for maternal and developmental toxicity was determined to be 1000 mg/kg BW.

Discussion

This present study firstly reveals the prenatal developmental toxicity of the bitter melon seed extract form $scCO_2$ extraction in Wistar rats in compliance with the OECD guideline No. 414. In the previous studies, most extracts of bitter melon seeds relied on water or organic extraction rather than supercritical fluid extraction [17–24]. Different extraction methods yield distinctive chemical profiles in botanical extracts [25]. $scCO_2$ extraction is recognized a highly safe and selective extraction technology, and it avoids the concern of toxic residues and reduces the risk of thermal degradation in products [26].

There were no mortality and notable clinical signs and toxic effects observed in all groups during this study. The decreases in the mean body weight at GD 20 and carcass weight were considered treatment related but non-adverse because the changes were less than 6% as compared with those of the control group. Body weight change within 10% is not considered adverse [27]. The lower body weight gains at GD 5–8 did not correspond with food consumption and recovered towards the end of gestation. Thus, the effects were considered incidental and did not have any toxicological relevance. The decreases in the food consumption were also considered treatment related but non-adverse since the changes in food consumption were not correlated with the body weight losses.

There were no statistical differences in the reproductive parameters and measures of fetal development between the control and experimental groups. The treatment-related skeletal and soft tissue malformations were no observed in this study. Some groups reported that a water extract of bitter melon seeds might result in malformed litters and congenital malformations, but the applied dosage was equivocal in this study [23]. The male sex ratios in the experimental groups were similar with the result of the control group, which suggests that the extract may not disrupt male sexual differentiation and interfere with the physiological action of either androgens or estrogens during foetal development [28]. Anogenital distance can used as an indicator for the reproductive toxicity, and shorter anogenital distance in males is connected to cryptorchidism, hypospadias, reduced sperm count, and infertility [29]. The results of anogential distance for the extract did not have positive findings. Moreover, the obvious findings in haemetoma in the high dose group and the number of unossified xiphisternum in the middle group were considered incidental and not treatment-related effects.

The gross pathological examination did not find any abnormal lesions in vital organs, so the histologic examination was only conducted in thyroid gland. It has been reported that the teratogenic and lethal effects of the water extract of bitter melon seeds were observed in zebrafish embryos [24]. The discrepancy may be contributed to the differences of study model and extraction method. Thyroid

hormones regulate several biological processes essential for growth, metabolism as well as brain maturation [30]. The extract did not affect the function of thyroid gland as evidenced by the fact that the levels of TSH, T3 and T4, thyroid gland weights, and microscopic examination of thyroid gland did not reveal significant findings. As such, the NOAEL of the extract was greater than 1,000 mg/kg BW.

Conclusion

In short, this exploring study unveils the negative findings in the prenatal developmental oral toxicity of the bitter melon seed extract from $scCO_2$ extraction in rats. Although past studies indicate the developmental toxicity of bitter melon seeds in rodent and zebrafish models, the reported toxicological effects were not observed in this research. It is challenging for us to objectively compare our results with others in light of the differences in the extraction method, the bitter melon used, and the study model. Nevertheless, we believe that our efforts can provide a difference view to the safety concern of bitter melon seeds in pregnant women.

Declarations

Declaration of Conflicting Interests

The authors declare no conflict of interest.

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Tables

Table 1. Reproductive performance.

	Control	250 mg/kg	500 mg/kg	1000 mg/kg
No. total females	25	25	25	25
No. pregnant females	23	25	23	22
No. non-pregnant females	2	0	2	3
No. pregnant with total litter loss	0	1	1	0
No. females with live fetuses	23	24	22	22

Table 2. Results of body weights and wight gains in rats treated with the bitter melon seed extract (mean value ± S.D.).

	Control (*n* = 23)	250 mg/kg (*n* = 24)	500 mg/kg (*n* = 22)	1000 mg/kg (*n* = 22)
Body weight				
GD 0	235.67 ± 12.98	241.23 ± 14.45	231.15 ± 11.14	234.10 ± 13.91
GD 20	330.45 ± 20.30	334.28 ± 21.81	316.45 ± 20.13	316.60 ± 25.22
Weight gain				
GD 0–3	10.45 ± 3.68	8.40 ± 4.01	9.61 ± 3.73	8.00 ± 3.18
GD 3–5	4.85 ± 2.37	6.38 ± 2.97	5.37 ± 2.29	4.45 ± 2.40
GD 5–8	6.79 ± 2.88	5.48± 3.85	2.97 ± 6.82*	0.37 ± 10.32**
GD 8–11	11.71 ± 4.06	10.98 ± 3.49	6.98 ± 8.02	8.13 ± 9.77
GD 11–14	10.98 ± 4.07	11.21 ± 3.11	11.20 ± 4.30	11.20 ± 4.10
GD 14–17	21.57 ± 4.93	22.34 ± 4.89	22.39 ± 4.02	22.12 ± 5.64
GD 17–20	28.43 ± 5.71	28.27 ± 6.77	26.78 ± 7.21	28.23 ± 6.14
GD 5–20	79.48 ± 14.52	78.26 ± 13.19	70.32 ±15.13	70.05 ± 18.71

Note: *n* indicated the sample number; *, $p < 0.05$; **, $p < 0.01$; the significance was compared with the control group.

Table 3. Reproductive parameters in rats treated with the bitter melon seed extract (mean value ± S.D.).

	Control (*n* = 23)	250 mg/kg (*n* = 25)	500 mg/kg (*n* = 23)	1000 mg/kg (*n* = 22)
No. corpora lutea	13.30 ± 1.72	13.16 ± 1.28	13.48 ± 1.65	12.95 ± 1.36
No. implantation sites	11.30 ± 2.49	11.68 ± 1.75	11.96 ± 2.01	11.36 ± 1.99
No. pre-implantation loss	2.00 ± 2.26	1.48 ± 1.78	1.52 ± 1.59	1.59 ± 1.68
No. total resorption	0.22 ± 0.52	0.88 ± 2.62	0.83 ± 2.69	0.05 ± 0.21
No. Fetal sex females	5.65 ± 2.27	5.72 ± 1.99	5.83 ± 2.35	5.50 ± 2.15
No. Fetal sex males	5.43 ± 2.29	5.04 ± 2.42	5.30 ± 1.96	5.82 ± 1.92
Male percent (%)	49.31 ± 15.93	42.87 ± 18.96	46.23 ± 17.50	51.86 ± 15.45
No. dead fetuses	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
No. live fetuses	11.09 ± 2.59	10.76 ± 3.33	11.13 ± 3.06	11.32 ± 1.96
No. post-implantation loss	0.22 ± 0.52	0.92 ± 2.61	0.83 ± 2.69	0.05 ± 0.21
Uterus weight (mg)	57.53 ± 11.43	56.40 ± 15.93	56.77 ± 14.11	58.82 ± 10.47
Anogential distance (mm)	2.06 ± 0.20	2.01 ± 0.22	2.04 ± 0.20	2.10 ± 0.24
Litter wieht (g)	36.34 ± 7.65	37.71 ± 5.68	38.12 ± 5.70	38.08 ± 7.66
Carcass weight (g)	272.671 ± 16.38	272.90 ± 20.85	258.24 ± 19.63**	257.41 ± 19.99**

Note: *n* indicated the sample number; **, $p < 0.01$; the significance was compared with the control group.

Table 4. Fetal observations in rats treated with the bitter melon seed extract (mean value ± S.D.).

	Control	250 mg/kg	500 mg/kg	1000 mg/kg
No. litters/No. fetuses examined				
External observations	23/255	24/269	22/256	22/249
Visceral observations	23/124	24/128	22/120	22/119
Skeleton observations	23/131	24/141	22/136	22/130
No. litters/No. fetuses affected (% litters/%fetuses affected in the group)				
External observations				
Haematoma	7/9 (30/3.60)	5/6 (21/2.15)	2/2 (9/0.79)	1/1 (5*/0.35)
Small (runt)	3/4 (13/1.30)	0/0 (0/0)	0/0 (0/0)	2/2 (9/0.87)
Visceral observations				
Convoluted ureter	1/1 (4/0.62)	0/0 (0/0)	0/0 (0/0)	0/0 (0/0)
Dilated ureter	2/2 (9/2.54)	0/0 (0/0)	1/1 (5/1.14)	0/0 (0/0)
Skeleton observations				
Frontal bone				
Incomplete ossification (bilateral)	1/1 (4/0.72)	0/0 (0/0)	0/0 (0/0)	0/0 (0/0)
Interparietal bone: incomplete ossification				
Incomplete ossification	2/3 (4/0.72)	0/0 (0/0)	0/0 (0/0)	0/0 (0/0)
Parietal bone				
Incomplete ossification (bilateral)	2/2 (9/1.27)	0/0 (0/0)	0/0 (0/0)	0/0 (0/0)
Incomplete ossification (unilateral)	1/1 (4/0.62)	0/0 (0/0)	0/0 (0/0)	0/0 (0/0)
Premaxilla				
Incomplete ossification (bilateral)	1/2 (4/1.09)	0/0 (0/0)	0/0 (0/0)	0/0 (0/0)
Pubis				
Unossified (left)	1/1	0/0 (0/0)	0/0 (0/0)	0/0 (0/0)

	(4/0.54)			
Ribs				
14th rib: extra ossification center (bilateral)	5/6 (22/4.08)	4/4 (17/2.68)	2/2 (9/1.52)	4/4 (18/2.92)
14th rib: extra ossification center (left)	6/13 (26/8.83)	10/15 (42/10.79)	9/10 (41/7.08)	11/15 (50/10.98)
14th rib: extra ossification center (right)	12/17 (52/15.47)	10/13 (42/9.12)	8/12 (36/9.16)	9/10 (41/7.18)
Full supernumerary (bilateral)	2/2 (9/1.35)	0/0 (0/0)	2/2 (9/1.41)	4/4 (18/3.12)
Full supernumerary (left)	2/3 (9/2.07)	0/0 (0/0)	2/2 (9/1.30)	1/1 (5/0.57)
Full supernumerary (right)	2/2 (9/1.35)	0/0 (0/0)	2/2 (9/1.30)	3/3 (14/1.87)
Short supernumerary (bilateral)	9/12 (39/9.95)	7/17 (29/4.80)	8/11 (36/8.51)	6/9 (27/6.31)
Short supernumerary (left)	7/10 (30/6.77)	5/5 (21/3.41)	10/11 (45/8.78)	6/9 (27/5.82)
Short supernumerary (right)	6/7 (26/5.52)	4/4 (17/2.72)	7/8 (32/5.67)	5/6 (22/3.99)
Vertebrae				
Misaligned axis	1/1 (4/0.87)	0/0 (0/0)	1/1 (5/1.14)	0/0 (0/0)
Sternum				
3rd sternebrae: misaligned	3/4 (13/3.42)	5/6 (21/3.77)	5/6 (23/4.33)	4/6 (18/4.68)
4th sternebrae: misaligned	7/8 (30/6.25)	6/9 (25/5.85)	9/11 (41/4.28)	6/9 (27/6.73)
4th sternebrae: unossified	2/3 (9/1.81)	1/1 (4/1.04)	0/0 (0/0)	0/0 (0/0)
4th sternebrae: incomplete ossification	1/1 (4/0.87)	1/1 (4/0.69)	0/0 (0/0)	0/0 (0/0)
5th sternebrae: dumbbell ossification	1/1 (4/0.72)	1/1 (4/0.69)	2/2 (9/1.41)	1/1 (5/0.65)
5th sternebrae: incomplete ossification	16/35 (70/26.45)	22/53 (92/38.86)	19/46 (86/32.94)	17/34 (77/25.65)
Xiphisternum	12/17 (52/11.51)	6/6 (25/4.80)	4/9 (18*/5.84)	5/8 (23/6.68)

Note: *, $p < 0.05$; the significance was compared with the control group.

Table 5. Terminal body and organ weights in rats treated with the bitter melon seed extract (mean value ± S.D.).

	Control (*n* = 23)	250 mg/kg (*n* = 24)	500 mg/kg (*n* = 22)	1000 mg/kg (*n* = 22)
Terminal body weight (g)	330.20 ± 20.33	334.01 ± 21.83	316.16 ± 20.28	316.24 ± 25.28
Weight of thyroid gland (mg)	18.63 ± 3.095	18.95 ± 3.584	19.13 ± 3.822	17.81 ± 3.044

Note: *n* indicated the sample number.

Table 6. Thyroid hormone analysis in rats treated with the bitter melon seed extract (mean value ± S.D.).

	Control (*n* = 23)	250 mg/kg (*n* = 25)	500 mg/kg (*n* = 23)	1000 mg/kg (*n* = 22)
T3 (ng/mL)	0.429 ± 0.098	0.438 ± 0.081	0.408 ± 0.092	0.401 ± 0.1
T4 (ng/mL)	24.286 ± 5.775	23.843 ± 4.927	21.502 ± 04.629	21.064 ± 5.424
TSH (ng/mL)	0.975 ± 0.381	0.894 ± 0.377	0.894 ± 0.393	1.033 ± 0.346

Note: *n* indicated the sample number.

Supplementary Files

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