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A subchronic oral toxicity study of *Salacia reticulata* extract powder in rats

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ABSTRACT

The safety of *Salacia* plant (*Salacia reticulata*) extract powder, which is used in Ayurvedic medical practices, was studied in a dose range-finding subchronic toxicity study in Crl:CD Sprague–Dawley rats. Male and female rats were randomly assigned to 4 treatment groups and were treated by oral gavage with 0, 10, 65, and 400 mg/kg body weight/day of the powder for 91 days. Body weight, food consumption, and clinical signs were assessed during the treatment period. Urinalysis, hematology, blood chemistry, and organ weights were determined one day after the final treatment. The animals were euthanized at the end of the treatment and were examined for necropsy and histopathological purposes. No adverse toxicity was observed in the *Salacia* powder-treated groups with a No Observed Adverse Effect Level of ≧400 mg/kg body weight/day in both male and female SD rats.

1. Introduction

Salacia is a genus of climbing plants of the Hippocrateaceae family and is widely distributed throughout Southeast Asia, including India, Sri Lanka, and Thailand. Extracts of *Salacia* species (such as *Salacia reticulata* and *Salacia oblonga*) contain salacinol, kotalanol, neosalacinol, neokotalanol, mangiferin, and catechin, and have been used in the Ayurvedic alternative medical practice to treat the symptoms of diseases, such as rheumatism and diabetes, for many years [1–3]. Recently, *Salacia* was reported to be a potential obesity treatment [4–6]. Typical components extracted from *Salacia* plants (salacinol, kotalanol, neosalacinol, and neokotalanol) inhibit α-glucosidase activity and increase blood glucose levels in rats and humans [1,7–10]. We previously reported that the *Salacia* plant extract might regulate intestinal immunity by altering the intestinal flora in rats [11].

S. reticulata is widely available in Japan. A water-based extraction process is used to produce *Salacia* extracts with an established safety profile [12]. *Salacia* plant extract powder has been tested in various safety studies, including acute toxicity, mutagenicity, and hepatotoxicity studies [13–16]. *S. reticulata* and *S. oblonga* powders developed by water-ethanol extraction have been investigated in subchronic toxicity studies, but none of these have used *S. reticulata* water extract powder. To determine if the subchronic use of *Salacia* powder derived from a water extraction process is safe, we carried out a dose range-finding subchronic toxicity study following 91 days of treatment at 0, 10, 65, or 400 mg/kg body weight/day.

2. Materials and methods

All animal use protocols were reviewed and approved by the Fuji Film Animal Experiment Committee. The study was conducted following the principles outlined in the 28-day repeat-dose toxicity study in mammalian animals described in the "Study methods for novel chemical substances" (Notification No. 1121002 of PFSB, 21st November 2003, MIB No. 2 and Notification No. 031121002 of EPB/EHD/PPB, 13th November 2003) and the OECD 408 guideline "Repeated Dose 90-day Oral Toxicity – Study in Rodents" (21st September 1998). The study was not conducted according to the requirements of Good Laboratory Practice, but was conducted in accordance with the Criteria for Reliability of Application Data (Article 43 of the Enforcement Regulations, Pharmaceutical Affairs Law, Japan).

2.1. Test materials and diet

S. reticulata trunks and roots were harvested in Sri Lanka by Eco Tech Create 21, Co. Ltd. (Sri Lanka) and purchased from Eco Tech

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Create 21, Co. Ltd. (Tokyo, Japan). The harvested *S. reticulata* trunks and roots were botanically compared with a reference specimen archived in a botanical specimen room at the Industrial Technology Institute in Sri Lanka using the TLC fingerprint method. *S. reticulata* trunks and roots were dried and chipped, and the compounds were extracted in boiled water for 1 h. The chips were removed by filtration, and the extract was cooled, powdered using a Spray Dryer ADL-310 (Yamato Science Co., Ltd., Tokyo, Japan), and stored at 4 °C [11]. The resulting powder was suspended in water at 0, 10, 65, and 400 mg/kg body weight/day oral gavage doses (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). The dosing volume was 5 mL/kg body weight. The *Salacia* plant extract consists of 60.9% carbohydrate including fiber, 17.4% polyphenol, 14.7% ash, 3.7% water, 2.6% protein, 0.7% lipids, and approximately 1.1% thiosugars (e.g., salacinol and kotalanol) [1,2].

2.2. Animals

Five-week-old male and female Sprague–Dawley (SD) rats (Charles River Laboratories Japan, Inc., Kanagawa, Japan) were quarantined/acclimated for 1 week prior to experimentation. The rats were kept under the following conditions: a room temperature of 23 °C ± 2 °C, relative humidity of 55% ± 10% (49.9–57.0%), air exchange frequency of 15 times/h, and a 12-h light/dark cycle. The rats were fed irradiated solid food (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and UV-sterilized, ultrafiltered (50 and 5 μm, AION Co., Ltd., Osaka, Japan) tap water that complied with the water quality standards pursuant to the Waterworks Act (Ordinance No. 101 of the Ministry of Health, Labor and Welfare). Food and water were provided ad libitum. Male and female rats (males: 190–211 g, females: 150–181 g) having favorable growth rates and without any clinical signs during the quarantine period were selected for this study (n = 10 females and males/treatment group). After acclimatization, the rats were randomly distributed into four treatment groups according to body weight using special grouping software (Dr. WinG; Human Life, Tokyo, Japan).

2.3. Experimental procedures

2.3.1. Animal dosing

The rats were assigned to four treatment groups: a control (distilled water; Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) group and three *Salacia* plant extract-treated groups, which received 10, 65, or 400 mg/kg body weight/day of the extract by oral gavage once-daily. According to the pharmacokinetic data, the active compounds (e.g., salacinol and kotalanol) were not absorbed by the rats (data not shown). We therefore referred to the Food for Specified Health Use (FOSHU, Japan) for the dose selection and chose a maximum dose (400 mg/kg body weight/day) hundred-fold of the oral dose (240 mg/day) used in humans [7].

2.3.2. Clinical observations

The rats were checked twice daily for mortality, signs of toxicity, and changes in general health and behavior. The body weights were measured with an electronic balance (PM4000; Mettler-Toledo, Greifensee, Switzerland) 3 times a week in study weeks 1 and 2, and 2 times a week after study week 3. Food consumption was measured weekly. Water consumption was measured at study weeks 1 and 13.

2.3.3. Clinical pathology

Urine was collected using metabolic cages (Shinano Manufacturing, Tokyo, Japan) on Day 85 (10:30–14:30). Urinalysis was performed with fresh urine for pH, protein, glucose, ketone bodies, urobilinogen, bilirubin, occult blood, nitrite, and urobilinogen analysis using Ames N-Multistix urinalysis strips (Bayer Medical Ltd., Tokyo, Japan) and an Ames Clinitek 100 urine analyzer (Bayer Medical Ltd., Tokyo, Japan). Twenty-four-hour urine was used for the assessment of color and cloudiness. The specific gravity was measured using a refractometer (URICON-JE; Atago, Co. Ltd., Tokyo, Japan). The urine volume was determined by adding one volume of fresh urine to the value calculated from the urine weight and specific gravity.

After the last dose administration and prior to necropsy, blood samples were collected via the posterior aorta under deep anesthesia for hematology and blood chemistry analysis. The hematological parameters assessed included white blood cell (WBC) counts, red blood cell (RBC) counts, hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), reticulocyte ratio (Reti), prothrombin time (PT), activated partial thromboplastin time (APTT), total protein concentration (TP), albumin concentration (Alb), A/G, glucose (Glu), triglyceride (TG), total cholesterol (T-CHO), blood urea nitrogen concentration (BUN), creatinine (Cre), calcium (Ca^{2+}), inorganic phosphorus (IP), aspartate aminotransferase (AST) activity, alanine aminotransferase (ALT) activity, serum creatine phosphokinase activity (CPK), total bilirubin (T-BIL), sodium (Na^+), potassium (K^+), and chloride (Cl^-). WBC, RBC, HGB, HCT, MCV, MCH, MCHC, PLT, and Reti were measured using the hematology analyzer XT-2000iV (Sysmex Co., Ltd., Hyogo, Japan) with plasma obtained by centrifugation for 10 min at 3000 rpm after adding the anticoagulant EDTA-2K to the blood. PT and APTT were measured using the automated coagulation analyzer (STA Compact; Roche Diagnostics K.K. Tokyo, Japan) using plasma obtained by centrifugation for 10 min at 3000 rpm after adding the anticoagulant sodium citrate. TP, ALB, A/G, Glu, TG, T-CHO, BUN, Cre, Ca^{2+}, IP, AST, ALT, GGT, ALP, CPK, T-BIL, Na^+, K^+, and Cl^- were measured using the automated biochemical analyzer H 7070 (Hitachi Ltd., Tokyo, Japan) with plasma obtained by centrifugation for 10 min at 3000 rpm after adding the anticoagulant sodium heparin.

2.3.4. Gross pathology and histopathology

After blood collection, the rats were euthanized under pentobarbital sodium anesthesia (1 mL/kg body weight, intraperitoneally (ip)), and the following tissues were collected for macroscopic and microscopic examination: the skin (ventral), liver, spleen, pancreas, kidneys, adrenals, urinary bladder, testes/epididymis/seminal vesicle/prostate (male) or ovary/uterus/vagina (female), submandibular gland, sublingual gland, cervical lymph node, axillary lymph node, trachea, thyroid and parathyroid glands, thymus, heart, aorta, lungs, gastrointestinal tract from the tongue to anus, mesenteric lymph nodes, femoral muscle, sciatic nerve, femur and bone marrow, auricle (bilateral), brain, spinal cord, pituitary gland, eyes, Harderian gland, and sternum. The testes were fixed in a mixture of formalin, glacial acetic acid, and distilled water for 1 day, and were stored in 10% neutral buffered formalin solution. All other organs were fixed and stored in 10% neutral buffered formalin solution. After organ weight measurement, the left lung was fixed in 10% neutral buffered formalin. Prior to fixation, the actual organ weight (absolute weight) of the brain, pituitary gland, thymus, lung, liver, kidneys, spleen, heart, adrenals, testes, epididymis, seminal vesicle, prostate (ventral lobe), ovary, and uterus were measured using an electronic balance AE-200 (PM4000; Mettler-Toledo, Greifensee, Switzerland) to calculate the organ weight ratio to body weight (relative weight). The organs were subsequently fixed in 10% neutral buffered formalin. Thin, 4 μm-thick sections were prepared from the liver, kidneys, heart, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, and rectum from all animals, and the sections were stained with hematoxylin–eosin (H&E) and were histopathologically examined by light microscopy.

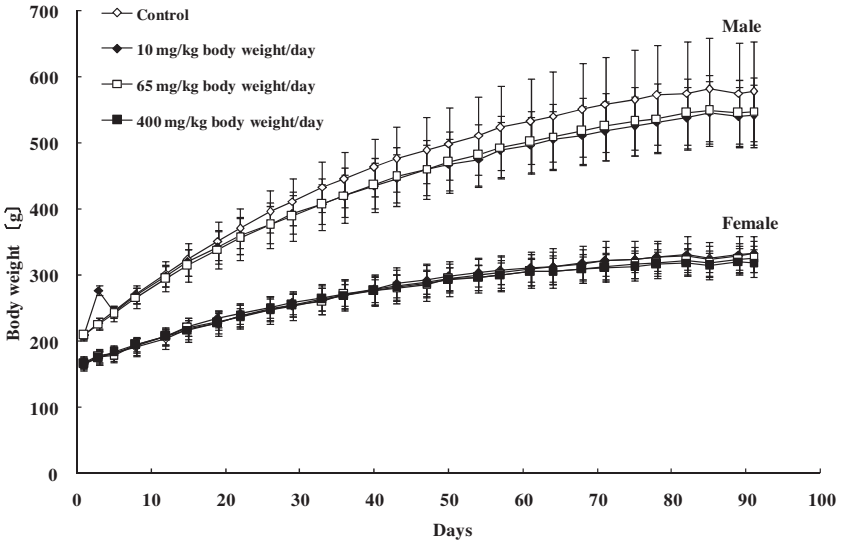

Fig. 1. Body weights of male and female rats treated with *Salacia* extract powder via oral gavage for 91 days.

2.4. Statistical analyses

The body weight, organ weights (absolute and relative weights), food consumption, water intake, and the urinalysis, hematology, and blood biochemistry values were measured for each rat in each group. First, Bartlett's test for homogeneity of variance was performed. If the variance was homogeneous, one-way analysis of variance was performed and significant results were tested using Dunnett's test. If the variance was not homogeneous, the Kruskal–Wallis rank test was applied and the rank test using Dunnett's method was used to determine significance. Statistical calculations were performed using data processing software (Dr. WinG; Human Life, Tokyo, Japan). The clinical signs, urinalysis point scale data, anatomical observation results, and histopathology results were not analyzed using statistical tests.

3. Results

3.1. Body weights

In all treatment groups, the body weight change was comparable to that of the control group in both male and female rats (Fig. 1, Table 1).

3.2. Food and water intake

Water intake significantly decreased from day 5 to 6 in the males in the 10-mg/kg body weight/day group. There was no change in food consumption in males in the treatment groups compared to that of the males in the control group. No differences in food consumption or water intake were observed in the females in any treatment group compared to that of the females in the control group (Figs. 2 and 3, Tables 2 and 3).

3.3. Clinical observations

No deaths or changes in clinical signs were observed in male or female rats in any of the treatment groups for the duration of the study. In males in the 400-mg/kg body weight/day group, soft stools were observed for 6 rats on day 87 and for 2 rats on day 88. For 1 female rat in the 65-mg/kg body weight/day group, no feces were observed from the previous day on day 85. No changes in environmental factors that might have affected the reliability of the data were observed.

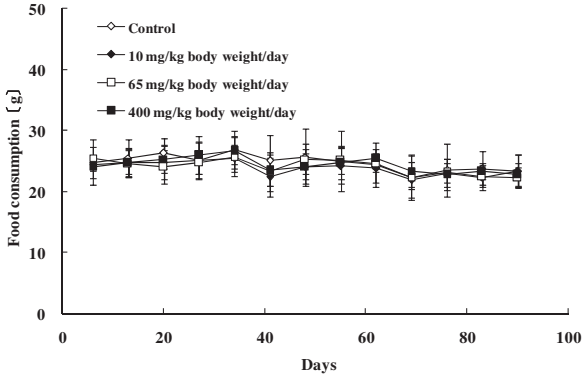

Fig. 2. Food consumption of male rats treated with *Salacia* extract powder via oral gavage for 91 days.

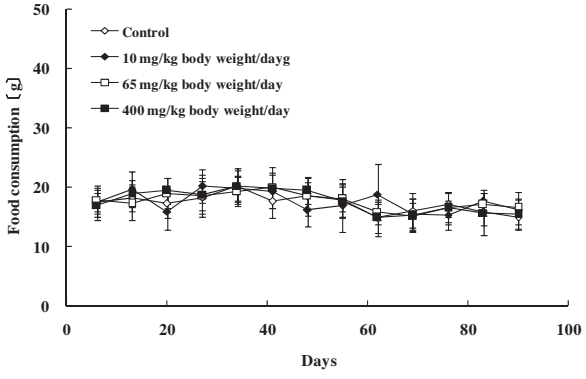

Fig. 3. Food consumption of female rats treated with *Salacia* extract powder via oral gavage for 91 days.

3.4. Clinical pathology

3.4.1. Urinalyses

Treatment did not alter the tested urinalysis parameters in both the male and female rats in any of the treatment groups. However,

Table 1
Body weights of male and female rats in each Salacia extract powder treatment group.

	Dose (mg/kg body weight/day)	Day	1	3	5	8	12	15	19	22	26	29	33	36
Male	0	Mean	209.0	228.0	245.2	270.8	302.5	323.5	351.3	370.9	396.0	411.4	433.6	445.0
		S.D.	5.5	8.1	8.9	13.2	19.3	23.8	28.9	30.3	32.2	35.7	38.6	42.1
	10	Mean	209.3	277.1	244.5	269.2	298.3	319.7	341.6	359.2	376.6	392.5	407.4	420.3
		S.D.	6.6	7.8	9.4	11.5	16.4	20.0	24.8	26.5	27.9	28.9	30.1	31.7
	65	Mean	208.5	225.8	241.0	265.9	294.0	314.3	338.6	355.8	375.5	389.3	406.7	420.6
		S.D.	6.7	8.8	11.5	15.2	19.4	24.7	29.0	32.3	35.4	36.6	38.8	41.5
	400	Mean	209.0	225.2	241.1	265.8	294.4	315.9	338.5	356.9	376.5	390.3	410.6	422.5
		S.D.	6.2	8.2	10.9	13.4	20.4	21.5	25.4	28.4	32.3	36.6	39.2	39.5
Female	0	Mean	166.3	175.2	181.6	190.8	203.5	217.3	227.3	237.9	247.5	253.1	262.1	269.9
		S.D.	10.2	10.5	12.3	13.2	14.5	17.6	19.6	18.7	21.3	21.0	22.0	23.1
	10	Mean	167.3	177.6	182.2	192.2	206.4	221.8	233.8	241.1	251.4	258.3	265.9	271.5
		S.D.	7.4	10.7	9.3	12.6	12.3	12.9	13.1	14.7	16.3	17.6	20.2	22.8
	65	Mean	165.6	176.4	178.0	194.3	207.3	220.3	229.6	236.7	248.0	254.6	260.6	270.8
		S.D.	8.2	7.9	9.1	10.0	11.4	11.9	13.2	14.0	16.7	18.0	19.5	20.5
	400	Mean	167.6	174.7	183.1	194.8	207.6	217.0	227.4	237.8	248.5	254.6	263.3	270.0
		S.D.	7.9	9.6	8.6	11.2	13.4	14.6	16.4	17.4	18.6	17.0	17.8	17.0

	Dose (mg/kg body weight/day)	Day	40	43	47	50	54	57	61	64	68	71	75	78	82	85	89	91
Male	0	Mean	462.8	475.8	488.9	499.0	511.5	523.2	532.4	540.5	550.1	558.7	565.4	572.3	575.2	580.9	575.1	578.2
		S.D.	43.3	48.6	50.4	54.5	59.0	63.5	65.1	67.8	70.9	72.1	74.8	75.7	77.6	77.8	76.1	76.0
	10	Mean	435.0	446.0	459.6	467.7	475.0	489.2	497.2	504.8	511.3	517.9	525.5	531.8	537.4	546.1	539.1	541.2
		S.D.	34.7	36.0	38.0	39.1	38.7	41.8	42.4	43.4	44.3	44.9	44.3	45.3	47.1	47.1	46.1	47.8
	65	Mean	436.4	449.2	460.4	470.8	481.2	493.4	501.5	509.6	518.2	524.8	533.0	536.8	545.1	548.4	545.6	548.0
		S.D.	41.4	44.2	44.6	46.3	46.8	47.3	47.5	50.2	50.4	51.4	51.6	52.9	52.5	53.8	50.6	51.1
	400	Mean	437.1	450.0	463.3	473.0	480.6	495.2	501.1	510.3	517.6	524.1	530.0	539.3	541.7	549.9	540.6	540.9
		S.D.	43.1	44.6	46.5	47.4	49.1	49.4	51.1	50.9	52.5	53.0	54.5	55.5	53.9	54.0	54.0	54.2
Female	0	Mean	277.4	283.7	289.4	294.7	300.3	303.5	308.3	313.6	318.4	322.0	324.1	327.4	331.5	324.7	331.5	332.2
		S.D.	23.7	27.3	28.6	25.9	26.9	27.3	26.6	27.1	26.3	30.0	28.2	25.3	26.7	25.6	26.9	27.4
	10	Mean	277.8	287.4	293.3	297.6	303.4	307.1	311.2	312.4	316.1	321.5	323.3	327.3	328.4	324.2	329.0	333.1
		S.D.	22.2	21.3	21.9	19.9	20.4	21.3	21.5	22.4	22.1	21.8	19.3	20.1	19.9	20.4	20.0	19.6
	65	Mean	276.3	280.6	286.3	294.1	296.8	299.5	306.1	305.8	310.0	313.5	316.3	318.4	321.6	317.7	323.0	324.6
		S.D.	20.7	20.2	20.7	18.6	19.3	22.4	20.9	24.5	21.9	20.1	20.5	20.0	20.2	20.2	20.9	20.3
	400	Mean	276.2	281.5	287.7	292.1	296.4	300.9	304.6	305.8	308.7	310.9	312.4	315.6	318.8	314.1	320.5	318.6
		S.D.	16.9	18.8	19.9	19.2	19.6	19.1	20.7	19.9	20.2	21.3	21.1	20.1	20.5	20.4	20.1	21.1

Table 2
Food consumption of male and female rats in each Salacia extract powder treatment group.

	Dose (mg/kg body weight/day)	Day	6	13	20	27	34	41	48	55	62	69	76	83	90
Male	0	Mean	24.8	25.5	26.3	25.1	26.8	25.1	25.7	25.0	24.4	22.3	23.5	23.7	23.4
		S.D.	3.7	3.0	2.5	3.0	3.1	4.2	4.7	4.9	3.7	3.6	4.3	2.9	2.7
	10	Mean	24.3	24.8	24.7	25.1	25.4	22.4	24.1	24.2	23.8	21.9	23.0	22.3	23.3
		S.D.	0.9	1.9	2.7	3.1	2.2	3.2	2.1	2.9	2.4	3.0	2.3	2.0	2.7
	65	Mean	25.5	24.6	24.0	24.7	25.7	23.3	25.3	25.1	24.6	22.2	23.1	22.4	22.3
		S.D.	1.8	2.3	2.8	1.9	3.2	3.2	2.5	2.2	1.3	1.8	1.6	1.3	1.6
	400	Mean	24.0	24.7	25.3	26.0	26.7	23.5	24.1	24.7	25.4	23.4	22.8	23.3	22.8
		S.D.	1.8	2.4	2.3	3.1	2.3	2.5	2.8	2.7	1.6	2.6	2.5	1.3	1.9
Female	0	Mean	17.4	18.1	17.2	18.2	20.1	17.5	18.4	17.7	14.8	16.0	17.0	15.7	14.8
		S.D.	1.6	2.3	2.5	3.2	3.0	2.7	2.4	2.7	2.6	2.9	2.1	3.8	1.1
	10	Mean	17.4	19.5	15.8	20.1	19.7	19.2	16.1	16.8	18.7	15.3	15.2	17.5	16.1
		S.D.	2.4	3.0	3.0	2.8	2.2	2.9	2.8	4.5	5.1	2.8	2.4	1.4	3.1
	65	Mean	17.8	17.2	18.9	18.5	19.2	20.0	18.4	17.9	15.8	15.0	16.5	17.0	16.5
		S.D.	2.4	2.8	2.6	2.4	2.4	2.3	3.2	2.1	1.4	2.3	2.4	1.9	1.6
	400	Mean	16.9	18.8	19.3	18.7	20.2	19.8	19.3	17.6	14.8	15.2	16.4	15.6	15.3
		S.D.	2.5	2.3	2.1	3.3	2.5	3.5	2.2	2.9	3.1	2.8	2.7	2.2	2.6

Table 3
Water consumption of male and female rats in each Salacia extract powder treatment group (n = 10/sex).

	Dose (mg/kg body weight/day)	Day	6	90	
Male	0	Mean	38.2	46.0	(ml/day)
		S.D.	5.1	11.7	
	10	Mean	32.2	37.6	
		S.D.	5.3*	10.3	
	65	Mean	33.8	40.9	
		S.D.	3.9	12.3	
	400	Mean	36.3	40.9	
		S.D.	3.5	7.1	
Female	0	Mean	27.6	30.1	
		S.D.	5.8	3.6	
	10	Mean	27.6	34.7	
		S.D.	6.3	11.7	
	65	Mean	26.2	34.8	
		S.D.	6.9	8.3	
	400	Mean	26.6	30.0	
		S.D.	6.4	6.8	

* Significantly different from the controls ($p < 0.05$).

Table 4
Urine volume of male and female rats in each Salacia extract powder treatment group ($n = 10$/sex).

Dose (mg/kg body weight/day)	Male				
	(mL/day)	0	10	65	400
Volume		11.5 ± 7.1	9.4 ± 3.3	14.1 ± 5.4	9.9 ± 3.2
Specific gravity		1.043 ± 0.017	1.043 ± 0.013	1.036 ± 0.019	1.041 ± 0.014
Dose (mg/kg body weight/day)	Female				
	0	10	65	400	
Volume	(mL/day)	12.3 ± 4.1	12.7 ± 5.4	12.1 ± 3.7	12.5 ± 4.1
Specific gravity		1.041 ± 0.010	1.040 ± 0.014	1.040 ± 0.013	1.043 ± 0.015

the urine volume could not be accurately measured for 2 females in the 400-mg/kg body weight/day group due to spillage (Tables 4–7).

3.4.2. Hematology/blood biochemistry

Treatment did not alter hematological parameters in both the males and females in any of the treatment groups. However, a significantly lower CPK activity was observed in males in the 400-mg/kg body weight/day group, and significantly higher Glu levels and lower T-BIL concentrations were observed in the females in this dose group (Tables 8 and 9).

3.4.3. Organ weights

We observed a significantly lower absolute testis weight in males in the 10-mg/kg body weight/day group and a significantly higher relative spleen weight in females in the 65-mg/kg body weight/day group (Tables 10–13).

3.4.4. Anatomical observations/histopathology

Macroscopic anatomical observations revealed the presence of liver cysts in 1 female in the 10-mg/kg body weight/day group. Pyelectasis in the right kidney and diffuse white spots in the kidney cross section were observed in 1 female in the 400-mg/kg body weight/day group.

Histopathological examinations revealed that the liver cysts in 1 female in the 10-mg/kg body weight/day group observed at necropsy were due to mild hepatocellular vacuolation. No additional histological changes were observed in the rat with pyelectasis and diffuse white spots. Other changes observed included mineral deposits in Peyer's patches of the jejunum,

Table 5
Urinalysis of male and female rats in each Salacia extract powder treatment group ($n = 10$/sex).

Dose (mg/kg body weight/day)		Male				Female			
		0	10	65	400	0	10	65	400
Color	Light yellow	5	7	7	5	6	4	7	7
	Yellow	5	3	3	5	4	5	2	3
	Light brown	0	0	0	0	0	1	1	0
	Brown	0	0	0	0	0	0	0	0
	Dark brown	0	0	0	0	0	0	0	0
	Red	0	0	0	0	0	0	0	0
Tubidity	−	4	4	4	2	2	2	3	1
	−	3	4	6	4	4	6	5	2
	+	3	2	0	4	4	2	2	6
	++	0	0	0	0	0	0	0	1
Glucose	−	10	10	10	10	10	10	10	10
	±	0	0	0	0	0	0	0	0
	+	0	0	0	0	0	0	0	0
	++	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0
Bilirubin	−	9	10	9	7	10	10	10	9
	+	1	0	1	3	0	0	0	1
	++	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0
Ketones	−	1	0	1	0	7	6	4	4
	±	1	1	4	3	2	4	6	6
	+	6	8	5	3	0	0	0	0
	++	2	1	0	4	0	0	0	0
	+++	0	0	0	0	0	0	0	0
pH	5.0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0
	6.0	0	0	0	0	0	2	2	0
	6.5	0	0	0	0	0	1	0	0
	7.0	0	0	0	0	0	0	2	0
	7.5	0	0	0	1	2	0	3	1
	8.0	0	0	0	0	1	1	0	0
	8.5	3	3	3	0	6	5	2	4
	≧9.0	7	7	7	9	1	1	1	5
Protein	−	0	0	0	0	4	3	4	1
	±	0	0	2	0	3	2	0	2
	+	3	1	3	2	1	4	4	5
	++	6	9	5	5	2	1	2	2
	+++	1	0	0	3	0	0	0	0
Urobilinogen	0.1	8	4	7	2	8	7	5	5
	1	2	6	3	8	2	3	5	5
	2	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0
	>8	0	0	0	0	0	0	0	0
Nitrite	−	10	10	9	10	10	10	10	10
	+	0	0	1	0	0	0	0	0
Occult blood	−	10	10	10	10	10	10	9	10
	±	0	0	0	0	0	0	0	0
	+	0	0	0	0	0	0	1	0
	++	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0
Cystals	−	7	7	8	9	8	10	10	9
	±	3	3	0	0	2	0	0	1
	+	0	0	1	1	0	0	0	0
	++	0	0	1	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0

Table 6
Urinary spermatozoa of male rats in each Salacia extract powder treatment group ($n = 10$).

Dose (mg/kg body weight/day)	Male			
	0	10	65	400
Spermatozoa				
−	2	4	5	5
±	4	5	2	2
+	2	1	3	2
++	2	0	0	1
+++	0	0	0	0

Table 7
Urinary bacteria of female rats in each Salacia extract powder treatment group ($n = 10$).

Dose (mg/kg body weight/day)	Female			
	0	10	65	400
Bacteria				
−	10	9	10	10
±	0	0	0	0
+	0	1	0	0
++	0	0	0	0
+++	0	0	0	0

Table 8
Hematology of male and female rats treated with Salacia extract powder for 91 days ($n = 10$/sex).

Dose (mg/kg body weight/day)		Male				Female			
		0	10	65	400	0	10	65	400
Leukocytes	($10^3/\mu L$)	7.827 ± 2.701	7.405 ± 2.268	7.172 ± 1.872	6.474 ± 2.339	4.503 ± 2.035	4.847 ± 2.098	4.027 ± 1.768	4.173 ± 1.421
Erythrocytes	($10^6/\mu L$)	9.103 ± 0.523	9.150 ± 0.396	9.169 ± 0.176	8.848 ± 0.318	8.211 ± 0.426	8.157 ± 0.431	7.975 ± 0.408	7.826 ± 0.367
Hemoglobin	(g/dL)	15.24 ± 0.49	15.54 ± 0.58	15.51 ± 0.55	15.41 ± 0.56	14.98 ± 0.70	15.00 ± 0.66	14.62 ± 0.65	14.75 ± 0.83
Hematocrit	(%)	46.43 ± 1.01	47.43 ± 1.28	47.06 ± 1.26	46.28 ± 1.67	45.49 ± 1.93	45.52 ± 1.67	44.23 ± 1.64	44.66 ± 2.18
MCV	(fL)	51.15 ± 2.86	51.91 ± 2.24	51.34 ± 1.63	52.38 ± 3.12	55.49 ± 2.53	55.90 ± 3.07	55.52 ± 2.07	57.08 ± 1.56
MCH	(pg)	16.77 ± 0.57	17.00 ± 0.39	16.91 ± 0.65	17.45 ± 1.05	18.24 ± 0.36	18.40 ± 0.73	18.35 ± 0.38	18.84 ± 0.44
MCHC	(g/dL)	32.84 ± 1.08	32.78 ± 1.06	32.96 ± 0.83	33.31 ± 0.82	32.94 ± 1.04	32.95 ± 1.06	33.06 ± 0.85	33.03 ± 0.73
PLT	($10^3/\mu L$)	1063.4 ± 163.5	1090.4 ± 104.4	1033.4 ± 90.5	1000.4 ± 110.2	1010.1 ± 88.6	1036.6 ± 91.0	1022.4 ± 93.9	1006.0 ± 84.0
Lymphocytes	(%)	82.1 ± 3.6	83.3 ± 1.8	79.0 ± 5.1	79.5 ± 5.4	79.8 ± 6.2	82.2 ± 3.9	79.0 ± 4.1	79.5 ± 4.4
Neutrophils	(%)	12.7 ± 3.9	11.9 ± 1.9	16.1 ± 4.5	15.3 ± 5.0	15.5 ± 5.5	13.4 ± 3.6	16.5 ± 3.2	15.3 ± 4.5
Eosinophils	(%)	1.9 ± 0.6	1.6 ± 0.5	1.8 ± 0.6	1.9 ± 0.9	1.7 ± 0.5	1.8 ± 0.4	1.8 ± 0.4	2.1 ± 0.6
Monocytes	(%)	3.3 ± 1.3	3.4 ± 1.0	3.2 ± 1.0	3.4 ± 1.1	3.2 ± 1.2	3.0 ± 0.9	3.2 ± 0.8	3.5 ± 1.1
Basophils	(%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Reticulocytes	(%)	2.57 ± 0.19	2.59 ± 0.40	2.44 ± 0.30	2.76 ± 0.51	2.83 ± 0.48	2.78 ± 0.53	3.07 ± 0.51	2.89 ± 0.31
PT	(s)	26.39 ± 9.59	25.16 ± 7.24	23.36 ± 6.63	28.06 ± 7.53	15.67 ± 0.48	15.20 ± 0.85	15.85 ± 0.61	15.39 ± 0.50
APTT	(s)	30.55 ± 4.50	33.64 ± 6.10	28.94 ± 4.53	30.83 ± 4.05	26.33 ± 5.57	24.06 ± 3.16	22.53 ± 2.38	23.64 ± 2.06

Table 9
Blood chemistry of male and female rats treated with Salacia extract powder for 91 days ($n = 10$/sex).

Dose (mg/kg body weight/day)		Male				Female			
		0	10	65	400	0	10	65	400
TP	(g/dL)	6.03 ± 0.33	6.01 ± 0.30	6.09 ± 0.26	6.19 ± 0.44	6.96 ± 0.60	6.98 ± 0.40	6.66 ± 0.31	6.80 ± 0.35
ALB	(g/dL)	3.05 ± 0.14	3.09 ± 0.14	3.04 ± 0.11	3.06 ± 0.21	3.92 ± 0.34	3.97 ± 0.25	3.72 ± 0.22	3.88 ± 0.18
A/G	(−)	1.028 ± 0.076	1.061 ± 0.056	1.000 ± 0.070	0.988 ± 0.126	1.296 ± 0.110	1.323 ± 0.093	1.269 ± 0.106	1.339 ± 0.136
Glu	(mg/dL)	165.1 ± 20.8	158.8 ± 22.0	154.8 ± 20.2	163.5 ± 19.7	134.7 ± 15.1	143.8 ± 14.6	136.5 ± 16.3	155.6 ± 15.5*
BUN	(mg/dL)	17.29 ± 1.42	17.79 ± 2.69	16.03 ± 2.82	15.21 ± 3.66	20.95 ± 3.69	16.98 ± 2.92	18.17 ± 2.93	18.66 ± 3.15
Cre	(mg/dL)	0.320 ± 0.076	0.294 ± 0.038	0.274 ± 0.045	0.260 ± 0.054	0.508 ± 0.108	0.455 ± 0.091	0.421 ± 0.031	0.401 ± 0.076
Ca^{2+}	(mg/dL)	9.54 ± 0.28	9.52 ± 0.20	9.60 ± 0.14	9.60 ± 0.32	10.04 ± 0.35	10.13 ± 0.34	10.12 ± 0.45	10.16 ± 0.25
IP	(mg/dL)	6.80 ± 0.53	6.56 ± 0.75	6.53 ± 0.53	6.34 ± 0.74	6.48 ± 1.89	6.19 ± 1.24	6.19 ± 1.19	5.87 ± 0.77
T-CHO	(mg/dL)	52.0 ± 12.7	48.9 ± 10.4	54.7 ± 8.5	58.4 ± 13.5	71.7 ± 18.8	73.1 ± 14.2	71.2 ± 18.2	79.8 ± 15.4
TG	(mg/dL)	49.9 ± 33.6	47.7 ± 29.6	45.5 ± 16.5	60.1 ± 22.1	19.2 ± 11.0	23.7 ± 8.9	19.8 ± 9.6	33.5 ± 23.9
T-BIL	(mg/dL)	0.232 ± 0.049	0.223 ± 0.064	0.240 ± 0.053	0.211 ± 0.067	0.226 ± 0.036	0.214 ± 0.025	0.207 ± 0.030	0.179 ± 0.030**
ALP	(U/L)	242.5 ± 41.6	258.5 ± 52.0	239.3 ± 27.8	227.3 ± 40.5	99.2 ± 23.4	108.4 ± 29.5	108.1 ± 21.2	97.3 ± 30.2
AST	(U/L)	61.0 ± 11.7	59.0 ± 4.5	55.5 ± 8.5	54.8 ± 6.8	92.9 ± 98.9	68.6 ± 25.1	72.6 ± 17.6	78.8 ± 25.1
ALT	(U/L)	24.6 ± 3.9	24.3 ± 6.2	22.8 ± 3.4	22.7 ± 4.9	28.6 ± 25.4	25.9 ± 14.1	29.3 ± 13.6	30.2 ± 13.2
GGT	(U/L)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	0.9 ± 0.3	1.0 ± 0.5
CPK	(U/L)	330.5 ± 300.7	280.1 ± 288.6	169.1 ± 36.5	151.1 ± 80.4*	316.0 ± 193.8	376.5 ± 295.3	462.7 ± 728.1	373.0 ± 224.5
Na^+	(mEq/L)	141.66 ± 2.21	141.64 ± 1.80	142.42 ± 1.51	142.80 ± 1.92	142.59 ± 2.05	142.95 ± 1.69	144.16 ± 1.98	144.07 ± 2.05
K^+	(mEq/L)	5.742 ± 1.136	5.888 ± 1.083	5.743 ± 1.103	5.983 ± 1.230	5.347 ± 0.966	5.377 ± 1.141	4.984 ± 1.071	5.221 ± 1.117
Cl^-	(mEq/L)	104.42 ± 1.56	103.40 ± 1.62	103.91 ± 1.30	103.93 ± 1.32	105.48 ± 1.81	106.13 ± 1.37	105.52 ± 1.24	105.82 ± 1.00

* Significantly different from the controls ($p < 0.05$).
** Significantly different from the controls ($p < 0.01$).

hepatocellular vacuolation and microgranuloma in the liver, and basophilic tubular epithelia, hyaline casts, eosinophilic bodies, and focal lymphocytic infiltration in the kidneys. However, these changes were also observed in the control group animals and were not considered test agent-related toxicities according to their incidence rates.

4. Discussion

In this study, a *Salacia* plant extract powder suspension was evaluated in a dose range-finding subchronic toxicity study following a 91-day oral administration regimen in male and female Crl:CD SD rats. Male and female Crl:CD SD rats (40 rats each, 6

Table 10
Absolute organ weights in male rats treated with Salacia extract powder for 91 days ($n = 10$).

Dose (mg/kg body weight/day)		Male			
		0	10	65	400
Final body weight	(g)	551.4 ± 73.3	515.8 ± 45.6	520.7 ± 51.1	515.2 ± 52.3
Hypophysis	(mg)	10.9 ± 2.7	10.1 ± 1.2	10.0 ± 1.4	9.1 ± 1.6
Adrenals	(mg)	60.5 ± 13.0	57.5 ± 8.9	53.1 ± 6.1	59.9 ± 9.5
Spleen	(mg)	768.5 ± 135.0	705.0 ± 75.5	734.8 ± 92.9	731.6 ± 147.6
Thymus	(mg)	289.4 ± 82.5	299.9 ± 38.9	275.0 ± 94.2	241.5 ± 51.8
Lungs	(mg)	1343.9 ± 108.5	1387.9 ± 148.8	1305.4 ± 63.3	1309.7 ± 147.0
Kidneys	(mg)	3068.2 ± 388.0	2881.9 ± 276.4	3009.1 ± 288.5	3047.0 ± 317.2
Testes	(mg)	3440.2 ± 91.1	3200.9 ± 220.7*	3359.3 ± 227.3	3430.5 ± 242.6
Brain	(mg)	2005.5 ± 93.9	2046.3 ± 56.6	1997.5 ± 101.1	2000.2 ± 129.7
Liver	(g)	14.141 ± 2.384	12.803 ± 1.625	13.077 ± 1.752	14.103 ± 1.627
Heart	(mg)	1523.7 ± 252.4	1460.7 ± 176.8	1417.9 ± 88.2	1414.3 ± 136.9
Epididymis	(mg)	1275.4 ± 100.5	1237.7 ± 156.1	1223.7 ± 89.4	1277.9 ± 115.8
Prostate	(mg)	649.1 ± 210.2	603.5 ± 154.4	588.6 ± 160.7	538.6 ± 150.1
Seminal vesicle	(mg)	1614.7 ± 270.5	1696.9 ± 358.4	1693.9 ± 258.5	1598.9 ± 361.5

* Significantly different from the controls ($p < 0.05$).

Table 11
Absolute organ weights in female rats treated with Salacia extract powder for 91 days ($n = 10$).

Dose (mg/kg body weight/day)		Female			
		0	10	65	400
Final body weight	(g)	315.9 ± 25.1	313.9 ± 18.4	307.6 ± 19.6	303.2 ± 18.9
Hypophysis	(mg)	15.1 ± 2.7	14.1 ± 2.0	14.9 ± 1.7	14.4 ± 1.7
Adrenals	(mg)	62.2 ± 6.9	66.9 ± 6.9	70.5 ± 5.5	65.8 ± 11.0
Ovaries	(mg)	80.6 ± 15.1	89.3 ± 17.9	91.7 ± 15.5	84.3 ± 8.5
Uterus	(mg)	634.0 ± 286.9	679.1 ± 266.3	730.9 ± 238.1	635.1 ± 162.6
Spleen	(mg)	496.4 ± 90.2	467.2 ± 71.4	597.7 ± 154.3	478.2 ± 109.7
Thymus	(mg)	269.2 ± 73.9	294.8 ± 85.8	283.4 ± 64.9	271.2 ± 47.0
Lungs	(mg)	1038.5 ± 72.5	1009.6 ± 79.2	1043.0 ± 92.2	1053.2 ± 121.4
Kidneys	(mg)	1803.2 ± 220.2	1769.7 ± 146.2	1901.6 ± 146.1	1826.9 ± 203.8
Brain	(mg)	1924.0 ± 106.3	1831.8 ± 104.0	1908.3 ± 106.8	1870.3 ± 69.7
Liver	(g)	7.595 ± 0.890	7.456 ± 0.500	7.720 ± 0.608	7.688 ± 0.878
Heart	(mg)	926.2 ± 82.9	919.6 ± 70.6	927.9 ± 55.6	898.5 ± 68.2

Table 12
Relative organ weights in male rats treated with Salacia extract powder for 91 days ($n = 10$).

Dose (mg/kg body weight/day)		Male			
		0	10	65	400
Hypophysis	(%)	0.0020 ± 0.0004	0.0020 ± 0.0002	0.0019 ± 0.0003	0.0018 ± 0.0004
Adrenals	(%)	0.0110 ± 0.0021	0.0111 ± 0.0015	0.0103 ± 0.0014	0.0118 ± 0.0023
Spleen	(%)	0.141 ± 0.028	0.138 ± 0.019	0.142 ± 0.018	0.142 ± 0.024
Thymus	(%)	0.052 ± 0.011	0.058 ± 0.008	0.053 ± 0.017	0.047 ± 0.010
Lungs	(%)	0.245 ± 0.018	0.270 ± 0.023	0.252 ± 0.021	0.255 ± 0.020
Kidneys	(%)	0.559 ± 0.048	0.560 ± 0.037	0.581 ± 0.063	0.594 ± 0.059
Testes	(%)	0.634 ± 0.085	0.623 ± 0.048	0.652 ± 0.089	0.673 ± 0.090
Brain	(%)	0.369 ± 0.051	0.400 ± 0.040	0.386 ± 0.034	0.392 ± 0.045
Liver	(%)	2.561 ± 0.235	2.479 ± 0.209	2.510 ± 0.220	2.744 ± 0.237
Heart	(%)	0.276 ± 0.026	0.284 ± 0.025	0.275 ± 0.032	0.276 ± 0.029
Epididymis	(%)	0.237 ± 0.047	0.242 ± 0.036	0.238 ± 0.036	0.251 ± 0.039
Prostate	(%)	0.122 ± 0.048	0.117 ± 0.029	0.113 ± 0.030	0.106 ± 0.033
Seminal vesicle	(%)	0.299 ± 0.074	0.332 ± 0.079	0.328 ± 0.060	0.317 ± 0.091

Table 13
Relative organ weights in female rats treated with Salacia extract powder for 91 days ($n = 10$).

Dose (mg/kg body weight/day)		Female			
		0	10	65	400
Hypophysis	(%)	0.0048 ± 0.0007	0.0045 ± 0.0006	0.0049 ± 0.0006	0.0048 ± 0.0005
Adrenals	(%)	0.0198 ± 0.0027	0.0214 ± 0.0030	0.0230 ± 0.0021	0.0216 ± 0.0030
Ovaries	(%)	0.0257 ± 0.0056	0.0287 ± 0.0066	0.0298 ± 0.0048	0.0278 ± 0.0025
Uterus	(%)	0.201 ± 0.092	0.217 ± 0.086	0.238 ± 0.076	0.210 ± 0.056
Spleen	(%)	0.157 ± 0.025	0.149 ± 0.020	0.194 ± 0.046*	0.157 ± 0.029
Thymus	(%)	0.085 ± 0.020	0.094 ± 0.026	0.092 ± 0.019	0.090 ± 0.016
Lungs	(%)	0.330 ± 0.028	0.322 ± 0.025	0.339 ± 0.021	0.348 ± 0.034
Kidneys	(%)	0.572 ± 0.063	0.565 ± 0.051	0.619 ± 0.044	0.601 ± 0.040
Brain	(%)	0.613 ± 0.058	0.585 ± 0.043	0.623 ± 0.053	0.619 ± 0.044
Liver	(%)	2.404 ± 0.209	2.376 ± 0.111	2.509 ± 0.104	2.529 ± 0.148
Heart	(%)	0.293 ± 0.012	0.293 ± 0.014	0.304 ± 0.035	0.296 ± 0.011

* Significantly different from the controls ($p < 0.05$).

weeks old) were assigned to 4 groups of 10 males and 10 females, and the test compound was administered once daily by oral gavage at doses of 0 (control), 10, 65, or 400 mg/kg body weight/day for 91 days. None of the males and females had died during the study and no changes in clinical signs were observed. No differences in body weight and food consumption were observed between the control group and the male and female treatment groups. Urinalysis and hematology evaluations showed no changes that were considered attributable to the *Salacia* extract treatment in the male and female treatment groups. In the males in the 10-mg/kg body weight/day group, water intake significantly decreased from day 5 to 6. However, this effect was not dose-dependent or sustained, rendering it unlikely to be caused by the *Salacia* plant extract powder treatment. Organ weight measurements revealed a significantly lower absolute testis weight in males in the 10-mg/kg body weight/day group and a significantly higher relative spleen weight in females in the 65-mg/kg body weight/day group. Nevertheless, these changes were not dose-dependent and were not attributed to the *Salacia* plant extract intake. Soft feces were observed for 6 males in the 400-mg/kg body weight/day group and no feces were observed for 1 female in the 65-mg/kg body weight/day group, although these changes were transient. The *Salacia* plant extract alters the intestinal flora, and the observed changes in the feces are common transient signs of alterations to the intestinal flora [11].

The observed reduction in CPK activity in males in the 400-mg/kg body weight/day group and the lower T-BIL concentration in females in the 400-mg/kg body weight/day group were within the normal range known for SD rats. The changes were not considered to be of toxicological significance. In addition, because the *Salacia* plant extract has a hepatoprotective effect, the intake of the *Salacia* plant extract improves liver function, reducing CPK activity and T-BIL concentrations [17]. The significant increase in Glu levels in females in the 400-mg/kg body weight/day group were within the normal range known for SD rats and were not considered toxic as no marked change in the liver and kidneys was observed in the histological examinations.

None of the changes observed during the anatomical observation and histopathology were dose-dependent, and have not been observed in our laboratory before, suggesting that these changes might not be attributable to the *Salacia* extract powder treatment.

5. Conclusion

Based on these results, no overt toxic change was observed in the current 91-day repeat-dose toxicity study, and the No Observed Adverse Effect Level of the *Salacia* plant extract powder was considered to be ≧400 mg/kg body weight/day in SD rats.

Conflict of interest

The authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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