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Data Report

A 90-day repeated oral dose toxicity study of p-cymene in rats

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ABSTRACT — p-Cymene, is a monocyclic monoterpene hydrocarbon, commonly used as a flavoring agent in food. A 90-day repeated oral toxicological study of p-cymene was conducted to examine the toxicological properties and determine the no-observed-adverse-effect level (NOAEL) of p-cymene in Crl:CD (SD) rats at the following doses: 0 (corn oil), 2.4, 12, and 60 mg/kg/day. No mortality or abnormal clinical signs were observed in the treatment groups. The body weight, food consumption, ophthalmoscopy, and gross pathology of the rats were also not affected by p-cymene treatment. However, in the 60 mg/kg group, certain parameters decreased in males, including hemoglobin and hematocrit, red blood cell count, triglyceride, total protein, and albumin. In females, urine volume and total potassium excretion increased, whereas specific gravity, and sodium, potassium, and chlorine concentrations decreased. Increased liver weight was observed in both males and females. Histopathological observations revealed centrilobular hepatocellular hypertrophy. In the 12 mg/kg group, no adverse effects of p-cymene treatment were observed in both sexes. In conclusion, the NOAEL of p-cymene was 12 mg/kg/day for both sexes under the present experimental conditions, considering the alterations in urinalysis, hematology, clinical biochemistry, and histopathology.

Key words: p-Cymene, Flavoring agent, Toxicity, Oral treatment, Rats

INTRODUCTION

p-Cymene, which is a monocyclic monoterpene hydrocarbon, is widely used as a flavoring agent in food. It is the main compound in essential oils from various aromatic plants. The Food and Agriculture Organization of the United Nation (FAO)/ World Health Organization (WHO) Joint Expert Committee on Food Additives (JECFA) (WHO, 2005), declared p-cymene under structure class I (acceptable daily intake > 1,800 µg per person per day), and is expected to be metabolized into a harmless product. The estimated daily per capita intakes of p-cymene in Europe and the USA is 1,085 µg per person per day (18 µg/kg body weight per day) and 472 µg per person per day (8 µg/kg body weight per day), respectively, which is below the tolerated level for structure class I compounds of 1,800 µg per person per day. Therefore, it is considered safe for use as a flavoring agent. p-Cymene

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has various pharmacological properties, including antimicrobial, antioxidant, anti-inflammatory, antiparasitic, antidiabetic antiviral, and antitumor activities (Balahbib *et al.*, 2021). Although several toxicological studies, such as genotoxicity, repeated dose toxicity with reproduction and developmental toxicity, have been conducted (Api *et al.*, 2021), evaluating the effects of long-term exposure to p-cymene is important to validate its safety to determine the criteria of food additive in Japan. Therefore, we evaluated the toxicity of p-cymene in rats using a 90-day repeated oral dose study to understand its toxicological properties and assess the no-observed-adverse-effect level (NOAEL) status.

MATERIALS AND METHODS

Test materials

The test chemical, p-cymene, 1-Methyl-4-(1-methylethyl) benzene, was purchased from Nippon Terpene Chemicals, Inc. (Hyogo, Japan; Lot no. 110901; purity 99.2%). It is a colorless or light yellowish liquid, and has been characterized using ultraviolet high-performance liquid chromatography (Fig. 1). As p-cymene can easily dissolve in corn oil, corn oil was used as the vehicle control and was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Animals and treatment

Four-week-old specific pathogen-free Crl:CD (SD) rats were purchased from Jackson Laboratories Japan, Inc. (Yokohama, Japan), and were housed at the DIMS Institute of Medical Science, Inc., under controlled temperature and relative humidity, with a 12-hr light/dark cycle. The animals were housed in the plastic cage (two rats per cage) with soft chip bedding (Japan SLC, Inc., Shizuoka, Japan). They received a CE-2 pellet diet (CLEA Japan, Inc., Tokyo, Japan) and tap water (Ichinomiya City) *ad libitum*. After quarantine and acclimation period, 5-week-old rats were randomly assigned to four groups (10 rats per group per sex) based on body weight. Each rat group received p-cymene at 0 (corn oil), 2.4, 12, and 60 mg/kg/day orally using a stomach gavage tube once daily at 4 mL/kg for 90 days.

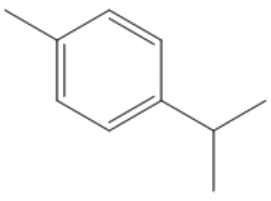

Fig. 1. Chemical structure of p-cymene.

In the previous 90-day repeated oral toxicity study in rats, which administered p-cymene to rats at doses of 12, 60, and 300 mg/kg, adverse effects were observed in the liver and kidneys in the groups administered with > 60 mg/kg group of p-cymene (Tachibana, 2018). The brain weight was slightly decreased in a dose dependent manner but without significance. NOAEL could not be assessed because the brain weight decreased in all p-cymene-treated groups. To confirm these changes in lower doses, the highest, middle, and low doses were set at 60, 12, and 2.4 mg/kg/day, respectively, with a proportional factor of 5. The animals were treated orally via gavage once daily for 90 days, and the dosing volume (4 mL/kg) was adjusted to the latest body weight of the individual rats.

All experiments were approved by the Animal Experimental Committee at the DIMS Institute of Medical Science, Inc., and conducted according to the "Act on Welfare and Management of Animals" (Law No. 39, June 2019); "Standards for Care and Use of Laboratory Animals of DIMS Institute of Medical Science, Inc." (October 8, 2021); "Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain" (Notice No. 84 of the Ministry of the Environment, September 2013); "Basic policies for the conduct of animal experiment in academic research institutions under the jurisdiction of the Ministry of Health, Labour, and Welfare (MHLW)" (Notice No. 0220-1 of MHLW, February 2015); and "Guidelines for Proper Conduct of Animal Experiments" (Science Council of Japan, June 2006).

Study design

The repeated oral dose toxicity study of p-cymene in rats was conducted for 90 days as per the Guidelines for Designation of Food Additives and Revision of Standards for the Use of Food Additives (MHLW, 1996) in compliance with Good Laboratory Practice (GLP) standard in the "Standard Concerning Testing Laboratories Implementing Tests for New Chemical Substances, etc." (MHLW, 2011).

Animal conditions were observed daily. Body weight and 24-hr food consumption were measured every week. The rat's eye appearance was observed on the 12th week of treatment for five rats per group per sex. After examining the response to light using dark adaptation, the rat's eyes were treated a mydriatic, Mydrin® P (Santen Pharmaceutical Co., Ltd., Osaka, Japan). Then, the anterior part, optic media, and ocular fundus were examined

using a slit-lamp, SL-5 (Kowa Co., Ltd., Nagoya, Japan), a fundus camera, and a Halogen binocular inversion ophthalmoscope, IO-α (Neitz Instruments Co., Ltd., Tokyo, Japan).

Urinalysis (five rats per group per sex) was performed on the 13th week (the final week of treatment). All animals were placed in urine-collection cages with food and water. Using fresh urine samples obtained within 4 hr after excretion, the qualitative parameters, including pH, occult blood (Ob), ketones (Ket), glucose (Glu), proteins (Pro), urobilinogen (Uro) and bilirubin (Bil), were examined using a test paper and an analyzer (Multistix, and CLINITEK Advantus, Siemens Healthcare Diagnostics K.K., Tokyo, Japan), and the color and turbidity of the urine were examined macroscopically. Moreover, fresh urine samples were centrifuged to obtain urinary sediments to examine erythrocyte (EC), leucocyte (LC), epithelial cells (EpiC), crystals (Cry), and casts (Casts). The specific gravity, urinary volume (Vol), urinary sodium (Na), potassium (K), and chloride (Cl) concentrations of the 24-hr urine sample were assessed. Urinary electrolytes were determined using an automated analyzer 3500 (Hitachi Ltd., Tokyo, Japan), and urinary sediments after New-Sternheimer staining were assessed microscopically. Specific gravity values were measured using an Atago Serum Protein Refractometer N (Atago Co., Ltd., Tokyo, Japan).

The hematology and serum biochemistry of the animals were evaluated using typical clinical methods. Before the autopsy, the animals fasted overnight. After being anesthetized with isoflurane (Mylan Seiyaku Co., Ltd., Tokyo, Japan), blood samples were collected from the abdominal aorta in EDTA-containing tubes for hematological examination to assay the whole blood. Red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular HGB (MCH), MCH concentration (MCHC), platelet (PLT), white blood cell (WBC), reticulocyte ratio (RET), and differential leukocyte counts were assessed using an automated hematology analyzer, Model XT-2000i (Sysmex Co., Hyogo, Japan). The samples were centrifuged with 3.2% sodium citrate to obtain the plasma, which was assayed using an automated coagulation analyzer CA-530 (Sysmex Co.) for prothrombin time (PT) and activated partial thromboplastin time (APTT). The serum samples obtained after centrifugation were assayed using an automatic analyzer 3500 (Hitachi Ltd., Tokyo, Japan), for aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (γ-GTP), total bilirubin (T-BIL), blood urea nitrogen (BUN), creatinine (CRE), glucose (Glu), total cholesterol (T-cho), triglyceride (TG), total proteins (TP), albumin (Alb), inorganic phosphorus (IP), calcium (Ca), sodium (Na), potassium (K), and chloride (Cl).

Pathological examinations

After euthanizing the rats from all groups, their organs and tissues were harvested to examine all gross lesions macroscopically. During autopsy, the brain, pituitary gland, heart, lung, liver, spleen, kidneys, adrenal glands, testes, prostate (ventral lobe), seminal vesicle including the coagulating gland, ovaries including the oviduct, uterus, salivary glands (submandibular and sublingual glands), thymus, and thyroid glands were weighed. The prostate, seminal vesicle, and pituitary and thyroid glands were weighed after fixation. Testes and eyes were pre-fixed in glutaraldehyde formalin acetic acid and glutaraldehyde-formaldehyde solution, respectively. The other organs and tissues were fixed in 10% neutralized buffered formalin, and their paraffin-embedded sections were stained with hematoxylin and eosin for histopathological examination. If histopathological changes were observed in the 60 mg/kg group, the relevant tissues from the lower-dose groups were examined. The liver samples from both sexes of all groups were subjected to histopathological examination. The uterus showing macroscopical changes in the 2.4 mg/kg group was also examined histopathologically. Tables 7 and 8 summarize the results for male and females, respectively.

Statistical analysis

For body weight, food consumption, urinalysis (excluding semiquantitative estimation, urinary sediment, and urinary appearance), hematology, clinical biochemistry, and organ weight data, the significance of the differences between the groups was assessed using Bartlett's test. Homogeneous and nonhomogeneous data were analyzed using Dunnett's multiple comparison test, and Steel's test, respectively. For the ophthalmoscopic, gross pathological, and histopathological lesion data, the significance of differences in frequency between the control and treated groups was evaluated using Fisher's exact probability test (one-sided). The levels of significance were set at $P < 0.05$ and 0.01. Categorical urinalysis data with grade, semiquantitative estimation, urinary sediments, and appearance were analyzed using the chi-squared test of independence for all the groups (two-sided). Statistical analysis was conducted using the Pharma LabSite (Fujitsu Ltd., Tokyo, Japan).

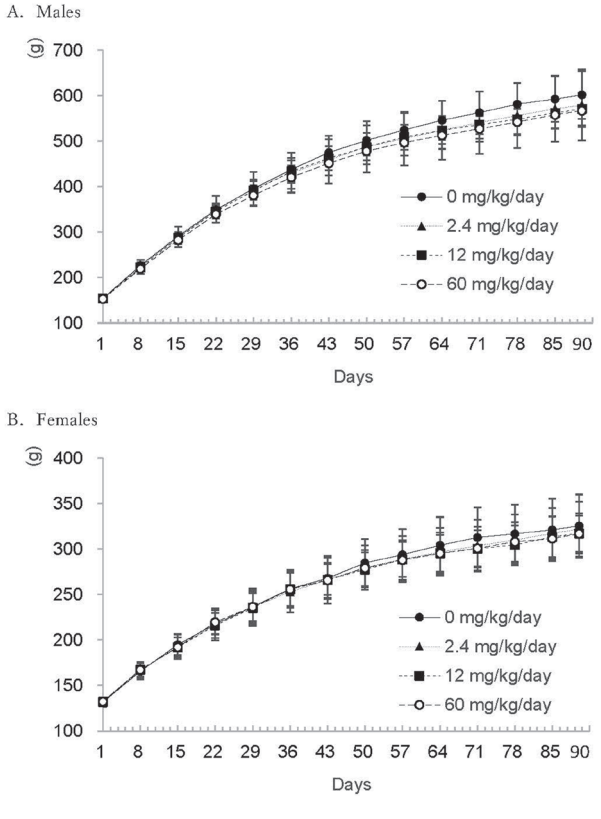

Fig. 2. Body weights of male (A) and female (B) rats treated orally with p-cymene for 90 days.

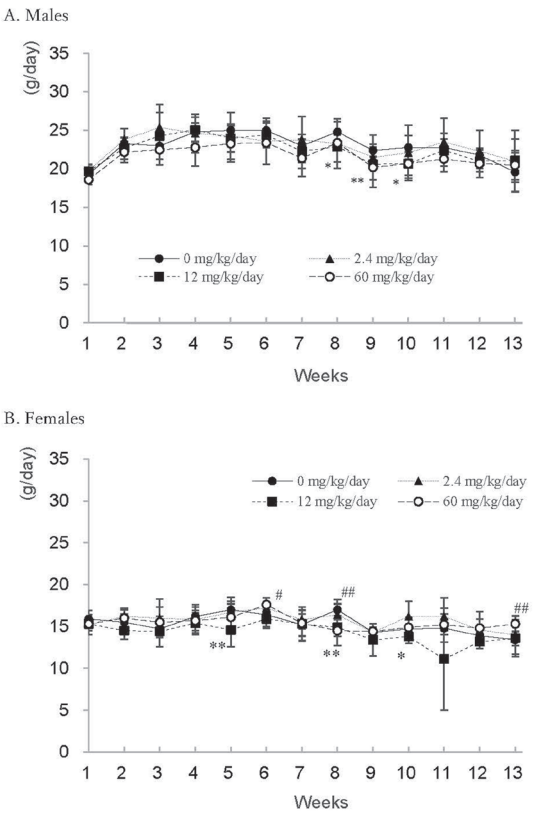

Fig. 3. Food intake of male (A) and female (B) rats treated orally with p-cymene for 90 days. *, **; Significantly different from the control group at $p < 0.05$ and $p < 0.01$, respectively (12 mg/kg/day). #, ##; Significantly different from the control group at $p < 0.05$ and $p < 0.01$, respectively (60 mg/kg/day).

RESULTS

General conditions

No deaths or clinical signs were observed in any groups during the study period. Moreover, the clinical signs, body weights (Fig. 2), food consumption (Fig. 3), and ophthalmological findings were unaffected by p-cymene treatment.

Urinary analysis

Tables 1 and 2 show the quantitative and qualitative urinary parameters, respectively. The female in the 60 mg/kg group displayed significantly higher urine volume but significantly decreased specific gravity, Na, K, and Cl concentrations, and increased total excretion of K concentration. Qualitative urinary parameters were unaffected by p-cymene. In males, none of the urine parameters were affected.

Hematology and serum biochemistry

Tables 3 and 4 show the hematological and serum biochemical data, respectively. HGB, HCT, TG, TP, and Alb were significantly decreased, whereas RBC was slightly decreased in the males in the 60 mg/kg group.

In the females, in this group, a subtle but significant decrease in Na concentration was observed. Significant changes were observed in other parameters as well, but degrees of changes were very subtle or not dose-dependent, or both, and thus had low toxicological significance.

Pathological examinations

At autopsy, the bilateral small testes and diverticulum of the small intestines were observed in one male each from the control and 60 mg/kg group. In females, the unilateral hypoplastic kidney and the bilateral dilation of the uterus horns were observed in one female each from the

control and 2.4 mg/kg group. On the basis of the frequency of findings, these changes were of low toxicological significance.

Tables 5 and 6 show the data for organ weights in males and females, respectively. In the 60 mg/kg group, the relative liver weight significantly increased in both sexes. In females, the absolute liver weight also statistically increased in this group. Furthermore, the relative spleen weight was increased in males.

The heart and pituitary gland weights were decreased, but not at relative values. These were due to the slightly low terminal body weight. None of these organs were accompanied by any unusual macroscopic and microscopic lesions, and thus were not to associated with any toxicological concern.

Histopathological assessment (Tables 7 and 8) indicated centrilobular hepatocellular hypertrophy in females in the 60 mg/kg group. No histological findings related to increased liver weight were observed in males in the 60 mg/kg group.

Metastatic tumor lesions were observed in the liver and spleen of one male in the 60 mg/kg group, but a primary lesion could not be identified. Histologically, it was assumed to be an early erythroleukemia lesion. The previous 90-day repeated oral study, conducted at a higher dose (300 mg/kg), showed no signs of erythroleukemia (Tachibana, 2018). Erythroleukemia is rare in rodents, as very few records of it in the laboratory-tested and facility-purchased animals. However, reports have shown that erythroleukemia appear spontaneously in SD rats, even at a relatively young age (Edamoto *et al.*, 2007; Nonoyama *et al.*, 1993), and p-cymene is unlikely to show genotoxicity (Api *et al.*,, 2021). Therefore, based on these results, we concluded that the correlation between tumor occurrence and p-cymene is insignificant. Several lesions were also sporadically detected in other tissues, but no significant treatment-mediated changes were obvious.

DISCUSSION

In this study, p-cymene adversely affected the urinary and hematopoietic parameters and the liver in the 60 mg/kg group. The brain weight remained unaffected by the p-cymene treatment. Although female rats exhib-

Table 1. Quantitative Urinary Parameters data for SD rats treated orally with p-cymene for 90 days.

Dose (mg/kg/day)		0[a)]	2.4	12	60
Male (No. of animals)		5	5	5	5
Vol	(mL/24 hr)	17.1 ± 10.0	16.4 ± 6.1	13.5 ± 9.4	15.7 ± 3.8
Specific gravity		1.037 ± 0.018	1.030 ± 0.011	1.050 ± 0.017	1.035 ± 0.010
Na	(mmol/L)	35.4 ± 18.4	36.2 ± 14.3	83.3 ± 32.0 *	51.5 ± 26.3
K	(mmol/L)	125.19 ± 56.26	106.03 ± 36.25	177.53 ± 57.38	132.61 ± 32.96
Cl	(mmol/L)	23.1 ± 20.8	17.1 ± 13.0	63.1 ± 40.8	39.1 ± 39.2
Na	(mmol/24 hr)	0.50 ± 0.17	0.54 ± 0.16	1.01 ± 0.52	0.85 ± 0.64
K	(mmol/24 hr)	1.70 ± 0.23	1.58 ± 0.23	2.02 ± 0.53	2.11 ± 0.92
Cl	(mmol/24 hr)	0.32 ± 0.22	0.23 ± 0.09	0.78 ± 0.50	0.71 ± 0.90
Female (No. of animals)		5	5	5	5
Vol	(mL/24 hr)	9.3 ± 2.7	12.6 ± 2.3	10.9 ± 2.3	17.8 ± 1.5 **
Specific gravity		1.057 ± 0.012	1.053 ± 0.013	1.051 ± 0.011	1.036 ± 0.004 *
Na	(mmol/L)	132.9 ± 23.8	120.6 ± 28.6	114.0 ± 36.7	77.0 ± 19.8 *
K	(mmol/L)	248.69 ± 36.54	239.21 ± 57.35	234.22 ± 45.06	164.47 ± 18.60 *
Cl	(mmol/L)	157.4 ± 33.7	146.7 ± 34.0	139.8 ± 27.1	100.9 ± 20.0 *
Na	(mmol/24 hr)	1.18 ± 0.19	1.48 ± 0.27	1.18 ± 0.26	1.36 ± 0.33
K	(mmol/24 hr)	2.23 ± 0.38	2.94 ± 0.60 *	2.46 ± 0.14	2.94 ± 0.43 *
Cl	(mmol/24 hr)	1.39 ± 0.15	1.80 ± 0.34	1.47 ± 0.19	1.79 ± 0.36

Values; Mean ± SD.
a); vehicle control, corn oil
*, **; Significantly different from the control group at $p < 0.05$ and $p < 0.01$, respectively.

Table 2. Qualitative urinary parameters data for SD rats treated orally with p-cymene for 90 days.

Item	Grade	Dose level (mg/kg/day)							
		Male				Female			
		0[a)]	2.4	12	60	0[a)]	2.4	12	60
No. of animals examined		5	5	5	5	5	5	5	5
pH	6.5	0	0	0	1	1	1	0	1
	7.0	1	0	0	0	0	1	2	1
	7.5	0	1	0	0	0	0	1	0
	8.0	0	3	1	1	0	3	0	2
	8.5	4	1	4	3	2	0	1	1
	>=9.0	0	0	0	0	2	0	1	0
Ob	-	3	3	3	4	5	5	5	5
	±	2	2	2	1	0	0	0	0
Ket	-	1	0	0	0	2	1	2	0
	±	1	0	0	0	3	3	2	2
	+	0	4	5	4	0	1	1	3
	2+	3	1	0	1	0	0	0	0
Glu	-	5	5	5	5	5	5	5	5
Pro	-	0	0	0	0	0	1	2	2
	±	1	0	2	1	2	3	3	2
	+	3	3	3	3	2	0	0	1
	2+	1	2	0	1	1	0	0	0
	3+	0	0	0	0	0	1	0	0
Uro	0.1	5	5	5	5	3	4	5	4
	1	0	0	0	0	2	1	0	1
Bil	-	4	4	5	5	4	4	5	5
	+	1	1	0	0	1	1	0	0
Casts	-	5	5	5	5	5	5	5	5
EpiC	-	4	4	2	3	3	5	1	4
	±	1	1	3	2	2	0	4	1
LC	-	5	5	5	5	5	5	5	5
EC	-	5	5	5	5	5	5	5	5
Cry	-	2	0	0	1	3	3	3	3
	±	0	0	1	1	1	0	0	1
	1+	1	1	2	0	0	1	1	0
	2+	2	1	0	1	1	1	1	0
	3+	0	3	2	2	0	0	0	1
Color	Pale yellow	5	5	5	5	5	5	5	5
Turbidity	-	4	4	5	5	5	5	4	5
	±	1	1	0	0	0	0	1	0

a); vehicle control, corn oil
Not significantly different from the control group.

Table 3. Hematology data for SD rats treated orally with p-cymene for 90 days.

Dose (mg/kg/day)		0[a)]	2.4	12	60
Male (No. of animals)		10	10	10	10
RBC	($\times 10^4/\mu L$)	879 ± 37	879 ± 53	871 ± 27	843 ± 23
HGB	(g/dL)	15.8 ± 0.5	15.8 ± 0.5	15.4 ± 0.5	15.1 ± 0.5 *
HCT	(%)	44.0 ± 1.5	44.3 ± 1.6	43.2 ± 1.8	42.3 ± 1.1 *
MCV	(fL)	50.1 ± 1.6	50.5 ± 2.7	49.6 ± 1.3	50.1 ± 1.3
MCH	(pg)	18.0 ± 0.5	18.0 ± 0.8	17.7 ± 0.4	17.9 ± 0.4
MCHC	(g/dL)	35.9 ± 0.6	35.7 ± 0.5	35.7 ± 0.5	35.7 ± 0.5
PLT	($\times 10^4/\mu L$)	113.8 ± 12.8	111.8 ± 14.8	111.9 ± 11.6	100.0 ± 12.5
RET	(%)	3.1 ± 0.5	3.1 ± 0.4	3.2 ± 0.6	3.1 ± 0.5
WBC	($\times 10^2/\mu L$)	90.2 ± 28.5	75.5 ± 12.1	93.2 ± 18.8	80.1 ± 14.6
LYMPH	(%)	81.9 ± 4.4	78.6 ± 5.7	79.1 ± 5.7	80.2 ± 4.8
NEUT	(%)	13.3 ± 3.9	16.8 ± 4.9	16.9 ± 5.0	14.6 ± 4.1
EO	(%)	1.5 ± 0.4	1.5 ± 0.7	1.0 ± 0.2 **	1.2 ± 0.2
BASO	(%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
MONO	(%)	3.2 ± 0.8	3.1 ± 0.8	3.0 ± 1.0	4.1 ± 0.9
PT	(sec.)	12.4 ± 1.4	11.7 ± 1.4	12.9 ± 2.2	11.6 ± 1.0
APTT	(sec.)	14.0 ± 2.2	13.3 ± 1.7	15.3 ± 1.9	12.4 ± 2.1
Female (No. of animals)		10	10	10	10
RBC	($\times 10^4/\mu L$)	784 ± 38	780 ± 26	776 ± 41	782 ± 27
HGB	(g/dL)	14.9 ± 0.4	15.0 ± 0.4	14.7 ± 0.7	14.7 ± 0.5
HCT	(%)	41.5 ± 1.4	41.5 ± 1.3	40.9 ± 1.3	41.2 ± 1.4
MCV	(fL)	53.1 ± 2.4	53.3 ± 1.5	52.8 ± 1.7	52.7 ± 2.1
MCH	(pg)	19.1 ± 0.8	19.2 ± 0.4	19.0 ± 0.3	18.8 ± 0.8
MCHC	(g/dL)	36.0 ± 0.5	36.0 ± 0.4	35.9 ± 0.7	35.7 ± 0.3
PLT	($\times 10^4/\mu L$)	104.4 ± 34.1	102.9 ± 8.6	104.5 ± 15.2	92.7 ± 12.6
RET	(%)	3.4 ± 0.5	2.9 ± 0.4 *	2.7 ± 0.4 **	3.0 ± 0.4
WBC	($\times 10^2/\mu L$)	45.8 ± 9.7	55.0 ± 17.8	47.9 ± 9.5	55.3 ± 18.6
LYMPH	(%)	78.1 ± 7.2	80.4 ± 5.5	83.9 ± 4.4	82.7 ± 4.4
NEUT	(%)	16.9 ± 6.5	14.8 ± 5.6	11.5 ± 4.1	12.9 ± 4.1
EO	(%)	1.6 ± 0.7	1.6 ± 0.6	1.4 ± 0.6	1.6 ± 0.4
BASO	(%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
MONO	(%)	3.4 ± 1.0	3.2 ± 0.6	3.2 ± 1.0	2.8 ± 0.9
PT	(sec.)	9.7 ± 0.2	9.7 ± 0.2	9.7 ± 0.1	9.9 ± 0.2
APTT	(sec.)	11.7 ± 1.6	11.5 ± 0.4	11.4 ± 1.9	11.4 ± 1.0

Values; Mean ± SD.
a); vehicle control, corn oil
*, **; Significantly different from the control group at $p < 0.05$ and $p < 0.01$, respectively.

Table 4. Blood Chemistry data for SD rats treated orally with p-cymene for 90 days.

Dose level (mg/kg/day)		0 [a)]	2.4	12	60
Male (No. of animals)		10	10	10	10
AST	(U/L)	89 ± 17	98 ± 29	97 ± 22	92 ± 20
ALT	(U/L)	29 ± 4	31 ± 6	31 ± 5	31 ± 7
ALP	(U/L)	91 ± 20	101 ± 26	101 ± 24	98 ± 19
γ-GTP	(U/L)	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.0
T-BIL	(mg/dL)	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.02	0.05 ± 0.02
BUN	(mg/dL)	12.9 ± 1.2	12.1 ± 1.8	12.7 ± 1.9	12.1 ± 1.7
CRE	(mg/dL)	0.28 ± 0.03	0.26 ± 0.04	0.26 ± 0.04	0.27 ± 0.04
Glu	(mg/dL)	162 ± 16	160 ± 32	165 ± 21	172 ± 23
T-cho	(mg/dL)	56 ± 8	63 ± 9	64 ± 9	52 ± 6
TG	(mg/dL)	47 ± 15	54 ± 35	44 ± 18	30 ± 7 *
TP	(g/dL)	6.0 ± 0.3	6.1 ± 0.2	6.0 ± 0.2	5.6 ± 0.2 **
Alb	(g/dL)	4.2 ± 0.2	4.2 ± 0.2	4.1 ± 0.2	3.9 ± 0.1 *
A/G		2.32 ± 0.20	2.28 ± 0.22	2.18 ± 0.25	2.46 ± 0.36
IP	(mg/dL)	6.3 ± 0.2	6.5 ± 0.5	6.3 ± 0.5	6.6 ± 0.5
Ca	(mg/dL)	10.2 ± 0.2	10.5 ± 0.3 *	10.2 ± 0.3	10.1 ± 0.2
Na	(mmol/L)	142.2 ± 0.6	142.5 ± 0.7	142.1 ± 1.0	141.8 ± 1.1
K	(mmol/L)	4.44 ± 0.20	4.45 ± 0.29	4.64 ± 0.25	4.47 ± 0.14
Cl	(mmol/L)	102.1 ± 1.0	102.6 ± 1.1	103.1 ± 1.4	102.4 ± 1.0
Female (No. of animals)		10	10	10	10
AST	(U/L)	84 ± 25	103 ± 36	97 ± 23	83 ± 13
ALT	(U/L)	23 ± 3	23 ± 3	24 ± 3	24 ± 4
ALP	(U/L)	54 ± 17	52 ± 12	51 ± 13	55 ± 13
γ-GTP	(U/L)	0.7 ± 0.3	0.6 ± 0.2	0.6 ± 0.1	0.7 ± 0.2
T-BIL	(mg/dL)	0.07 ± 0.02	0.09 ± 0.03	0.07 ± 0.01	0.07 ± 0.01
BUN	(mg/dL)	15.8 ± 3.4	13.9 ± 1.8	15.4 ± 2.4	14.8 ± 3.3
CRE	(mg/dL)	0.34 ± 0.05	0.30 ± 0.03	0.34 ± 0.05	0.35 ± 0.06
Glu	(mg/dL)	127 ± 14	124 ± 16	124 ± 14	135 ± 7
T-cho	(mg/dL)	65 ± 7	74 ± 16	69 ± 15	75 ± 15
TG	(mg/dL)	43 ± 22	41 ± 14	49 ± 27	24 ± 7
TP	(g/dL)	6.4 ± 0.3	6.4 ± 0.4	6.4 ± 0.3	6.2 ± 0.2
Alb	(g/dL)	4.9 ± 0.2	5.0 ± 0.3	4.9 ± 0.4	4.7 ± 0.3
A/G		3.36 ± 0.35	3.65 ± 0.43	3.39 ± 0.49	3.21 ± 0.89
IP	(mg/dL)	5.1 ± 0.5	5.6 ± 0.9	5.4 ± 0.8	5.4 ± 0.5
Ca	(mg/dL)	10.2 ± 0.3	10.3 ± 0.3	10.3 ± 0.4	9.9 ± 0.2
Na	(mmol/L)	141.4 ± 0.8	140.6 ± 0.6	140.9 ± 1.1	140.3 ± 0.8 *
K	(mmol/L)	3.91 ± 0.40	4.04 ± 0.27	4.06 ± 0.30	3.86 ± 0.26
Cl	(mmol/L)	103.9 ± 1.2	102.6 ± 1.1	103.7 ± 1.2	103.4 ± 1.3

Values; Mean ± SD.
a); vehicle control, corn oil
*, **; Significantly different from the control group at $p < 0.05$ and $p < 0.01$, respectively.

Table 5. Organ Weight for SD male rats treated orally with p-cymene for 90 days.

Dose level (mg/kg/day)	0[a)]	2.4	12	60	
Male (No. of animals)	10	10	10	10	
Body weight (g)	574.7 ± 50.8	555.4 ± 74.4	546.0 ± 32.6	544.0 ± 32.8	
Absolute (g)					
Brain (g)	2.257 ± 0.119	2.226 ± 0.074	2.167 ± 0.059	2.173 ± 0.101	
Pituitary (mg)	15.8 ± 1.5	14.7 ± 1.6	14.7 ± 1.9	13.6 ± 1.1	**
Salivary glands (g)	0.780 ± 0.053	0.788 ± 0.118	0.746 ± 0.104	0.735 ± 0.060	
Thymus (g)	0.285 ± 0.034	0.314 ± 0.089	0.303 ± 0.046	0.305 ± 0.073	
Thyroids (mg)	26.2 ± 3.8	28.3 ± 4.7	28.5 ± 4.5	26.6 ± 5.0	
Heart (g)	1.713 ± 0.147	1.590 ± 0.187	1.585 ± 0.129	1.531 ± 0.132	*
Lungs (g)	1.591 ± 0.164	1.497 ± 0.119	1.457 ± 0.099	1.490 ± 0.126	
Liver (g)	15.379 ± 1.698	15.783 ± 3.465	15.379 ± 1.595	15.823 ± 1.797	
Spleen (g)	0.799 ± 0.129	0.848 ± 0.150	0.834 ± 0.126	0.875 ± 0.128	
Kidneys (g)	3.678 ± 0.332	3.574 ± 0.521	3.555 ± 0.221	3.492 ± 0.306	
Adrenals (mg)	63.3 ± 7.5	62.6 ± 10.7	60.8 ± 7.4	55.7 ± 7.5	
Testes (g)	3.580 ± 0.519	3.668 ± 0.261	3.574 ± 0.290	3.587 ± 0.332	
Ventral Prostate (g)	0.909 ± 0.174	0.869 ± 0.240	0.855 ± 0.147	0.782 ± 0.245	
Seminal vesicle (g)	1.802 ± 0.112	1.791 ± 0.210	1.760 ± 0.186	1.700 ± 0.207	
Male (No. of animals)	10	10	10	10	
Rerative (%)					
Brain	0.395 ± 0.032	0.407 ± 0.053	0.398 ± 0.022	0.401 ± 0.035	
Pituitary ($x10^{-3}$)	2.76 ± 0.24	2.67 ± 0.31	2.69 ± 0.28	2.51 ± 0.17	
Salivary glands	0.137 ± 0.014	0.143 ± 0.020	0.137 ± 0.021	0.135 ± 0.012	
Thymus	0.050 ± 0.004	0.057 ± 0.015	0.056 ± 0.008	0.056 ± 0.014	
Thyroids ($x10^{-3}$)	4.59 ± 0.80	5.10 ± 0.59	5.21 ± 0.66	4.89 ± 0.91	
Heart	0.299 ± 0.021	0.287 ± 0.018	0.291 ± 0.022	0.281 ± 0.011	
Lungs	0.278 ± 0.025	0.272 ± 0.021	0.267 ± 0.019	0.274 ± 0.015	
Liver	2.673 ± 0.136	2.818 ± 0.278	2.815 ± 0.205	2.902 ± 0.184	*
Spleen	0.139 ± 0.013	0.152 ± 0.016	0.152 ± 0.017	0.161 ± 0.021	*
Kidneys	0.641 ± 0.042	0.643 ± 0.028	0.653 ± 0.046	0.643 ± 0.052	
Adrenals ($x10^{-3}$)	11.13 ± 1.88	11.30 ± 1.44	11.12 ± 0.92	10.24 ± 1.17	
Testes	0.629 ± 0.114	0.668 ± 0.074	0.656 ± 0.051	0.662 ± 0.079	
Ventral Prostate	0.158 ± 0.029	0.158 ± 0.046	0.158 ± 0.032	0.145 ± 0.050	
Seminal vesicle	0.316 ± 0.037	0.325 ± 0.039	0.324 ± 0.043	0.313 ± 0.044	

a); vehicle control, corn oil
*, **; Significantly different from the control group at $p < 0.05$ and $p < 0.01$, respectively.

Table 6. Organ Weight for SD female rats treated orally with p-cymene for 90 days.

Dose level (mg/kg/day)	0[a)]	2.4	12	60
Female (No. of animals)	10	10	10	10
Body weight (g)	310.6 ± 34.1	307.4 ± 27.4	301.3 ± 20.7	301.7 ± 20.0
Absolute (g)				
Brain (g)	2.035 ± 0.072	2.079 ± 0.099	2.071 ± 0.092	2.029 ± 0.053
Pituitary (mg)	17.2 ± 2.4	16.9 ± 3.0	18.3 ± 2.2	18.2 ± 3.6
Salivary glands (g)	0.454 ± 0.031	0.489 ± 0.070	0.462 ± 0.044	0.433 ± 0.047
Thymus (g)	0.273 ± 0.070	0.280 ± 0.065	0.309 ± 0.072	0.289 ± 0.074
Thyroids (mg)	22.6 ± 2.1	23.7 ± 6.2	22.9 ± 3.1	20.2 ± 3.6
Heart (g)	0.960 ± 0.077	0.961 ± 0.103	0.920 ± 0.049	0.932 ± 0.072
Lungs (g)	1.083 ± 0.075	1.117 ± 0.099	1.094 ± 0.063	1.137 ± 0.110
Liver (g)	7.700 ± 0.754	8.268 ± 0.887	7.853 ± 0.383	9.226 ± 0.915 **
Spleen (g)	0.515 ± 0.059	0.581 ± 0.072	0.506 ± 0.073	0.542 ± 0.069
Kidneys (g)	1.889 ± 0.218	2.030 ± 0.154	1.897 ± 0.188	1.924 ± 0.164
Adrenals (mg)	66.9 ± 4.7	72.4 ± 10.2	65.4 ± 6.7	64.4 ± 8.8
Ovaries (mg)	113.7 ± 10.0	128.5 ± 13.9	115.2 ± 19.8	117.7 ± 8.7
Uterus (g)	0.613 ± 0.144	0.589 ± 0.198	0.620 ± 0.207	0.571 ± 0.180
Female (No. of animals)	10	10	10	10
Rerative (%)				
Brain	0.661 ± 0.064	0.681 ± 0.065	0.690 ± 0.057	0.674 ± 0.034
Pituitary ($x10^{-3}$)	5.58 ± 0.94	5.48 ± 0.71	6.11 ± 0.97	6.03 ± 1.22
Salivary glands	0.147 ± 0.015	0.159 ± 0.016	0.154 ± 0.016	0.144 ± 0.014
Thymus	0.089 ± 0.024	0.091 ± 0.019	0.102 ± 0.020	0.096 ± 0.023
Thyroids ($x10^{-3}$)	7.32 ± 0.84	7.68 ± 1.80	7.63 ± 1.08	6.70 ± 1.20
Heart	0.311 ± 0.027	0.313 ± 0.025	0.306 ± 0.019	0.309 ± 0.014
Lungs	0.351 ± 0.034	0.364 ± 0.017	0.364 ± 0.022	0.377 ± 0.020
Liver	2.485 ± 0.143	2.692 ± 0.217	2.612 ± 0.144	3.060 ± 0.246 **
Spleen	0.167 ± 0.024	0.190 ± 0.019	0.168 ± 0.026	0.180 ± 0.020
Kidneys	0.609 ± 0.050	0.662 ± 0.037	0.631 ± 0.067	0.639 ± 0.053
Adrenals ($x10^{-3}$)	21.75 ± 2.70	23.52 ± 2.21	21.87 ± 3.14	21.36 ± 2.71
Ovaries ($x10^{-3}$)	36.88 ± 4.01	41.98 ± 4.81 *	38.19 ± 6.02	39.11 ± 3.04
Uterus	0.198 ± 0.044	0.192 ± 0.064	0.207 ± 0.069	0.189 ± 0.060

a); vehicle control, corn oil
*, **; Significantly different from the control group at $p < 0.05$ and $p < 0.01$, respectively.

Table 7. Histopathological findings of SD male rats treated orally with p-cymene for 90 days.

Organ: Findings	0 mg/kg/day [a)]							2.4 mg/kg/day							12 mg/kg/day							60 mg/kg/day						
	-	1+	2+	3+	4+	5+	P	-	1+	2+	3+	4+	5+	P	-	1+	2+	3+	4+	5+	P	-	1+	2+	3+	4+	5+	P
	n=10																					n=10						
Liver	n=10							n=10							n=10							n=10						
Metastatic tumor	10							10							10							9						1
Fatty change, Periportal	2	4	4					3	3	4					5	5						3	3	4				
Infiltration, Mononuclear	8	2						10							10							8	2					
Tension lipidosis	10							10							10							7	1	2				
Pancreas	n=10																					n=10						
Infiltrate, Mononuclear cell	9	1																				10						
Jejunum	n=10																					n=10						
Diverticulum	10																					9						1
Heart	n=10																					n=10						
Hemorrhage	10																					9	1					
Mononuclear cell infiltrate/ fibrosis, Myocardium	7	2	1																			9	1					
Pituitary	n=10																					n=10						
Pseudocyst, Pars distalis	10																					9						1
Thyroid	n=10																					n=10						
Infiltrate, Inflammatory cell	9	1																				10						
Adrenal	n=10																					n=10						
Vacuolation, Cortical, Increased, focal	9	1																				10						
Testis	n=10																											
Atrophy, Tubular	9			1																		10						
Epididymis	n=10																					n=10						
Reduced sperm, Luminal	9			1																		10						
Prostate	n=10																					n=10						
Infiltrate, Inflammatory cell, Lymphocytic	4	2	4																			1	2	7				
Spleen	n=10																					n=10						
Metastatic tumor	10																					9						1
Femur	n=10																					n=10						
Cyst, Bone	10																					8	2					
Sternum	n=10																					n=10						
Chondromucinous degeneration	9	1																				10						
Lung/bronchial	n=10																					n=10						
Alveolar macrophage aggregation	9	1																				9	1					
Kidney	n=10																					n=10						
Cyst	10																					9	1					
Basophilia, tubule	10																					9	1					

Data are presented as the number of animals showing each finding.
Grade: - Normal, 1+ Minimal, 2+ Slight, 3+ Moderate, 4+ Marked, 5+ Severe, P: Present,
a); vehicle control, corn oil
Not significantly different from the control.
Submandibular gland, Sublingual gland, Tongue, Esophagus, Stomach, Duodenum, Ileum, Cecum, Colon, Rectum, Aorta, Parathyrid Seminal vesicle, Coagulation gland, Thymus, Mandibular lymph node, Mesenteric lymph node, Bone marrow, Skin/subcutis, Mammary gland, Skeletal muscle, Brain, Spinal cord, Optic nerve, Sciatic nerve, Nasal cavity, Trachea, Eye, Harderian gland, Zymbal gland, and Urinary bladder were not observed any changes related to the treatment of p-cymene.

Table 8. Histopathological findings of SD female rats treated orally with p-cymene for 90 days.

Organ: Findings	0 mg/kg/day [a)]							2.4 mg/kg/day							12 mg/kg/day							60 mg/kg/day						
	-	1+	2+	3+	4+	5+	P	-	1+	2+	3+	4+	5+	P	-	1+	2+	3+	4+	5+	P	-	1+	2+	3+	4+	5+	P
Liver	n=10							n=10							n=10							n=10						
Angiectasis	10							10							10							9	1					
Hypertrophy, Hepatocellular, Centrilobular	10							10							10												10	**
Fatty change, Periportal	6	3	1					10						*	6	3	1					8	1	1				
Infiltration, Mononuclear	7	3						7	3						10							7	2	1				
Tension lipidosis	8		2					10							7	1	2					10						
Thyroid	n=10																					n=10						
Ultimobranchial cyst	9					1																10						
Ovary	n=10																					n=10						
Cyst	8	1		1																		10						
Uterus	n=10							n=1														n=10						
Dilatation, Luminal	7		3								1											8		2				
Harderian	n=10																					n=10						
Infiltrate, Inflammatory cell, Lymphocytic	10																					9	1					
Kidney	n=10																					n=10						
Mineralization, Cortico-medullary junction	4	3	3																			4	4	2				
Agenesis	9					1																10						
Infarct, Cortex	9		1																			10						

Data are presented as the number of animals showing each finding.
Grade: - Normal, 1+ Minimal, 2+ Slight, 3+ Moderate, 4+ Marked, 5+ Severe, P: Present,
a); vehicle control, corn oil
*, **; Significantly different from the control at $p < 0.05$ and $p < 0.01$ in the Fisher's exact test , respectively.
Submandibular gland, Sublingual gland, Tongue, Esophagus, Pancreas, Stomach, Duodenum, Jejunum, Ileum, Cecum, Colon, Rectum, Aorta, Heart, Pituitary, Parathyroid, Adrenal, Oviduct, Vagina, Thymus, Spleen, Mandibular lymph node, Mesenteric lymph node, Bone marrow, Skin/subcutis, Mammary gland, Femur, Sternum, Skeletal muscle, Brain, Spinal cord, Optic nerve, Sciatic nerve, Nasal cavity, Trachea, Lung/bronchial, Eye, Zymbal gland, and Urinary bladder were not observed any changes related to the treatment of p-cymene.

ited increased urine volume, total excretion of K, and decreased specific gravity and electrolyte concentration, no renal alteration was observed histopathologically, or biochemically. In males, HGB and HCT were significantly decreased, with a moderate decrease in RBC, suggesting the animals were slightly anemic. A previous study (Tachibana, 2018) reported that at higher dose (300 mg/kg), p-cymene increased kidney weight and blood urea nitrogen (BUN). Histopathological observations revealed renal alterations, such as papillary stasis and necrosis, and vacuolar cytoplasmic changes of collective tubes. Hematopoietic changes were also more detected in these rats. Thus, our results demonstrate that p-cymene might affect renal and hematopoietic functions.

The liver weight increased in both sexes. Histopathological observation revealed centrilobular hypertrophy of the hepatocytes in females. At a higher p-cymene dose (300 mg/kg), as shown in a previous study (Tachibana, 2018), along with centrilobular hypertrophy of the hepatocytes, increased incidence of the single hepato-cell necrosis in the centrilobular area was also observed. Thus, the observed hypertrophy of hepatocytes might indicate the initial stage of hepatocytic necrosis. Biochemically, TG, TP, and Alb were decreased in males, which is related to hepatic dysfunction (Klassen C.D. (edu), 2019). Several groups of experts, including the Flavor and Extract Manufacturers Association (FEMA), the WHO, and the European Chemicals Agency (ECHA), reported that the major metabolic pathways for p-cymene are catalyzed through cytochrome 450, alcohol dehydrogenase, and aldehyde dehydrogenase enzymes. The metabolites formed are expected to conjugate with glycine, glucuronic acid, or glutathione and be excreted via the urine or bile (Adams *et al.*, 2011; ECHA, 2008; WHO, 2006). The changes observed in the liver could be due to the metabolic effects of p-cymene.

The brain weight in all p-cymene groups, whereas the absolute brain weight decreased in a dose-dependent

manner in groups treated with > 12.5 mg/kg of p-cymene in a previous study (Tachibana, 2018). A study showed that p-cymene had an antioxidant effect on the hippocampus of adult mice (de Oliveira *et al.*, 2015), and may act as a neuroprotective agent in the brain. After 4 weeks of inhalation exposure to p-cymene, the regional and synaptosomal neurochemistry in rats were altered (Lam *et al.*, 1996). Thus, p-cymene can potentially act on the central nervous system (CNS). However, the effects of p-cymene on the weight and histopathology of the brain and signs of CNS dysfunction were not observed in this study.

In conclusion, p-cymene exerted toxic effects on the urinary, hematopoietic systems, and the liver. On the basis on these changes, the NOAEL was judged at 12 mg/kg/day in both sexes under this study's condition.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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