[Chem. Pharm. Bull. 27(4) 874—879 (1979)]

UDC 615.918.011.5.076.9 : 581.192

Subchronic Toxicity of Ericaceous Toxins and *Rhododendron* Leaves

Hiroshi Hikino, Yasushi Ohizumi, Chohachi Konno,[1a] Kazuo Hashimoto,[1b] and Haruki Wakasa[1c]

Pharmaceutical Institute, Tohoku University,[1a] College of Medical Sciences, Tohoku University,[1b] and Tohoku University School of Medicine[1c]

(Received August 29, 1978)

The extracts of two species of *Rhododendron* leaves and their poisonous principles, grayanotoxin I and III, have been daily administered *p.o.* to mice and rats for 12 weeks. Behavior, general appearance, mortality, body weight, organ weight, hematology, blood biochemistry, as well as gross and microscopic findings have not revealed any significant effects from the extracts and the toxins in both animals, except for the following changes. Thus, the body weights and the liver weights have generally decreased in the treated mice and in the treated animals, respectively. Decrease of the spleen weights and increase of the serum glutamic-oxalacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) levels have been also found in the animals given the extracts and the toxins at a higher dose which, however, has been concluded to be within a normal range of function from pathological examinations. Accumulated data demonstrate that despite of the intense acute toxicity, grayanotoxin I and III exhibit rather weak subchronic toxicity as far as the parameters selected in this study concern, which is possibly due to rapid metabolism and/or excretion.

Keywords——acute toxicity; Ericaceae; grayanotoxin I; grayanotoxin III; *Rhododendoron* leaves; subchronic toxicity

Some members of the ericaceous family, which fall in the botanically related tribes Andromedeae and Rhodoreae, are well known to be toxic and certain species often produce livestock loss. The poisonous principles of these plants are now clarified to be diterpenic polyalcohols possessing the andromedane skeleton, the grayanotoxins, asebotoxins, rhodojaponins, and lyoniatoxins (lyoniols).[2] The acute toxicity of 36 samples of the ericaceous toxins and their derivatives has been recently determined using mice, the *i.p.* LD_{50} values of the most toxic congeners, asebotoxin III, rhodojaponin III, asebotoxin I, desacetyllyoniatoxin, rhodojaponin V, grayanotoxin VI, and grayanotoxin III, being very low ($<$ 1 mg/kg).[2] On the other hand, extracts of *Rhododendron* leaves are being consumed in Japan as a folklore remedy for hypertension. Since *Rhododendron* leaves contain grayanotoxin I and III of high toxicity,[3,4] the human may not be tolerant of relatively large quantities of the leaves without developing symptoms. An instance of human poisoning has been recently recorded in which long-term ingestion of a decoction made from the leaves of *R. metternichii* Siebold *et* Zuccarini var. *pentamerum* Maximowicz brought about toxic symptoms (particularly numbness of hands) but not death. The present paper deals with the study conducted to determine the subchronic oral toxicity of *Rhododendron* leaves and their principal poisonous ingredients, grayanotoxin I and III, in two species of laboratory animals.

Experimental

Materials——Grayanotoxin I and III were isolated from the leaves of *Leucothoe grayana* Maximowicz. The dried leaves of *Rhododendron brachycarpum* D. Don (collected at Mt. Hakkoda, Aomori prefecture) and

1) Location: *a) Aoba-yama, Sendai*; *b*), *c) 2-1 Seiryomachi, Sendai.*
2) H. Hikino, T. Ohta, M. Ogura, Y. Ohizumi, C. Konno, and T. Takemoto, *Toxicol. Appl. Pharmacol.*, 35, 303 (1976) and references cited theirin.
3) T. Takemoto, N. Nishimoto, H. Meguri, and K. Katayama, *Yakugaku Zasshi*, 78, 110 (1958).
4) H. Ohue, M. Ohnishi, and H. Meguri, *Yakugaku Zasshi*, 94, 756 (1974).

R. metternichii SIEBOLD *et* ZUCCARINI var. *pentamerum* MAXIMOWICZ (collected at Sakunami, Miyagi prefecture) were respectively extracted 6 times with refluxing 50% ethanol for 5 hr (each extraction) and the combined aqueous ethanol solutions were concentrated to give the extracts, the yields being 23.3% and 23.8%, respectively.

Acute Toxicity——Acute toxicity test was conducted in adult male mice of dd strain weighing approximately 25 g. Grayanotoxin I or III suspended in 3% gum arabic solution was orally administered to mice at various dose levels, and accumulated deaths were recorded up to 72 hr. The *p.o.* LD_{50} determined by means of the up and down method.

Subchronic Toxicity——Five 3 week old male mice (dd strain, weighing 13—16 g at the start of the experiment) and five 6 week old male rats (Wistar strain, weighing 110—140 g at the start) per group were housed at 23—25° and given free access to food and drinking water for a period of 12 weeks. Selected doses of grayanotoxin I and III, and the *Rhododendron* extracts (doses shown in the tables) were administered *p.o.* by intubation to animals 6 days per week as suspension in 0.5% gum arabic solution in the volume of 0.05 ml/10 g body weight for mice and of 0.1 ml/100 g body weight for rats. Control animals received equal volumes of 0.5% gum arabic solution. All animals were examined 6 days per week for clinical signs of toxicity and weighed twice weekly.

Surviving animals at the termination of the 12 weeks experiment were sacrificed. Hematologic examinations included determinations of total leucocyte counts and erythrocyte counts. Serum biochemical examinations including quantitative tests for glutamic-oxalacetic transaminase (GOT) activity, glutamic-pyruvic transaminase (GPT) activity, alkaline phosphatase (AlP) activity, and lactic acid dehydrogenase (LDH) activity were performed at the same time.

At necropsy, gross examinations were made of all animals. Livers, kidneys, and spleens were excised and immediately weighed. The tissues from livers, kidneys, and spleens were fixed in 3% formalin, processed in routine fashion, stained with hematoxylin and eosin, and examined microscopically.

Results

In the acute toxicity test, signs induced in the animals by *p.o.* administration of grayanotoxin I and III were similar to those in the case of *i.p.* administration.[2] Deaths from poisoning of the animals occurred mostly within the lethal criterion time of 60 min. The *p.o.* LD_{50} values were 5.1 mg/kg for grayanotoxin I and 4.9 mg/kg for grayanotoxin III.

In the subchronic toxicity test, no significant differences were observed between the control animals and those given the toxins or the extracts at all dosage levels with respect to physical signs and behaviors throughout the test period, with the exception that the administration of grayanotoxin I and III at the highest dose 1 mg/kg/day (*ca.* 1/5 of *p.o.* LD_{50}) caused a considerable degree of flaccidity in mice initially but this effect lessened later in the study.

Weight data for control and test level animals are presented in Table I.

The mean body weights of all the treated mice exhibited a trend to be lower than those of the control mice in the period of study. Particularly, the body weight decrease was significant in the *R. brachycarpum* treated group ($p<0.01$). In rats, no significant differences were noted between the control and test level animals.

The liver weights generally decreased in both toxin- and extract-treated mice and rats. Daily administration of the *Rhododendron* extracts and the toxins at 1 mg/kg/day caused remarkable decrease in the weights of spleens ($p<0.01$ except for *R. brachycarpum* in rats). No other significant changes of organ weights and organ/body weight ratios occurred in mice and rats.

The results of hematologic examinations are shown in Table II. A marked decrease in total leucocyte counts occurred in mice fed grayanotoxin III at 1 mg/kg/day ($p<0.05$), while the administration of grayanotoxin I at 0.05 and 0.25 mg/kg/day was accompanied by increases in total leucocyte counts in mice and rats. The total leucocyte counts and erythrocyte counts in the other test level animals were found to be within normal limits.

The data in serum enzymes are presented also in Table II. Among serum GOT, GPT, AlP, and LDH activity determined as an index of hepatotoxicity, GOT, GPT, and LDH values were in general elevated in the mice and rats tested. In particular, GPT values, and

TABLE I. Body Weight, Organ Weight, and Organ/Body Weight Ratio for Male Mice and Rats Receiving Daily Grayanotoxins and *Rhododendron* Leaves for 12 Weeks

Animal species	Substance[a]	Daily *p.o.* dose (mg/kg)	Initial body wt. (g)	Terminal body wt. (g)	Liver		Kidney		Spleen	
					Wt. (g)	Ratio (%)	Wt. (g)	Ratio (%)	Wt. (g)	Ratio (%)
Mouse	Control	—	14.0± 2.1	31.0± 0.6	1.68±0.02	5.4	0.53±0.03	1.7	0.11±0.02	0.34
	G-I	0.05	14.8± 0.8	28.6± 1.0*	1.46±0.14	5.1	0.51±0.04	1.8	0.11±0.02	0.38
		0.25	14.6± 0.4	29.2± 1.4	1.47±0.02**	5.0	0.46±0.01**	1.6	0.10±0.01	0.35
		1.0	14.3± 0.8	30.0± 1.0	1.64±0.07	5.5	0.48±0.04	1.6	0.08±0.01**	0.27
	G-III	0.05	14.4± 0.6	30.0± 0.5	1.56±0.14	5.2	0.52±0.02	1.7	0.09±0.00**	0.29
		0.25	14.6± 1.0	29.6± 1.3	1.60±0.06	5.4	0.51±0.02	1.7	0.09±0.00**	0.29
		1.0	14.1± 0.8	29.0± 1.0	1.50±0.11	5.2	0.43±0.03**	1.5	0.07±0.01**	0.25
	R.b.	400	14.4± 1.0	28.4± 0.6**	1.37±0.03**	4.8	0.46±0.01**	1.6	0.08±0.00	0.28
	*R.m.*v.*p.*	400	14.2± 0.3	29.2± 0.9	1.63±0.03	5.6	0.51±0.03	1.8	0.09±0.00**	0.30
Rat	Control	—	122.8±11.3	380.5±12.5	13.25±0.09	3.5	2.28±0.09	0.60	1.14±0.26	0.30
	G-I	0.05	120.8± 9.5	311.3±37.5	9.70±0.89**	3.1	2.01±0.22	0.69	1.21±0.12	0.39
		0.25	127.4±11.3	364.4±25.0	13.71±0.82	3.8	2.51±0.07	0.69	1.07±0.14	0.29
	G-III	0.05	128.6± 9.3	323.0±33.6	10.45±0.70**	3.2	2.03±0.12	0.63	1.10±0.03	0.34
		0.25	127.6± 5.1	331.5±45.9	12.50±1.89	3.8	2.07±0.26	0.62	1.25±0.11	0.38
	R.b.	400	121.8± 6.6	362.7±18.1	12.15±1.09	3.4	2.37±0.10	0.65	0.95±0.59	0.26
	*R.m.*v.*p.*	400	121.2± 3.4	341.7±43.1	10.73±0.97*	3.1	2.01±0.09*	0.59	0.85±0.02**	0.25

a) 5 animals/group except for the two groups (G-I and G-III, 1.0 mg/kg) in which the experiment was started with 5 mice but terminated with 4 mice.

Abbreviations: G-I=grayanotoxin I, G-III=grayanotoxin III, *R.b.*=*Rhododendron brachycarpum* leaves, *R.m.*v.*p.*=*R. metternichii* var. *pentamerum* leaves.

* Significantly different from the control, $p<0.05$.

** Significantly different from the control, $p<0.01$.

TABLE II. Biological and Biochemical Blood Data of Male Mice and Rats after Daily Feeding with Grayanotoxins and *Rhododendron* Leaves for 12 Weeks

Animal species	Substance[a)]	Daily *p.o.* dose (mg/kg)	Blood biologicals		Plasma biochemicals			
			T. leucocytes ($\times 10^3$/mm^3)	Erythrocytes ($\times 10^6$/mm^3)	GOT (IU/l)	GPT (IU/l)	AlP (IU/l)	LDH (IU/l)
Mouse	Control	—	5.63±0.83	9.76±0.51	40.5± 8.2	18.8±4.6	41± 5	510± 84
	G-I	0.05	8.52±2.07	9.64±0.47	40.5± 3.6	17.8±2.1	38± 3	448± 52
		0.25	8.33±1.39	9.86±0.35	49.6± 3.9*	18.3±1.3	36± 2	650± 36
		1.0	6.50±1.55	8.83±0.47	45.8± 4.0	28.9±1.0**	43± 3	448± 36
	G-III	0.05	4.58±0.73	9.05±0.13	44.8± 5.8	19.3±1.3	41± 3	508±102
		0.25	5.80±1.04	9.49±0.22	40.5± 3.3	20.7±2.1	38± 4	548± 95
		1.0	2.90±0.84*	8.94±0.37	56.9± 4.6**	25.1±2.4*	45± 4	547± 69
	R.b.	400	5.75±0.00	9.31±0.41	48.7± 3.9	25.1±3.9	46± 1**	913±489
	*R.m.*v.*p.*	400	7.54±1.60	9.49±0.13	51.6± 5.9	24.6±5.6	46± 3	600± 15
Rat	Control	—	15.21±2.67	8.93±0.28	43.9± 1.9	18.8±1.7	214±19	435± 27
	G-I	0.05	22.69±6.85	8.84±0.54	46.8± 1.0	17.4±1.2	209±17	351± 44
		0.25	17.83±5.02	8.79±0.40	56.4± 6.6	22.7±1.7	223±25	265± 36
	G-III	0.05	14.42±2.15	9.30±0.36	50.1± 6.9	19.3±1.2	227±29	414±164
		0.25	14.66±1.49	8.57±0.49	44.3± 3.2	20.2±2.0	268±33	324±105
	R.b.	400	15.28±0.42	8.19±0.34	44.3± 1.3	21.2±2.0	242±81	297± 43
	*R.m.*v.*p.*	400	12.00±3.07	7.53±0.24**	62.2±13.3**	28.0±1.5**	128±22**	603±159

a) 5 animals/group except for the two groups (G-I and G-III, 1.0 mg/kg) in which the experiment was started with 5 mice but terminated with 4 mice.
For abbreviations see Table I.
* Significantly different from the control, $p<0.05$.
** Significantly different from the control, $p<0.01$.

GOT and GPT values were slightly elevated in mice receiving grayanotoxin I and III at 1.0 mg/kg/day, respectively, when compared to the gum arabic treated mice. Administration of both the *Rhododendron* extracts to mice resulted in increases in the GOT and GPT values as compared with the control levels. It is worthy to note that the rats dosed with the *R. metternichii* extract gave higher values of GOT, GPT ($p<0.01$), and LDH, and a lower value of AlP ($p<0.01$) than the gum arabic treated controls.

Necropsies performed on the animals showed no alterations of appearance in the organs. The results of the histological examinations on the organs of the animals fed for 12 weeks were as follows: Control mice: The liver showed normal architecture except a slight activation of stellate cells which revealed slight to moderate nuclear atypia. Mice which received grayanotoxin I at 0.05 mg/kg/day: In the liver, a slight limphocytic infiltration intermingled with mononuclear cells and granulocytes were observed in Glisson's sheath in two cases, and microgranuloma was formed in one case accompanying a slight swelling of liver cells in adjacent portion. The spleen and kidneys were normal. Mice which received grayanotoxin I at 0.25 mg/kg/day: In all cases of this group, a small number of microgranulomas were present in the liver. However, it is unlikely to consider that these lesions may be produced any functional disturbance. The spleen and kidneys were normal. Mice which received grayanotoxin I at 1.0 mg/kg/day: The liver cells showed slight swelling with clear cytoplasm. The spleen and kidneys were normal. Mice which received grayanotoxin III at 0.05 mg/kg/day: In one case, the arrangement of liver cells was slightly dissociated in association with lymphocytic infiltration. Other cases showed normal hepatic architecture. The spleen and kidneys were normal. Mice which received grayanotoxin III at 0.25 mg/kg/day: In two cases, a few microgranulomas were present in the liver, and one case showed a slight nuclear stypia of liver cells. No other particular changes were observed in the liver. The spleen and kidneys showed no particular changes. Mice which received grayanotoxin III at 1.0 mg/kg/day: In two cases, small microgranulomas were present in a few number in the

liver. Other spleen and kidneys were normal. Mice which received the *R. brachycarpum* and *R. metternichii* extracts: The liver, spleen, and kidneys contained normal histologic pattern. Control rats: The liver showed normal architecture with slight eosinophilic cell infiltration in Glisson's sheath. The spleen contained several megakaryocytes and different types of granulocytic cells, and the kidneys revealed normal structure. Rats which received grayanotoxin I at 0.05 mg/kg/day: The liver had normal architecture with a small number of microgranulomas in acini and mild lymphocytic infiltration in Glisson's sheath. The spleen showed similar finding as those of control, but the kidneys had slight degree of lymphoid cell infiltration in the interstitial tissue. Rats which received grayanotoxin I at 0.25 mg/kg/day: The liver showed similar findings as those of the above group. There was no cellular damage in the liver. The spleen and kidneys were normal. Rats which received grayanotoxin III at 0.05 mg/kg/day: The liver, kidneys, and spleen were almost normal. Rats which received grayanotoxin III at 0.25 mg/kg/day: A slight infiltration of the lymphocytes was observed in the liver of a rat, and several microgranulomas were formed in the liver of another rat. The spleen and kidneys were normal. Rats which received the *R. brachycarpum* and *R. metternichii* extracts: In both groups, the liver, kidneys, and spleen showed normal structure and cellular components.

Discussion

Rhododendron leaves have been employed as a cure for hypertension in a folk remedy in Japan. However, since they are known to contain grayanotoxins which exhibit high acute toxicity, their repeated dosage might bring about some side effects. In order to examine the possibility, subchronic toxicity of the leaves of *R. brachycarpum* and *R. metternichii* var. *pentamerum* as well as of their poisonous constituents grayanotoxin I and III was examined.

The long-term administration of the extracts of the two species of the *Rhododendron* plants resulted in elevation of serum GOT and/or GPT values in mice and rats though no other significant toxicologic effects were found, suggesting that ingestion of these plants induces slight liver lesions. Since *Rhododendron* leaves contain the grayanotoxins in the order of 0.01%, administration of the extracts of the dried leaves (0.4 g/kg) is thought to be corresponding to that of a grayanotoxin (0.05 mg/kg). However, according to the criteria used in the evaluation, the serum parameters as well as behavior, general appearance, body weight, organ weight (except for the weights of the spleens as discussed below), and hematologic findings did not reveal no discernible toxicologic effects on mice and rats receiving the aforementioned dose (0.05 mg/kg/day) and its 5-fold dose (0.25 mg/kg/day) of grayanotoxin I or III, a fact which indicates that the hepatotoxic effects of the *Rhododendron* leaves reflected in the blood biochemical data are mediated by other principles rather than the grayanotoxins.

The daily administration to mice of grayanotoxin I or III at a dose of 1.0 mg/kg (the 1/5 amount of the acute LD_{50} which might cause subacute death of the animals) led to the results that only one of the mice died in each group during 12 weeks and there were no severe differences between the control and test animals (survivors) regarding body weights, organ weights, and hematologic and blood chemical evaluations except for the decrease of the spleen weights and the increase of the GOT and GPT values in both groups and the reduction of the total leucocytes in the grayanotoxin III-treated group.

Pathological examinations on the organs excised from the animals showed that in the livers of several cases in the treated mice, only a slight infiltration of the lymphocytes and formation of microgranulomas was observed, while in the livers of some cases in the treated rats only a slight infiltration of the lymphocytes or microgranulomas was present, no essential differences between the control and treated animals, however, being noticed. Although slight liver function lesions in the toxin-fed animals were suggested by the above biochemical evidence, the histological evidence indicated that the liver function maintained within normal state.

When the animals were fed with the *Rhododendron* extracts and the toxins at a higher dose, the weights of the spleens were significantly reduced. However, histological examinations of the spleens in question revealed no discernible changes, demonstrating that the administration of the *Rhododendron* extracts and their constituents at the doses employed caused no appreciable functional disorders of the spleen. These findings, therefore, were unseemingly considered to give any significant influence to the function.

On the basis of the above evidence, it is concluded that despite of the high acute toxicity, graynotoxin I and III show rather weak subchronic toxicity as far as the parameters selected in this study concern, which is possibly due to rapid metabolism and/or excretion.

Acknowledgement Thnaks are due to Ms. T. Tamada, this laboratory, for technical assistance, to Dr. O. Miura, Botanical Garden of Tohoku University, and to Mr. T. Kitahara, Sendai District Forest Office, for donation of the *Rhododendron* leaves.