Korean J. Toxicol.
Vol 4, No. 2, pp. 85-94, 1988

EFFECTS OF AQUEOUS EXTRACT OF A POISONOUS MUSHROOM, *AMANITA PANTHERINA* ON MICE AND ASSAY OF TOXIC ISOXAZOLE DERIVATIVES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Yoshio Yamaura and Il-Moo Chang*

Nagano Research Institute of Public Health, Nagano 380, Japan
**Natural Products Research Institute, Seoul National University, Seoul 110-460, Korea*

ABSTRACT: *In order to elucidate the mechanism of toxic action of a pisonous mushroom, Amanita pantherina, biochemical effects of the mushroom extracts on mice were studied. A hot-water extract of Amanita pantherina injected intraperitoneally into male ICR mice evoked signs similar to those observed clinically upon acute poisoning by the mushroom and also changed the levels of component enzyme activities in blood, liver and urine. The serum cholinesterase activity was decreased significantly during 1-3 h after injection. The blood glucose level was increased markedly at 1 h and then decreased at 3 h after the injection, and the hepatic glycogen level was decreased to 73% and 57% of the controls at 1 and 3 hr after the injection, respectively. The levels of ammonia and urea nitrogen in blood was increased to approximately 2-3 fold of the control levels by 3 h after the injection. Some nitrogen compounds in urine were affected by the treatment of mushroom extract. The activities of serum trans aminases did not change for 12 h after the injection. The obtained results suggest that the mushroom extract caused toxicity in the nervous and kidney systems but not in the liver. Isoxazole derivatives in the mushroom, ibotenic acid and muscimol were determined by a newly developed high performance liquid chromatography method. The contents of ibotenic aci and muscimol in the mushroom were 0.066% (W/W) and 0.028% (W/W) of the fresh mushroom, respectively.*

Keywords: *Amanita pantherina, Neurological effect, Blood glucose, Renal toxicity, Ibotenic acid, Muscimol, High performance liquid chromatography.*

INTRODUCTION

In Japan, there has been a notable increase in the consumption of wild mushrooms and this has resulted in a considerable increase in mushroom poisoning cases (The Sta-

tistics, 1980-1987). The treatment of the mushroom poisoning should be different with the species of mushrooms, since symptoms of the poisoning are different depending on the species of mushrooms ingested. The treatment of the patients with mushroom poisoning has not sufficiently been established, because the mechanism of mushroom poisoning has not been fully elucidated and most of the toxic constituents in the mushrooms have not been identified.

To provide useful information to the clinical treatment of mushroom poisoning, we have studied the changes of components and enzyme activities in blood, liver and urine of mice showing clinical signs induced by the poisonous mushrooms of various species (Yamaura, 1981, 1982, 1983, 1984, 1986a, 1986b).

In the present study, the biochemical effects caused by an aqueous extract of *Amanita pantherina* in mice were investigated. A new high performance liquid chromatography (HPLC) method for the simultaneous determination of isoxazole derivatives in the mushroom was developed.

EXPERIMENTAL

Materials

Amanita pantherina were collected in Nagano, Japan in October 1986. As mushroom are normally cooked by heating, an extract of the mushroom was prepared as follows. The fresh mushrooms were cut into small pieces and boiled in 4 vols. of distilled water for 10 min and filtered. The filtrate was used as the source of aqueous extract of the mushroom in the animal experiment.

Ibotenic acid (α-amino-3-hydroxy-5-isoxazoleacetic acid) and muscimol (5-aminomethyl-3-hydroxy-isoxazole) were purchased from Aldrich Chemical Co., Milwaukee, U.S.A. and Sigma Chemical Co., St. Louis, MO, U.S.A., respectively.

Animals and treatment

Male ICR mice of 5 weeks old (Shizuoka Lab. Animals Co. Ltds., Shizuoka, Japan) were injected intraperitoneally at a dose of 1.5 g of the fresh mushroom per kg body weight. Control animals received an equal volume of 0.9% saline. Diet was withdrawn after injection, but water was provided *ad libitum* until the time of sacrifice.

Assay o f blood parameters

Cholinesterase (Gomi, 1977), glucose (Cawley *et al.*, 1959), glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT)(Reitman and Frankel, 1957), uric acid (Lorentz and Berndt, 1967), and creatinine and creatine (Bonsnes and Taussky, 1945) were determined using freshly prepared serum. Urea nitrogen and ammonia were also assayed by commercially available kits (Wako Pure Chemical Co. Ltd., Osaka, Japan).

Assay of hepatic components and enzymes

Glycogen, protein and glutathione contents were assayed by the method of Morris (1984), by the modification (Miller, 1961) of the method of Lowry (1951), and by the DTNB[5,5′-dithiobis(2-nitro-benzoic acid)](Ellman, 1959), respectively. For the assay of heaptic enzymes, the fresh liver was homogenized with 1.15% KCl/50 mM Tris-HCl (pH 7.4) and the homogenate was centrifuged at 105,000 g for 60 min to obtain microsomal and cytosol fractions, which were then used for the assay of glucose-6-phosphatase (G-6-Pase) (Swanson, 1972), and glucose-6-phosphate dehydrogenase (G-6-P DH)(Glock and McLean, 1953), respectively.

Assay of urinary parameters

Urine was collected using metabolic cages (Sugiyamagen Co. Ltd., Tokyo). Glucose, protein and urobilinogen in urine were assayed by Urinary Ana.yzer (Ames Co. Ltd., U.S.A.). Urea nitrogen, ammonia, uric acid, creatinine and creatine were also assayed by the same methods of those of the blood parameters.

Determination of isoxazole derivatives in the mushroom

A method which ibotenic acid and muscimol can be determined simultaneously has been developed using HPLC with ultraviolet photometric detection (Shimazu LC-3A, Shimazu Co. Ltd., Japan).

The fresh mushroom (10g) was homogenized with 100 m*l* of 50% aqueous methanol and filtered. The 10 m*l* of the filtrate was poured onto a Dowex 1-X8 column (acetate form) and eluted with 15 m*l* of water. Ibotenic acid was adsorbed and muscimol passed through the column. After the fraction containing muscimol was concentrated, it was added on bi-layer column (upper: alumina, lower: silica gel) and eluted with 60 m*l* of acetone-methanol-water (2:2:1). Ibotenic acid was eluted with 70 m*l* of 0.1 M acetic acid from the Dowex 1-X8 column. The eluates containing ibotenic acid and muscimol were combined and evaporated to dryness at 40°C. The residue was

dissolved in 1 ml of 50% methanol and injected into a Shim-pack PNH_2-10/S2504 column (10 m, 4.0 × 250mm) at 40°C. The sufficient resolution of these constituents was obtained using 0.01 M phosphate buffer (pH 4.5)-methanol (30:70) as mobile phase with a flow rate of 1.0 ml/min and with detection at 220 nm.

Statistical analysis

Statistical significance between the control and the treated groups was determined using Student's t-test.

RESULTS

General

The minimum lethal dose of a single intraperitoneal injection of the aqueous extract of *A. pantherina* on male ICR mice during 24 h was 21.5 g fresh mushroom/kg body weight. The clinical signs observed in early phase after injection in the treated mice were dilated pupils and salivation. Muscle spasm of back legs was observed about 2 h after injection.

Blood and hepatic parameters

As shown in Fig. 1, serum cholinesterase activity was decreased in early phase after injection, attaining 70% of the control levels at 3 h after the injection and recovered gradually with time. Blood glucose level was increased markedly at 1 h and then decreased by 3 h after injection (Fig. 2). Effects of the mushroom extract on hepatic com-

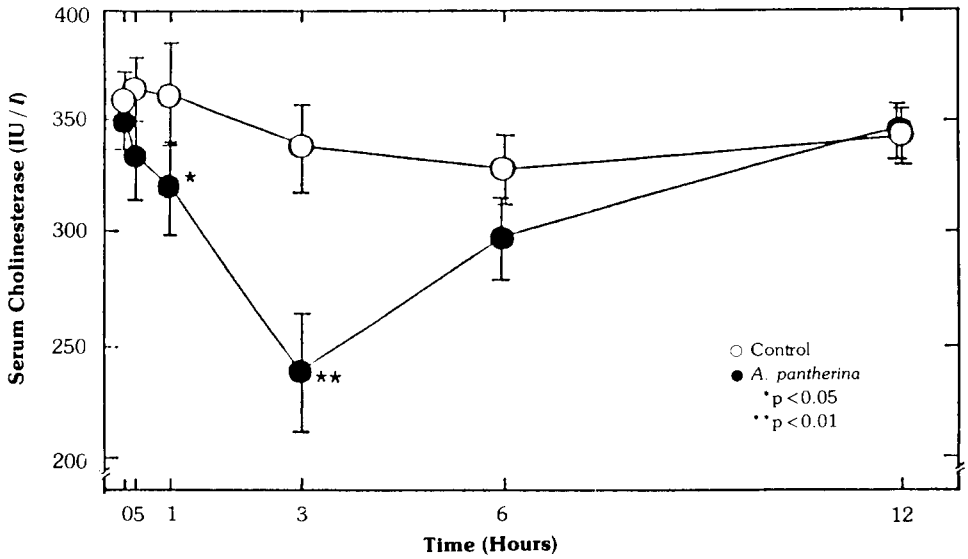


Fig. 1. Time related change of serum cholinesterase activity after an injection of a dose of 1.5 g of the fresh mushroom per kg body weight (Mean ± S.E. of 5 mice).

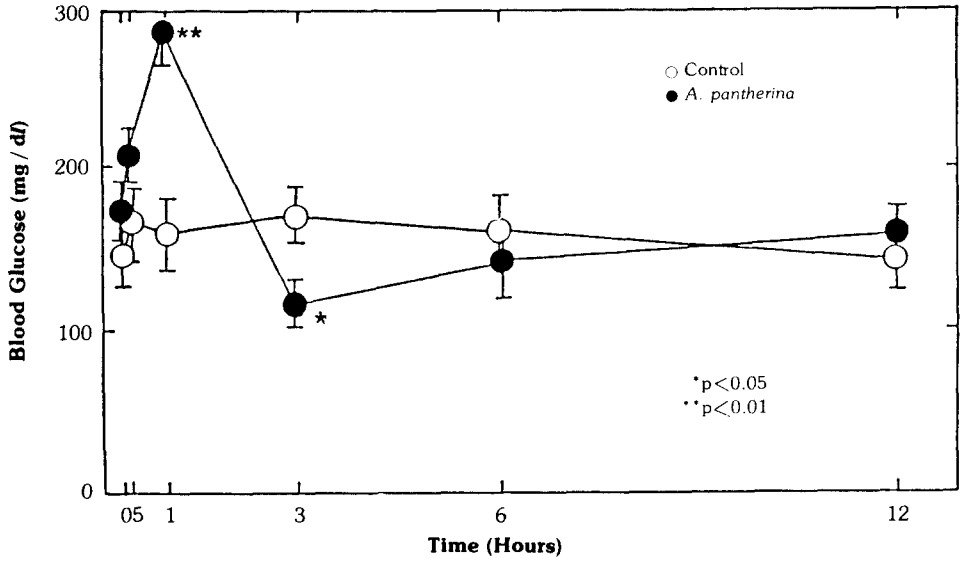


Fig. 2. Time related change of blood glucose content after an injection of a dose of 1.5 g of the fresh mushroom per kg body weight (Mean ± S.E. of 5 mice).

ponents and enzymes are summarized in Table 1. Hepatic glycogen level decreased to 73% (1 h) and 57% (3 h) of the controls, respectively. A slight increase, but not significant, was observed in the G-6-Pase and G-6-P DH activities at 1 h after the injection, and both enzyme activities tended to decrease by 3 h after the injection. The levels of microsomal protein and glutathione did not change.

Time related change of BUN level in blood is shown in Fig. 3. A significant increase in BUN level was observed during 1-12 h after injection and the maximal level was reached by 3 h after the injecion. Change of nitrogen compounds in blood are shown in Table 2. Ammonia level was increased twice that of the controls at 3 h after the injection. Levels of uric acid and creatinine were also increased significantly. The activities of SGOT and SGPT did not change for 12 h after the injection.

Table 1. Effect of the *A. pantherina* extract on hepatic components and enzymes in mice after an i.p. injection of a dose of 1.5 g the fresh mushroom per kg body weight. Values are mean ± S.E. of 5 mice. Asterisks indicate the statistical significances at $P<0.05(*)$ and $P<0.01(**)$.

Time after injection	Protein (mg/g)	Glycogen (mg/g)	Glucose-6-phosphatase (Piμg/min/g)	Glucose-6-phosphate dehydrogenase (μmol/min/g)	Glutathione (μmol/g)
1 h					
Control	24.3 ± 1.5	11.5 ± 1.02	6.60 ± 1.07	714 ± 96	6.38 ± 0.21
Treated	23.7 ± 1.3	8.47 ± 0.62*	7.77 ± 0.68	812 ± 87	6.12 ± 0.16
3 h					
Control	27.3 ± 2.1	12.5 ± 0.65	6.85 ± 0.53	786 ± 63	5.34 ± 0.36
Treated	28.2 ± 1.4	7.12 ± 0.92**	6.03 ± 0.47	627 ± 79	5.48 ± 0.34

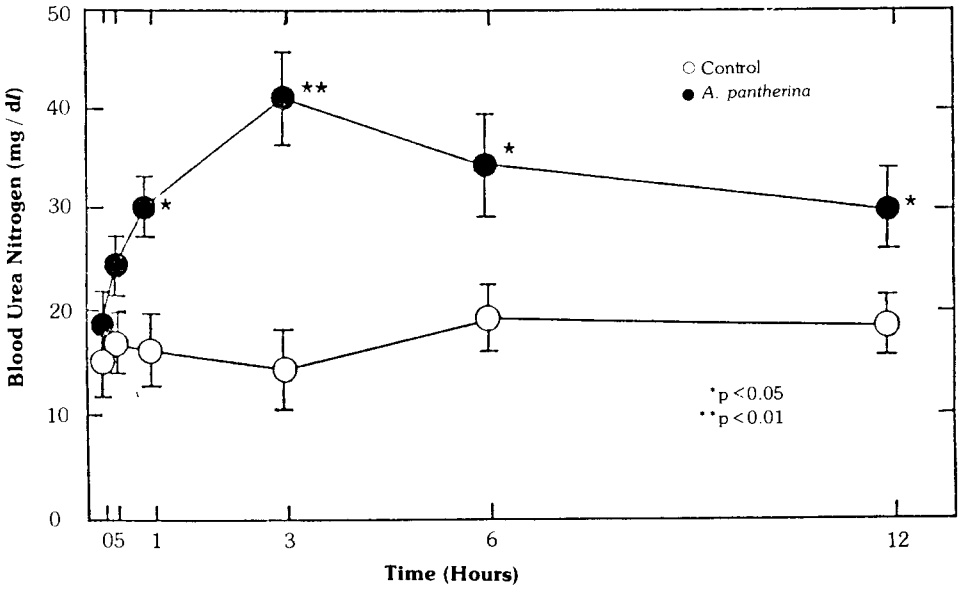


Fig. 3. Time related change of blood urea nitrogen content after an injection of a dose of 1.5 g of the fresh mushroom per kg body weight (Mean ± S.E. of 5 mice).

Table 2. Effect of the *A. pantherina* extract on nitrogen compounds in blood at 3 h after i.p. injection of a dose of 1.5 g of the fresh mushroom per kg body weight. Values are means ± S.E. of 5 mice. Asterisks indicate the statistical significances at $P<0.05$(*) and $P<0.01$(**).

	Control	Treated
Ammonia(μg/dl)	53 ±6.1	117 ±7.2**
Uric acid(mg/dl)	1.42±0.06	2.81±0.09**
Creatinine(mg/dl)	0.41±0.01	0.62±0.03*
Creatine(mg/dl)	2.46±0.15	2.71±0.02

Urinary parameters

Change of urinary parameters is shown in Table 3. The levels of urea nitrogen and uric acid were lower in the treated mice than those of the controls. Creatinine level was higher than that of the control. The contents of glucose, protein and urobilinogen were equal to those of the controls.

Assay of isoxazole derivatives

As active constituents, ibotenic acid and muscimol were identified in the mushroom extract as shown in the chromatograms of HPLC (Fig. 4). The recoveries of ibotenic acid and muscimol were 99.3% and 90.6%, respectively. The method can be applicable for the determination of ibotenic acid and muscimol in the mushroom with determination limits of 18 and 10 ppm, respectively. The contents of ibotenic acid and

Table 3. Effect of the *A. pantherina* extract on urinary parameters. Urine was collected for 24 h after an i.p. injection of a dose of 1.5 g of the fresh mushroom per kg body weight. Values were obtained from the pooled samples of 5 mice.

Parameters	Control	Treated
Total urine(ml)	20	12
Glucose(mg/dl)	0	0
Protein(mg/dl)	30	30
Urobilinogen(Eu/dl)	0.1	0.1
Urea nitrogen(mg/day)	186	103
Uric acid(mg/day)	4.46	2.24
Creatinine(mg/day)	2.68	1.86

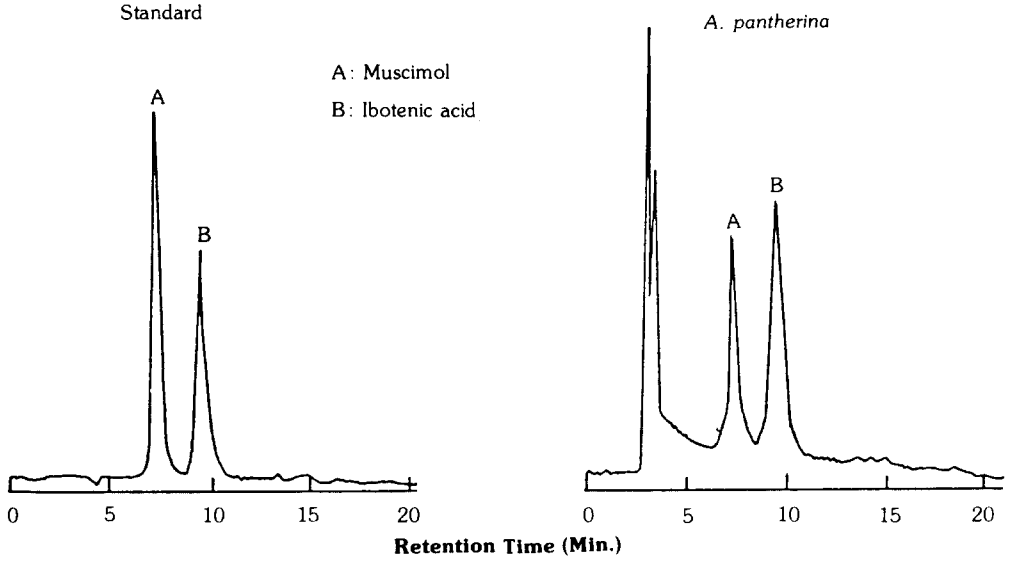


Fig. 4. High performance liquid chromatograms of the standard of ibotenic acid and muscimol and the extract of *A. pantherina*.

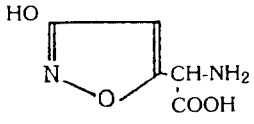
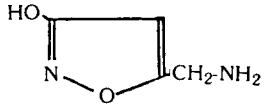
Chemical name	Ibotenic acid	Muscimol
Formula		
Content (ug/g-wet wt.)	660 ± 69	285 ± 49

Fig. 5. Structural formulas and contents of ibotenic acid and muscimol in *A. pantherina* (Mean ± S.D. of 3 mushrooms).

muscimol were approximately 0.066% (W/W) and 0.028% (W/W) of the fresh mushroom (Fig. 5).

DISCUSSION

Though there have been a few reports on the mushroom poisoning cases caused by *A. pantherina* (Hatson, 1934; Kawahara, 1978; Wucke, 1983), little is known about the mechanisms of toxic effects of its aqueous extract on human. *A. pantherina* is not deadly such as the *Amanita virosa* poisoning. That is, the lethal dose of *A. pantherina* was 1/5 of *Amanita virosa* reported previously (Yamaura, 1981).

In Nagano, Japan, the poisonings by *A. pantherina* occurred in 1985 and 1988, which involved four patients. The clinical symptoms observed in these cases were the neurological manifestations including salivary secretion, visual disturbances, confusion, muscle spasms, coma, and delirium followed. Some of these symptoms are similar to the clinical signs of the treated mice. The salivation observed in mice might be caused by stimulation of parasympathetic nerve ending, which induced the inhibition of serum cholinesterase activity is generally observed in organophosphorus pesticides intoxications (Ikeda *et al.*, 1975). In the present study, the serum cholinesterase activity was also depressed rapidly in the early phase after injection, indicating acetylcholine levels may be altered in the circulation.

Significant changes of components related to glucose metabolism, such as blood glucose and hepatic glycogen might be to due to an adrenal discharge or sympathetic discharge. The dilated pupils in clinical observations are consistent with such a mechanism.

The levels of nitrogen compounds such a BUN, ammonia, uric acid and creatinine in blood were increased significantly and the urinary parameters were also affected. The results indicate that kidney dysfunction might be provoked by the mushroom extract. The marked increase of ammonia level in blood may be the cause for a comatose state, which was observed in the poisoning cases of human (Hashimoto, 1983).

As the active constituents, ibotenic acid and muscimol have been identified in the *A. pantherina*. There have been a few methods for determination of ibotenic acid and muscimol by paper chromatography and thin-layer chromatography (Benedict *et al.*, 1966; Stijive, 1981). We have developed the precise method which ibotenic acid and muscimol are determined simultaneously by HPLC. The content of ibotenic acid in the *A. pantherina* was much higher than that of muscimol which is 5-fold potent than ibotenic acid in its effects on the nervous systems (Lincoff and Mitchel, 1977).

The present study thus shows that the clinical signs and biochemical effects observed in the treated mice were partly caused by these isoxazole derivatives.

ACKNOWLEDGEMENTS

We express our appreciation to Prof. Takashi Hashimoto of Shinshu University, School of Medicine and Dr. Morio Fukuhara of the Institute of Public Health for their valuable advices, and Prof. Kazumasa Yokoyama (Dept. of Biology Faculty of Education, Shiga University) for identification of the harvested mushroom. This work was

supported in part by research grants from Daido Seimei Social Welfare and the Nagano Society for the Promotion of Science.

REFERENCES

Benedict, R.G., Tyler, V.E. Jr. and Brady, L.R. (1966); Chemotaxonomic significance of isoxazole derivatives in *Amanita* species, *Lloydia*, 29: 333-342.

Bonsnes, R.W. and Taussky, H.H. (1945); On the colorimetric determination of creatinine by the Jaffe reaction, *J. Biol. Chem.*, 158: 581-591.

Cawley, L.P., Spear, F.E. and Kendall, R. (1959); Ultramicrochemical analysis of blood glucose with glucose oxidase, *Am. J. Clin. Pathol.*, 32: 195-200.

Ellman, G.L. (1959); Tissue sulfhydryl groups, *Arch. Biochem. Biophys.*, 82: 70-77.

Food Hygiene Devision, Envirometal Health Bureau, Japanese Ministry of Health and Welfare (1980-1987); The Statistics Department of Public Health and Wel fare.

Glock, G.E. and McLean, P. (1953); Further studies on the properties and assay of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver, *Biochem. J.*, 55: 400-408.

Gomi, K. (1977); The determination of GLDH, cholinesterase and their clinical significance, *Jpn. J. Clin. Path. Suppl.*, 29: 145-156.

Hashimoto, Y. (1983); Clinical pharmacology, p. 181, Asakura Press Co. Tokyo.

Hatson, J.W. (1934); Mushroom poisoning at Seattle, *Mycologia*, 26: 194-195.

Ikeda, Y., Hasegawa, Y. and Iwata, K. (1975); Toxicosis, pp. 175-185, Asakura Press Co., Tokyo.

Kawahara, I. and Nagasawa, T. (1978); An illustrated book of mushrooms and edible natural vegetables in Nagano, pp. 274-279, The Shinanomainichi, Nagano, Japan.

Lincoff, G. and Mitchel, D.H. (1977); Ibotenic acid-muscimol poisoning, in *Hallucinogenic Mushroom Poisoning*, ed. by W.K. Williams, pp. 85-89, Van Nostrand Reinhold Co., New York.

Lorentz, K. and Berndt, W. (1967); Enzymic determination of uric acid by a colorimetric method, *Anal. Biochem.*, 18: 58-63.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951); Protein determination with the Folin phenol reagent, *J. Biol. Chem.*, 193: 265-275.

Miller, G.L. (1961); Protein determination for large number of samples, *Anal. Chem.*, 31: 964.

Morris, D.L. (1948); Quantitative determination of carbohydrate with Dreywood's anthrone reagent, *Science*, 107: 254-255.

Reitman, S. and Frankel, S. (1957); A colorimetric method for the determination of serum glutamic-oxaloacetic and glutamic-pyruvic transaminases, *Am. J. Clin. Pathol.*, 28: 56-59.

Stijive, T. (1981); High performance thin-layer chromatographic determination of the toxic principales of some poisonous mushrooms, *Mitt Gebiete Lebensm, Hyg.*, 72: 44-54.

Swanson, M.A. (1972); Phosphatase of liver. I. Glucose-6-phosphatase, *J. Biol. Chem.*, 184: 647-659.

Wucke, K. (1983); Toadstool *Amanita pantherina* poisoning, *Gesamte Hyg.*, 29: 81-83.

Yamaura, Y., Maezawa, H., Takabatake, E. and Hashimoto, T. (1981); Biochemical effects of *Amanita virosa* extract on the components and enzymes in the liver and blood of mice, *Jpn. J. Food Hyg. Soc.*, 22: 203-208.

Yamaura, Y., Maezawa, H., Takabatake, E. and Hashimoto, T. (1982); Biochemical effects of poisonous mushrooms suspected of causing cholera-like symptoms in mice, *Jpn. J. Food Hyg. Soc.*, 23: 314-318.

Yamaura, Y., Komiyama, S., Fukuhara, M., Takabatake, E. and Hashimoto, T. (1983); Biochemical effects of *Amanita muscaria* extract in mice, *Jpn. J. Food Hyg. Soc.*, 24: 459-464.

Yamaura, Y., Wada, M., Komiyama, S., Fukuhara, M., Takabatake, E. and Hashimoto, T. (1984); Biochemical effects in mice and cytotoxicity in a cultured cell line of the extract of *Amanita abrupta, Jpn. J. Hyg.*, 39: 855-861.

Yamaura, Y., Fukuhara, M., Takabatake, E., Ito, N. and Hashimoto, T. (1986a); Hepatotoxic action of a *poisonous mushroom, Amanita abrupta* in mice and its toxic component, *Toxicology*, 38: 161-173.

Yamaura, Y., Fukuhara, M., Kawamata, S., Satsumabayashi, H. Takabatake, E. and Hashimoto, T. (1986b); Effects of *Clitocybe clavipes* extract on the components and enzymes related to ethanol metabolism in mice, *Jpn. J. Food Hyg. Soc.*, 27: 522-527.