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Edible Plants Containing Naturally Occurring Carcinogens in Japan

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Introduction

It is now clear from epidemiologic data that most human cancers are induced by environmental causes. Some of these environmental carcinogens are naturally occurring carcinogens. The Japanese have long used the young buds and seeds of a variety of native wild plants as special seasonal foods. Certain wild plants have also been used as folk medicines. However, some of these wild plants have been found to be carcinogenic to experimental animals. This review article deals with green plants used as human foods or folk medicines in Japan and found to be carcinogenic, and describes their utilization, their carcinogenic activity, and epidemiology.

Cycasin (Methylazoxymethanol-β-D-glucoside)

1. Discovery of the carcinogenicity of cycasin

Cycads (So-te-tsu in Japanese) are widely distributed in tropical and subtropical regions, and the species indigenous to Amami, Okinawa and Yaeyama, the southwestern islands of Japan is *Cycas revoluta* Thunb. In these islands, the seeds of cycads were used as a source of starch and as a constituent of the bean paste "miso" in some parts of the islands before and during World War II. An unusually high incidence of the neurological disease amyotrophic lateral sclerosis on the island of Guam was first reported by Kurland and Mulder.[1] It has also been reported that ingestion of cycad plant material in the tropics and subtropics causes paralysis of the hindlegs and ataxia in cattle.[2,3] Thus, studies on the cause of this disease focused on *Cycas circinalis* L., which the Guamanians had long utilized as a source of food starch. In an exploratory study at the National Institutes of Health on the possible existence of neurotoxins in cycads, Laqueur *et al.*[4] found that rats fed crude cycad meal developed hepatocellular carcinomas, kidney tumors of both epithelial and mesenchymal types, and intestinal tumors, though no neurological disorder was observed. Laqueur[5] subsequently found that the carcinogen in cycad is cycasin (Fig. 1), which was first isolated from the seeds of *C. revoluta* Thunb., identified in 1955 by Nishida *et al.*,[6] and later found by Riggs[7] in *C. circinalis* L. from Guam.

2. Metabolism of cycasin and its carcinogenicity

Cycasin was only toxic when given orally, and its toxicity appeared after a period of about 12 h.[8] Thus, it seemed likely that a metabolite of cycasin was the toxic compound. Since cycasin was apparently not toxic to germ-free rats, its fate in germ-free and conventional rats was compared by measuring the oral cycasin intake and its fecal and urinary excretion by these animals. Results showed that in germ-free rats excretion of cycasin was 97% of the intake, whereas in conventional rats it was only 26%.[9] These observations strongly suggested that intestinal microorganisms contained the enzyme that hydrolyzed cycasin *in vivo*. Germ-free rats were then infected with pure strains of microorganisms with or without β-glucosidase activity, as determined by *in vitro* assay. After these had successful colonized the intestine, cycasin was given by stomach tube. Good accordance was found between the enzymic activity of the microorganisms and extent of cycasin hydrolysis.[10] Whereas cycasin was toxic and carcinogenic only after passage through the GI tract, its aglycone methylazoxymethanol (MAM) (Fig. 1) was toxic and induced tumors in conventional and germ-free rats irrespective of the route of its administration.[11–17] MAM was, therefore, the proximate carcinogen. It has also been shown that the synthetic MAM acetate induced tumors in germ-free rats.[18]

The skin of newborn rats and of rats during early postnatal life was found to contain a β-glucosidase capable of hydrolyzing cycasin. Its activity was highest during the first few days after birth, then decreased from day 5 to day 8, and was no longer detectable by day 25 after birth.[19,20] The demonstration of β-glucosidase activity in subcutaneous tissue of newborn and early postnatal rats provided an explanation for the toxic[21] and carcinogenic[22–26] effects of cycasin which were observed after a single s.c. injection of cycasin into newborn animals.

Histological types of tumors in rats induced by cycasin or MAM were classified by Laqueur *et al.*[4,12] as follows: (1) liver — liver cell adenoma, liver cell carcinoma, bile duct adenoma, and cystadenoma; (2) kidney — adenoma, interstitial tumor, nephroblastoma, and sarcoma; (3) intestine — adenoma and adenocarcinoma. There are many reports on the carcinogenicity of cycasin in rats.[5,22,27,28] Cycasin is carcinogenic not only in rats but

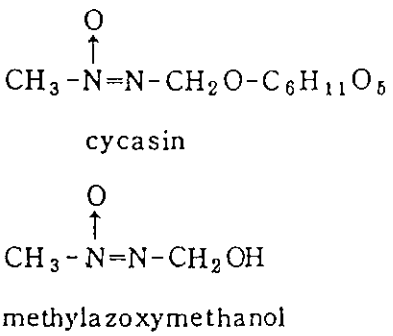


Fig. 1. Chemical structures of cycasin and its aglycone, methylazoxymethanol.

also in mice,[23, 24, 26] hamsters,[25] guinea pigs,[29] rabbits,[30] and aquarium fish.[31] Even on single administration, cycasin and MAM induced tumors of the kidney and large intestine in rats, tumors of the liver and lung in mice, and intrahepatic bile duct tumors in hamsters.[32]

3. Mutagenicity and other short-term tests

MAM prepared from crystalline cycasin induced reverse mutation in *Salmonella typhimurium*, although the parent compound was inactive.[17] Gabridge *et al.*[33] showed that cycasin and MAM increased the mutant frequency of *S. typhimurium* when tested in a host-mediated assay. Cycasin was also demonstrated to be mutagenic using a modification of the Ames *Salmonella* test in which cycasin was preincubated with β-glucosidase and the tester strain liquid medium.[34] Teas and Dyson[35] reported the results of tests for mutagenesis in *Drosophila* using the induction of sex-linked recessive lethals in males fed cycasin, MAM and MAM-acetate. Cycasin was not mutagenic but MAM and MAM-acetate were potent mutagens.

Bracken Fern

Bracken fern, *Pteridium aquilinum*, is widely distributed in many parts of the world. The toxic effect of bracken fern on livestock has attracted the attention of veterinary scientists since the end of the last century. The predominant feature in affected cattle is depressed bone marrow activity, named "bracken poisoning," which gives rise to severe leukopenia, especially of granulocytes, thrombocytopenia, the hemorrhagic syndrome, and hematuria.[36] Affected cattle nearly always die. The carcinogenicity of bracken fern was demonstrated most clearly by the experiment of Evans and Mason,[37] showing that rats fed diet containing bracken fern developed multiple ileal adenocarcinomas. Subsequently, the simultaneous induction of urinary bladder and ileal tumors[38] and the occurrence of mammary carcinoma (papillary carcinoma and adenocarcinoma) in addition to ileal and urinary bladder tumors in female Charles River Sprague-Dawley rats (CD rats) fed a bracken diet were reported.[39]

In Japan, young bracken fern in the fiddlehead or crosier stage of growth is used as human food. However, when rats were given a diet containing powdered young bracken fern, they developed ileal and urinary tumors. The most common site for ileal tumors was the terminal 20 cm of the ileum. Histologically, they were not only epithelial tumors, such as adenomas and adenocarcinomas, but also sarcomas (mainly, malignant fibrous histiocytoma).[40] The relationship between the administration of a diet containing bracken powder (33%) and tumor incidence in ACI rats was studied. The tumor incidence in these animals fed for 120 days, 60 days, and 20 days was 100%, 78%, and 50%, respectively. Thus, as short a period as 20 days caused intestinal tumors in 50% of the animals, suggesting that the carcinogenicity of bracken fern is considerable. Young bracken fern is generally used as a food in Japan after being processed by one of the following treatments: 1, fresh bracken is immersed in plain boiling water or in boiling water containing wood ash or sodium bicarbonate; 2, fresh bracken is pickled in salt, and then immersed in boiling water and washed with running water before use. Although the carcinogenicity was reduced markedly, processed bracken thus prepared retained weak carcinogenic activity.[41] Whereas tumor incidence in rats fed a diet containing unprocessed bracken was 78.5%, it was 25%, 10% and 4.7% in rats fed diets containing processed bracken treated with wood ash, sodium bicarbonate, and sodium chloride, respectively. The tumor incidence in rats fed a diet containing processed bracken treated with plain boiling water was 66.6%, while diet containing the same bracken without processing induced tumors in 100% of the animals. Carcinogenicity of bracken fern in laboratory animals and farm livestock is shown in Table I. The highest susceptibility to the bracken fern was observed in rats, and the lowest in hamsters.

1. Isolation of the bracken fern carcinogen

The historical background of studies on the nature of the bracken fern carcinogen is shown in Table II. The nature of the carcinogen remained unclear for a long time. Flavonoids,[67, 68] indanones,[54, 55] and pterolactam[57] have been isolated from bracken fern. However, there were no data to indicate that these chemicals are carcinogenic.[44, 56] Pamukcu *et al.*[52] implanted pellets containing a methanol extract of bracken fern into the urinary bladder of mice, and their findings suggested that the carcinogenic substance is soluble in methanol. Evans and Osman[53] reported that shikimic acid contained in bracken fern is carcinogenic in mice. However, rats fed

Table I. Carcinogenicity of Bracken Fern

Animal[a]	Target organs and histological findings
Rat [37, 38, 42]	ACI & CD: ileum, cecum (adenoma, adenocarcinoma, sarcoma, malignant fibrous histiocytoma)[40] Urinary bladder (papilloma, carcinoma) Liver (hyperplastic nodules)[43] CD: mammary cancer (papillary carcinoma, adenocarcinoma)[39]
Mouse[44, 45, 46]	Swiss & dd: lung adenoma, lymphatic leukemia C57BL/6: jejunal adenoma
Quail[45]	Cecum, colon, ileum (adenocarcinoma)
Hamster[45, 47]	Cecum, ileum (adenocarcinoma)
Guinea pig[45, 48]	Small intestine (adenoma, adenocarcinoma) Urinary bladder (transitional cell carcinoma)
Cattle[49, 50]	Urinary bladder (papilloma, carcinoma, hemangioendothelioma)

a) All animals were fed a diet containing dry bracken powder, except quail, which were given ethanol extract of bracken fern.

Table II. Historical Background of Studies of Bracken Carcinogen

Extract	Carcinogenicity test	Result	Reference
Acidic fraction of urine from cattle fed on bracken fern	Implantation of pellets containing the fraction into the urinary bladder of mice	+	Pamukcu *et al.*[51] (1966)
Methanol extract of bracken fern	〃	+	Pamukcu *et al.*[52] (1970)
Shikimic acid	Intraperitoneal injection or intragastric administration in mice	+	Evans & Osman[53] (1974)
Pterosin and pteroside	Feeding experiment in rats	−	Hikino *et al.*[54] (1970) Fukuoka *et al.*[55] (1972) Saito *et al.*[56] (1975)
Pterolactam	Urinary bladder implantation in mice, feeding or intragastric administration in rats	−	Takatori *et al.*[57] (1972) Hirono *et al.*[44] (1975)
Tannin	Urinary bladder implantation in mice	+	Wang *et al.*[58] (1976)
Shikimic acid	Feeding experiment in rats	−	Hirono *et al.*[59] (1977)
Boiling water extract of bracken fern	〃	+	Hirono *et al.*[60] (1978)
Tannin and tannin-free fraction of bracken fern	Subcutaneous injection of tannin fraction	+	Pamukcu *et al.*[61] (1980)
	Diet containing tannin fraction,	−	〃
	chloroform fraction, and	+	〃
	tannin-free fraction given to rats	+	〃
Quercetin	Feeding experiment (Rats)	+	Pamukcu *et al.*[62] (1980)
	(Mice)	−	Saito *et al.*[63] (1980)
	(Rats)	−	Hirono *et al.*[64] (1981)
	(Hamsters)	−	Morino *et al.*[65] (1982)
	(Rats)	−	Takanashi *et al.*[66] (1983)

a diet containing shikimic acid did not develop tumors.[59] Hikino *et al.*,[54] Fukuoka *et al.*,[55] and Saito *et al.*,[56] isolated indanones, pteroside and pterosin from the fronds and rhizomes of bracken, but these compounds were not carcinogenic. Wang *et al.*[58] isolated a carcinogenic tannin from bracken. The tannin induced bladder tumors in mice when intravesically implanted. However, neither intestinal nor bladder tumors were induced when a diet containing tannin was fed to rats.[61] Pamukcu *et al.*[62] reported that quercetin is contained in bracken fern and rats fed a diet containing 0.1% quercetin developed intestinal and urinary bladder tumors in high incidence. However, carcinogenic activity of quercetin has not been found in other experiments[63–66] including a recent study reported by Ito *et al.*[69] Thus, the nature of the carcinogen contained in bracken fern has long been uncertain. One important reason is the absence of specific acute toxicity of bracken in small laboratory animals such as rats and mice. The presence of acute toxicity of a certain substance does not necessarily denote carcinogenic activ-

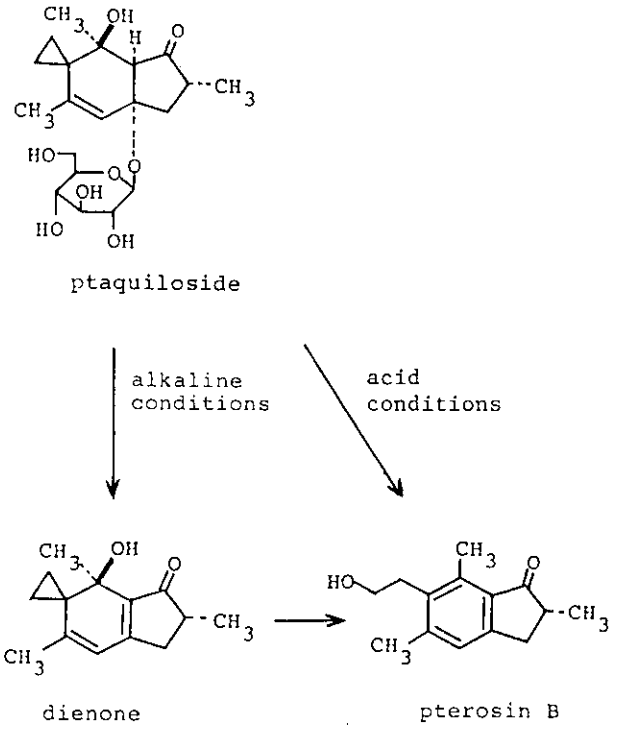


Fig. 2. Chemical structure of ptaquiloside and its reactions.

ity. Nevertheless, large numbers of carcinogens, such as cycasin and carcinogenic pyrrolizidine alkaloids, do produce signs of acute toxicity. The isolation of a carcinogen from a plant such as bracken fern, which produces no acute toxicity in laboratory animals, will obviously be difficult. The most reliable method was to look for carcinogenicity in each fraction using experimental animals as efficiently as possible. Each fraction from the boiling water extract of bracken was freeze-dried and mixed with rat basal diet. A diet thus prepared was given to female CD rats and the occurrence of mammary cancer was used as an early indicator for the presence of a carcinogen. We were successful in the isolation of a carcinogenic principle, ptaquiloside (Fig. 2), a novel norsesquiterpene glucoside of the illudane type.[70-73] Ptaquiloside is unstable at room temperature under both acidic and basic conditions and undergoes aromatization to give 1-indanone derivatives such as pterosin B and pterosin O, depending on the solvent used. Under particular alkaline conditions, ptaquiloside was converted with concomitant elimination of D-glucose into an unstable dienone. The extreme instability of the dienone can be ascribed to the presence of the highly reactive cyclopropane ring, which reacts quite readily with various nucleophiles (e.g., water, methanol, amines, thiols, etc.) (Fig. 2).

2. Carcinogenicity of ptaquiloside[74, 75]

Female CD rats were used in the carcinogenicity test of ptaquiloside. They were given an intragastric administration of ptaquiloside which was freshly dissolved in physiological saline each time. The incidences of mammary cancer (papillary carcinoma and adenocarcinoma) were 100 and 91% in two groups, respectively. Ileal tumor was also induced in high incidence, and the terminal 20 cm of the ileum was the most common site, as in the case of rats fed bracken diet. From these results, it is evident that ptaquiloside is the carcinogenic principle of bracken fern. However, urinary bladder tumor was induced in only one rat. In the next experiment, female ACI rats were given a diet containing ptaquiloside throughout the 210-day experimental period. Both ileal and urinary bladder tumors developed in all rats in the experimental group. The ileal tumors were adenomas, adenocarcinomas, and malignant fibrous histiocytomas. The urinary bladder tumors were transitional cell carcinomas, squamous cell carcinomas, and sarcomas.[75] Furthermore, it was demonstrated that ptaquiloside is not only the carcinogenic principle, but also the causative principle of cattle bracken poisoning.[76] Very recently, it was found that the progressive retinal degeneration, bright blindness, in sheep due to the ingestion of bracken fern is also induced by ptaquiloside.[77]

3. Mutagenicity and other short-term tests

Van der Hoeven *et al.*[78] isolated from bracken a new mutagenic compound which has the same planar structure as ptaquiloside and named it aquilide A. Aquilide A was found to be responsible for >50% of the mutagenic activity observed after incubation of the methanol extract under alkaline conditions. Mori *et al.*[79] reported that ptaquiloside elicited clear and dose-dependent unscheduled DNA synthesis by means of the hepatocyte primary culture/DNA-repair test. It was also reported that ptaquiloside was mutagenic in *S. typhimurium* TA100 and TA98 under weakly basic conditions (pH 8.5), and a novel bioassay for ptaquiloside was developed using a preincubation mutation test at pH 8.5 with *S. typhimurium* tester strains.[80, 81]

4. Epidemiology of the carcinogenicity of bracken fern

Kamon and Hirayama[82] conducted an epidemiologic survey of cancer in a mountainous area of central Japan where residents eat large amounts of bracken fern. They reported a significantly higher risk of esophageal cancer in people who ate either hot tea gruel or bracken fern every day; the risk was particularly high when both types of food were consumed. We[83] also reported that rats fed a diet containing 30% bracken powder for 33 weeks developed tumors of the pharynx and esophagus. How-

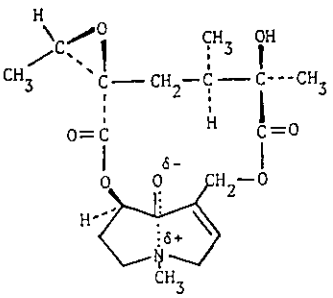
ever, the old eating customs in this area may be changing. The possible human hazard of bracken fern carcinogen has been indicated by several researchers,[84-86] especially as a consequence of its transfer into milk. A calf given milk from a cow receiving a sublethal dietary bracken supplement showed a hematological response which would be typical of a calf directly consuming bracken at a low rate.[85] Pamukcu *et al.*[86] studied the carcinogenicity of the milk of bracken fern-fed cows. The milk obtained was fed to rats as either fresh or freeze-dried powdered milk mixed with a grain diet. Groups receiving both fresh and powdered diets developed small intestinal, renal, or urinary bladder carcinomas, while none of the rats fed either fresh or powdered milk from cows receiving a normal (non-bracken fern) diet displayed neoplasia of those organs. Milk from cows that have eaten bracken fern may thus be hazardous to humans. In order to assess the epidemiological significance of the carcinogen as a human and animal hazard, establishment of an assay method for ptaquiloside was necessary. Natori and his group[87] developed a chemical assay of ptaquiloside and related compounds based on two-dimensional TLC-densitometry.

Edible Plants Containing Carcinogenic Pyrrolizidine Alkaloids in Japan

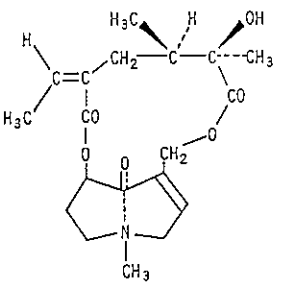
Pyrrolizidine alkaloids were first found as carcinogenic natural products of plant origin. They are contained in various kinds of plant all over the world, such as the tribe Senecioneae of the family Compositae, the tribe Crotalatia of the family Leguminosae, and the tribe Heliotropium of the family Boraginaceae. However, pyrrolizidine alkaloids are not always hepatotoxic. The number of pyrrolizidine alkaloids for which carcinogenic activity has been definitely proved is relatively few, e.g., retrorsine, isatidine, monocrotaline, and lasiocarpine.[88-90] We found that the young flower stalk of petasites, buds of coltsfoot, and leaf and root of comfrey are carcinogenic and that carcinogens contained in these plants are pyrrolizidine alkaloids, petasitenine, senkirkine, and symphytine.

1. Carcinogenicity of Petasites japonicus Maxim., a kind of coltsfoot[91]

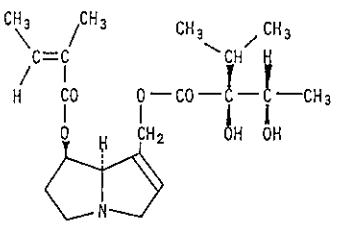
The carcinogenicity of young flower stalks of wild petasites, *P. japonicus* Maxim. (Japanese name "Fuki-no-toh"), which has long been used as a food or a herbal remedy (cough cure, expectorant, or stomachic) was studied in rats. The young flower stalk of *P. japonicus* was dried, then milled, and mixed with rat basal diet. Rats fed diet containing dry petasites powder developed hemangioendothelial sarcomas of the liver, hepatocellular adenomas, and hepatocellular carcinomas. *P. japonicus* is a herb of the tribe Senecioneae in the family Compositae.



petasitenine



senkirkine



symphytine

Fig. 3. Chemical structures of petasitenine, senkirkine, and symphytine.

Many species of this tribe commonly contain hepatotoxic pyrrolizidine alkaloids. The histologic feature of the liver injury induced in rats fed the flower stalk was quite similar to that induced by hepatotoxic pyrrolizidine alkaloids. Therefore, we[92] studied the nature of the alkaloids contained in the young flower stalk and were successful in the isolation of petasitenine, a new hepatotoxic pyrrolizidine alkaloid. Petasitenine is a stereoisomer of otosenine; its structure is shown in Fig. 3. Carcinogenic activity of petasitenine was tested using rats. They were given petasitenine solution as drinking water *ad libitum* and developed hemangioendothelial sarcomas of the liver and hepatocellular adenomas.[93] Thus, it was evident that the carcinogenic activity of the flower stalk could be attributed to petasitenine.

2. Carcinogenicity of coltsfoot, Tussilago farfara L.[94]

The dried buds of coltsfoot which is a herb of the tribe Senecioneae, family Compositae, are known as "Kan-to-ka" in Japanese and are imported into Japan as a cough cure or expectorant from China. Rats fed coltsfoot diet developed hemangioendothelial sarcoma in the liver. Chemical studies on the dried buds used in this experiment revealed that the pyrrolizidine alkaloid was present

in a low concentration (0.015%) and that the only constituent detectable was senkirkine (Fig. 3).[95] Senkirkine has been found to be hapatotoxic by Schoental,[96] and it seems most probable that the carcinogenicity of coltsfoot is due to senkirkine. When male ACI rats were given i.p. injection of freshly prepared senkirkine, 9 of 20 rats (45%) developed hepatocellular adenoma. These findings supported the conclusion that the carcinogenicity of buds of *T. farfara* can be attributed to senkirkine.[97]

3. Carcinogenicity of comfrey, Symphytum officinale L.[98]

S. officinale L. is a herb of the family Boraginaceae. This plant is called comfrey or Russian comfery and is cultivated for use in Japan as a green vegetable or tonic. The fresh leaves are used in salads, and their juice is used as a drink. Sliced roots are also eaten. Rats fed a diet containing dry powder of comfrey developed hepatocellular adenomas and hemangioendothelial sarcomas of the liver. It is known that comfrey contains symphytine (Fig. 3) and echimidine, which are pyrrolizidine alkaloids.[99, 100] To test the carcinogenic activity of symphytine, rats were given the chemical by i.p. injection. They developed hemangioendothelial sarcoma of the liver and hepatocellular adenoma.[97] These results strongly support the conclusion that the carcinogenicity of leaves and roots of comfrey, *S. officinale*, can be attributed to symphytine. The carcinogenicity of echimidine another pyrrolizidine alkaloid present in comfrey, has not yet been studied.

4. Carcinogenicity of Farfugium japonicum and Senecio cannabifolius

F. japonicum (Japanese name, Tsuwabuki) and *S. cannabifolius* (Japanese name, Hangon-so), which belong to the genus Senecioneae in the family Compositae, have been used as a human food. These plants were also hepatocarcinogenic in rats.[101] It has already been reported that petasitenine and senkirkine, which are hepatocarcinogenic pyrrolizidine alkaloids,[93, 97] are contained in *F. japonicum*.[102, 103] The carcinogenicity of *F. japonicum* is considered due to these pyrrolizidine alkaloids. This plant is a popular edible plant in the Kyushu area in Japan. It is usually used as human food after the bitterness has been removed by immersion in boiling water. It was reported by Niwa *et al.*[104] that petasitenine is extractable by boiling water. Carcinogenic activity of this plant may also be reduced by cooking with boiling water.

Judging from our experimental results so far obtained, it seems most probable that hepatotoxic pyrrolizidine alkaloids are simultaneous hepatocarcinogens.

5. Mutagenicity and other short-term tests of carcinogenic pyrrolizidine alkaloids

Both petasitenine and senkirkine induced mutations in *S. typhimurium* TA100 in the presence of S9 mix, using a preincubation assay.[105] These two alkaloids caused a significant increase in unscheduled DNA synthesis in rat hepatocytes.[106] They also induced chromosomal aberrations and forward mutations to 8-azaguanine resistance in V79 Chinese hamster cells.[107]

Symphytine induced mutation in *S. typhimurium* TA100 in the presence of S9 mix and also induced forward mutations to 8-azaguanine resistance in V79 Chinese hamster cells.[108]

Conclusions

It is generally difficult to estimate the human cancer risk of a carcinogen based on the results of carcinogenicity examinations of laboratory animals for the following reasons: the differences in life span and susceptibility to carcinogens between animals and human beings, multiple causal factors of human cancer, and the presence of factors inhibiting human carcinogenesis. Moreover, the dose and duration of exposure of human beings to a certain carcinogen cannot be accurately assessed. It is now clear from epidemiological data that most human cancers are induced by environmental causes. The possibility of cancer development by a certain carcinogen is increased when modifiers, such as the habitual use of a specific food or medicine or occupational conditions, provide favorable conditions for that development, even though the amount of carcinogen exposure is small. Thus, it is considered to be best to avoid the use of these plants as food or herbal remedies. Reduction of the total amount of carcinogens in the diet, including carcinogenic natural products, may eventually contribute to the prevention of cancer.

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