J. Pharm. Dyn., **3**, 236–244 (1980)

CHARACTERIZATION OF MUTAGENIC PRINCIPLES AND CARCINOGENICITY TEST OF DILL WEED AND SEEDS*

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(Received November 21, 1979)

Among the various kinds of spices tested, the aqueous extracts of dill weed from *Anethum graveolens* L. and dill seeds from *A. sowa* D.C. (Umbelliferae), exhibited a mutagenicity to *Salmonella typhimurium*, strains TA98 and TA100. The aqueous methanol extracts were fractionated by the mutation assay using the strain TA98 with S-9 Mix. Isorhamnetin 3-sulfate (persicarin) and quercetin 3-sulfate were characterized as the mutagenic principles.

Carcinogenicity was not observed for dill weed and seeds when the diets containing these in 33% were administered for 450 and 410 days, respectively, to the inbred strain ACI rats.

Keywords—flavonols; quercetin 3-sulfate; isorhamnetin 3-sulfate; dill weed; dill seeds; *Anethum graveolens* L; *Anethum sowa* D.C.; mutagen test; *Salmonella typhimurium*; carcinogenicity test

Spices and flavours contain large and heterogeneous groups of substances. They are generally accepted as safe as far as the amount used does not exceed a conventional level. However further assessment for safety including examination of carcinogenicity is required under current trend.

Recently mutagenicity test of the *Salmonella*/microsome system[1-3] is widely employed in a preliminary screening for carcinogens. In the course of examination of the mutagenicity for various spices at one of our laboratories, aqueous extracts of dill weed and seeds from *Anethum graveolens* L. and *A. sowa* D.C., respectively (Umbelliferae), which are generally recognized as safe,[4] showed a mutagenicity for *Salmonella typhimurium* strains TA98 and TA100. This paper reports the characterization of the mutagenic principles and carcinogenicity examination of dill weed and seeds.

MATERIALS AND METHODS

Plant Materials—Dill seeds and weed were commercial products from *Anethum sowa* D.C. cultivated at India and *A. graveolens* L. at California, U.S.A., respectively.

Fractionation of Boiling Aqueous Methanol Extract of Dill Weed and Seeds—The weed (100 g) was extracted three times with a boiling 50% methanol (2 l) for 1 hr. The combined solution was concentrated to 400 ml and extracted successively three times with 1 l each of hexane, methylene chloride, ethyl acetate and saturated aqueous butanol as shown in Chart 1. The seeds (6

*1 Presented partly at the 99th Annual Meeting of Pharmaceutical Society of Japan, Sapporo, August 1979, Abstracts of Paper, p. 203.

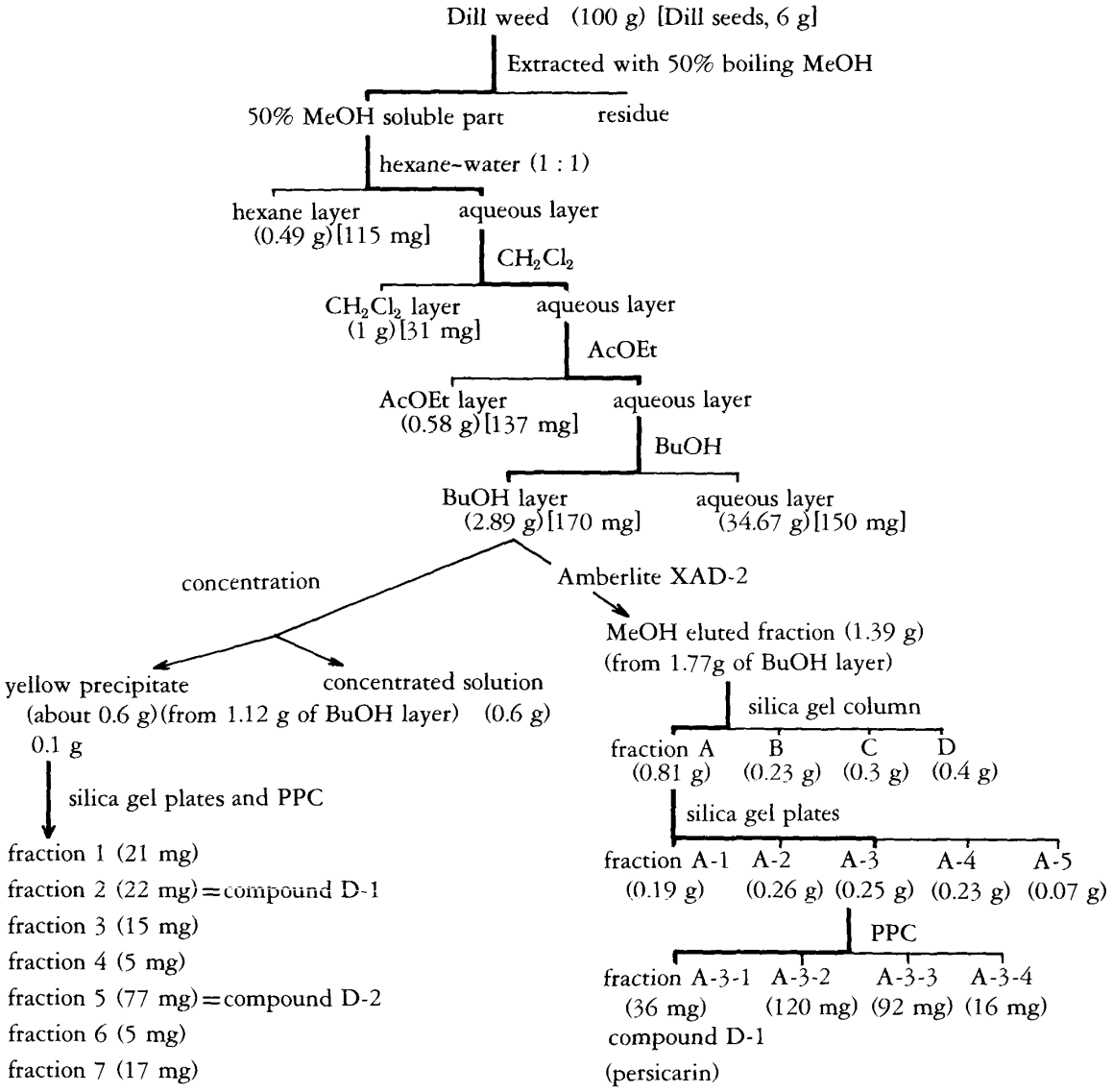


CHART 1

g) were extracted under the same condition.

Isolation of Mutagenic Principles from the Butanol Extract (Chart 1 and Table I) — 1) The butanol extract (1.77 g) was dissolved in water (100 ml), passed through Amberlite XAD-2 (300 ml) and the resin was successively eluated with water (2 l) and methanol (1 l). The methanol eluate (1.39 g) exhibiting mutagenicity was chromatographed on a silica gel column (Merck, silica gel 60, 70—230 mesh, 320 g, 2.7ϕ × 120 cm) and eluted with a mixture of butanol-acetic acid-water (4 : 1 : 1) to give a mutagenic fraction A (0.81 g), and three nonmutagenic fractions, B, C, and D. Fraction A was rechromatographed on silica gel thin-layer plates (Merck, HF-254) in the above solvent system to afford a mutagenic fraction (0.25 g), fraction A-3, which was further purified with paper chromatography (PPC) on Toyoroshi No. 514 (butanol-acetic acid-water = 4 : 1 : 5) to give a mutagenic compound, D-1 (36 mg) and non-mutagenic fractions, A-3-2, -3, and -4.

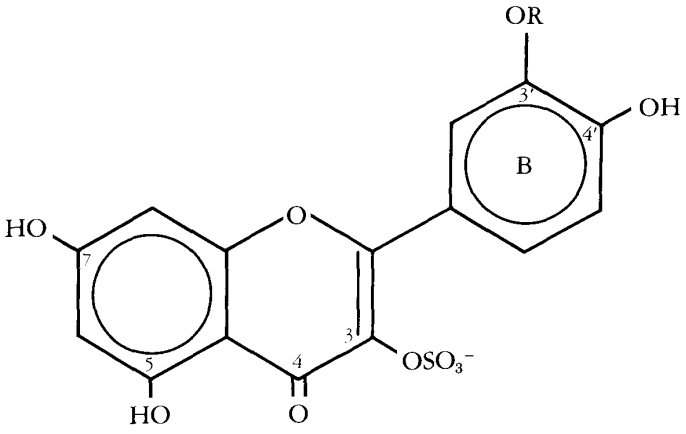
2) The butanol extract (1.12 g) was dissolved in saturated aqueous butanol and concentrated to afford yellow precipitates (about 0.6 g), which decomposed upon drying. The precipitates (0.1 g)

TABLE I. *Mutagenicity of 50% Methanol Extract of Dill (see Chart 1)*

	Mutagenic activities in *Salmonella typhimurium* TA 98				
	+S-9 Mix.	−S-9 Mix.		+S-9 Mix.	−S-9 Mix.
Dill seeds			Water eluted fraction	0	0
50% MeOH soluble part	477 *a)*	0	Methanol eluted fraction	134	0
Hexane layer	0	0	Fraction A	143	0
Methylene chloride layer	0	0	Fractions B,C,D	0	0
Ethyl acetate layer	135	0	Fraction A-3	212	0
Butanol layer	317	0	Fractions A-1,2,4,5	0	0
Aqueous layer	0	0	Fraction A-3-1 (compound D-1)	650	0
Dill weed			Fractions A-3-2,-3-3,-3-4	0	0
50% MeOH soluble part	136	0	Yellow precipitate	1050	0
Hexane layer	0	0	Concentrated solution	0	0
Methylene chloride layer	0	0	Fraction 2 (compound D-1)	1050	0
Ethyl acetate layer	18	0	Fraction 5 (compound D-2)	1740	0
Butanol layer	1025	0	Fractions 1,3,4,6,7	0	0
(After four months)	(512)	(0)	Quercetin	37000	—
Aqueous layer	0	0	Isorhamnetin	5700 *b)*	—

a) Number of revertant colonies/mg extract or fraction per plate. Spontaneous revertant colonies were not included.
b) See the reference 25).

were chromatographed on Merck Kieselgel 60 F_{254} plates using the solvent system of butanol-acetic acid-water (4 : 1 : 1) to give seven fractions and each fraction was purified by PPC using a mixture of butanol-acetic acid-water (4 : 1 : 5); fraction 2 afforded a compound identical with compound D-1 (22 mg) and fraction 5 (77.3 mg) gave another mutagenic compound (compound D-2).



1: R=CH_3 compound D-1 (persicarin)
2: R=H compound D-2

Compound D-1 (1) and D-2 (2) — Both compounds are yellow crystalline powder under wet conditions, but unstable to drying and heating. They gave olive-green color in $FeCl_3$ and magenta color with Mg-HCl, characteristic of flavonoids. The infrared (IR) spectra; ν_{max}^{KBr} 3400, 1650, 1605 cm^{-1} for compound D-1 and 3400−3150, 1650, 1605 cm^{-1} for compound D-2. The ultraviolet (UV) spectra; λ_{max}^{MeOH} 355 (band I), 324 (inf), 298 (inf), 267 (inf), 255 (band II) nm for compound D-1 and 358 (band I), 322 (inf), 298 (inf), 257 (band II) nm for compound D-2 (Table II).*

Acid Hydrolysis of the Flavonoids — Compound D-1 (20 mg) was heated with 2N HCl (1 ml) for 30 min and the reaction mixture was

* The molecular extinction coefficients were not measured because the compounds were decomposed by drying.

allowed to stand overnight. The mixture was extracted with ethyl acetate. The aqueous layer gave white precipitates with $BaCl_2$ solution and the ethyl acetate layer gave a hydrolysate, mp 304–306° (dec.) (from EtOH), mass spectrum: *m/e* 316.0580 (M^+, Calcd. for $C_{16}H_{12}O_7$, 316.0582), which was identified with isorhamnetin by mixed mp, IR, PPC, and thin-layer chromatography (TLC).

Compound D-2 (30 mg) was treated with the same method to give the hydrolysate, a yellow compound, from the ethyl acetate layer, mp 310–312° (dec.) (from aq. EtOH), mass spectrum: *m/e* 302.0447 (M^+, Calcd. for $C_{15}H_{10}O_7$, 302.0425), which was identified with quercetin by mixed mp, IR, PPC, and TLC. The aqueous layer obtained from compound D-2 also gave white precipitates with $BaCl_2$ solution.

The presence of the sulfate anion in the aqueous layers was established by PPC (*vide infra*) and the fact that the white precipitates of barium sulfate were insoluble in 10% nitric acid.

Preparation of Quercetin 5,7,3',4'-Tetramethyl Ether from Compound D-2 —Compound D-2 (18.72 mg) was treated with methanol (2 ml) and potassium carbonate (anhydrous, 200 mg) for 1 min at 90°. To the mixture was added dimethyl sulfate (0.2 ml) and then heated for 10 min at 90°. The reaction mixture was diluted with 2N HCl (3 ml), boiled for 30 min, and extracted with ethyl acetate. Preparative layer chromatography of the extract gave mainly two compounds. The less polar compound was recrystallized from benzene to give quercetin 5,7,3',4'-tetramethyl ether (1.85 mg), mp 195–196°, mass spectrum: *m/e* 358.1015 (M^+, Calcd. for $C_{19}H_{18}O_7$, 358.1051), which was identified with the synthetic specimen prepared from rutin according to the known method[5] by a mixed mp, IR and TLC.

The more polar compound (9.4 mg), mp 193–196° (from MeOH), showed its IR absorptions at 1680 and 1600 cm^{-1}, UV absorptions at 252 (sh) and 286 nm (log ϵ, 3.80, 3.56) and a mass spectrum (*m/e*: 330.1094, M^+, Calcd. for $C_{18}H_{18}O_6$, 330.1102). The physical properties suggested that the compound was 4,6,3',4'-tetramethoxy-3(2H)-benzofuranone.[6,7]

Identification of Flavonoids by Chromatographic and Electrophoretic Methods —Paper Chromatography (PPC): Paper, Toyoroshi No. 514 and Whatman No. 1; solvent, benzene-acetic acid-water (125 : 72 : 3)[8] for isorhamnetin and quercetin, butanol-acetic acid-water (4 : 1 : 5), water, and 15% acetic acid[9] for isorhamnetin 3-sulfate (persicarin) and quercetin sulfate (*vide infra*); detection, colors under UV light (364 nm) with ammonia and by Pauly's reagent.

Thin-Layer Chromatography (TLC): Merck Kieselgel 60 F-254, 5 cm × 20 cm × 0.25 mm; solvent, benzene-acetone (9 : 1)[10] for quercetin 5,7,3',4'-tetramethyl ether, benzene-dioxane-acetic acid (90 : 25 : 4)[11] for isorhamnetin and quercetin, and butanol-acetic acid-water (4 : 1 : 1) for isorhamnetin 3-sulfate (persicarin) and quercetin sulfate (*vide infra*); detection, UV light (254 nm), iodine-vapors and Pauly's reagent.

Electrophoresis: Paper, Toyoroshi No. 514 and Whatman No. 1; solvent, formate-acetate buffer, pH 2.2[9] for 2.5 hr at constant voltage of 400 mV; detection, colors under UV light (364 nm) with ammonia and Pauly's reagent.

Identification of Inorganic Ions in the Aqueous Hydrolysates —Paper chromatographic survey was carried out by the following conditions. Paper, Toyoroshi No. 514 and Whatman No. 1; solvent, isopropanol-1.5 N ammonium hydroxide (7 : 3)[12]; detection, 0.1% aqueous bromocresol green-ammonium hydroxide solution[13] for ions of calcium, potassium and sulfate; solvent, butanol-hydrobromic acid[14]; detection, ammoniacal 8-hydroxyquinoline-ethanol solution for calcium ion and sodium cobaltinitrite solution for potassium ion.[14]

Flame photometric survey was carried out by Hitachi 170–50 A Atomic Absorption Spectrophotometer at 422.8 nm for calcium and 766.5 nm for potassium. Calcium and potassium in the aqueous hydrolysate were in the ratio 1 : 2 for compound D-1 and 10 : 1 for compound D-2.

Isorhamnetin and the Sulfate —Isorhamnetin and its 3-sulfate (persicarin) were supplied by Prof. M.Takahashi, Tohoku College of Pharmacy,

and Prof. N.Morita, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, respectively. Quercetin is a commercial product obtained from Tokyo Kasei Kogyo Co., Ltd.

Preparation of Quercetin 3-Sulfate from Quercetin —Quercetin (1.1 g), sulfamic acid (0.8 g) and pyridine (2.2 ml) were treated according to Harborne's[15] and Yamaguchi's methods[16] to afford a crystalline mixture of quercetin sulfates (0.5 g). The mixture (0.11 g) was separated by PPC under the known conditions[17,18] into quercetin 3-sulfate (1.8 mg), 3′-sulfate (67.9 mg), and a mixture of unidentified compounds (42.2 mg). The synthetic quercetin 3-sulfate was also very unstable to drying and heating and was not directly compared with the natural compound by a mixed mp and IR.

Mutation Assay — *Salmonella typhimurium* strains TA98 and TA100 were used for mutation assay. The S-9 fraction of liver homogenate (2 ml 0.15M KCl/g wet liver) of male Sprague-Dawley rats treated with polychlorinated biphenyl was prepared by the known method.[19] S-9 Mix contained 50 μmol sodium phosphate buffer (pH 7.4), 4 μmol $MgCl_2$, 16.5 μmol KCl, 2.5 μmol glucose-6-phosphate, 0.25 unit glucose-6-phosphate dehydrogenase, 2 μmol NADPH and 150 μl of S-9 fraction in a total of 0.5 ml. Mutation assay was carried out as described by Ames *et al.*[19] with some modification.[20] The test substance, S-9 Mix or 0.1 M phosphate buffer (pH 7.4) and the bacteria were incubated at 37° for 20 min, added with 2 ml of molten top agar, and then poured into a plate of minimal glucose agar. Colonies of revertants were counted after incubation for 2 days at 37°.

Carcinogenicity Examination —Inbred strain ACI rats, 4—6 weeks old, of both sexes were used in this study. Dried powders of dill weed and dill seeds were mixed with the rat basal diet CE-2 (CLEA Japan Inc., Tokyo).

For 450 days, 12 male and 12 female rats were given the diet containing 33% of dill weed, and 14 male and 14 female rats were given diet containing 33% of dill seeds for 410 days. Thirty male and 22 female rats were fed with the normal CE-2 diet as control. After the termination of feeding with the diet containing these materials, rats were returned to the normal CE-2 diet. The experiments were terminated at 480 days after the start of experiments. All animals were autopsied at death, or killed due to their moribund condition or at the termination of experiment. All organs were fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin.

RESULTS AND DISCUSSIONS

In the course of the mutagenicity tests of aqueous extracts of various spices, dill seeds and weed showed positive results for *Salmonella typhimurium* TA98 and TA100 after metabolic activation by S-9 Mix. Consequently hexane, dichloromethane, ethyl acetate, and 50% aqueous methanol extracts of the dill weed were prepared for a preliminary mutagenicity test. The mutagenic activity was most remarkable in the 50% aqueous methanol extracts after microsomal activation. Thus the aqueous methanol extract obtained by direct extraction of dill weed or seeds, was partitioned to separate the known ingredients such as essential oil, oleoresins,[21] and glycosides[22] as shown in Chart 1 monitored by mutation assay (Table I) for the strain TA98.* The mutagenic principles were concentrated into the butanol layer as shown in Table I. The butanol layer was chromatographed on Amberlite XAD-2 to give a methanol eluate showing the activity. The eluate was chromatographed on silica gel column and plates followed by paper partition to afford four fractions, one of which, compound D-1 (**1**), was proved to have the mutagenicity. However the activity of the butanol layer could not be explained only by the action of compound D-1. When the total mutagenic activity of the layer was compared with that of compound D-1, the

* The strain TA98 was proved to be more sensitive than TA100 in the preliminary test.
** The activity was proved to be reduced to half during storage for a couple of months.

presence of other mutagenic compound in the layer was speculated. Therefore, the butanol layer was concentrated to one tenth of its volume to afford yellow precipitates which exhibited the mutagenic activity.** The precipitate mainly consisted of two ingredients having UV absorption when developed on thin-layer plate and paper chromatography. The less polar ingredient was identified with the mutagenic compound D-1 obtained by the column chromatography. The other polar mutagenic ingredient, compound D-2 (**2**), was shown to be very unstable to drying, heating or chromatographic separation. By such procedures, the compound changed from yellow to black in color, becoming insoluble in hot water. The polar compound was the more predominant component than the less polar compound in the freshly prepared precipitates, but reduced from day to day in amounts and finally disappeared from the precipitates so far as examined in thin-layer plates.

Both compounds gave olive-green color in $FeCl_3$ and magenta color with Mg-HCl, characteristic of flavonoids. The IR spectra showed the presence of hydroxyl and α, β-unsaturated carbonyl groups at 3400-3100 and 1660-1650 cm^{-1}. Acid hydrolysis of compounds D-1 and D-2 gave isorhamnetin and quercetin, respectively, and both compounds were identified by every means of identification. The aqueous layers of the hydrolysates did not show any sign of the presence of a sugar residue but gave white precipitates with $BaCl_2$ solution. The precipitates were not soluble in 10% nitric acid and the paper chromatography of the aqueous layer showed the presence of a sulfate ion. Thus the compounds D-1 and D-2 were suspected to be flavonol sulfates.[9]

The position of the sulfate residue of both compounds was suggested by the bathochromic effects on the UV spectra in the presence of NaOMe, NaOAc, H_3BO_3, $AlCl_3$, and $AlCl_3$-HCl[23] as shown in Table II. The bathochromic shift of band I of the compounds on addition of NaOMe without a decrease in intensity as well as the effect of NaOAc on band II indicated the presence of a free 4′-hydroxyl group, while the effect of NaOAc on band I also indicated a free 7-hydroxyl group of isorhamnetin and quercetin.[23] The presence of an ortho-dihydroxyl groups in the B ring of compound D-2 was assignable by a comparison of the spectra of quercitrin and rutin (Table II) in the presence of $AlCl_3$ with or without HCl and by a bathochromic shift of band I in the presence of NaOAc/H_3BO_3.[23] Moreover from the above UV evidences and the stability in NaOMe, the sulfate residue was inferred to locate at the 3 position of isorhamnetin or quercetin.[23] Therefore, compound D-1 was assignable to be isorhamnetin 3-sulfate (**1**). The sulfate, designated persicarin, was first isolated from *Persicaria hydropiper* Opiz.[24] and then from several of the Umbelliferae and other families.[17,18] The comparison with an authentic specimen was carried out by PPC, TLC, and electrophoresis.

Compound D-2 was finally proved to be quercetin 3-sulfate (**2**) by the following evidences;

TABLE II. *The Bathochromic Shift of The UV Spectra of Compound D-1 and D-2*

Substance	NaOMe		AcONa		AcONa/H_3BO_3		$AlCl_3$		$AlCl_3$/HCl	
	Band I	II	Band I	II	Band I	II	Band I	II	Band I	II
Compound D-1	+55	+20	+19	+18	0	0	+2	+1	+1	+1
Isorhamnetin-3-O-rutinoside[a]	+58	+17	—	+17						
Isorhamnetin-3-O-galactoside[a]	+58	+19	—	+19						
Compound D-2	+49	+16	+21	+14	+19	+4	+50	+14	+34	+10
Quercitrin[b]	+46	+15	+13	+13	+16	+3	+63	+8	+38	+4
Rutin[b]	+50	+15	+26	+15	+21	+4	+76	+17	+41	+11

a) Data of the reference 23).
b) Authentic samples of quercitrin and rutin were obtained from Tokyo Kasei Kogyo Co., Ltd.

i.e. compound D-2 was subjected to permethylation followed by acid hydrolysis to afford quercetin 5,7,3′,4′-tetramethyl ether, which was identified with an authentic specimen prepared from rutin by the known method.[5] Additionally compound D-2 showed an identical *Rf*-value on PPC, TLC and electrophoresis with a synthetic specimen prepared from quercetin and sulfamic acid according to Harborne's[15] and Yamaguchi's method.[16] Although compounds D-1 and D-2 were shown to be isorhamnetin 3-sulfate and quercetin 3-sulfate respectively, both compounds were assumed to exist as a mixture of calcium and potassium salts from the results of flame photometric surveys of the hydrolysates.

Isorhamnetin and quercetin themselves have been known as the strong mutagens in systematic surveys for mutagenicity of flavonoids.[25-29] Although the presence of the flavone, kaempferol, which exhibited mutagenicity,[25] in *Anethum graveolens* L, has been reported,[30] the presence of the free flavonols, such as isorhamnetin, kaempferol and quercetin, was not observed in the butanol layer or in the yellow precipitates as far as examined by consuming 5 mg of the samples on TLC. It is known that sulphation in animals is one of the major detoxification mechanism of foreign phenols.[31] Nevertheless the mutagenic activity of the extracts of dill weed and seeds was proved to be due to the flavonol sulfates, which exhibit the activities probably by hydrolysis with arylsulfatase[32] contained in S-9 Mix preparation.*

So far, the separation of plant metabolites by mutation assay in the *Salmonella*/microsomal activation system has culminated in the isolation of flavonol.[33,34] Flavonol sulfates are contained in many edible plants especially of Umbelliferae.[17,35]

Since an overlapping of mutagens and carcinogens is now widely accepted,[1-3] dill weed and seeds were tested by long-term feeding experiments in rats.

In the group of dill weed fed animals, two out of 24 rats died before 7 months after the start of experiment and another 7 rats died between 7 and 15 months. The remaining 15 rats survived until the termination of the experiment. However, no tumors were observed. In the group of dill seeds fed animals, one out of 28 rats died before 7 months after the start of experiment, and another 5 rats died without tumors between 7 and 15 months. The remaining 22 rats which survived until the termination of experiment had interstitial cell tumor of the testis, leukemia, and adrenal pheochromocytoma each in one rat, respectively. In control group, 1 pituitary adenoma, 1 adrenal pheochromocytoma, 1 urinary bladder sessile papilloma, 2 adrenal cortical adenomas, and 3 interstitial cell tumors of the testis developed in 4 rats.

Interstitial cell tumor of the testis developed in 1 rat fed diet containing dill seeds and 3 control rats. This type of tumor seems to be spontaneous, inasmuch as it is known that old male ACI rats are prone to develop interstitial cell tumor of the testis.[36] Adrenal pheochromocytoma and leukemia observed in rats fed diet containing dill seeds were also considered to be spontaneous. Therefore, it may be said that the carcinogenic activity of dill weed and dill seeds could not be detected when the diet containing 33% of these materials were administered. These negative results obtained in the carcinogenicity examination may show that the amount of the flavonol sulfates contained in dill weed and dill seeds was not sufficient to produce tumors, or the flavonol sulfates wee not carcinogenic *in vivo,* whereas they were mutagenic *in vitro.*

Acknowledgement The authors are indebted to Dr.K.Kanohta and Mr.H.Suzuki for mass spectral determinations. This work was supported in part by Grants-in Aid for Cancer Research from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare.

* The mutagenic activity sometimes varied with the degradation of the sulfates or with a recipe of S-9 Mix.

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