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Antimutagenic Effect of Plant Flavonoids in the *Salmonella* Assay System

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The antimutagenic effects of 27 kinds of plant flavonoids on the mutagenicity of aflatoxin B_1(AFB_1) and N-methyl-N'-nitro-N-nitrosoguanidine(MNNG) in *Salmonella typhimurium* TA100 were investigated. In the mixed applications of AFB_1 (1 μg/plate) with the flavonoids (300 μg/plate) in the presence of a mammalian metabolic activation system (S9 mix), chrysin, apigenin, luteolin and its glucoside, kaempferol, fisetin, morin, naringenin, hesperetin, persicogenin, (+)-catechin and (−)-epicatechin showed the antimutagenic effect against AFB_1 with more than 70% inhibition rate. A little or no antimutagenicities except flavone against MNNG (0.5 μg/plate) were observed. For the antimutagenicity of the flavonoids on AFB_1, the flavonoid structure that contains the free 5-, 7-hydroxyl group seemed to be essential. However, saturation of the 2,3-double bond or elimination of the 4-keto group did not affect the activity.

Key words: Antimutagenicity, Flavonoid, *Salmonella* assay system

INTRODUCTION

Flavonoids,which are among the most ubiquitously distributed compounds in the plant kingdom (as well as some mosses,liverworts,fungi and ferns) have been shown to possess a variety of biochemical and pharmacological activities. In addition to their biological activities,several kinds of naturally occurring plant flavonoids have been implicated as genotoxic agents (Bjeldanes *et al.*, 1977; Brown, 1980; Nagao *et al.*, 1981). Since flavonoids occur in all higher plants they are,and always have been, a common constituent of diet. It has been estimated that the "average" daily diet contains about 1 gram of flavonoids (Kuhnau, 1976). Thus the significance of their biological activities with regard to the toxicities has attracted much attention by several workers.

In the course of search for antimutagenic principles from Chinese medicines, we demonstrated that the methanol extract obtained from the whole plants of *Orostachys japonicus* showed the antimutagenic activity (Park *et al.*, 1991a). And also we reported the active principles as flavonoids (Park *et al.*, 1991b). This kind of result is contradictory to the previous results that flavonoids exhibit the mutagenicities. The results that flavonoids suppress/or reduce the mutagenicity of various chemicals are reported by several workers (Hwang *et al.*,1983, Francis *et al.*, 1989; Heo,*et al.*, 1992).

Since as naturally occurring substances they are thought to be harmless, we screened the mutagenic and antimutagenic activities of 27 flavonoids isolated from various plants using a microsuspension technique of the *Salmonella*/microsome assay (Ames test). The antimutagenic activity was assayed against mutagens of aflatoxin B_1(AFB_1) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). The present report gives data of chemical structures of flavonoids and their antimutagenicity.

MATERIALS AND METHODS

Chemicals

Most of the flavonoids tested were extracted from various plants,purified and characterized according to the previously described procedures (Choi *et al.*, 1986, 1987, 1990).

AFB_1 (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in spectrophotometric grade dimethylsulfo-

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xide (DMSO). MNNG (Aldrich Chemical Co. Milwaukee, WI, USA) was dissolved in sterile distilled water. Flavonoid samples (flavone, chrysin, diosmin and hesperidin) were obtained from Sigma Chemical Co., and all of the flavonoids were dissolved in DMSO for the test.

Ames Mutagenicity/antimutagenicity Assay

Salmonella typhimurium strain TA100 histidine requiring mutants were used as baterial strains. They were maintained as described by Maron and Ames (19 83).

S9 mix to activate the mutagen was also prepared by the method of Maron and Ames (1983). Aroclor 1254 in corn oil (200 mg/ml) was injected to male Sprague-Dawley rats(body weight about 200 g) for inducing the liver enzymes. Five days later, liver was excised, homogenized with 0.15 M KCl (3 ml/g liver) using homogenizer (Potter-Elvehiem apparatus, USA) and the homogenate was centrifuged at 9,000 g for 10 min (4℃). Supernatant (S9 fraction) was kept frozen at −80℃ until use. The S9 fraction (10%) was mixed with $MgCl_2$-KCl salts (2%), 1 M glucose 6-phosphate (0.5%), 1 M NADP (4%), 0.2 M phosphate buffer (pH 7.4), and sterilized water prior to use for the Ames assay.

Mutagenicity/antimutagenicity test was carried out by a modified plate incorporation test (liquid preincubation of the organisms with the test compounds) (Matsushima *et al.*, 1980). A 0.5 ml of S9 mix (or 0.5 ml of phosphate buffer for-MNNG) distributed in sterile capped-tubes in an ice bath. Then 0.1 ml of testers from an overnight culture ($1\sim2\times10^9$ cells/ml) and 0.1 ml of test compounds (50 μl of mutagen and 50 μl of 0.5~5% (catechins) or 300 μg/plate (all test flavonoids) were added. The tubes were gently vortexed and preincubated at 37℃ for 30 min. The top agar (2 ml) kept at 45℃ were added to each tube and vortexed for 3 seconds. The entire mixture was overlaid on the minimal agar plate. The plates were incubated at 37℃ for 48 hrs and then the revertant baterial colonies on each plate were counted. Toxicity tests for the different levels of flavonoid samples were also carried out (Maron and Ames, 1983), and the flavonoid concentrations employed for the antimutagenic test in this study did not show any toxicity to the tester strain.

RESULTS

Flavones

Flavone and five related compounds were tested for (anti) mutagenicities and their antimutagenic activities are given in Table I. These compounds except diosmin revealed strong antimutagenic effect against AFB_1, however, a little or no antimutagenicities except flavone against MNNG were observed.

Table I. Antimutagenic effect of flavones and their glycosides against aflatoxin B_1 (AFB_1, 1 μg/plate) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 0.5 μg/plate) in *Salmonella typhimurium* TA100[a]

Compounds	Substitution	Inhibition%	
		AFB_1	MNNG
Flavone	−	51%	79%
Chyrisin	5,7-diOH	90%	−12%
Apigenin	5,7,4'-triOH	88%	6%
Luteolin (Lt)	5,7,3',4'-tetraOH	89%	7%
Cynaroside	Lt-7-O-Glu	80%	−14%
Diosmin	5,3'-diOH,4'-OMe,7-O-Rut	3%	31%

[a] 300 μg/plate of flavonoid solutions was employed for the test. The results are the means of triplicate. Glu; glucoside, Rut; rutinoside

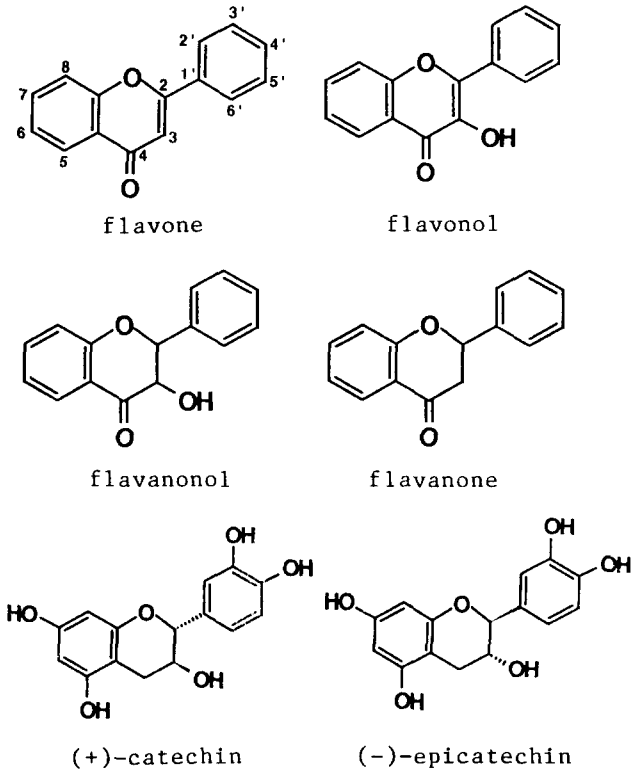
Table II. Antimutagenic effect of flavonols and their glycosides against aflatoxin B_1 (AFB_1, 1 μg/plate) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 0.5 μg/plate) in *Salmonella typhimurium* TA100[a]

Compounds	Substitution	Inhibition%	
		AFB_1	MNNG
Kaempferol (kp)	5,7,4'-triOH	75%	14%
Quercetin (Qc)	5,7,3',4'-tetraOH	55%	2%
Fisetin	7,3',4'-triOH	80%	31%
Morin	5,7,2',4'-tetraOH	82%	8%
Populnin	Kp-7-O-Rha	66%	8%
Hyperin	Qc-3-O-Gal	4%	31%
Isoquercitrin	Qc-3-O-Glu	13%	17%
Quercitrin	Qc-3-O-Rha	0%	13%
Avicularin	Qc-3-O-Ara(f)	26%	15%

[a] 300 μg/plate of flavonoid solutions was employed for the test. The results are the means of triplicate. Rha; rhamnoside, Gal; galactoside, Ara(f); arabinofuranoside

Flavonols

The antimutagenicities of four flavonols and their five glycosides were tested (Table II). All the flavonols tested showed antimutagenic activity against AFB_1, but little antitagenicity except fisetin against MNNG was observed. Only fisetin which does not have a hydroxyl group at positon 5 was antimutagenic at concentrations tested against both AFB_1 and MNNG. Thus, the hydroxyl group at position 5 seems to be important for antimutagenicity in this case. Morin which is the 2'-hydroxy derivative of kaempferol, showed more enhanced antimutagenicity than kaempferol against AFB_1. Therefore, hydroxy groups in the B ring also affect the antimutagenicity. In plants, flavonols are often present as glycosides. Interestingly the quercetin glycosides (hyperin, quercitrin, isoquercitrin, and avicularin)



Scheme 1. Structure and numbering of flavonoids.

were not antimutagenic against AFB_1 or MNNG compared to those of flavonols.

Flavanones and Flavanonols

The antimutagenicities of three flavanones, five flavanone glycosides and two flavanonols were tested (Table III). Among them, naringenin, hesperetin, and persicogenin revealed strong antimutagenic activities against AFB_1. Methylation or glycosylation of the 7-hydroxyl group does affect the antimutagenic potential. However, aromadendrin and taxifolin showed weak antimutagenic effect against AFB_1. They are the 2,3-dihydro derivatives of kaempferol and quercetin, respectively.

(+)-Catechin and (−)-Epicatechin

The antimutagenicities of (+)-catechin and (−)-epicatechin structurally related to flavonoids were tested (Tables IV and V). (+)-Catechin and (−)-epicatechin showed strong antimutagenic activity against AFB_1 with dose-dependant manners. Little or no antimutagenicity was observed against MNNG.

Discussion

In this work, we tested the antimutagenicities of various naturally occurring flavonoid aglycones, flavonoid glycosides and some synthetic flavonoids. Among the 27 flavonoids tested, flavones and some of their glycoside showed strong antimutagenic effect against AFB_1. Among them only flavone revealed strong antimutagenic activity to both AFB_1 and MNNG.

Although the mutagenicities of various flavonoids have been reported by many workers, Nagao *et al.* (1981) found that flavones having no hydroxyl group at position 3 were not mutagenic to TA100 or TA98 at concentrations of up to 200~300 μg/plate. Thus, absence of the hydroxyl group at position 3 seems to be essential for the antimutagenicity of flavones.

Naturally occurring flavonol aglycones, kaempferol, quercetin, fisetin and morin showed strong antimutagenic effect against AFB_1 in this study, but Pamukcu *et al.* (1980) reported that quercetin was carcinogenic.

Table III. Antimutagenic effect of flavanones and their glycosides against aflatoxin B_1 (AFB_1, 1 μg/plate) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 0.5 μg/plate) in *Salmonella typhimurium* TA100[a]

Compounds	Substitution	Inhibition%	
		AFB_1	MNNG
Naringenin (Nr)	5,7,4'-triOH	85%	9%
Hesperetin (He)	5,7,3'-triOH,4'-OH	88%	20%
Persicogenin (Pe)	5,3'-diOH,7,4'-diOMe	71%	15%
Hesperetin 5-Glu	He-5-O-Glu	49%	8%
Hesperidin	He-7-O-Glu	−21%	24%
Prunin	Nr-7-O-Glu	−4%	13%
Naringin	Nr-7-O-Hes	10%	−25%
Persiconin	Pe-5-O-Glu	59%	2%
Aromadendrin	3,5,7,4'-tetraOH	27%	12%
Taxifolin	3,5,7,3',4'-pentaOH	59%	27%

[a] 300 μg/plate of flavonoid solutions was employed for the test. The result are the mean of triplicate. Hes; hesperidoside.

Table IV. Effects of (+)-catechin and (−)-epicatechin on the inhibition of aflatoxin B_1 (AFB_1, 1 μg/plate) mutagenicity in *Salmonella typhimurium* TA100

Treatment	Revertants/plate	Inhibition rate (%)
AFB_1	941± 44	
Spontaneous	106± 9	
(+)-catechin		
0.5%	598± 57	41
1.0%	494± 27	54
2.5%	286± 27	78
5.0%	195± 9	89
AFB_1	787± 18	
Spontaneous	132± 7	
(−)-epicatechin		
0.5%	709± 20	12
1.0%	546± 6	37
2.5%	458± 21	50
5.0%	282± 42	77

Table V. Effects of (+)-catechin and (−)-epicatechin on the inhibition of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 0.5 μg/plate) mutagenicity in *Salmonella typhimurium* TA100

Treatment	Revertants/plate	Inhibition rate (%)
MNNG	962± 27	
Spontaneous	142± 4	
(+)-catechin		
0.5%	938± 40	3
1.0%	920± 2	5
2.5%	933± 52	4
5.0%	888± 21	9
MNNG	1032± 71	
Spontaneous	169± 20	
(−)-epicatechin		
0.5%	1041± 168	−
1.0%	1114± 131	−
2.5%	1029± 44	0
5.0%	1105± 119	−

Quercetin was found to induce transformations of all cryopreserved Syrian golden hamsters *in vitro*, sister Chromatid exchange in Chinese hamster cells *in vitro*, and mutation in mouse lymphoma cells L5178Y (Amacher *et al.*, 1980; Sugimura, 1979; Umezawa *et al.*, 1977). Quercetin was also shown to induce intestinal and bladder carcinoma in rats. However, there is a report (Sugimura, 1979) that it was noncarcinogenic in mice, hamsters, and rats. Though the results on quercetin are contradictory, our result showed this compound was antimutagenic toward AFB_1. It seems that dose levels or different experimental systems might cause the differences. In the case of flavonol compounds, glycosylation at position 3 or 7 reduced the activity significantly.

Flavanones and flavanonols which are the 2,3-dihydro derivatives of flavones and flavonols, respectively, revealed strong antimutagenic activities toward AFB_1. Their antimutagenic activities are similar to those of flavones, but they are stronger than flavonols.

As flavonol compounds did, glycosylation at position 7 of flavanones was not antimutagenic. Methylation of the 7-hydroxyl group reduced the activity. However the magnitiude was not significant than glycosylation of the 7-hydroxyl group. Glycosylation at position 5 of flavanones also reduced the activity significantly. Thus, the free hydroxyl group at position 5 or 7 seems to be important for the antimutagenicity.

Our results define several structural features essential for the antimutagenic activity of flavonoids in the strain TA100 against AFB_1 and MNNG. From the above data, it appears that the following generalization may be made regarding the structural features essential for the antimutagenicity of flavonoids. Those with a free hydroxyl group at position 5 and 7 were active unless the 3 position of the C ring was glycosylated. Saturation of the 2,3-double bond or elimination of the 4-keto group does not affect the activity for AFB_1.

Since the precise mechanism of the antimutagenic activity of flavonoids in the bacterial strain system is uncertain, the possible antimutagenic mechanism of flavonoids was demonstrated as inhibitory action on DNA-adduct formation through interaction with microsomal activating enzymes (Francis *et al.*, 1989).

Although Macgregor and Jurd (1978) described that structural features which appear essential for mutagenic activity of the flavonoid in TA98 strain are a basic flavonoid ring structure with (1) a free hydroxyl group at the 3 position, (2) double bond at the 2,3 position, (3) a keto group at the 4 position, and (4) a structure which permits the proton of the 3-hydroxyl group to tautomerise to a 3-keto compound, the essential feature of the hypothesis proposed from our results is that it seems not necessary to have more structural requirement for antimutagenic activity compared to the mutagenicity.

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