Antimutagenic Activity of Flavonoids from *Pogostemon cablin*

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A methanol extract from *Pogostemon cablin* showed a suppressive effect on *umu* gene expression of SOS response in *Salmonella typhimurium* TA1535/pSK1002 against the mutagen 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (furylfuramide). The methanol extract was re-extracted with hexane, dichloromethane, butanol, and water. A dichloromethane fraction showed a suppressive effect. Suppressive compounds against furylfuramide in the dichloromethane fraction were isolated by SiO_2 column chromatography and identified as 7,4′-di-*O*-methyleriodictyol (**1**), 7,3′,4′-tri-*O*-methyleriodictyol (**2**), and 3,7,4′-tri-*O*-methylkaempferol (**3**). In addition, three flavonoids, ombuine (**4**), pachypodol (**5**), and kumatakenin (**6**), were isolated and identified from the dichrolomethane fraction. Compounds **1** and **3** suppressed >50% of the SOS-inducing activity at <0.6 μmol/mL, and the ID_{50} values of both compounds were 0.25 μmol/mL. Compound **2** showed a weakly suppressive effect (17%) at a concentration of 0.6 μmol/mL, and compounds **4**−**6** did not. These compounds were also assayed with 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), which requires liver metabolizing enzymes. Compounds **3**−**6** suppressed >80% of the SOS-inducing activity of Trp-P-1 at <0.06 μmol/mL, and compounds **1** and **2** suppressed 87 and 63% at a concentration of 0.3 μmol/mL. In addition, these compounds were assayed with activated Trp-P-1, and the suppressed effects of these compounds were further decreased when compared to Trp-P-1. The antimutagenic activities of these compounds against furylfuramide, Trp-P-1, and activated Trp-P-1 were assayed by the Ames test using *S. typhimurium* TA100.

Keywords: *Pogostemon cablin; Labiatae; flavonoid; SOS response; umu test; antimutagenic activity; Ames test*

INTRODUCTION

Several short-term tests for screening of environmental mutagens and carcinogens have been developed and used widely in many laboratories (Ames et al., 1975; Kada, 1981). The Ames test is a convenient method to evaluate the mutagenic activities of these chemicals (Ames et al., 1975), and several lines of evidence have suggested that the mutagenic activities of a number of chemicals correlate well with the carcinogenic activities reported so far (McCann et al., 1975; Shugimura et al., 1976).

The *umu* test system was developed to evaluate the genotoxic activities of a wide variety of environmental carcinogens and mutagens, using the expression of one of the SOS genes to detect DNA-damaging agents (Oda et al., 1985; Nakamura et al., 1987). The results of this test are in agreement with the results of the Ames test and may be more useful with respect to simplicity, sensitivity, and rapidity (Reifferscheid and Heil, 1996).

Pogostemon cablin (Labiatae) is the aerial part of *Pogostemon cabin* Bentham and has been used against the common cold and as an antifungal agent in traditional medicine. This plant is cultivated extensively in Indonesia, Malaysia, China, and Brazil for its essential oil (patchouli oil), important to the perfumery industry. Thus, the constituents of patchouli oil have frequently been investigated, and the presence of a number of mono- and sesquiterpenoids has been reported (Terhune et al., 1973; Hikino et al., 1968; Tsubaki et al., 1967). Also, several flavonoids and alkaloids have been isolated and identified from *P. cablin* (Itokawa et al., 1981; Buchi et al., 1966). In our search for new naturally occurring antimutagenic compounds in plants, with a history of safe use as Chinese crude drugs (Miyazawa et al., 1997, 1998a,b), we found that the methanol extract of *P. cablin* ("kakkou" in Japanese) exhibited a suppression of the SOS-inducing activity of furylfuramide. In this paper, we report the isolation and identification of antimutagenic compounds against the mutagen in *P. cablin.*

EXPERIMENTAL PROCEDURES

General Procedure. Gas chromatography (GC) was performed on a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector (FID). GC−mass spectrometry (GC-MS) was performed on a Hewlett-Packard 5972 series mass spectrometer interfaced with a Hewlett-Packard 5890 gas chromatograph fitted with a column (HP-5MS, 30 m × 0.25 mm i.d.). IR spectra were determined with a Perkin-Elmer 1760-x infrared Fourier transform spectrometer. Nuclear magnetic resonance (NMR) spectra (δ, *J* in hertz) were recorded on a JEOL GSX 270 NMR spectrometer. Tetramethylsilane (TMS) was used as the internal reference (δ 0.00) for ^1H NMR spectra measured in $CDCl_3$, acetone-d_6, or DMSO-d_6. This solvent was used for ^{13}C NMR spectra.

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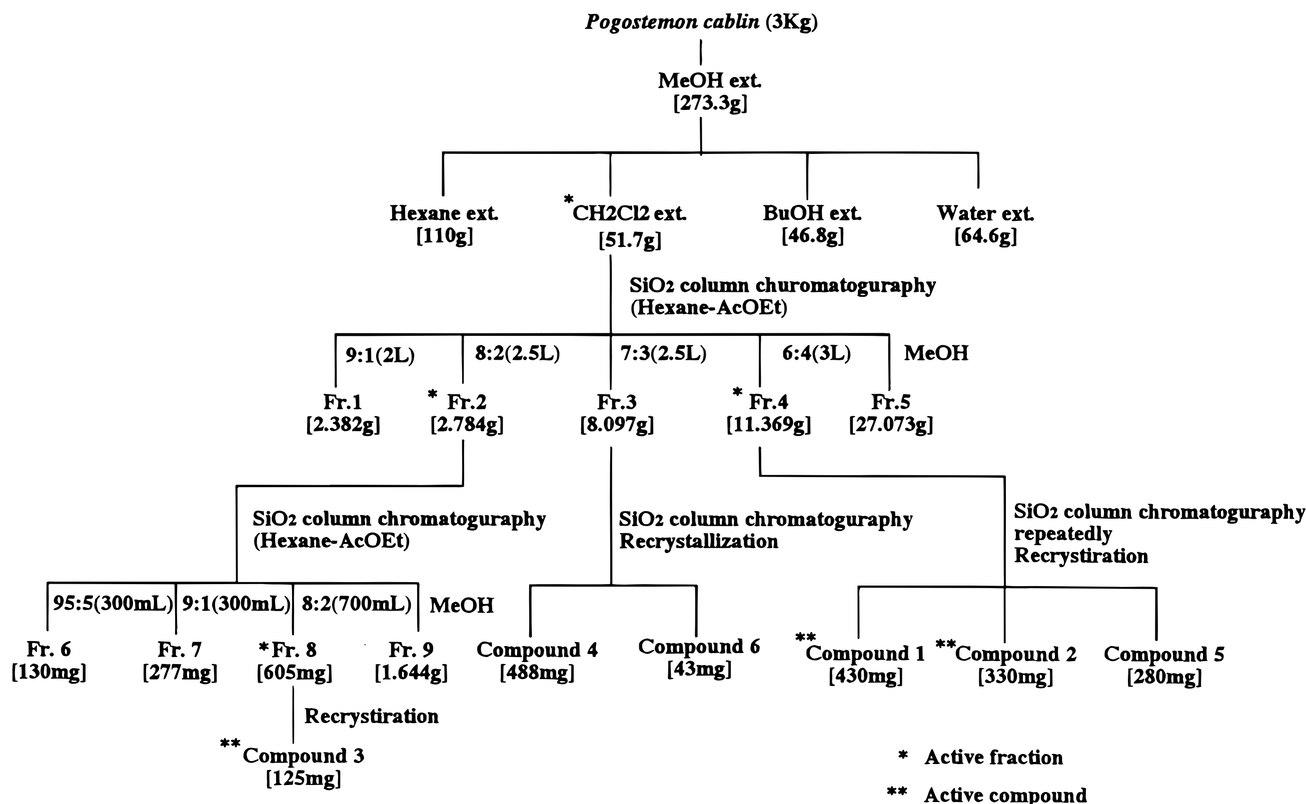


Figure 1. Isolation scheme for the suppressive compounds from *P. cablin*.

Materials. Commercially available air-dried tips of *P. cablin* were obtained from Yamada Yakken Co., Ltd. Furylfuramide and Trp-P-1 were purchased from Wako Pure Chemical Co. S9 (supernatant of 9000*g*), and coenzyme, NADPH, NADH, and G-6-P were purchased from Oriental Yeast Co.

***Umu* Test**. The *umu* test for detecting the SOS-inducing activity of chemicals was carried out according to the method of Oda et al. (1985) using *S. typhimurium* TA1535/pSK1002, the plasmid pSK1002 of which carries an *umuC′-′lacZ* fused gene.

UV Irradiation. The overnight cultured cells (*S. typhimurium* TA1535/pSK1002) were diluted 50-fold with fresh TGA medium and incubated at 37 °C until the bacterial density at 600 nm reached 0.25−0.30. The cultured cells were centrifuged to collect them and were suspended with 5 mL of 0.1 M phosphate buffer. They were removed into a Petri dish (4 cm) and UV irradiated for 20 s (0.5 J/m^2) with a germicidal lamp at room temperature. The culture induces SOS response by UV and was diluted into TGA medium until the bacterial density reached 025−030 at OD_{600}, and it used the *umu* test as test strain.

Ames Test. The mutation test was carried out according to the preincubation method (Yahagi et al., 1977), which is a modification of the Ames method (Ames et al., 1975).

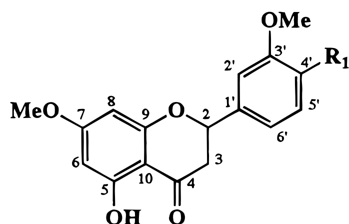
Preparation of Activated Trp-P-1. Preparation of activated Trp-P-1 was carried out according to the method of Arimoto et al. (1980).

Fractionation and Identification of the Suppressive Compounds from *P. cablin*. To prepare the suppressive compounds, fractionation of *P. cablin* was carried out as described in Figure 1 using the *umu* test as a guide. The dry powder (4 kg) of *P. cablin* was refluxed with methanol for 12 h to give a methanol extract (273.3 g). This extract was suspended in water (3 L) and partitioned between hexane (3 L) and water, dichloromethane (3 L) and water, and butanol (3 L) and water, successively. Each soluble fraction was concentrated under reduced pressure to give hexane (110 g), dichloromethane (51.7 g), butanol (46.8 g), and water (64.6 g) fractions. The dichloromethane fraction showed a suppressive effect. This was fractionated to fractions 1−5 by SiO_2 column chromatography with hexane and ethyl acetate as eluents. Fractions 2 and 4 showed suppression of the SOS-inducing activity of furylfuramide in the *umu* test, and these fractions were refractionated by SiO_2 column chromatography and recrystallized. Finally, suppressive compounds **1** (430 mg), **2** (330 mg), and **3** (125 mg) were isolated and identified as 7,4′-di-*O*-methyleriodictyol (**1**), 7,3′,4′-tri-*O*-methyleriodictyol (**2**), and 3,7,4′-tri-*O*-methylkaempferol (**3)** by GC, GC-MS, and ^{1}H and ^{13}C NMR spectroscopy, respectively. In addition, three flavonoids, ombuine (**4**, 488 mg), pachypodol (**5**, 280 mg), and kumatakenin (**6**, 43 mg), were isolated and identified.

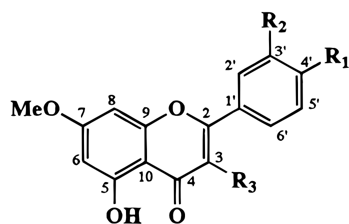
Compound 1. Compound **1** was a colorless needle: mp 148−150 °C; MS, *m*/*z* 316 (M^+ 75), 180 (60), 167 (100), 150 (37), 137 (71); IR γ_{max} KBr (cm^{-1}) 3417, 1645, 1575, 1519, 1269, 1158; ^{1}H NMR and ^{13}C NMR spectra of compound **1** were compared with those of 7,3′-di-*O*-methyleriodictyol [2,3-dihydro-5-hydroxy-2-(4′-hydroxy-3′-methoxyphenyl)-7-methoxy-4*H*-1-benzopyran-4-one] (Rauter et al., 1989; Marambio and Silva, 1989). Compound **1** was identified as 7,3′-di-*O*-methyleriodictyol from these spectral data and physical properties.

Compound 2. Compound **2** was a colorless needle: mp 155−157 °C; MS, *m*/*z* 330 (M^+ 83), 164 (72), 151 (100), 138 (18); IR γ_{max} KBr (cm^{-1}) 3451, 1640, 1575, 1515, 1267, 1158; ^{1}H NMR and ^{13}C NMR spectra of compound **2** were compared with those of 7,3′,4′-tri-*O*-methyleriodictyol [2-(3′,4′-dimethoxyphenyl)-2,3-dihydro-5-hydroxy-7-methoxy-4*H*-1-benzopyran-4-one] (Fernandez et al., 1988; Adelakun and Okogun, 1996). Compound **2** was identified as 7,3′,4′-tri-*O*-methyleriodictyol from these spectral data and physical properties.

Compound 3. Compound **3** was a yellow crystal: mp 138−140 °C; MS, *m*/*z* 328 (M^+ 100), 327 (98), 299 (15), 285 (58), 150 (22), 135 (28); IR γ_{max} KBr (cm^{-1}) 3332, 1664, 1597, 1559, 1504, 1258, 1165; ^{1}H NMR and ^{13}C NMR spectra of compound **3** were compared with those of 3,7,4′-tri-*O*-methylkaempferol [5-hydroxy-3,7-dimethoxy-2-(4′-methoxyphenyl)-4H-1-benzopyran-4-one] (Rossi et al., 1997). Compound **3** was identified as



	R
7,4'-di-*O*-methyleriodictyol(1)	OH
7,3',4'-tri-*O*-methyleriodictyol(2)	OMe



	R_1	R_2	R_3
3,7,4'-tri-*O*-methylkaempferol(3)	OMe	H	OMe
ombuine(4)	OMe	OH	OH
pachypodol(5)	OH	OMe	OMe
kumatakenin(6)	OH	OMe	OMe

3,7,4′-tri-*O*-methylkaempferol from these spectral data and physical properties.

Compound 4. Compound **4** was a yellow needle: mp 221−223 °C; MS, *m*/*z* 330 (M^+ 100), 329 (14), 299 (15), 287 (9), 167 (6), 151 (10); IR γ_{max} KBr (cm^{-1}) 3469, 1656, 1590, 1504, 1469, 1215, 1156; ^1H NMR and ^{13}C NMR spectra of compound **4** were compared with those of ombuine [3,5-dihydroxy-2-(3′-hydroxy-4′-methoxyphenyl)-7-methoxy-4*H*-1-benzopyran-4-one] (Itokawa et al., 1981; Wagner et al., 1976). Compound **4** was identified as ombuine from these spectral data and physical properties.

Compound 5. Compound **5** was a yellow needle: mp 163−166 °C; MS, *m*/*z* 344 (M^+ 100), 343 (63), 329 (51), 301 (53), 167 (10), 151 (12); IR γ_{max} KBr (cm^{-1}) 3434, 1664, 1601, 1516, 1495, 1351, 1210, 1159; ^1H NMR and ^{13}C NMR spectra of compound **5** were compared with those of pachypodol [5-hydroxy-2-(4′-hydroxy-3′-methoxyphenyl)-3,7-dimethoxy-4*H*-1-benzopyran-4-one] (Itokawa et al., 1981; Valesi et al., 1972). Compound **5** was identified as pachypodol from these spectral data and physical properties.

Compound 6. Compound **6** was a yellow crystal: mp; MS, *m*/*z* 314 (M^+ 100), 313 (99), 295 (22), 285 (20), 271 (47), 167 (22), 143 (27), 121 (53); IR γ_{max} KBr (cm^{-1}) 3252, 1667, 1602, 1587, 1504, 1226, 1168; ^1H NMR and ^{13}C NMR spectra of compound **6** were compared with those of kumatakenin [5-hydroxy-2-(4′-hydroxyphenyl)-3,7-dimethoxy-4*H*-1-benzopyran-4-one] (Parsons et al., 1993; Wang et al., 1989). Compound **6** was identified as kumatakenin from these spectral data and physical properties.

RESULTS

Fractionation of the Extract from *P. cablin* and Isolation of Compounds 1−6. The methanol extract of *P. cablin* was fractionated to search for suppressive compounds using the *umu* test as a guide (Figure 1). To obtain dose−response data, test samples were evaluated at dose levels of 0.2, 0.1, and 0.04 mg/mL. The dichloromethane extract exhibited a suppressive effect on *umu* gene expression of SOS-inducing activity in *S. typhimurium* TA1535/pSK1002 against furylfuramide. To prepare the suppressive compounds, fractionation of the dichrolomethane extract was carried out as described in Figure 1. Finally, suppressive compounds **1** (1.6 g), **2** (330 mg), and **3** (125 mg) were isolated. Compounds **1**, **2**, and **3** were identified as 7,4′-di-*O*-methyleriodictyol (**1**), 7,3′,4′-tri-*O*-methyleriodictyol (**2**), and 3,7,4′-tri-*O*-methylkaempferol **(3)** by GC, GC-MS, and ^1H NMR and ^{13}C NMR spectroscopy, respectively. In addition, three flavonoids, ombuine (**4**, 488 mg), pachypodol (**5**, 280 mg), and kumatakenin (**6**, 43 mg), were isolated and identified.

Table 1. Suppression of Furylfuramide[a]-Induced SOS Response by Compounds 1−6 Using *S. typhimurium* TA1353/pSK1002

compound	furylfuramide	control[b]	dose response[b] (μmol/mL) 0.6	0.3	0.1	0.05	LD_{50}[d]
1	650	184	344	384	496	605	0.25
2	650	184	571	605	643	668	
3	650	184	392	382	473	602	0.25
4	650	184	638	700	673	655	
5	650	184	613	587	584	584	
6	650	184	695	639	676	644	

[a] Furylfuramide (1 μg/mL in DMSO) was added at 50 μL. [b] β-Galactosidase activity (units). [c] Control was treatment without mutagen and compounds. [d] 50% inhibition dose.

Inhibition of Compounds 1−6 on the SOS-Inducing Activity. The suppressive effects of compounds **1**−**6** were determined in the *umu* test. Compounds **1**−**3** inhibited the furylfuramide-induced SOS response. Compounds **1**−**3** suppressed 66, 20, and 56% of the SOS-inducing activity at a concentration of 0.6 μmol/mL, respectively. ID_{50} (50% inhibition dose) values of compounds **1** and **3** were 0.25 μmol/mL (Table 1). Compounds **1**−**6** showed suppression of the SOS-inducing activity of Trp-P-1, which requires metabolic activation. Compounds **3**−**6** suppressed >80% of the SOS-inducing activity of Trp-P-1 at <0.06 μmol/mL, and compounds **1** and **2** suppressed 87 and 63% at a concentration of 0.3 μmol/mL, respectively (Table 2). From these results, there is a great difference between the suppressive effects of these compounds against furylfuramide and those of Trp-P-1. Compounds **1**−**6** showed more potent suppressive effects on Trp-P-1-induced SOS response than furylfuramide.

Suppressive Effects of Compounds 1−6 on Metabolic Activation of Trp-P-1. The suppressive effects of compounds **1**−**6** on metabolic activation of Trp-P-1 were tested by the *umu* test. As shown in Table 2, suppressive effects of these compounds on activated Trp-P-1 decreased compared with those of Trp-P-1. Especially compounds **2** and **4**−**6** showed very little suppressive effects against activated Trp-P-1. This result suggested the possibility that the inhibition of SOS-inducing activity of Trp-P-1, which was caused by compounds **1**−**6**, was due to the inhibition of metabolic activation by S9.

Suppressive Effects of Compounds 1−6 on UV Irradiation. The suppressive effects of compounds **1**−**6** on UV irradiation-induced SOS response were determined in the *umu* test using *S. typhimurium* TA1535/pSK1002. Compound **1** suppressed 53% of the SOS-inducing activity at a concentration of 0.6 μmol/mL with an ID_{50} value of 0.52 μmol/mL. Compound **3** suppressed 80% of the SOS-inducing activity at a concentration of 0.6 μmol/mL with an ID_{50} value of 0.35 μmol/mL (Figure 2).

Table 2. Suppression of Trp-P-1[a] and Activated Trp-P-1[b]-Induced SOS Response by Compounds 1−6 Using *S. typhimurium* TA1353/pSK1002

compound	Trp-P1	activated Trp-P-1	control[d]	dose response[c] (μmol/mL) 0.60	0.30	0.15	0.06	0.03	0.015	0.006	LD_{50}[e]
1	580		152		211	255	333	346	439	563	0.027
		760	156	549	672	748	731				0.52
2	580		152		312	318	324	366	403	419	0.03
		760	156	727	735	757	760				
3	580		152		231	231	231	279	364	352	0.012
		760	156	500	507	626	717				0.35

compound	Trp-P1	activated Trp-P-1	control[d]	dose response[c] (μmol/mL) 0.60	0.03	0.01	0.006	0.003	0.0006	0.0003	LD_{50}[e]
4	580		152	241	236	233	252	273	322	336	0.00021
		760	156	627	637	653	645				
5	580		152	231	226	249	283	315	378	384	0.0007
		760	156	751	695	804	721				
6	580		152	217	225	235	257	296	415	482	0.0024
		760	156	706	625	678	617				

[a] Trp-P-1 (40 μg/mL in DMSO) was added at 50 μL. [b] Activated Trp-P-1 (10 μg/mL in DMSO) was added at 100 μL. [c] Control was treatment without mutagen and compounds. [d] β-Galactosidase activity (units). [e] 50% inhibition dose.

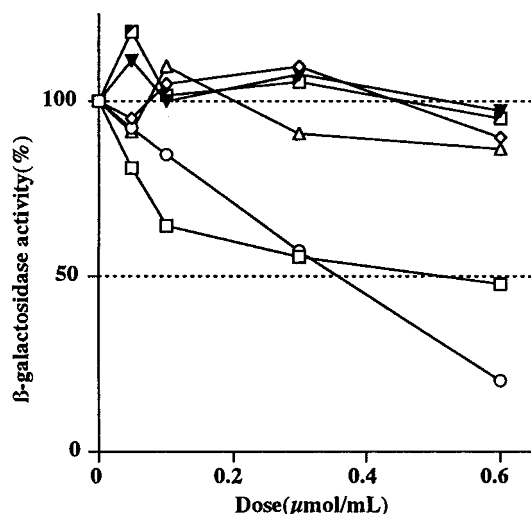


Figure 2. Suppression of UV-induced SOS response by compounds **1**−**6**: (□) effect of **1** of UV irradiation; (◇) effect of **2** of UV irradiation; (○) effect of **3** of UV irradiation; (△) effect of **4** of UV irradiation; (▼) effect of **5** of UV irradiation; (◪) effect of **6** of UV irradiation. UV irradiation was done at 0.5 J/m^2 for 20 s.

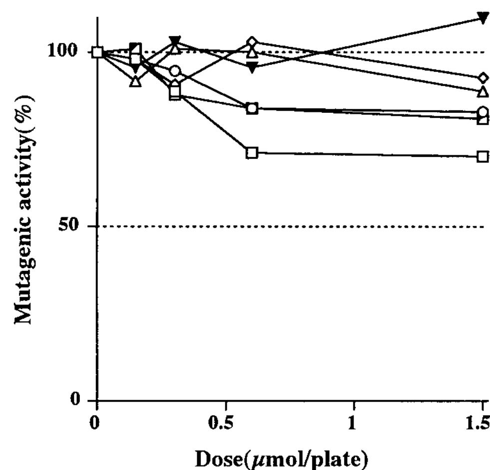


Figure 3. Effects of compounds **1**−**6** on the mutagenicity of furylfuramide in *S. typhimurium* TA100: (□) effect of **1** of furylfuramide; (◇) effect of **2** of furylfuramide; (○) effect of **3** of furylfuramide; (△) effect of **4** of furylfuramide; (▼) effect of **5** of furylfuramide; (◪) effect of **6** of furylfuramide. Furylfuramide (0.5 μg/mL in DMSO) was added at 50 μL/plate.

Antimutagenic Activity of Compounds 1−6. The antimutagenic activity of these compounds against furylfuramide, Trp-P-1, and activated Trp-P-1 was also demonstrated by the Ames test using *S. typhimurium* TA100. Compounds **1** and **3** suppressed 30 and 22% of the mutagenicity of furylfuramide at a concentration of 1.6 μmol/plate, respectively (Figure 3) As shown in Figure 4, these compounds showed antimutagenic activity against Trp-P-1, but the antimutagenic activity against activated Trp-P-1 was remarkably decreased. From this result, it is suggested that the antimutagenic activity of these compounds on Trp-P-1 is due to the inhibition of metabolic activation of Trp-P-1 by S9.

DISCUSSION

The antimutagenic compounds in *P. cablin* were clearly identified as compounds **1**−**6**. These compounds were assayed with chemical mutagens, furylfuramide and Trp-P-1, using *S. typhimurium* TA1535/pSK1002 in the *umu* test. In the former mutagen, compounds **1**−**3** exhibited suppressive effects on *umu* gene expression of the SOS response, but compounds **4**−**6** did not. As shown in Table 1, compounds **1** and **3** had greater suppressive potency than compound **2**. Compounds **1** and **2** are flavanone derivatives, without a double bond at the 2−3 position. The difference in structure between compounds **1** and **2** is a substituent group at the 4′-position. This result indicated that a hydroxy group at the 4′-position is an important factor for suppressing the SOS-inducing activity of furylfuramide in flavanones, whereas in flavones (compounds **3**−**6**) it is indicated that the only methoxy group at the 4′-position of the B ring probably contributed to the appearance of suppression effects in *umu* test. In the litter mutagen, all compounds showed potent inhibition of the SOS induction at a lower concentration than those of furylfuramide. Especially compounds **3**−**6** suppressed >80% of the SOS-inducing activity at $^{1}/_{10}$-fold the concentration of furylfuramide. Compounds **1**−**6** were examined for their ability to suppress the metabolic activation of Trp-P-1 by S9. As shown in Table 2, these compounds did not suppress the SOS induction of activated Trp-P-1. These results indicated two possibilities: (i) the hydroxy or methoxy group at the 3-position enhances the suppressive effect against Trp-P-1; (ii) inhibition of

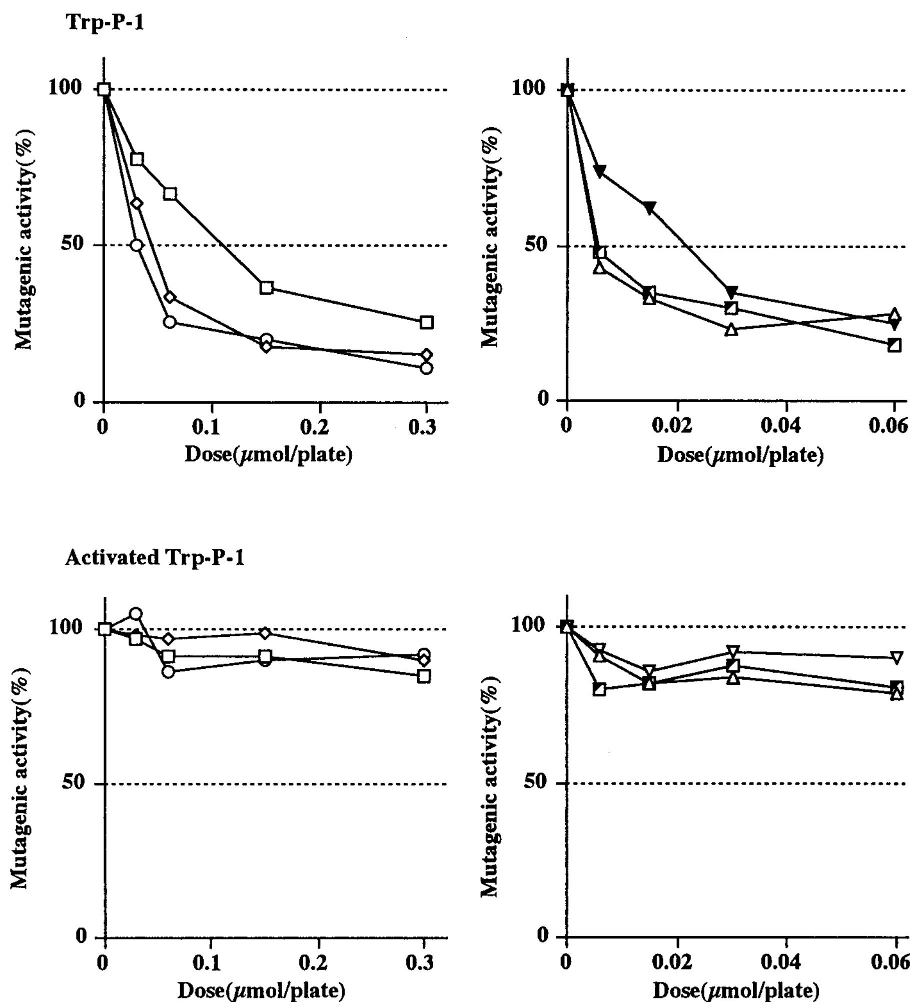


Figure 4. Effects of compounds **1**–**6** on the mutagenicity of Trp-P-1 and activated Trp-P-1 in *S. typhimurium* TA100: (□) effect of **1**; (◇) effect of **2**; (○) effect of **3**; (△) effect of **4**; effect of **5**; (◪) effect of **6**. Trp-P-1 (40 μg/mL in DMSO) was added at 50 μL/plate. Activated Trp-P-1 (10 μg/mL in DMSO) was added at 50 μL/plate.

SOS-inducing activity of Trp-P-1 is due to the inhibition of metabolic activation of S9. The antimutagenic effect of flavonoids against heterocyclic amines has been reported (Lee et al., 1994; Edenharder et al., 1993; Alldrick et al., 1986). Kanazawa et al. (1995) reported that luteolin, galangin, and qurecetin showed antimutagenic effects against Trp-P-1. They indicated that the mechanism of the antimutagnic effect is especially due to the inhibition of activation of Trp-P-2 by the ultimate carcinogenic metabolite in the P450 monooxygenase system, regardless of the OH group.

On the other hand, compounds **1** and **3** had suppressive effects on *umu* gene expression of SOS response in *S. typhimurium* TA1535/pSK1002 against UV irradiation. The antimutagenic factors are divided into two main classes: one type, desmutagen, inactivates or destroys mutagens directory or indirectly out of the cell, and the other type of factor is called bioantimutagen, which suppresses the process of mutagenesis itself in the cells. From these results, compounds **1** and **3** may have potency as bioantimutagens.

In the Ames test using *S. typhimurium* TA100, these compounds similarly inhibited the mutagenicity of Trp-P-1, whereas compounds **1** and **3** showed a weak suppressive effect of the mutagenicity of furylfuramide compared with the *umu* test (Figure 3). Recent studies have shown it is well-known that flavonoids exhibit inhibition of mutagenicity induced by chemical mutagens (Wall et al., 1988; Francis et al., 1989a,bb; Choi et al., 1994; Ohtuka et al., 1995). In addition, a large variety of pharmacological activities have been reported, for example, anticarcinogenic activity (Verman et al., 1988; Bon et al., 1992), antioxdative activity (Cholbi et al., 1991), and anti-inflammatoric activity (Abad et al., 1993). However, inhibition of mutagen-induced SOS response by flavonoids and bioactivity of methoxy flavonoids has not been reported. In summary, this research suggests that antimutagenic compounds in *P. cablin* were primarily 7,4′-di-*O*-methyleriodictyol (**1**), 7,3′,4′-tri-*O*-methyleriodictyol (**2**), 3,7,4′-tri-*O*-methylkaempferol (**3**), ombuine (**4**), pachypodol (**5**), and kumatakenin (**6**). Compounds **1** and **3** showed potent suppressive effects of SOS-inducing activity by chemical mutagen and UV irradiation, and these two compounds may have potency as bioantimutagens.

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