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Fundamental and Molecular Mechanisms of Mutagenesis

Emodin inhibits the mutagenicity and DNA adducts induced by 1-nitropyrene

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Abstract

Polygonum cuspidatum S. (PC) is frequently used as a laxative and an anticancer drug in Chinese medicine. The inhibitory effect of this herb and its component, emodin, on the direct-acting mutagenicity of 1-nitropyrene (1-NP) was examined using the Ames/microsomal test with *Salmonella typhimurium* TA98 and the genotoxicity of 1-NP was evaluated using the SOS chromotest with *E. coli* PQ37. Emodin and water extracts of PC markedly decreased the mutagenicity of 1-NP in a dose-dependent manner in both assay systems. Furthermore, emodin and the extracts of PC significantly inhibited the formation of 1-NP DNA adducts in *S. typhimurium* TA98 in the ^{32}P-postlabeling study. The results suggest that PC extracts and emodin act as blocking and/or suppressing agents to reduce the direct-acting mutagenicity of 1-NP.

Keywords: Emodin; *Polygonum cuspidatum* S.; 1-Nitropyrene; DNA adduct; Mutagenicity

1. Introduction

Polygonum cuspidatum S. (PC) is frequently used in Chinese medicine to treat bacterial and viral infections, cough, asthma, hypertension, and cancer (Chang and But, 1987). Anthraquinone derivatives, which constitute 3–5% of PC extracts, are considered to be the major biologically active components. Among them, emodin has been extensively investigated. This compound exhibits a variety of biological effects such as inhibition of growth of murine leukemia virus (Kawai et al., 1984), antimicrobial activity (Brown, 1980), immunosuppressive effect (Huang et al., 1992), and vasorelaxant activity (Huang et al., 1991). Emodin is mutagenic to *Salmonella typhimurium* TA1537, TA2637, and TA100 with hepatic microsomal activation (Brown and Brown, 1976; Liberman et al., 1982; Tikkanen et al., 1983; Krivobok et al., 1992). It has also been found to be active in the V79 HGPRT mutagenicity assay, the DNA repair assay and the C3H/M2 transformation assay (Westendorf et al., 1990). On the other

Abbreviations: 1-NP, 1-nitropyrene; PC, *Polygonum cuspidatum* S.; B[a]P, benzo[*a*]pyrene; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole; 4-NQO, 4-nitroquinoline *N*-oxide; β-gal, β-galatosidase; Ap, alkaline phosphatase; ONPG, *O*-nitrophenyl-β-D-galactopyranoside; PNPP, *p*-nitrophenyl phosphate

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hand, extracts of PC demonstrate the greatest antimutagenic effects against 1-nitropyrene (1-NP) and benzo[*a*]pyrene (B[a]P) among 41 Chinese anticancer herbs (Lee et al., 1989). Moreover, emodin reduces the mutagenicity of IQ by direct inhibition of hepatic microsomal activation instead of interaction with proximate metabolites of IQ (Lee and Tsai, 1991).

According to Wattenberg's criterion, the inhibitors of carcinogenesis can be divided into three categories (Wattenberg, 1985). The first category consists of compounds that interfere with the formation of carcinogens from their precursor substances. The second category contains compounds that prevent complete carcinogens from reaching or reacting with critical sites in target cells. These inhibitors are called 'blocking agents' and they act primarily by retarding the activation or facilitating the detoxification and removal of xenobiotics. The third category contains 'suppressing agents', which either impair, delay or reverse the expression of malignancy after exposure to carcinogens. In this study, we investigated the effect of PC extracts and emodin on the bacterial genotoxicity induced by 1-NP in the Ames test and SOS chromotest. Furthermore, the effect of the herb and emodin on the formation of 1-NP DNA adducts was examined by ^{32}P-post-labeling.

2. Materials and methods

Materials and chemicals

Polygonum cuspidatum S. was obtained from a local Chinese herb store in Taichung City, Taiwan. PC extracts were prepared using the method described previously (Lee et al., 1989). $NADP^+$, glucose 6-phosphate (G-6-P), *p*-nitrophenyl phosphate (PNPP), 4-nitroquinoline *N*-oxide (4-NQO), lysozyme, micrococcal endonuclease, and potato apyrase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 1-NP was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). The purity of 1-NP was 99%. [γ-^{32}P]ATP was purchased from Amersham International Plc. (Amersham, UK). *O*-Nitrophenyl-β-D-galactopyranoside (ONPG), proteinase K, RNase A, RNase T1, and spleen phosphodiesterase were provided by Boehringer-Mannheim GmbH (Mannheim, Germany).

The Salmonella / microsomal test

The procedures were basically the same as the plate incorporation method described by Maron and Ames (1983). To 2 ml molten top agar (45° C) was added 5 nmol 1-NP in 50 μl dimethyl sulfoxide (DMSO), varying concentration of PC extracts and emodin in 50 μl DMSO, and 100 μl of an overnight culture of TA98. The mixture was gently mixed and poured onto minimal glucose agar plates. The plates were incubated at 37° C for 48 h and revertant colonies were counted. All experiments were performed at least twice with three plates for each concentration. The results are expressed as means with standard deviation (SD). The percent inhibition (PI) was calculated as described previously (Lee et al., 1989).

The SOS chromotest

The procedures were basically the same as those described previously (Sato et al., 1991) which were modified for the SOS chromotest by Quillardet and Hofnung (1985). An overnight culture of *E. coli* PQ37 (1.0 ml) was diluted 10-fold with 9.0 ml L-medium. For simultaneous treatment, 1.3 ml L-medium containing *E. coli* PQ37 in each tube was incubated with 5.0 μg 1-NP (10 μl DMSO) and antimutagen (PC extracts or emodin, 40 μl DMSO) at 37° C for 2 h. For the post-treatment, mixtures of 5.0 μg 1-NP in 10 μl DMSO and 1.3 ml diluted culture were pre-incubated at 37° C for 20 min. The antimutagen was added to the mixtures for another 2 h incubation. After incubation, 0.3-ml aliquots of the culture were added to four test tubes. Two test tubes were used for the assay of β-galactosidase and the other two tubes were used for the assay of alkaline phosphatase. The enzyme units, *R* values, and induction factors (IF) were calculated as described previously (Quillardet and Hofnung, 1985). The modification factor (MF) was calculated according to the formula given by Sato et al. (1991). Percent inhibition was calculated as described above.

Nitroreductase activity assay

The nitroreductase assay was basically according to the procedures described by Kinouchi et al. (1986) and slightly modified as previously described (Lee et al., 1994). An overnight culture of strain TA98 (200 ml) was centrifuged at 10000 rpm for 10 min at 4° C. The bacterial pellet was washed with phosphate buffered saline (PBS) and resuspended in 10 ml PBS. The bacterial cells were broken by sonifier (Model-250, Branson, USA) at 180 W for 10 min at 4° C. The mixture contained 0.2 ml of the bacterial homogenate, 4 μmol $NADP^+$, 5 μmol G-6-P, 33 μmol KCl, 8 μmol $MgCl_2$, 100 μmol sodium phosphate buffer (pH 7.4), 100 nmol 1-NP (10 μl DMSO), and either did or did not contain PC extracts or emodin (50 μl DMSO) for a total volume of 1.0 ml. The mixtures were incubated at 37° C for 4 h, which was the optimal incubation time observed from our preliminary experiment (Lee et al., 1994). The 1-AP in the resultant solution was extracted with 2 ml ethyl acetate and the extracts were diluted 100-fold with the same solvent. The amounts of 1-AP in the extracts were quantified by fluorescence spectrophotometry (Model F-2000, Hitachi Co., Japan) at an excitation wavelength of 363 nm and an emission wavelength of 420 nm. The nitroreductase activity of strain TA98 was calculated by the production of 1-AP.

^{32}P-postlabeling method

Previous procedures (Gupta et al., 1982; Gupta, 1985) with slight modifications (Lee et al., 1994) were used. Briefly, 10 ml of an overnight culture of strain TA98 was diluted to 260 ml and incubated at 37° C for 2 h. To each cultured sample was added 1.0 μmol 1-NP in 100 μl DMSO with or without PC extracts and emodin. The cultures were then incubated with shaking in a water bath at 37° C for 21 h. The cells were recovered by centrifugation at 10000 rpm for isolation of DNA. DNA samples were dissolved in sodium succinate buffer and hydrolyzed by micrococcal endonuclease and spleen phosphodiesterase at 38° C for 4 h. The hydrolysates were dissolved in 100 μl water containing 10 mM ammonium formate (pH 3.5) and 1 mM tetrabutylammonium chloride. To concentrate the adducts, the mixture was extracted twice with 1 volume of 1-butanol. The butanol extract was then evaporated using a Speed Vac concentrator (Savant Instrument, Inc., Hicksville, NY). For ^{32}P labeling, the residue was dissolved in a mixture of 18 μl water and 6 μl kinase and incubated at 37° C for 1 h. Adducts were analyzed by four-directional PEI-cellulose TLC. The conditions for the development of the TLC plates were described previously (Roy et al., 1989). The adducts were located by screen-enhanced autoradiography at

Table 1
Inhibitory effect of PC extracts and emodin on the mutagenicity of 1-NP with *S. typhimurium* TA98

Sample	Concentration (μg/plate)	Number of revertants/plate		PI (%) [a]
		− 1-NP	+1-NP	
PC extracts	500	22 ± 1 [b]	1559 ± 229	31.1
	1000	22 ± 1	1362 ± 3	40.0
	2000	19 ± 6	1052 ± 82	54.0
	4000	25 ± 1	643 ± 65	72.5
Emodin	6.25	24 ± 1	1586 ± 208	29.9
	12.5	21 ± 1	1392 ± 91	38.6
	25.0	21 ± 1	1238 ± 52	45.6
	50.0	21 ± 1	792 ± 47	65.7
	100.0	24 ± 1	475 ± 25	80.0
1-NP alone	1.2		2247 ± 189	
Spontaneous revertants		33 ± 6		

[a] PI (%), percent inhibition = 100 − [(No. of revertants induced by 1-NP in the presence of inhibitor − spontaneous revertants)/(no. of revertants induced by 1-NP in the absence of inhibitor − spontaneous revertants)] × 100.
[b] Values are mean ± SD. All the tested groups are significantly different from the control (F-test, $P < 0.01$).

Table 2
Genotoxicity of PC extracts and emodin in *E. coli* PQ37

Sample	Concentration (μg/assay)	β-gal (units)	Ap (units)	R	IF
PC extracts	0	12.50	36.25	0.35	1.00
	125	10.25	32.71	0.31	0.91
	250	7.40	30.24	0.25	0.71
	500	4.85	23.60	0.21	0.60
	1000	1.15	18.38	0.06	0.18
Emodin	0	12.5	36.25	0.35	1.00
	0.63	9.35	32.99	0.28	0.82
	1.25	7.90	29.74	0.27	0.77
	2.50	5.85	24.85	0.24	0.68
	5.00	4.15	21.90	0.19	0.55
	10.00	4.15	20.71	0.20	0.58
Positive control, 4-NQO	20 ng	40.40	39.75	1.02	1.90

IF, induction factor; R values, β-gal/Ap.

Table 3
Inhibitory effect of PC extracts and emodin on the genotoxicity of 1-NP with *E. coli* PQ37

Sample	Dose (μg/1.3 ml)	β-gal (units)	Ap (units)	R	MF	PI [a] (%)
Simultaneous treatment						
PC extracts	0	30.25	37.18	0.81	1.00	
	125	24.20	34.60	0.70	0.86	14.0
	250	17.55	30.86	0.57	0.70	30.0
	500	7.00	23.10	0.30	0.37	63.0
	1000	2.65	16.83	0.16	0.19	81.0
Emodin	0	30.25	37.18	0.81	1.00	
	0.63	29.70	35.61	0.83	1.03	−3.0
	1.25	33.50	35.83	0.94	1.15	−15.0
	2.50	17.25	30.77	0.56	0.69	31.0
	5.00	7.65	22.89	0.33	0.41	59.0
	10.00	7.80	22.65	0.34	0.42	58.0
Post-treatment						
PC extracts	0	32.10	38.18	0.84	1.00	
	125	26.15	34.35	0.76	0.95	5.50
	250	20.70	32.11	0.65	0.77	23.0
	500	8.15	24.48	0.33	0.40	60.0
	1000	1.90	19.33	0.10	0.12	88.0
Emodin	0	32.10	38.18	0.841	1.00	
	0.63	54.15	38.11	1.42	1.69	−69.0
	1.25	36.10	34.70	1.40	1.24	−24.0
	2.50	20.45	31.14	0.66	0.78	22.0
	5.00	8.55	24.76	0.35	0.41	59.0
	10.00	8.35	23.28	0.359	0.43	57.0

R values, β-gal/Ap; MF, modification factor.
[a] PI (%) = (the value of MF in the absence of PC extracts or emodin − the value of MF in the presence of PC extracts or emodin) × 100.

Table 4
Inhibitory effect of PC extracts and emodin on the nitroreductase activity of TA98 strain

Sample	Production of 1-AP		PI (%) [a]
	Total amount (pmol)	Specific activity (pmol/h/mg of protein)	
1-NP alone	351.48 ± 21.38 [b]	35.63 ± 2.17	–
PC extract (mg)			
0.4	140.75 ± 1.50	14.27 ± 0.15	59.95
0.6	54.64 ± 2.51	5.54 ± 0.25	84.45
0.8	26.38 ± 4.25	2.67 ± 0.43	92.50
1.0	21.68 ± 5.34	2.20 ± 0.54	93.83
Emodin (μg)			
40	309.81 ± 29.60	31.41 ± 3.00	11.84
60	207.93 ± 8.32	21.08 ± 0.84	40.84
80	46.76 ± 8.74	4.74 ± 0.89	86.70
100	8.45 ± 8.78	0.86 ± 0.89	97.59

[a] PI (%) = 100 − (the specific activity in the presence of PC extracts or emodin/the specific activity in the absence of PC extracts or emodin) × 100.
[b] Values are mean ± SD.
All the tested groups are significantly different from the control (F-test, $P < 0.01$) except for emodin at the dose of 40 μg.

−80° C and their levels were calculated according to the formula of Gupta (1985).

3. Results

The mutagenicity of 1-NP (1.2 μg/plate) to TA98 was significantly and dose-dependently inhibited by PC extracts and emodin (Table 1). Emodin exerted a much greater inhibitory effect than did PC extracts. No genotoxicity in *E. coli* PQ37 was observed with PC extracts (≤ 1000 μg/assay) and emodin (≤ 10 μg/assay) (Table 2). We then evaluated the inhibitory effects of PC extracts and emodin on 1-NP-induced genotoxicity in the SOS chromotest. They demonstrated similar inhibitory effects on SOS induction by simultaneous and post-treatments with 1-NP (Table 3). The results suggest that both PC extracts and emodin act as blocking agents (at simultane-

Table 5
Inhibitory effect of PC extracts and emodin on the formation of 1-NP DNA adducts in *S. typhimurium* TA98

Sample	Concentration (mg/assay)	DNA adducts/10^8 nucleotides	PI (%) [a]
PC extracts	1.0	4.43 ± 0.56 [b]	70.5
	5.0	4.53 ± 2.24	70.0
	25.0	ND	100.0
Emodin	0.05	9.38 ± 0.69	37.5
	0.25	8.18 ± 1.09	45.5
	1.25	7.54 ± 1.78	49.7
1-NP alone	0.19	15.0 ± 2.66	

[a] PI (%) = 100 − (the DNA adduct level in the presence of PC extracts or emodin)/(the DNA adduct level in the absence of PC extracts or emodin) × 100.
[b] Values are mean ± SD.
ND, not detected.
All the tested groups are significantly different from the control (F-test, $P < 0.05$).

ous treatment) and suppressing agents on SOS repair function (at post-treatment). Surprisingly, the enhancement of SOS induction by 1-NP was observed with a low dose of emodin in both treatments. The reason remains unknown. Table 4 shows that the nitroreductase activity of strain

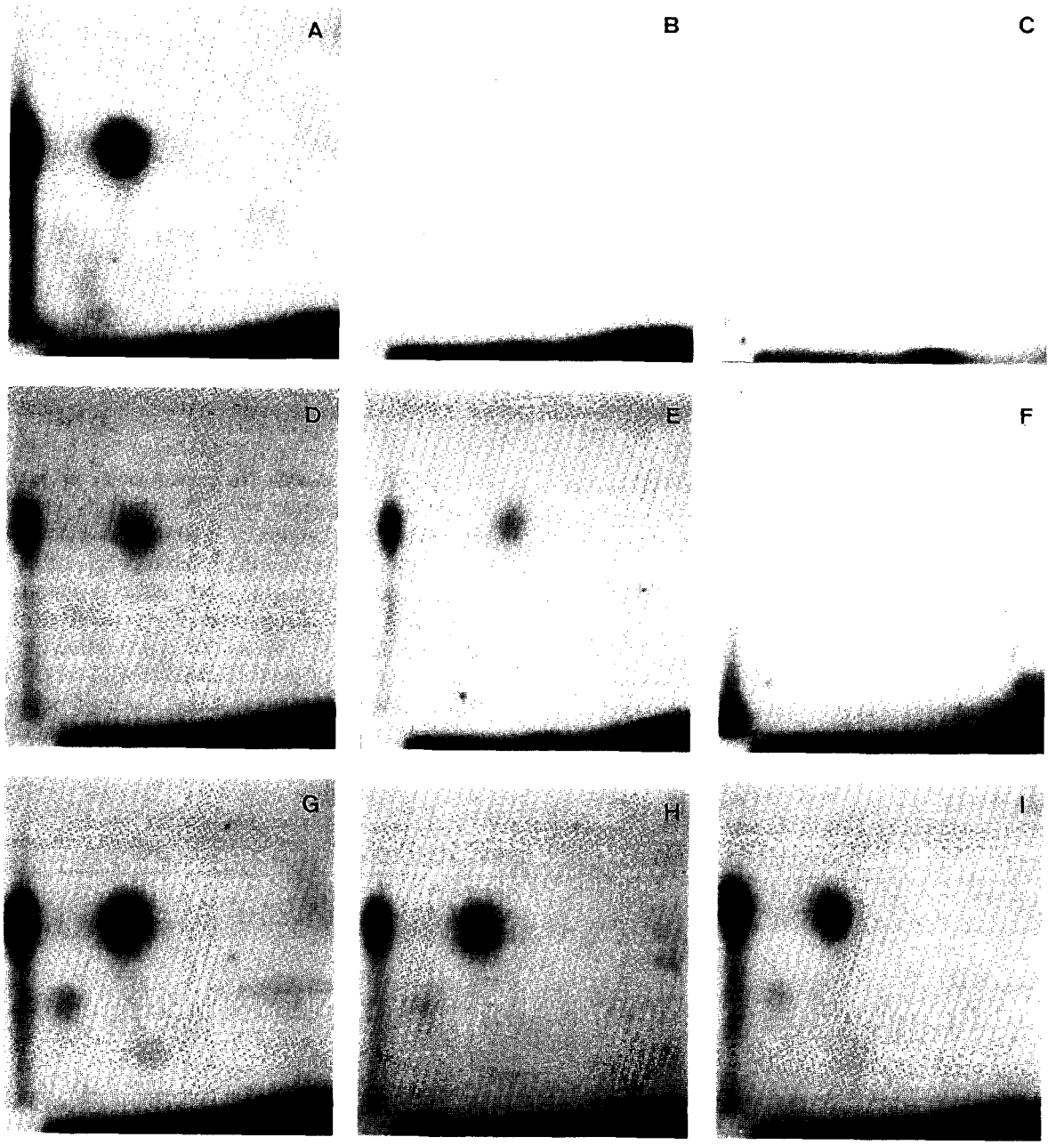

Fig. 1. ^{32}P-postlabeling assay autoradiograms of 1-NP in the presence and absence of various concentrations of PC extracts and emodin. (A) 1-NP alone, (B) PC extracts alone, (C) emodin alone, (D) 1 mg PC extracts and 1-NP, (E) 5 mg PC extracts and 1-NP, (F) 25 mg PC extracts and 1-NP, (G) 0.05 μg emodin and 1-NP, (H) 0.25 μg emodin and 1-NP, (I) 1.25 μg emodin and 1-NP.

TA98 was significantly reduced by PC extracts and emodin in a dose-dependent manner.

The ^{32}P-postlabeling assay was used to determine the inhibitory effect of PC extracts and emodin on the formation of 1-NP DNA adducts from mutagenesis induced by 1-NP in *S. typhimurium* TA98. PC extracts and emodin showed a strong ability to inhibit the formation of the 1-NP DNA adducts (Table 5). In this assay, a dose of PC extracts of 25 μg/assay completely reduced DNA adduct formation. This effect of PC extracts and emodin seemed to be more potent than the effects in the mutagenicity test. The autoradiograms of 1-NP in the presence and absence of PC extracts and emodin are shown in Fig. 1. No DNA adducts were detected after treatment with PC extracts and emodin alone (Fig. 1B,C).

4. Discussion

1-NP is the most abundant nitro-polycyclic aromatic hydrocarbon, and it has been detected at low levels in ambient urban air (Gibson, 1982), broiled chicken (Kinouchi et al., 1986), and diesel engine exhaust (Schuetzle, 1983). It has been shown to be a mutagenic and carcinogenic compound in various systems (Fu, 1990; Heflich et al., 1986; Eddy et al., 1986; Hecht and El-Bayoumy, 1990).

We have previously examined the antimutagenicity of extracts of 41 Chinese herbs against 1-NP to understand the potential protection of human health from the hazard of pollutants. Among them, we had found that PC extracts possessed the most potent inhibitory effect on the direct-acting mutagenicity of 1-NP. The potent antimutagenic effect of emodin (a major constituent of PC extracts) against 1-NP was also observed in this study. The results indicate that emodin contributed at least partly to the inhibitory effect of PC extracts on 1-NP mutagenicity. Previous studies showed that emodin reduces the mutagenicity of indirect-acting mutagens (B[a]P, IQ and Trp-P-2) by directly inhibiting the hepatic microsomal activation (Lee and Tsai, 1991). In addition, emodin does not interact with proximate metabolites of IQ and/or by modification of DNA repair processes in the Salmonella bacteria (Lee and Tsai, 1991; Hao et al., 1995). Thus, we can conclude that emodin acts as a desmutagen to affect IQ mutagenicity.

The mutagenicity of 1-NP requires the involvement of classical nitroreductase (Fu, 1990). We found that the antimutagenicity of PC extracts and emodin was probably due to an alteration of the ability of the *S. typhimurium* TA98 cultures to nitroreduce as in the case of B[a]P on 1-NP mutagenicity (Table 4, Lee et al., 1994). In addition, the inhibitory effects of PC extracts and emodin in SOS induction were shown by either simultaneous or post-treatments with 1-NP. The inhibitory effect of PC extracts and emodin on 1-NP-induced mutation in the SOS chromotest appeared to be more potent than in the Ames test. This suggests that PC extracts and emodin probably act as a blocking agent and/or suppressing agent to prevent 1-NP from reacting with DNA. Nitroreduction of 1-NP followed by DNA binding, both in vitro and in vivo, leads mainly to C8-substituted deoxyguanosine adducts (Howard et al., 1983; Djuric et al., 1988). The mutagenic potency of 1-NP correlates with the degree of C8-modified deoxyguanosine adduct formation (Beland et al., 1983). To understand the function of PC extracts and emodin on the inhibitory effect against the mutagenicity of 1-NP, formation of DNA adducts in the repair-deficient strain *S. typhimurum* TA98 was determined using ^{32}P-postlabeling. This study demonstrated that PC extracts and emodin were able to inhibit the formation of the C8-substituted deoxyguanosine adduct of 1-NP. This phenomenon may have resulted from lowered nitroreduction of 1-NP. Thus, emodin is not only a desmutagen against indirect-acting mutagens, but also a blocking agent and/or suppressing agent against direct-acting mutagens. However, the differences in the formation of the 1-NP-DNA adducts between *S. typhimurium* TA98 and *E. coli* PQ37 need further investigation.

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