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In Vitro Antimutagenic Effects of Alaternin and Isorubrofusarin Gentiobioside from roasted *Cassia tora*

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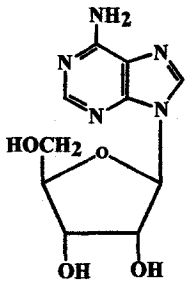
Abstract – The antimutagenic activity of a methanol extract of roasted *Cassia tora* seeds against aflatoxin B_1(AFB_1) was demonstrated with the *Salmonella typhimurium* assay. The numbers of revertants per plate decreased significantly when this extract was added to the assay system using *Salmonella typhimurium* TA100 and or TA98. The MeOH extract was then sequentially partitioned with CH_2Cl_2, EtOAc, *n*-BuOH, and H_2O. The CH_2Cl_2 and *n*-BuOH fractions possessed antimutagenic activity, but the EtOAc and H_2O fractions were inactive. Both the MeOH extract and its fractions were capable of inhibiting the indirect-acting mutagen AFB_1, suggesting that these fractions may prevent the metabolic activation of AFB_1 or scavenge the electrophilic intermediate capable of inducing mutations. Column chromatography using silica gel yielded pure alaternin from the CH_2Cl_2 fraction, and adenosine and isorubrofusarin gentiobioside from the *n*-BuOH fraction. Alaternin and isorubrofusarin gentiobioside demonstrated significant antimutagenic activities.

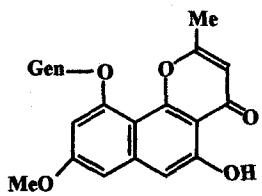
Key words – roasted, *Cassia tora*, Leguminosae, antimutagenicity, anthraquinone, naphthopyrone glycoside, Ames test

Introduction

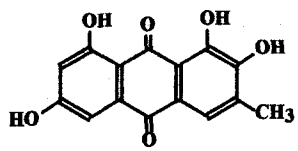
The seeds of *Cassia tora* L. (Leguminosae) are used to improve vision in Chinese herbal medicine and are reputed for their medicinal value as an asperient, antiasthenic, and diuretic agent (Namba, 1980). Previously we reported that the MeOH extract of the seeds of *C. tora* has shown to exert an antimutagenic activity against aflatoxin B_1 (AFB_1) induced mutagenicity with the *Salmonella typhimurium* assay (Choi *et al.*, 1997). From the MeOH extract anthraquinones (chrysophanol, aurantio-obtusin and ch-



adenosine



iso-rubrofusarin gentiobioside



alaternin

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ryso-obtusin) and naphthopyrone glycosides (cassiaside and rubrofusarin gentiobioside) were isolated as active principles (Choi *et al.*, 1994, 1997). Since the roasted Cassia seeds are frequently consumed as a tea or Chinese herbal drug preparations in Korea, we studied the antimutagenic activities of the MeOH extract of the seeds of the roasted *C. tora*, several fractions purified from the MeOH extract, and isolated compounds using the *Salmonella*/microsome assay (Ames test). The antimutagenic activity was assayed against mutagens of aflatoxin B_1 (AFB_1).

Experimental

Plant materials – The seeds of *Cassia tora* were purchased from a commercial supplier, in 1993 and authenticated by Prof. H. J. Chi of the Natural Products Research Institute, Seoul National University. A voucher specimen has been deposited in the Herbarium of the Natural Products Research Institute.

Extraction, fractionation and isolation – The powdered seeds (0.8 kg) of commercially available *C. tora* roasted at 250°C for 10 min was grounded and extracted with MeOH under reflux. The extracts were concentrated to dryness *in vacuo* at 40°C to render the MeOH extract (62 g, yield 7.75%), and then partitioned with CH_2Cl_2 (40.2 g), EtOAc (2.3 g) *n*-BuOH (8.4 g) and H_2O (11.0 g) in sequence to yield the corresponding dried extracts. The CH_2Cl_2 fraction (20 g) was chromatographed on silica gel (CH_2Cl_2/MeOH, gradient) to give alaternin{**1**, 350 mg}. The *n*-BuOH fraction (8.4 g) was chromatographed on silica gel (CH_2Cl_2/MeOH, gradient) to give isorubrofusarin gentiobioside{**2**, 80 mg} and adenosine{**3**, 10 mg}. These compounds were identified by direct comparison with authentic samples (mp, ir, ^1H and ^{13}C NMR) (Lee *et al.*, 1997).

Mutagens – Aflatoxin B_1 (AFB_1) was purchased from the Sigma Chemical Co., St. Louis, MO, USA.

Bacterial strains – *Salmonella typhimurium* strains TA100 and TA98, which are histidine-requiring mutants, were kindly provided by Dr. B. N. Ames, University of Califonia, Berkeley, CA, USA, and maintained as described by Maron and Ames (1993). The genotypes of the test strains were checked routinely for their histidine requirement, deep rough (*rfa*) character, UV sensitivity (*uvr* B mutation) and presence of the R factor.

S9 fraction and S9 mix – According to the method described by Maron and Ames (1993), male Sprague-Dawley rats were injected intraperitoneally with Aroclor 1254 dissolved in corn oil (500 mg/kg body weight). Five days after the injection, the rats were destroyed, their livers removed and minced in 0.15 M KCl, and then homogenized with a Potter-Elvehjem apparatus. The homogenate was centrifuged at 9,000×g for 10 min in a refrigerated centrifuge, and the supernatant S9 fraction was distributed in 1.8~2.0 ml portions in plastic Nunc tubes, frozen quickly in a bed of crushed dry ice, and stored immediately at -80°C until use. The S9 required for the preparation of the S9 mix was thawed at room temperature and placed in a container of crushed ice. The S9 mix was prepared as soon as the S9 had thawed. The components of the S9 mix were 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, 100 mM sodium phosphate, pH 7.4, and S9 at a concentration of 10% per ml of the mix. The S9 mix was freshly prepared for each antimutagenicity assay.

Antimutagenicity test – A modified plate incorporation procedure (Matsushima *et al.*, 1980) was employed to determine the antimutagenic effect of the MeOH extract of roasted *C. tora*, its subfraction, isolated alaternin, isorubrofusarin gentiobioside and adenosine on AFB_1-induced mutagenicity. In brief, 0.5 ml of the S9 mix was distributed in sterile capped tubes in an ice bath, and then 0.1 ml of the test bacterial suspension from an overnight culture ($1\sim2\times10^9$ cells/ml)

and 0.1 ml of the test compound (50 μl of the mutagen and/or 50 μl of 0.05~5.0 mg test compounds) were added. After gently vortexing and preincubating at 37°C for 30 min, 2 ml of the top agar kept at 45°C was added to each tube and vortexed for 3s. The resulting complete mixture was overlaid on the minimal agar plate. The plates were incubated at 37°C for 48 h and the revertant bacterial colonies on each plate were counted. The inhibition rate (%) of mutagenicity was calculated relative to those in the control group with the mutagen by the following equation : the inhibition rate (%)=100×[(A-B)/(A-C)] where A is the number of revertants in the control group, B is the number of revertants in the test group with the mutagen, and C is the number of spontaneous revertants. Dose response test of AFB_1 on the test strain was carried out to determine the regions of revealing mutagenicity induced by mutagen. Toxicity tests for the different levels of samples were also carried out, and the sample concentrations employed for the antimutagenic test did not show any toxicity to the tester strain (Maron and Ames, 1983).

Statistics – Data were collected with a mean±standard deviation of three plates (n =3), and their significances were analyzed by Student's t-test.

Results and Discussion

As shown in Table 1, AFB_1 proved to be mutagenic in the *Salmonella typhimurium* TA100 with the S9 mix. However, when a 1.25 mg/plate MeOH extract of roasted *C. tora* was added to the assay system containing *Salmonella typhimurium* TA100, the numbers of revertant per plate decreased by about 41% of those obtained in the absence of the extract. The inhibitory effect was further demonstrated when the concentration of the extract was raised to 2.50~5.00 mg/plate. The antimutagenic activity of this MeOH extract showed less potent when compared with the previous results of the MeOH extract obtained from raw seeds described by us (Choi *et al.*, 1997). This can be explained by the fact that a structural modification of active compounds affecting antimutagenic activity may be occurred during this roasting process.

Since the MeOH extract exerted antimutagenic activity as shown in Table 1, the effect of each fraction purified from the MeOH extract was examined. As shown in Table 2, when a 0.5 mg/plate fraction of CH_2Cl_2 and *n*-BuOH obtained from the MeOH extract was added to the assay system, the numbers of the TA100 revertants decreased by 86% and 66%, respectively. The numbers of revertants per plate for *Salmonella typhimurium* TA98 also showed significant decreases by 85% and 95%, respectively. The inhibitory effect was further increased when the concentration of the fractions was raised to 1.25 mg/plate. These antimutagenic activities of the fractions were higher than that of the MeOH extract. Among these fractions, the CH_2Cl_2 fraction showed the highest activity. On the other hand, the EtOAc and H_2O fractions did not display any activity. The MeOH extract and its fractions were capable of inhibiting the indirect-acting mutagen AFB_1, suggesting that these

Table 1. Effect of an MeOH extract of roasted *Cassia tora* on the mutagenicity induced by aflatoxin B_1 (AFB_1, 0.3 mg/plate) in *Salmonella typhimurium* TA 100 in the presence of S9

Treatment	Concentration (mg/plate)	Revertants per plate TA 100
Spontaneous	-	115±8
AFB_1	-	1,216±65[b]
MeOH extract	1.25	761±14[a,c] (41)
	2.50	485± 4[a,d] (66)
	5.00	137±10[b,d] (98)

Figures in parenthesis indicate inhibition rate.
Significantly different from the spontaneous value, [a]$p<0.01$, [b]$p<0.001$
Significantly different form the AFB_1-treated value, [c]$p<0.01$, [d]$p<0.001$

Table 2. Effect of fractions obtained from an MeOH extract of roasted *Cassia tora* on the mutagenicity induced by aflatoxin B_1 (AFB_1, 1.0 μg/plate) in *Salmonella typhimurium* TA 100 or TA 98 in the presence of S9

Treatment	Concentration (mg/plate)	Revertants per plate	
		TA 100	TA 98
Spontaneous		123± 1	20±5
AFB_1		980±35[a]	925±74[a]
CH_2Cl_2 fraction	0.50	241±11[b] (86)	160±17[b] (85)
	1.25	140±19[b] (98)	27±10[b] (99)
EtOAc fraction	0.50	1,341±113 (-)	670±4[c] (28)
	1.25	1,214± 18 (-)	561±57 (40)
n-BuOH fraction	0.50	414±11[c] (66)	69±25[b] (95)
	1.25	224±53[b] (88)	20±3[b] (100)
H_2O fraction	0.50	1,220±274 (-)	856±49 (8)
	1.25	1,420± 8 (-)	688±47 (26)

Figures in parenthesis indicate inhibition rate.
[a] Significantly different from the spontaneous value, $p<0.001$
[b] Singificantly different from the AFB_1-treated value, $p<0.001$
[c] Significantly different form the AFB_1-treated value, $p<0.05$

fractions may prevent the metabolic activation of AFB_1 or scavenge the electrophilic intermediate capable of inducing mutations. Column chromatography of the CH_2Cl_2 and *n*-BuOH fraction over silica gel resulted in the isolation of alaternin, adenosine and isorubrofusarin gentiobioside, which were identified instrumentally.

The present study was also carried out to investigate whether the isolated compounds from roasted *C. tora* would be effective on the inhibition of AFB_1 mutagenicity in the *Salmonella typhimurium* strains. The isolated isorubrofusarin gentiobioside and alaternin markedly and significantly reduced the numbers of revertants in dose dependent manners in comparison with the non-treated group, as shown in Fig. 1. Among these compounds, alaternin was more effective than that of isorubrofusarin gentiobioside. On the other hand, nucleoside adenosine did not showed any activity.

In the present study, the antimutagenic effects of the MeOH extract and its components of roasted *C. tora* were observed in both TA100 and TA98, which are *Salmonella typhimurium* strains showing a base-pair substitution mutation in gene G of the histidine operon, and a frame-shift mutation due to the lack of a base pair in the GC-pair region of gene D (Matsushima *et al.*, 1980), respectively. The above observations suggest that the MeOH extract and its components exert an influence on hereditary factors.

Anthraquinone compounds are the largest class of naturally occurring quinones and are widely distributed in lower and higher plants. Several research workers have revealed that some of those compounds are genotoxic in both procaryotic and mammalian systems (Westendorf *et al.*, 1988, 1990,

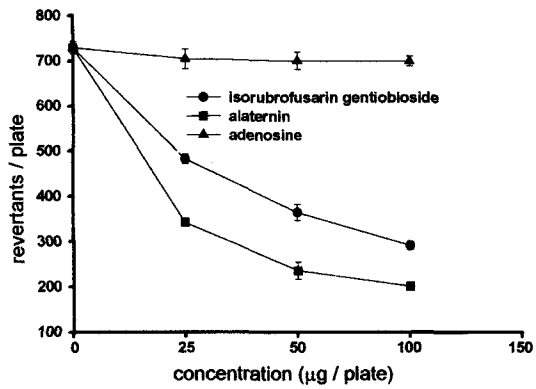

Fig. 1. The inhibitory effect of isorubrofusarin gentiobioside, adenosine and alaternin on mutagenesis induced by AFB_1 in *S. typimurium* TA 100. Assays were performed with the 0.3 μg/plate of AFB_1 as described in Materials and Methods. Each point represents the mean±SD. Spontaneous revertants were subtracted.

Morooka *et al.*, 1990, Brown and Dietrich, 1979, Kawasaki *et al.*, 1992). In contrast to the mutagenicity of these compounds *in vitro*, we previously demonstrated that isolated anthraquinones from *C. tora* have the capacity to suppress the mutagenic activity of AFB_1 when added in low concentrations to the rat liver microsomal activation system in the presence of *Salmonella* tester strains. The previous study also indicated that the number and position of the hydroxyl and methoxyl groups is the most critical factor on the antimutagenicity from the structure-activity relationships between the chemical structures of anthraquinones and their antimutagenicity.

In this study, the antimutagenic activity of alaternin having four hydroxyls was demonstrated. Since alaternin was also isolated from raw seeds, the antimutagenic activity of raw and roasted *C. tora* may be possibly due to this compound as well as anthraquinones isolated before.

Although we (Choi *et al.*, 1997) have reported that linear naphthopyrone glycosides isolated from *C. tora* exhibited significant antimutagenic activity against AFB_1, no report on the antimutagenicity of non-linear naphthopyrone glycoside have yet appeared.

The mechanism of the antimutagenic activity of the anthraquinone and non-linear naphthopyrone glycoside from roasted *C. tora* in the bacterial system is uncertain. The possible mechanism of these compounds may be mediated through interaction with a microsomal activating system since AFB_1 was shown to be completely dependent upon the enzyme activation being an ultimate carcinogen.

A detailed report of the structure-antimutagenicity of the anthraquinones and naphthopyrone glycosides is further needed.

References

Brown, J. P. and Dietrich, P. S., Mutagenicity of anthraquinone and benzanthrone derivatives in the Salmonella/microsome test : Activation of anthraquinone glycosides by enzyme extracts of rat cecal bacteria, *Mutation Res.* **66**, 9-24 (1979).

Choi, J. S., Lee, H. J. and Kang, S. S., Alaternin, cassiaside and rubrofusarin gentiobioside, radical scavenging principles from the seeds of *Cassia tora* on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, *Arch. Pharm. Res.* **17**(6), 462-466 (1994).

Choi, J. S., Lee, H. J., Park, K. Y., Ha, J. O. and Kang, S. S., In vitro antimutagenic effects of anthraquinone aglycones and naphthopyrone glycosides from *Cassia tora*, *Planta Medica* **63**(1), 11-14 (1997).

Kawasaki, Y., Goda, Y. and Yoshihira, K., The mutagenic constituents of *Rubia tinctorum*, *Chem. Pharm. Bull.* **40**(6), 1504-1509 (1992).

Lee, H. J., Jung, J. H., Kang, S. S. and Choi, J. S., A rubrofusarin gentiobioside isomer from roasted *Cassia tora*, *Arch. Pharm. Res.* **20**(5), 513-515 (1997).

Maron, D. M. and Ames, B. N., Revised methods for the Salmonella mutagenicity test, *Mutation Res.* **113**, 173-215 (1983).

Matsushima, T., Sugimura, T., Nagao, M., Yahagi, T., Shirai, A. and Sawamura, M., Factors modulating mutagenicity in microbial test, in Norpoth, K. H. and Garnei, R. C. (eds), *Short Term Test System for Detecting Ccarcinogens*, Springer Verlag, Berlin, 1980, pp. 273-285.

Morooka, N., Nakano, S., Itoi, N. and Ueno, Y., The chemical structure and the mutagenicity of emodin metabolites, *Agric. Biol. Chem.* **54**(5), 1247-1252 (1990).

Namba, T., *Colored Illustrations of Wakan-Yaku*, Hoikusha Publishing Co. Ltd., Vol. 1. pp. 226 (1980).

Westendorf, J., Marquardt, H., Poginsky, B., Dominiak, M., Schmidt, J. and Marquardt, H., Genotoxicity of naturally occurring hydroxyanthraquinones, *Mutation Res.* **240**, 1-12 (1990).

Westendorf, J., Poginsky, B., Marquardt, H., Groth, G. and Marquardt, H., The genotoxicity of lucidin, a natural component of *Rubia tintorum* L. and lucidinethylether, a component of ethanolic Rubia extracts, *Cell. Biol. Toxicol.* **4**, 225-239 (1988).

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