Ursolic Acid Inhibits Aflatoxin B₁-Induced Mutagenicity in a *Salmonella* Assay System

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An attempt was made to isolate the active component of *Eriobotrya japonica*, which inhibits aflatoxin B₁-induced mutagenicity in the *Salmonella* assay system. The number of revertants per plate was significantly decreased when a MeOH extract of *Eriobotrya japonica* was added to the assay system using *Salmonella typhimurium* TA100 or TA98. Furthermore, we examined the effect of each fraction purified from the MeOH extract, and an EtOAc fraction was found to be the most effective. Ursolic acid isolated from the EtOAc fraction markedly and significantly decreased the numbers of *Salmonella typhimurium* TA100 revertants per plate, thus showing antimutagenic activity.

Keywords *Eriobotrya japonica*; ursolic acid; antimutagenic activity

Many human diseases are known to be attributable to genetic abnormalities, *i.e.*, gene mutation, structural chromosome aberration and numerical chromosome aberration. It has been pointed out that, unless exposure to chemical substances in the environment decreases, a higher incidence of hereditary diseases can be expected.¹⁾ The results of previous experimental studies have shown that mutagenic substances exist in various forms in our environment. These substances include food additives, drugs, insecticides, cosmetics, contaminants in the water and air, and various chemical substances used in the home or in industry.^{2,3)} In addition, a close correlation between mutagenicity and carcinogenicity has been demonstrated by Brookes and Serres,⁴⁾ who carried out experiments using bacteria, fungi and cultured cells from insects and mammals. McCann *et al.*²⁾ also found in a study using *Salmonella* that 90% of the carcinogenic substances examined had mutagenicity. Thus, it has been suggested that chemical substances having mutagenicity not only exert influences on hereditary factors but also have carcinogenicity.

On the other hand, various studies have been carried out to discover inhibitors of these mutagenic substances, and Kada and colleagues^{5–7)} and Ames³⁾ have reported that peroxidase, vitamins A, C and E, sulfites and potassium metabisulfite act as such inhibitors. Prompted by the fact that some crude drugs, which have been used empirically for a long time, act against influenza or other viruses, inflammation and carcinoma, Park⁸⁾ screened aqueous extracts of these crude drugs, and found high antimutagenic activity in the leaf of *Eriobotrya japonica*.

In the present study, we attempted to isolate the active component of *Eriobotrya japonica*, and found antimutagenic activity in ursolic acid, a component obtained at a relatively high yield.

MATERIALS AND METHODS

Plant Material The *Eriobotrya japonica* used in the experiments was purchased from a local market. A voucher

specimen is deposited in the herbarium of the Pusan National University.

Extraction, Fractionation and Isolation Dried and powdered leaves of *Eriobotrya japonica* (4.5 kg) were extracted with 95% MeOH under reflux. The extract was concentrated under reduced pressure to give a MeOH extract (570 g), and then partitioned with *n*-hexane (41.5 g), CHCl₃ (74.5 g), EtOAc (25.5 g) and *n*-BuOH (102 g) in sequence to yield corresponding fractions. Each fraction was dried under reduced pressure and applied to the mutagenicity test. Among them, the EtOAc fraction, which showed the strongest antimutagenic effect, was subjected to column chromatography on silica gel (Kiesel gel 60, E. Merck, Germany) using a solvent system of benzene–ether (4:1→2:1) to yield the main terpenoidal compound (4.3 g). This compound was identified as ursolic acid by direct comparison with an authentic sample (mixed m.p., co-TLC, and superimposable IR and NMR spectra).

Mutagen Aflatoxin B₁ (AFB₁) was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Bacterial Strains *Salmonella typhimurium* strains TA100 and TA98, which are histidine-requiring mutants, were kindly provided by Dr. B.N. Ames, University of California, Berkeley, CA, U.S.A., and maintained as described by Maron and Ames.⁹⁾ The genotypes of the test strains were checked routinely for their histidine requirement, deep rough (*rfa*) character, UV sensitivity (*uvr* B mutation) and the presence of R factor.

S9 Fraction and S9 Mix⁹⁾ According to the method described by Maron and Ames,⁹⁾ 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 sacrificed, their livers were removed and minced in 0.15 M KCl, and then homogenized with a Potter-Elvehjem apparatus. The homogenate was centrifuged at 9000 × *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 0.04 ml per ml of mix. The S9 mix was prepared freshly for each mutagenicity assay.

Mutagenicity Test A modified plate incorporation procedure¹⁰⁾ was employed to determine the effect of the MeOH extract, its subfraction and ursolic acid on AFB₁-induced mutagenicity. In brief, 0.5 ml of S9 mix (0.5 ml of phosphate buffer in the case of the spontaneous group) was distributed in sterile capped tubes in an ice bath, and then 0.1 ml of test bacterial suspension from an overnight culture ($1-2 \times 10^9$ cells/ml) and 0.1 ml of test compound (50 μl of 2% mutagen and/or 50 μl of 0.25–5.0% test compound) were added. After vortexing gently 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 3 s. The resulting complete mixture was overlaid on the minimal agar plate. The plates were incubated at 37°C for 48 h and then the revertant bacterial colonies on each plate were counted. Toxicity tests for the different cells of the samples were also carried out, and the sample concentrations employed for the antimutagenic test did not show any toxicity.

RESULTS AND DISCUSSION

As shown in Table I, AFB₁ proved to be mutagenic in both of the mutagenic strains examined, i.e., *Salmonella typhimurium* TA100 and TA98. However, when a 2.5% MeOH extract of *Eriobotrya japonica* was added to the assay system containing *Salmonella typhimurium* TA 100, the number of revertants per plate decreased to about 20% of those obtained in the absence of the extract. The number of revertants per plate for *Salmonella typhimurium* TA 98 also showed a significant decrease, the value being 85% lower than that in the absence of the extract. The inhibitory effect was further increased when the concentration of the extract was raised to 5%. These antimutagenic activities of the MeOH extract were higher than that of an aqueous extract reported previously.⁸⁾

Since the MeOH extract had antimutagenic activity, as shown in Table I, the effect of each fraction purified from the MeOH extract was examined.

As shown in Table II, hexane, CHCl_3 , EtOAc and *n*-BuOH fractions decreased the numbers of TA100 revertants by 60, 61, 74 and 65%, respectively. Among these fractions, the EtOAc fraction showed the highest activity. This fraction showed a positive Liebermann–Burchard reaction, indicating the presence of a triterpenoidal compound. Column chromatography of the EtOAc fraction over silica gel resulted in the isolation of ursolic acid, which was identified instrumentally.

The isolated ursolic acid reduced the number of TA100 revertants markedly and significantly, the value being 64% lower at 0.25% concentration, 65% lower at 0.5 and 70% lower at 1% in comparison with the non-treated

TABLE I. Effect of a MeOH Extract from *Eriobotrya japonica* on the Mutagenicity Induced by AFB₁ in *Salmonella typhimurium* TA100 or TA98

Treatment	Concentration (%)	Revertants per plate	
		TA100	TA98
Spontaneous	—	125 ± 12	28 ± 2
AFB ₁	—	2038 ± 7 ^{c)}	1731 ± 71 ^{c)}
AFB ₁ + MeOH extract	2.5	441 ± 12 ^{c,d)}	258 ± 74 ^{a,d)}
AFB ₁ + MeOH extract	5.0	206 ± 11 ^{b,d)}	98 ± 14 ^{b,d)}

Statistical significance: a) $p < 0.05$, b) $p < 0.01$, c) $p < 0.001$ vs. spontaneous value, d) $p < 0.001$ vs. AFB₁-nontreated value.

TABLE II. Effect of Fractions Obtained from a MeOH Extract of *Eriobotrya japonica* on the Mutagenicity Induced by AFB₁ in *Salmonella typhimurium* TA100

Treatment	Concentration (%)	Revertants per plate
Spontaneous	—	161 ± 2
AFB ₁	—	1106 ± 47 ^{b)}
AFB ₁ + hexane fraction	2.5	438 ± 66 ^{a,c)}
AFB ₁ + CHCl_3 fraction	2.5	428 ± 55 ^{a,c)}
AFB ₁ + EtOAc fraction	2.5	293 ± 4 ^{b,c)}
AFB ₁ + <i>n</i> -BuOH fraction	2.5	391 ± 2 ^{b,c)}

Statistical significance: a) $p < 0.01$, b) $p < 0.001$ vs. spontaneous value, c) $p < 0.001$ vs. AFB₁-nontreated value.

TABLE III. Effect of Ursolic Acid from the Leaf of *Eriobotrya japonica* on the Mutagenicity Induced by AFB₁ in *Salmonella typhimurium* TA100

Treatment	Concentration (%)	Revertants per plate
Spontaneous	—	119 ± 9
AFB ₁	—	1550 ± 126 ^{a)}
AFB ₁ + ursolic acid	0.25	559 ± 8 ^{a,b)}
AFB ₁ + ursolic acid	0.5	544 ± 2 ^{a,b)}
AFB ₁ + ursolic acid	1	472 ± 26 ^{a,b)}

Statistical significance: a) $p < 0.001$ vs. spontaneous value, b) $p < 0.001$ vs. AFB₁-nontreated value.

group, as shown in Table III.

Eriobotrya japonica is a persistent tall tree which grows naturally in Korea, Japan and China, and its leaves have been used as a Chinese crude drug for the treatment of inflammation, tumor, influenza, cough and asthma. In the present study, antimutagenic activity was found in the MeOH extract of the leaf of *Eriobotrya japonica*. This effect was 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,⁹⁾ respectively. The above observations suggest that the MeOH extract also exerts an influence on hereditary factors.

We also separated this MeOH extract into hexane, CHCl_3 , EtOAc and *n*-BuOH fractions, and examined their effects. Antimutagenic activity was also found in the EtOAc fraction, and ursolic acid was isolated from this fraction. These findings suggest that the antimutagenic activity of *Eriobotrya japonica* is due in part to ursolic

acid.

On the other hand, it has been reported that ursolic acid isolated from *Gleboma bederacea* inhibits not only Epstein-Barr virus early antigen activation *in vitro*, but also tumor promotion *in vivo*.^{11,12)} Lee *et al.*¹³⁾ have also reported that ursolic acid shows significant cytotoxicity against lymphocytic leukemia cells P-388 and L-1210, as well as the human lung carcinoma cell A-549. Taking these results together, we consider that ursolic acid has antitumorigenic activity.

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