SUPPLEMENTARY MATERIAL

Antimutagenic Components in *Glycyrrhiza* against *N*-Methyl-*N*-nitrosourea in the Ames Assay

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Antimutagenesis against *N*-nitroso compounds contributes to prevention of human cancer. We have found that *Glycyrrhiza aspera* ethanolic extract exhibits antimutagenic activity against *N*-methyl-*N*-nitrosourea (MNU) using the Ames assay with *Salmonella typhimurium* TA1535. In the present study, eight purified components from *Glycyrrhiza*, namely, glabridin, glycyrrhetic acid, glycyrrhizin, licochalcone A, licoricesaponin H2, licoricesaponin G2, liquiritigenin, and liquiritin, were evaluated for their antimutagenicity against MNU in the Ames assay with *S. typhimurium* TA1535. Glycyrrhetic acid, glycyrrhizin, licoricesaponin G2, licoricesaponin H2 and liquiritin did not show the antimutagenicity against MNU in *S. typhimurium* TA1535. Glabridin, licochalcone A and liquiritigenin reduced revertant colonies derived from MNU in *S. typhimurium* TA1535 without showing cytotoxic effects, indicating that these compounds possess antimutagenic activity against MNU. The inhibitory activity of glabridin and licochalcone A was more effective than that of liquiritigenin. *Glycyrrhiza* contains antimutagenic components against DNA alkylating, direct-acting carcinogens.

Supplemental file legends

Experimental

Table S1. Antimutagenic activity of glycyrrhetic acid against MNU in *S. typhimurium*

Table S2. Antimutagenic activity of glycyrrhizin against MNU in *S. typhimurium* TA1535.

Table S3. Antimutagenic activity of licoricesaponin H2 against MNU in *S. typhimurium* TA1535.

Table S4. Antimutagenic activity of licoricesaponin G2 against MNU in *S. typhimurium* TA1535.

Table S5. Antimutagenic activity of liquiritin against MNU in *S. typhimurium* TA1535.

Table S6. Antimutagenic activity of glabridin against MNU in *S. typhimurium* TA1535.

Table S7. Antimutagenic activity of licochalcone A against MNU in *S. typhimurium* TA1535.

Table S8. Antimutagenic activity of liquiritigenin against MNU in *S. typhimurium* TA1535.

References

Experimental

3.1. Materials

Glabridin, licochalcone A, licoricesaponin H2, licoricesaponin G2, liquiritigenin, and liquiritin were kindly provided by Tokiwa Phytochemical Co., Ltd. (Chiba, Japan). Glycyrrhizin was purchased from Wako (Osaka, Japan). β -Glycyrrhetic acid was prepared by hydrolysis of glycyrrhizin, as previously described (m.p. 294–296 °C) (Merck Index, 13 ed.).

Sodium ammonium hydrogen phosphate tetrahydrate and iron(II) chloride tetrahydrate were purchased from Merck (Darmstadt, Germany). Ammonium hexafluorophosphate was obtained from Kanto Chemical Co., Ltd. (Tokyo, Japan). Bacto agar and bacto nutrient broth were obtained from Becton Dickinson Microbiology Systems (Sparks, USA). The other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

3.2. Bacterial Mutation assay

The antimutagenic effect of each compound was assayed according to the Ames method with a plate-incorporation protocol (Maron & Ames. 1983; Mortelmans & Zeiger. 2000). Professor B. N. Ames (University of California, Berkeley, USA) kindly provided the *S. typhimurium* TA1535.

A solution of MNU (1.5 μ mol/50 μ L DMSO) was placed into the test tube, and then the following were added: 0.1 M sodium phosphate buffer (pH 7.4, 0.5 mL), a solution with each concentration of compound (50 μ L in DMSO), and a culture of the *S. typhimurium* TA1535 (0.1 mL). Then, the solution was thoroughly mixed. Afterwards, top agar (2 mL) was added, and the mixture was poured onto a minimal-glucose agar plate. The revertant colonies were counted after incubation at 37°C for 44 h. Each sample was assayed using duplicate plates and the results are expressed as the mean revertant colonies per plate. Plates with neither MNU nor plant extract were considered as negative controls. MNU (1.5 μ mol/50 μ L) gave 1581 ± 312 colonies, and the activity was similar with the previously reported (Bartsch et al. 1982). DMSO (50 μ L/plate) gave 11 ± 2 colonies. All tested plates were microscopically examined for thinning or the absence of the background lawn and/or presence of micro colonies, which are considered to be indicators of the toxicity induced by the test material. Neither MNU

nor the compounds displayed toxicity in the *S. typhimurium* TA1535 in the antimutagenicity test.

Mutagenic activity in the presence of compounds is expressed as the percentage of mutagenicity ($\% = R_s/R \times 100$), where R_s is the number of his⁺ revertants/plate in plates exposed to MNU and compounds and R is the numbers of his⁺ revertants/MNU plate. The number of spontaneous revertants was subtracted to give R_s and R . As a result, the mutagenicity of the MNU in the absence of compounds is defined as 100% of the MNU mutagenicity.

Toxicity assays, which were performed under the same conditions as those used for the Ames test, were used to determine the maximum concentrations of the compounds that could be added without exerting toxic effects on the bacteria used in the Ames test. A solution of MNU (1.5 $\mu\text{mol}/50 \mu\text{L}$ DMSO) was added to the test tube, and the following were added to the test tube: 0.1 M sodium phosphate buffer (pH 7.4, 0.5 mL), each compound concentration (50 μL), and a culture of *S. typhimurium* TA1535 (0.1 mL). A portion of the mixture was diluted 10^5 times in 1/15 M PB. A histidine-free top agar (2 mL) was added to 200 μL of the diluted solution, while the solution was poured on the NB agar plate. The colonies were counted after incubation at 37°C for 20 h. Each sample was assayed using duplicate plates. A substance was considered cytotoxic when the bacterial survival was less than 80% that observed in the negative control. The mutation frequency is established by calculating the number of mutants per 10^7 surviving bacterial cells.

Table S1. Antimutagenic activity of glycyrrhetic acid against MNU in *S. typhimurium* TA1535.

Concentration (μg/plate)	Revertants/plate±SE			Relative activity (%)
glycyrrhetic acid				
0	831	±	70	100.0
100	845	±	42	94.3
250	917	±	5	102.3
500	797	±	48	89.0
1000	861	±	54	96.1
DMSO	7	±	2	

Table S2. Antimutagenic activity of glycyrrhizin against MNU in *S. typhimurium* TA1535.

Concentration (µg/plate)	Revertants/plate±SE			Relative activity (%)
glycyrrhizin				
0	1280	±	51	100.0
100	1100	±	1	85.8
250	1322	±	63	103.3
500	1274	±	90	99.5
1000	1137	±	43	96.1
DMSO	12	±	1	

Table S3. Antimutagenic activity of licoricesaponin H2 against MNU in *S. typhimurium* TA1535.

Concentration (µg/plate)	Revertants/plate ± SE	Relative activity (%)
licoricesaponin H2		
0	881 ± 118	100.0
50	950 ± 62	107.9
100	975 ± 36	110.8
250	1016 ± 41	115.5
500	1009 ± 54	114.6
1000	949 ± 10	96.1
DMSO	6 ± 1	

Table S4. Antimutagenic activity of licoricesaponin G2 against MNU in *S. typhimurium* TA1535.

Concentration (µg/plate)	Revertants/plate ± SE	Relative activity (%)
licoricesaponin G2		
0	1113 ± 163	100.0
50	1046 ± 190	93.9
100	1392 ± 95	125.2
250	1067 ± 26	95.8
500	966 ± 49	86.7
DMSO	6 ± 0	

Table S5. Antimutagenic activity of liquiritin against MNU in *S. typhimurium* TA1535.

Concentration (μg/plate)	Revertants/plate±SE			Relative activity (%)
liquiritin				
0	953	±	202	100.0
50	860	±	139	90.1
100	765	±	25	80.0
250	841	±	68	88.1
500	879	±	39	92.1
DMSO	12	±	3	

Table S6. Antimutagenic activity of glabridin against MNU in *S. typhimurium* TA1535.

Concentration (μg/plate)	Revertants/plate ±SE			Survival (%)		MF (%)	
				Colonies ±SE	Survival rate (%)		
glabridin							
0	1891	±	90	419	± 14	100.0	100.0
5	1930	±	100	428	± 14	102.1	99.9
10	1796	±	25	424	± 17	101.1	93.9
25	1322	±	199	407	± 1	97.1	71.7
30	1270	±	312	374	± 1	89.3	74.9
37.5	992	±	260	420	± 16	100.2	51.9
40	828	±	114	408	± 9	97.4	44.5
50	389	±	195	402	± 11	95.9	20.7
75	276	±	144	400	± 17	95.5	14.5
100	96	±	78	302	± 83	72.1	5.9
DMSO	16	±	4	410	± 19	97.9	

Table S7. Antimutagenic activity of licochalcone A against MNU in *S. typhimurium* TA1535.

Concentration (μg/plate)	Revertants/plate ±SE			Survival (%)			MF (%)
				Colonies ±SE	Survival rate (%)		
licochalcone A							
0	3282	±	76	545	± 4	100.0	100.0
1	3128	±	4	517	± 35	94.8	100.6
5	3228	±	34	591	± 31	108.4	90.7
10	2605	±	129	573	± 23	105.1	75.4
25	1907	±	250	567	± 60	104.0	55.7
50	1555	±	160	536	± 27	98.3	48.0
75	1231	±	127	535	± 19	98.2	38.0
100	991	±	177	526	± 8	96.5	31.0
DMSO	12	±	5	514	± 2	94.2	

Table S8. Antimutagenic activity of liquiritigenin against MNU in *S. typhimurium* TA1535.

Concentration (μg/plate)	Revertants/plate ±SE			Survival (%)		MF (%)	
				Colonies ±SE	Survival rate (%)		
liquiritigenin							
0	2420	±	188	411	± 1	100.0	100.0
50	2231	±	114	395	± 23	96.1	95.8
100	1832	±	251	404	± 2	98.3	76.8
250	1759	±	54	412	± 23	100.4	72.2
500	1392	±	42	399	± 12	97.1	58.9
1000	713	±	110	381	± 10	97.1	58.9
DMSO	18	±	1	404	± 14	98.3	

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