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Antimutagenic Compounds Identified from Perilla Leaf

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Abstract

The methanol extract of perilla leaves contained compounds to reduce the mutagenicity of aflatoxin B_1 (AFB_1) in *Salmonella typhimurium* TA98 and 100. They were separated by solvent extractions and identified by GC and GC-MS as 2-ethoxy acetate, 1,2,3,4-tetramethyl-cis-cyclobutene, two isomers of methyl 11,14,17-eicosatrienoate, 12-acetyl-9-octadecanoic acid, and phytol. The antimutagenicities of phytol and methyl 11,14,17-eicosatrienoate were dependent on the mutagens tested. Phytol reduced the mutagenicity of Trp-p-2 but not of AFB_1 and methyl 11,14,17-eicosatrienoate reduced the activities both of the mutagens.

Key words : antimutagenic, perilla leaves, identification, methyl 11,14,17-eicosatrienoate, phytol

INTRODUCTION

Perilla (*Perilla frutescens Britton, Perilla ocymoides L.*) has been cultivated for the production of its seed oil used in food additives and medicinal ingredients as well. Recently, fresh perilla leaves are consumed with grilled red meats etc. in Korea, and its consumption has been greatly increased by increasing red meat consumption. Perilla leaves are known to be good nutritional sources due to high contents of Ca, K, lysine, and linolenic acid[1~4]. Sixteen flavonoids including the glucosides of cyanin (shisonin), luteolin, and apigenin have been identified from perilla leaves[5]. They also contain caffeylmalonylcyanin and malonyl-cis-shisonin used as food colorants in Japan[6~8]. 1-Perillaldehyde and l-limonene are known to be important volatile compounds in the essential oil of perilla leaves[10~12]. It has been reported that rosmarinic acid found in raw perilla leaves demonstrates anti-inflammatory activity and antioxidant effects as well[13].

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Perilla leaves are used as folk medicine to cure and to prevent cancer in Korea. However, it has not been reported any scientific evidence or data for the cancer remedy effect of perilla leaves. Among thirty different yellow-green vegetables, methanol extract of perilla leaves showed the highest antimutagenic activity against AFB_1 with 61% inhibition in *Salmonella typhimurium* TA98[14]. It was necessary to isolate and identify the compounds inhibiting mutagenicity of AFB_1 in *Salmonella typhimurium* TA98 and 100.

To identify antimutagenic compounds the methanol extract was fractionated into hexane, chloroform, and aqueous fractions which were further purified by column chromatography packed with silica gel and thin layer chromatography. The compounds isolated from the most active fraction of the solvent extraction and silica gel column eluents were tentatively identified by GC-MS. Some authentics of major compounds among those identified from active fractions were tested for the antimutagenicity. We report tentatively identified antimutagenic compounds from perilla leaves in this paper.

MATERIALS AND METHODS

Antimutagenicity test

Antimutagenicity test was carried out by using Ames assay system[15]. *Salmonella typhimurium* strains TA98 and TA100 histidine requiring mutants were used as bacterial strains. They were maintained as described by Maron and Ames[15]. Antimutagenic activities were tested using aflatoxin B_1 (AFB_1) and 3-amino-1-methyl-5H-pyrido [4,3-b] indole (Trp-p-2). AFB_1 was purchased from Sigma Chemical Co. (St. Louis, Mo. USA), and Trp-p-2 was obtained from Wako Pure Chemical Ind. Co. Ltd (Tokyo, Japan). Aflatoxin B_1 was dissolved in DMSO and Trp-p-2 was dissolved in methanol before they were used.

S9 mix to activate the mutagens was also prepared by the method of Maron and Ames[15]. Aroclor 1254 in corn oil (200mg/1ml) was injected to male Sprague-Dawley (body weight about 200g) rats five days before killed to induce the liver enzyme. Five days later, liver was homogenized with 0.15M KCl (3ml/g liver) using homogenizer (Potter-Elvehiem apparatus, USA) and then the homogenate was centrifuged at 9000g for 10 min (4° C). Supernatant (S9 fraction) was kept frozen at -80° C until use. The S9 fraction (10%) was mixed with $MgCl_2$-KCl salts (2%), 1M glucose-6-phosphate (0.5%), 1M NADP (4%), 0.2M phosphate buffer (pH 7.4), and sterillized water prior to be used for Ames assay.

Mutagenicity test was carried out by a modified plate incorporation test (liquid preincubation of the organisms with the test compound)[14,15]. A 0.5ml of S9 mix was distributed in sterile capped-tubes in an ice bath and then 0.1ml of testers from an overnight culture ($1 \sim 2 \times 10^9$ cells/ml) and 0.1ml of test compounds were added. The tubes were gently vortexed and preincubated at 37° C for 30min. 2ml of the top agar kept at 45° C were added to each tube and vortexed for 3 seconds. The entire mixture was overlaid on the minimal agar plate. The plates were incubated at 37° C for 48hrs and then the revertant bacterial colonies on each plate were counted.

Fractionation and isolation of antimutagenic compounds

Preparation of methanol extract

Perilla leaves (1Kg) were freeze dried and powdered. The powder was extracted with glass distilled methanol (10L) by shaking for 12hrs according to the method of Takahashi et al.[17]. After decanting the supernant of the methanol extracts, an additional 10L of methanol was added to the residue and shaken for 12 hrs, followed by separating the supernatant again. The extraction step of the residue was repeated one more time. The combined methanol extract was concentrated to about 300 ml under a vacuum rotary evaporator (Hedolph Co. model W 2000) at 60° C.

Fractionation by solvent extraction

Two different solvent extraction systems were used to isolate antimutagenic compounds. One procedure for the fractionation of methanol extract includes the following steps. Three hundreds ml of the mixture of hexane/methanol/H_2O (10 : 1 : 9) were added to the concentrated methanol extract, and then shaken. After the hexane layer was removed, the aqueous layer was fractionated into the chloroform layer and aqueous layers by 150ml of chloroform. After the chloroform extraction was separated, the residual aqueous layer was further fractionated into ethyl acetate and aqueous layers. The last aqueous layer was fractionated into butanol and aqueous layers. A total of five fractions were obtained from the methanol extract. Since antimutagenic activity was distributed to the chloroform, ethyl acetate, and butanol fractions, we attempted to collect active compounds into one fraction. Therefore, we extracted the three combined organic layers with chloroform/H_2O (1 : 1) and the chloroform extract was used for silica gel column chromatography.

Silica gel column chromatography

The chloroform fraction was mixed with silica gel (10g) and then placed on the column (100cm × 5cm

i.d.) packed with silica gel (245g), followed by elution with two solvent systems of chloroform/methanol (10:1, v/v) and chloroform/methanol (1:10, v/v).

Compound identification by GC-MS

GC-MS analysis of antimutagenic fractions were carried out with a HP 5970 Mass spectrometer connected with HP 5890 Gas chromatograph using a bonded polyethylene glycol fused silica capillary column (HP-5 fused silica WCOT capillary column). The mass spectra were recorded at an electron energy of 70eV and the ion source temperature was 280°C. The column was operated with a temperature program from 100°C to 280°C at 4°C/min, and then held for 15 min at 280°C. Helium was used as a carrier gas (1ml/min, split ratio 1/25). Each peak was identified based on Chamstation mass spectral data base (HP 91153C, NBS-REVEL) and/or mass spectrum of authentic compounds.

RESULTS AND DISCUSSION

About 20g of methanol extract was obtained from 100g of freeze dried perilla leaves. Each 2.5%, 5%, 10% of methanol extract was tested for the effect on the mutagenicity of aflatoxin B1 (AFB_1, 1 μg/plate). Methanol extract inhibited mutagenicity of AFB_1 in *S. typhimurium* TA98 and 100 in the fashion of dose responses (Table 1). The inhibition ratios were 38%, 54%, and 65% for *S. typhimurium* TA98, with treatment of the methanol extract 2.5%, 5%, and 10%, respectively. Therefore, the methanol extract was further fractionated into hexane, chloroform, ethyl acetate and butanol, and then each fraction was concentrated in order to examine their antimutagenicity. The yields of concentrated fractions from methanol extract was 26.3% (hexane), 3.4% (chloroform), 8.2% (ethyl acetate), 7.3% (butanol), and 54.8% (aqueous phase). Antimutagenicity was tested at 2.5% and 5.0% of concentrates of each fraction. The antimutagenic activity against AFB_1 was almost equally distributed to the chloroform, ethyl acetate, and butanol fractions.

Table 1. Effect of methanol extracts of perilla leaf on the mutagenicity of aflatoxin B1 (AFB_1, 1μg/plate) in *Salmonella typhimurium* TA 98 and TA100

	Revertants/plate	
	TA98	TA100
Spontaneous	28 ± 4	121 ± 1
AFB_1	1051 ±36	824 ±86
AFB_1 + MeOH extract		
2.5%	666 ±57(38)*	654 ±82(24)
5.0%	503 ±66(54)	559 ±45(38)
10.0%	384 ±30(65)	463 ± 7(51)

*The values in parentheses are the inhibition rate (%)

The methanol extract was prepared with chloroform to collect these active compounds and tested for the mutagenic activity against AFB_1. As shown in Fig. 1, most activity was in the chloroform fraction and the fraction was further fractionated by silica gel column chromatography. The fraction number 14 from the silica gel column chromatography eluted with chloroform/methanol (10:1, v/v) showed the strongest antimutagenicity against AFB_1 in *Salmonella typhimurium* TA98 and TA100 (Fig. 2).

Compounds in the fraction were separated and identified by GC-MS and their total ion chromatogram (TIC) is shown in Fig. 3. Six compounds

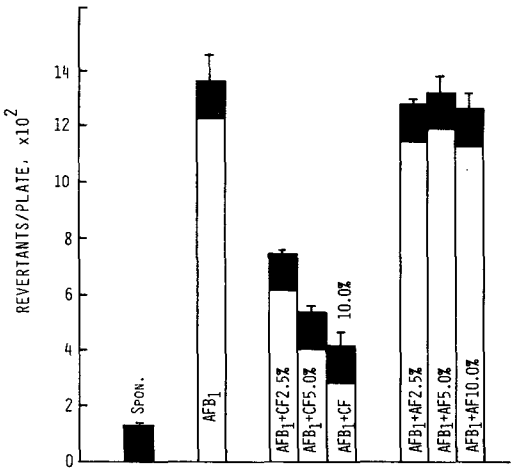

Fig. 1. Effect of chloroform fraction (CF) and aqueous fraction (AF) from methanol extract of perilla leaf on the mutagenicity induced by aflatoxin B1 (AFB_1, 1 μg/plate) in *Salmonella typhimurium* TA100.

of 2-ethoxy acetate, 1,2,3,4-tetramethyl-cis-cyclobute- ne, methyl 11,14,17-eicosatrienoate, 12-acetyl-9-octadecanoic acid, and phytol were identified from fraction number 14 (Table 2). Among three major authentic compounds, two isomers of methyl 11,14,17-eicosatrienoate and phytol were obtained from commercial sources (Aldrich Chemical Co., Milwaukee WI, USA). Phytol and methyl 11,14,17-eicosatrienoate were tested for antimutagenic activity against AFB1 in *Salmonella typhimurium* TA100 and 98. Antimutagenic activity of phytol

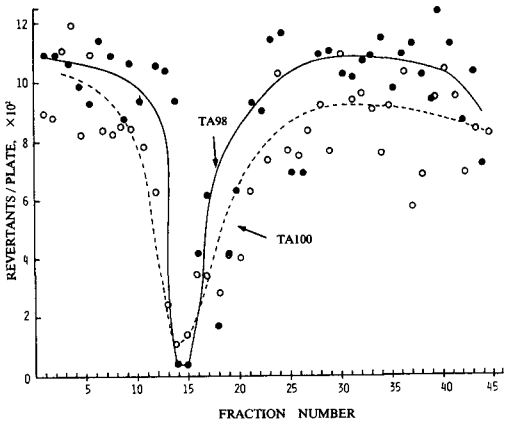

Fig. 2. Revertant numbers obtained from various fractions from chloroform fraction of methanol extracts of perilla leaf on the mutagenicity induced by aflatoxin B1 (1μg/plate) in *Salmonella typhimurium* TA98 and TA100.

*The spontaneous number and the revertants from aflatoxin B1 in TA98 and TA100 were 34±4 ; 1143 ±55 and 109±10 ; 822±116, respectively.

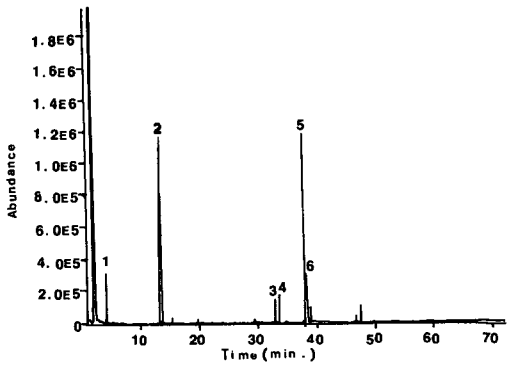

Fig. 3. Total ion chromatogram (TIC) of GC-MS of antimutagenic fraction number 14 obtained from silica gel column chromatography.

was dependent on the mutagens used. 5% of phytol showed antimutagenic activity against Trp-p-2, but not against AFB1 (Table 3). The methyl 11,14,17-

Table 2. Compounds identified from the antimutagenic fraction number 14 of silica gel column chromatography of chloroform extracts of perilla leaf by GC-MS

Peak[1] no.	Compounds	Retention time (min.)
1	2-Ethoxyethyl acetate	4.325
2	Cyclobutene, 1,2,3,4-tetramethyl-, cis,	13.770
3	Methyl 11,14,17-eicosatrienoate	32.643
4	9-Octadecanoic acid, 12-acetyl,	33.544
5	Phytol	36.270
6	Methyl 11,14,17-eicosatrienoate	37.561

[1]Shown in Fig. 3

Table 3. Inhibitory effect of phytol on the mutagenicity of Trp-P-2(0.02μg/plate) and aflatoxin B1 (AFB1, 1μg/plate) in *Salmonella typhimurium* strains of TA98 and TA100, respectively

	Revertants/plate	
	Trp-P-2 (TA98)	AFB1 (TA100)
Spontaneous	33 ± 2	141 ± 11
Control	1402 ± 50	1233 ± 12
1.0%	1206 ± 6	1260 ±157
2.5%	1118 ±129	1246 ± 16
5.0%	650 ± 54	1118 ± 59

Table 4. Inhibitory effect of methyl 11,14,17-eicosatrienoate on the mutagenicity of Trp-P-2 (0.02μg/plate) and aflatoxin B1 (AFB1, 1μg/plate) in *Salmonella typhimurium* strains of TA98 and TA100, respectively

Concentraion	Revertants/plates	
	Trp-p-2 (TA98)	AFB1 (TA100)
Spontaneous	38 ±1	161 ±10
Control	2069 ±9	1969 ±86
0.5%*	698 ±141	588 ±53
1.0%	61 ±13	228 ±37
2.5%	–	206 ±35

eicosatrienoate (0.5% and 1%) showed strong antimutagenicity against Tryp-p-2 and AFB_1 in TA98 and TA100 (Table 4).

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들깻잎에서 동정된 항돌연변이 물질

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요 약

들깻잎의 메탄올 추출물이 *S. typhimurium* TA98과 100에서 aflatoxin B_1 (AFB_1)의 돌연변이 유발성을 억제시키는 효과가 있었다. 메탄올 추출물을 silica gel column을 사용하여 더욱 분획하여 항돌연변이 효과를 시험하였으며, 그중 효과가 가장 컸던 fraction 중의 화합물을 분리, 동정한 결과 2-ethoxy acetate, 1,2,3,4-tetramethyl-cis-cyclobutene, 두 개의 methyl 11,14,17-eicosatrienoate 이성체, 12-acetyl-9-octadecanoic acid, 그리고 phytol이 있음이 잠정적으로 밝혀졌다. Phytol과 methyl 11,14,17-eicosatrienoate의 표준품을 사용하여 AFB_1 및 Trp-P-2에 대한 항돌연변이 효과를 시험한 결과 그 효과가 컸음이 밝혀졌다.