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Antimutagenic Compounds Identified from Chloroform Fraction of Persimmon Leaves

Suk-Hee Moon†, Jeong-Ok Kim* and Kun-Young Park**

Dept. of Food and Nutrition, Kyungnam Junior College, Pusan 617-012, Korea
**Dept. of Chemistry, Pusan Women's University, Pusan 607-737, Korea*
***Dept. of Food Science and Nutrition, Pusan National University, Pusan 609-735, Korea*

Abstract

Methanol extract of dried persimmon leaves was refractionated using hexane, chloroform, ethylacetate, *n*-butanol and aqueous fractions. Among these, chloroform fraction showed the highest inhibition rate on the mutagenicities of aflatoxin B_1(AFB_1) and 3,2'-dimethyl-4-amino-biphenyl(DMAB) in *Salmonella typhimurium* TA100. Chloroform fraction was further fractionated into eight fractions by silica gel column chromatography and thin layer chromatography(TLC). The fraction 5 on TLC exhibited the highest antimutagenic activities on AFB_1 and DMAB. 2,4-Decadienal, dihydro-4-methyl-2(3H)-furanone, hexanoic acid, 1,4-bis(1-methyl ethyl)benzene, heptanoic acid, phenol, octanoic acid, nonanoic acid and benzoic acid were tentatively identified from this antimutagenic fraction by GC-MS.

Key words: persimmon leaves, antimutagenic compounds

INTRODUCTION

Green tea or black tea is one of the most widely consumed beverage. A large amount of research on the antimutagenic, anticarcinogenic and pharmacological activity of the tea were carried out by many scientist(1,2). But green tea is more expensive than other teas and the quality of domestic black tea is not so good in Korea. Therefore, it is necessary that another good tea is developed. The persimmon tree(*Diospyros kaki* Thunberg) is grown all over Korea, China and Japan(3). Persimmon leaves have been traditionally used for the treatment of hypertensive disease in Japan(4). The fruit of a persimmon have been used for treatment of apoplexy, hematemesis, chilblain and burns(4). Persimmon leaves are known to be good nutritional tea due to high content of vitamin A, C, D and chlorophyll(5). Also, above nutrients are known to have antimutagenic and anticarcinogenic effects.

By screening 49 kinds of medicinal plants having cancer remedy effect, methanol extract of persimmon leaves showed strong antimutagenic activities against aflatoxin B_1(AFB_1), 3,2'-dimethyl-4-amino-biphenyl (DMAB), N-methyl-N'-nitro-N-nitrosoguanidine(MNNG), and 4-nitroquinoline-1-oxide(4-NQO) in *Salmonella typhimurium* TA100(6). To identify antimutagenic compounds, the methanol extract was fractionated into hexane, chloroform, ethylacetate, *n*-butanol and aqueous fractions which were further purified by column chromatography packed with silica gel and thin layer chromatography(TLC). The compounds isolated from the most active fraction of the TLC were tentatively identified by GC-MS. Among those identified from active fractions, some authentics were tested for the antimutagenicity.

MATERIALS AND METHODS

Antimutagenicity test

Salmonella typhimurium strain TA100, histidine requiring mutant, was provided by Dr. B. N. Ames, University of California(Berkley, CA, USA) and were maintained as described by Maron and Ames(7). The genotypes of tester strain was checked routinely for their histidine requirements, deep rough(*rfa*) character, UV sensitivity(*uvr* B mutation) and for the presence of R factor.

Aflatoxin B_1(AFB_1) and 3,2'-dimethyl-4-amino-biphenyl(DMAB) from Sigma Chemical Co., Milwaukee,

† Corresponding author

aWI, USA were dissolved in spectrophotometric grade dimethylsulfoxide(DMSO).

Preincubation test was employed(7,8) to determine the antimutagenic effect of persimmon leaves. S9 mix (0.5ml) prepared by the method of Maron and Ames(7) was distributed to sterile capped tubes kept in an ice bath and then 0.1ml of testers from overnight culture(1 ~2×10^9cells/ml) and 0.1ml of test compounds respectively were added. The tubes were gently vortexed and preincubated at 37°C for 30min. 2ml of the top agar in each tube kept at 45°C were added and vortexed for 3 seconds. The resulting entire mixture was over-laid 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(7).

Toxicity tests for the samples from the persimmon leaves were also carried out and the samples employed for the antimutagenic test in this study did not show any toxicity to the tester strain.

Fractionation and isolation of antimutagenic compounds

Solvent extraction and fractionation

Persimmon leaves were harvested at Top-lee Hoegae-myeon Hadong-gun Kyeongnam in June 1992. Dried and then powdered leaves(1kg) were extracted with glass distilled methanol(10L) by shaking for 12hrs according to the method of Takahashi et al.(9). After decanting the supernatant of the methanol extract, additional 10L glass-distilled methanol was added to the persimmon leaves residue and shaken for 12hrs, followed by separating the supernatant again. The combined methanol extracts(20L) were concentrated to 200 ml under a vacuum rotary evaporator(Hedolph Co. model W 2000) at 60°C. The concentrated extract was further fractionated into hexane and aqueous soluble fractions using 800ml of hexane : methanol : H_2O(10 : 1 : 9). Aqueous layer was extracted with 400ml of chloroform fractioning into chloroform and aqueous fractions. Further fractionation of the aqueous phase with ethylacetate resulted ethylacetate and aqueous fractions that fractionated into *n*-butanol and aqueous phase.

Silica gel column chromatography

The chloroform fraction was further fractionated using silica gel column chromatography. The sample was mixed with silica gel(10g) and then placed on the column(10cm×5cm, i.d.) packed with silica gel(245g). The sample was eluted with chloroform-methanol(9 : 1, v/v) and each 50ml of eluent was collected in each test tube. After testing antimutagenicity of eluent in each test tube, eluents that had high antimutagenicity were combined, and then refractionated on silica gel column II(100cm ×2cm, i.d.).

Thin layer chromatography

The fractions collected from silica gel column chromatography(column II) showing antimutagenic activity were further fractionated on precoated TLC silica gel plates(Kiesel gel 60 F_{254} plate, Art No. 5735, Merck). The plates were developed with chloroform-methanol (9 : 1, v/v).

Separation and identification of compounds in antimutagenic fraction 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, 25m×0.2mm, 0.33μm thickness). 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 60°C to 210°C at 2°C/min, and then held for 15min. at 210°C. Helium was used as a carrier gas(1ml/min. split ration 1/25). Each peak was identified based on Chamstation mass spectral data base(HP 91153C, NBS-REVFL) and/or mass spectrum of authentic compounds.

RESULTS AND DISCUSSION

In previous study, methanol extract of persimmon leaves exhibited strong antimutagenic effects against AFB_1, DMAB, MNNG and 4-NQO in *Salmonella typhimurium* TA100 in a dose dependent fashion(6). So, the methanol extract was further fractionated into hexane, chloroform, ethylacetate, *n*-butanol and aqueous fractions. Among these fractions, the chloroform fraction showed strong antimutagenic activity against more indirect mutagens(AFB_1, DMAB) than direct mutagens (MNNG, 4-NQO)(Table 1 and 2). The inhibition ratios were 89% for AFB_1, 85% for DMAB, 33% for MNNG and 37% for 4-NQO with treatment of the 5% chloroform extract(Table 1 and 2). This indicated that antimutagenic activity of the chloroform fraction against indirect mutagens could be related to the inhibition of

Table 1. Effects of solvent fractions from the methanol extract of persimmon leaves on the inhibition of aflatoxin B_1(AFB_1, 1μg/plate) and 3,2′-dimethyl-4-amino-biphenyl(DMAB, 10μg/plate) mutagenicities in *Salmonella typhimurium* TA100

Treatment	Concentration (%)	AFB_1	DMAB
Control		1087±133	992± 3
Spontaneous		199± 16	195± 8
Hexane fr.	1.25	821± 83(30)[1]	901± 28(11)
	2.50	631± 36(51)	812±107(23)
	5.00	532± 69(62)	699± 52(37)
Chloroform fr.	1.25	688± 5(45)	847± 37(18)
	2.50	427± 25(74)	663± 14(41)
	5.00	294± 10(89)	312± 56(85)
Ethylacetate fr.	1.25	628± 78(52)	770± 90(28)
	2.50	456± 13(71)	536± 42(57)
	5.00	322± 1(86)	337± 33(82)
Butanol fr.	1.25	1004±209(9)	935± 32(7)
	2.50	883±137(23)	914± 84(10)
	5.00	583± 2(57)	873± 35(15)
Aqueous fr.	1.25	1032± 62(6)	946± 28(6)
	2.50	1049±100(4)	975± 24(2)
	5.00	1176±110 -	1038± 22 -

[1]The values in parentheses are the inhibition rate(%)

Table 2. Effects of solvent fractions from the methanol extract of persimmon leaves on the inhibition of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG, 0.5μg/plate) and 4-nitroquinoline-1-oxide(4-NQO, 0.25μg/plate) mutagenicities in *Salmonella typhimurium* TA100

Treatment	Concentration (%)	MNNG	4-NQO
Control		1096± 6	1251± 4
Spontaneous		135± 1	83± 15
Hexane fr.	1.25	870± 80(24)[1]	900± 8(30)
	2.50	781± 70(33)	837± 11(35)
	5.00	603± 57(51)	684± 1(49)
Chloroform fr.	1.25	1070± 74(3)	929± 68(28)
	2.50	977± 73(12)	908± 7(29)
	5.00	781± 33(33)	813± 7(37)
Ethylacetate fr.	1.25	776± 3(33)	975± 30(24)
	2.50	666± 0(45)	951± 45(26)
	5.00	621± 21(49)	910± 5(29)
Butanol fr.	1.25	884± 60(22)	1010±121(21)
	2.50	742± 18(37)	1007±115(21)
	5.00	639± 62(48)	952± 45(26)
Aqueous fr.	1.25	737± 81(37)	1074± 36(15)
	2.50	664± 56(45)	1000± 5(21)
	5.00	616± 8(50)	1020± 69(20)

[1]The values in parentheses are the inhibition rate(%)

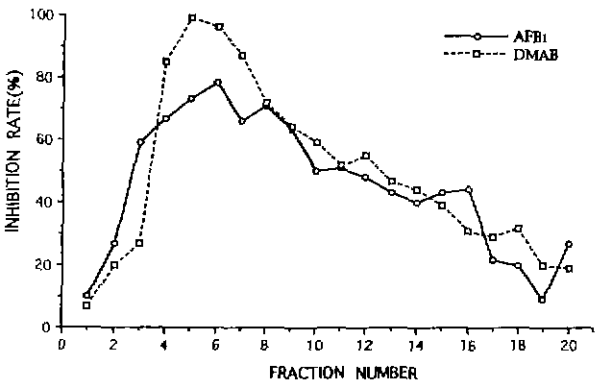

Fig. 1. Effects of the fractions obtained by using column I(100cm×5cm, i.d.) from chloroform fraction of methanol extract of persimmon leaves on the inhibition rate of the mutagenicities induced by aflatoxin B_1(AFB_1, 1μg/plate) and 3,2′-dimethyl-4-amino-biphenyl(DMAB, 10μg/plate) in *Salmonella typhimurium* TA100.

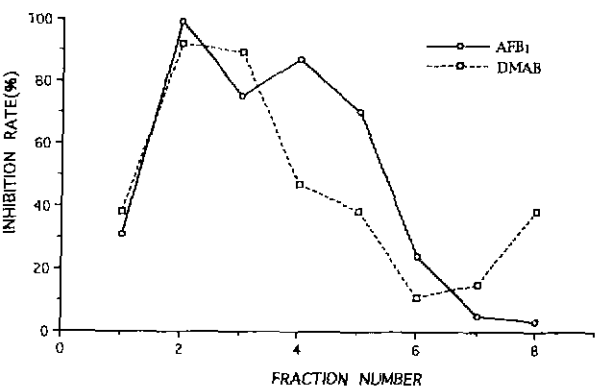

Fig. 2. Effects of the fractions obtained by using column II(100cm×2cm, i.d.) from fraction number 5~8 of column I(Fig. 1) on the inhibition rate of the mutagenicities induced by aflatoxin B_1(AFB_1, 1μg/plate) and 3,2′-dimethyl-4-amino-biphenyl (DMAB, 10μg/plate) in *Salmonella typhimurium* TA100.

some enzymes involved the conversion of AFB_1 and DMAB to the ultimate carcinogens. Further separation of the chloroform fraction was carried out by silica gel column chromatography using two different size of column(column I : 100cm×5cm, column II : 100cm ×2cm). Fraction number 5 and 6 from the silica gel column I eluted with chloroform-methanol(9 : 1, v/v) showed the strong antimutagenicities against AFB_1 and DMAB(Fig. 1). These antimutagenic fractions(5 and 6) collected from column I were combined, concentrated and then refractionated on silica gel column II. Fraction number 2 inhibited mutagenicities of AFB_1 and DMAB with up to 90~100% inhibition ratio with

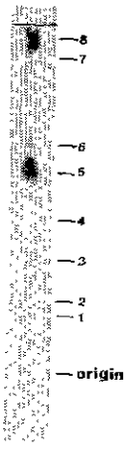

Fig. 3. Thin layer chromatographic fractionation of active fractions obtained by using column II (100cm×2cm, i.d.) from hexane fr.(Solvent system; Chloroform: Methanol=9: 1, v/v) of persimmon leaves.

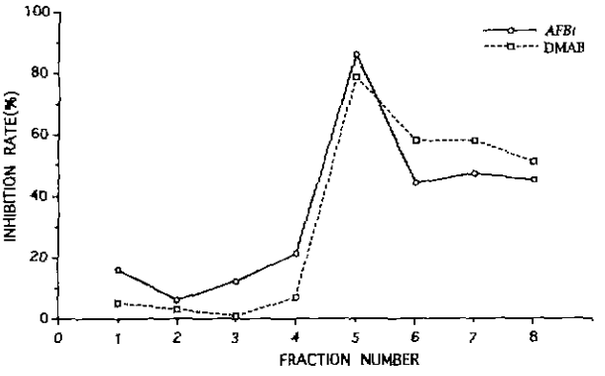

Fig. 4. Effects of the fractions obtained by using TLC from fraction number 2 of column II(Fig. 2) on the inhibition rate of the mutagenicities induced by aflatoxin B_1(AFB_1, 1μg/plate) and 3,2'-dimethyl-4-amino-biphenyl(DMAB, 10μg/plate) in *Salmonella typhimurium* TA100.

treatment of concentrated eluents(1μg/plate for AFB_1, 10μg/plate for DMAB) (Fig. 2). Antimutagenic fractions (No. 2) from column II were further separated on silica gel TLC plate and 8 bands were observed(Fig. 3). Antimutagenic activities of these 8 bands separated on TLC are shown in Fig. 4. TLC fraction 5 among these 8 bands showed the highest inhibition rate on the mutagenicities of AFB_1 and DMAB(Fig. 4). TLC fraction 5 was eluted with chloroform-methanol(9 : 1, v/v) and then subjected to the GC-MS analysis. Peaks were separated on HP-5 capillary column as shown in Fig. 5 and they were identified as 2,4-decadienal(peak No. 1), dihydro-4-methyl-2(3H)-furanone(peak No. 2), hexanoic acid(peak No. 3), 1,4-bis(1-methyl ethyl)benzene (peak No. 4), heptanoic acid(peak No. 5), phenol(peak No. 6), octanoic acid(peak No. 7), nonanoic acid(peak No. 8) and benzoic acid(peak No. 9)(Table 3).

Among 9 compounds identified from the chloroform fraction of persimmon leaves, 2,4-decadienal and benzoic acid were tested for the antimutagenic activities against AFB_1 and MNNG using their authentic compounds. The results are shown in Table 4. Benzoic acid reduced mutagenic activity of AFB_1 in *Salmonella typhimurium*

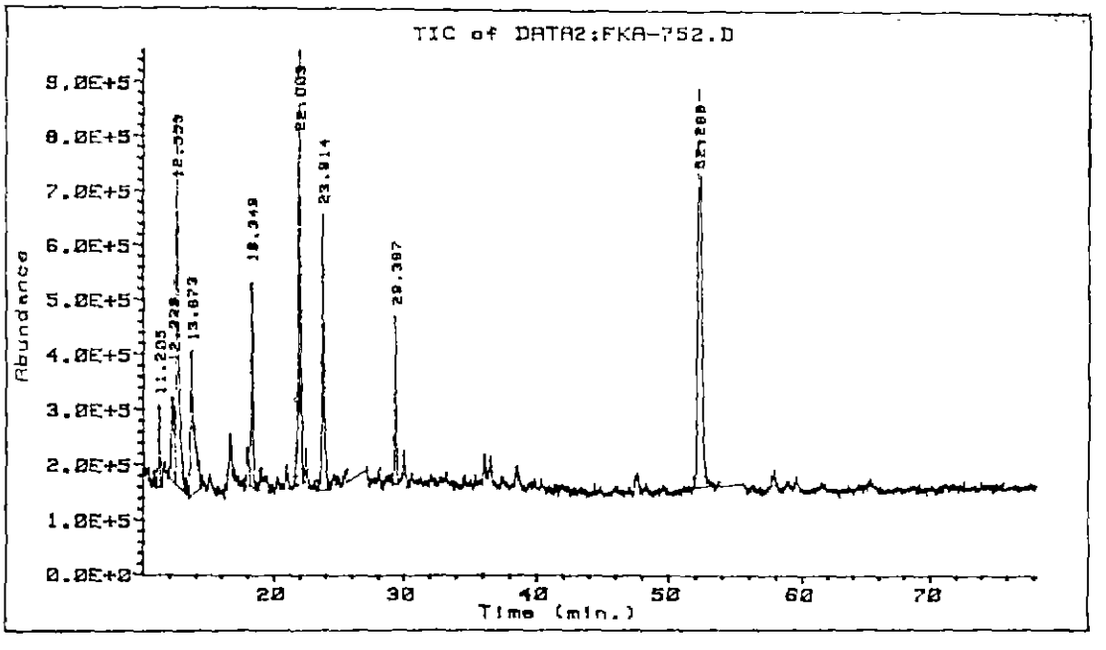

Fig. 5. Gas chromatogram of TLC fraction 5 that fractionated from the persimmon leaves.

Table 3. Compounds identified from the antimutagenic fraction number 5 in thin layer chromatography from the chloroform fraction of persimmon leaves

Peak No.	Compounds	Retention time(min.)*
1	2,4-Decadienal	11.205
2	Dihydro-4-methyl-2(3H)-furanone	12.228
3	Hexanoic acid	12.553
4	1,4-Bis(1-methyl ethyl)benzene	13.673
5	Heptanoic acid	18.349
6	Phenol	22.003
7	Octanoic acid	23.814
8	Nonanoic acid	29.367
9	Benzoic acid	52.288

*Retention time is in accordence on Fig. 5

Table 4. Effects of benzoic acid and 2,4-decadienal identified from persimmon leaves on the inhibition of aflatoxin B_1(AFB_1, 1μg/plate) and N-methyl-N'-nitro-N-nitrosoguanidine(MNNG, 0.5μg/plate) mutagenicities in *Salmonella typhimurium* TA100

Treatment	Concentration (%)	AFB_1	MNNG
Control		787± 18	1032± 71
Spontaneous		132± 7	169± 20
Benzoic acid	0.5	796± 17	1078± 11 -
	1.0	748± 66(.6)*	1058± 14 -
	2.5	276± 30(78)	740± 34(34)
	5.0	198± 24(90)	559± 13(55)
2,4-decadienal	0.010	741± 34(7)	911± 35(14)
	0.050	568± 41(33)	299± 14(85)
	0.075	493± 15(45)	229± 1(93)
	0.100	335± 16(69)	113± 10(106)

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

TA100 in a dose dependent fashion. The inhibition ratio was 90% for AFB_1 with treatment of the 5% benzoic acid. But 2,4-decadienal showed stronger antimutagenic activity against MNNG than that against AFB_1.

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