J. Korean Soc. Food Nutr.
22(3), 307 ~ 312(1993)

한국영양식량학회지

Antimutagenic Effects and Compounds Identified from Hexane Fraction of Persimmon Leaves

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Abstract

Methanol extract of dried persimmon leaves was fractionated to hexane, chloroform, ethyl acetate, butanol, and aqueous fractions. Hexane, butanol, and aqueous fractions had high yields of extracts. Hexane fraction among these fractions showed the highest inhibition rate on the mutagenicities of aflatoxin (AFB_1), dimethyl-amino-biphenyl (DMAB), N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), and 4-nitroquinoline-1-oxide (4-NQO) in *Salmonella typhimurium* TA100. Hexane 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 activity on AFB_1, DMAB, and MNNG. 1′-oxocannabinol, 3B-acetoxy-17-methyl-5a-18(13-17) abeoardrost-13-ene, 4-methoxy-2′6′-dinitro-3,5-di-*t*-butylbiphenyl, 8, 9-dihydro-5, 6-dimethoxy-dibenz[c, h]isoquino[2, 1, 8-1ma]carbazole-11,16-dione were tentatively identified from this antimutagenic fraction by GC-MS.

Key words : persimmon leaves, hexane fraction, antimutagenic compounds

INTRODUCTION

The persimmon tree (*Diospyros kaki* Thunberg) is grown all over Korea, China, and Japan, and its fruit is one of the major fruits consumed in Korea[1]. Its leaves have been used as a herb medicine to cure coagulation, asthma, angiotensin, hypertension, and showed anticarcinogenic activity[2] ; its usage is expanded for the preparation of herb tea. Four flavonoids, astragalin, kaempferol-3-O-(2″-O-galloyl)-glucoside, isoquercitrin and quercetin-3-O-(2″-O-galloyl)-glucoside have been isolated from persimmon leaves and shown biological activities[3]. Kaempferol inhibits the synthesis of nucleic acid in tumor cells and transcription by RNA polymerase II[4].

During screening herb medicine having a cancer remedy effect, methanol extract of persimmon leaves inhibited mutagenic activities of aflatoxin B_1 (AFB_1), dimethyl-amino-biphenyl(DMAB), N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), and 4-nitroquinoline-1-oxide (4-NQO) in *Salmonella typhimurium* TA100[5]. To identify antimutagenic compounds the methanol extract was fractionated into hexane, chloroform, ethyl acetate, 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.

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 decribed by Maron and Ames[6]. The genotypes of tester strain was checked routinely for their histidine requirments, deep rough (*rfa*) character, UV sensitivity (*uvr* B mutation) and for the presence of R factor.

Aflatoxin B_1 (AFB_1) and dimethyl-amino-biphenyl (DMAB) from Sigma Chemical Co., Milwaukee, aWI,

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USA were dissolved in spectrophotometric grade dimethylsulfoxide (DMSO). N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) and 4-nitroquinoline-1-oxide (4-NQO) from Aldrich Chemical Co., Milwaukee, WI, USA were dissolved in distilled water and 95% ethanol, respectively.

Preincubation test was employed[6,7] *to determine* the antimutagenic effect of persimmon leaves. S9 mix(0.5ml) prepared by the method of Maron and Ames[6] was distributed to sterile capped tubes kept in an ice bath and then 0.1ml of testers from overnight culture ($1\sim2\times10^9$ cells/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[6].

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.[8]. 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 200ml 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 ethyl acetate resulted ethyl acetate and aqueous fractions that fractionated into butanol and aqueous phase.

Silica gel column chromatography

The hexane fraction was further fractionated using *silica gel column chromatography. The sample was* mixed with silica gel (10g) and then placed on the column (100cm × 5cm, i.d.) packed with silica gel (245g). The sample was eluted with hexane-ethlyacetate (1 : 4) 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 hexane-ethylacetate (4 : 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 coulmn, 25m × 0.25mm, 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 100° C to 280° C at 4° C/min, and then held for 15min 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-REV-EL) and/or mass spectrum of authentic compounds.

RESULTS AND DISCUSSION

Twenty one grams of methanol extract was obtain-

ed from 100g of dried persimmon leaves (Table 1). Each 1.25%, 2.5%, and 5% of methanol extract was tested for the effect on the mutagenicities of AFB_1 (1μg/plate), DMAB (10μg/plate), MNNG (0.5μg/plate), and 4-NQO (0.25μg/plate) in *Salmonella typhimurium* TA100. Methanol extract of persimmon leaves inhibited mutagenicities of AFB_1, DMAB, MNNG, and 4-NQO in *Salmonella typhimurium* TA100 in the fashion of dose responses (Table 2). The inhibition ratios were 95% for AFB_1, 93% for DMAB, 77% for MNNG, and 45% for 4-NQO with treatment of the 5% methanol extract (Table 2). This indicates that the methanol extract of persimmon leaves inhibits more strongly on the indirect mutagens (AFB_1, DMAB) than direct mutagens (MNNG, 4-NQO). Antimutagenic activity of the methanol extract against indirect mutagens could be related to the inhibition of some enzymes involved the conversion of AFB_1 and DMAB to the ultimate carcinogens.

The methanol extract was further fractionated into hexane, chloroform, ethyl acetate, butanol, and aqueous fraction. The yields of concentrated fractions from methanol extract were high in the order of butanol (22.4%), aqueous phase (22.4%), hexane (20.5%), chloroform (13.8%), and ethyl acetate (11.9%) (Table 1). Among these fractions, the hexane fraction had relatively high yields (20.5%) and antimutagenicity against AFB_1 showing 62% inhibition rate by treatment of 5% hexane concentrate in DMSO. Therefore, we further investigated its antimutagenic activities against AFB_1, DMAB, MNNG, 4-NQO at concentrations of 1.25%, 2.5%, and 5.0%. Higher antimutagenic activity of hexane fraction was shown against AFB_1 than other three mutagens tested in this study (Table 3). Further separation of the hexane fraction was carried out by silica gel column chromatography using two different size of columns (column I : 100cm × 5cm, column II : 100cm × 2cm). Fraction

Table 1. Yields of methanol extract and solvent fractions from methanol extract of persimmon leaves

Fractions	Yields(%)
Methanol extract	21[1]
Fractions of methanol extract[2]	
Hexane	20.5
Chloroform	13.8
Ethyl acetate	11.9
Butanol	22.4
Aqueous	22.4

[1] g/100g dried persimmon leaves
[2] % of methanol extract

Table 2. Effects of methanol extract of persimmon leaves on the mutagenicities of aflatoxin B_1 (AFB_1, 1μg/plate), 3, 2′-dimethyl-4-amino-biphenyl (DMAB, 10μg/plate), N-methyl-N′-nitro-N-nitrosoguanidine(MNNG, 0.5μg/plate) and 4-nitroquinoline-1-oxide (4-NQO, 0.25μg/plate) in *Salmonella typhimurium* TA100

Concentrations of methanol extract in DMSO	AFB_1	DMAB	MNNG	4-NQO
	Revertants/plate (Inhibition rate, %)			
Spontaneous	199± 16	195± 8	135± 6	83±15
0%	1087±133	992± 3	1096± 6	1251±44
1.25%	593± 56(56)	759±24(30)	542±34(58)	903±81(30)
2.50%	439± 36(70)	507±34(61)	432±17(69)	890±82(31)
5.00%	239± 27(95)	250±27(93)	352±45(77)	727±35(45)

Table 3. Effects of hexane fraction from methanol extract of persimmon leaves on the mutagenicities of aflatoxin B_1 (AFB_1, 1μg/plate), 3, 2′-dimethyl-4-amino-biphenyl(DMAB, 10μg/plate), N-methyl-N′-nitro-N-nitrosoguanidine(MNNG, 0.5μg/plate), 4-nitroquinoline-1-oxide (4-NQO, 0.25μg/plate) in *Salmonella typhimurium* TA100

Concentrations of methanol extract in DMSO	AFB_1	DMAB	MNNG	4-NQO
	Revertants/plate (Inhibition rate, %)			
Spontaneous	199± 16	195± 8	135± 6	83±15
0%	1087±133	992± 3	1096± 6	1251±44
1.25%	821± 83(30)	901± 28(11)	870±80(24)	900± 8(30)
2.50%	631± 36(51)	812±107(23)	781±70(33)	837±11(35)
5.00%	532± 69(62)	699± 52(37)	603±57(51)	684± 1(49)

number 8 to 10 from the silica gel column I eluted with hexane-ethylacetate (1 : 4) showed the strong antimutagenicities against AFB_1, DMAB, and MNNG in *Salmonella typhymurium* TA100 (Fig. 1). These antimutagenic fractions (8~10) collected from column I were combined, concentrated, and then refractionated on silica gel column II. Fraction number 4 and 5 from column II inhibited mutagenicities of AFB_1, DMAB, and MNNG with up to 90% inhibition ratio with treatment of concentrated eluents (1μg/plate for AFB_1, 10μg/plate for DMAB, 0.5μg/plate for MNNG) (Fig. 2). Antimutagenic fractions (No. 4 and 5) from column II were further separated on silica gel TLC plate and 8 bands were observed (Fig. 3). Antimutagenic activities of fractions (No. 4 and 5 from column II) separated on TLC are shown in Fig. 4, and TLC fraction 5 had antimutagenicities against AFB_1 and MNNG.

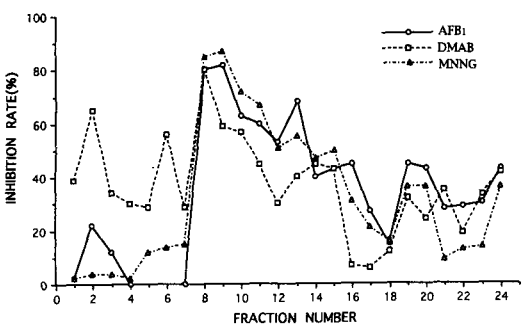

Fig. 1. Inhibition rate(%) of the mutagenicities induced by AFB_1(1μg/plate), DMAB(10μg/plate) and MNNG(0.5μg/plate) in *Salmonella typhimurium* TA100 by the fractions from the hexane fraction of methanol extract of persimmon leaves fractionated by using silica gel column I (100cm×5cm, i.d.).

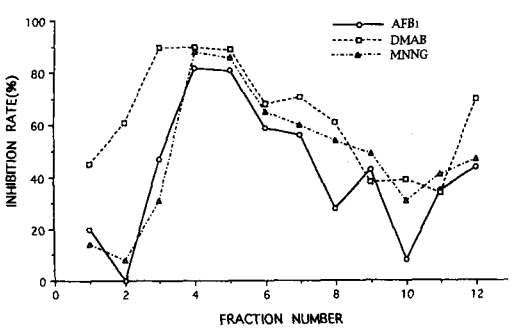

Fig. 2. Inhibition rate(%) of the mutagenicities induced by AFB_1(1μg/plate), DMAB(10μg/plate) and MNNG(0.5μg/plate) in *Salmonella typhimurium* TA100 by the fractions collected from silica gel column II (100cm× 2cm, i.d.).

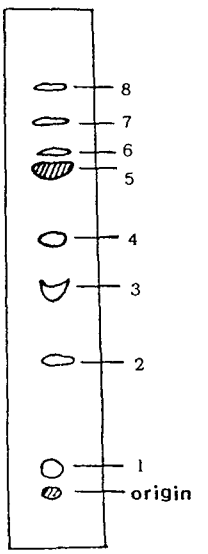

Fig. 3. Thin layer chromatographic fractionation of active fractions (Fraction number of 4 and 5) obtained by using silica gel column II (100cm×2cm, i.d.) from the hexane fraction of persimmon leaves.
Eluent ; hexane : ethylacetate=4 : 1(v/v).

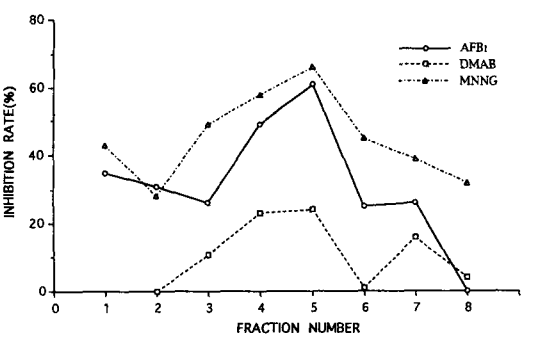

Fig. 4. Inhibition rate(%) of the mutagenicities induced by AFB_1(1μg/plate), DMAB (10μg/plate) and MNNG (0.5μg/plate) in *Salmonella typhimurium* TA100 by the TLC fractions.

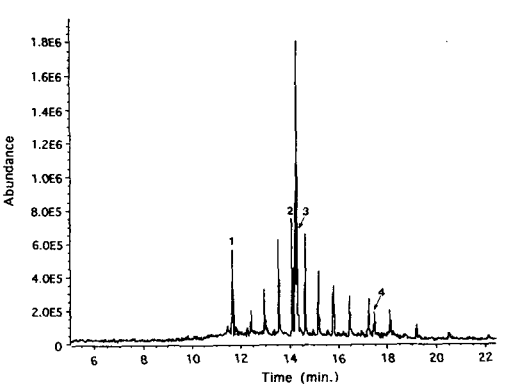

Fig. 5. Total ion chromatogram (TIC) of GC-MS of antimutagenic fraction number 5 obtained from TLC.

Table 4. Compounds identified from the antimutagenic fraction number 5 of TLC of the hexane fraction of persimmon leaves by GC-MS

Peak[1] No.	Compounds	Retention time (min.)
1	1′ – oxocannabinol	11.643
2	3B-acetoxy-methyl-5a-18 (13-17) abeoandrost-13-ene	14.202
3	4-methoxy-2′ 6′ -dinitro-3,5 -di-*t*-butylbiphenyl	14.281
4	8,9-dihydro-5,6-dimethoxy-dibenz[c, h]isoquino[2,1,8-1ma] carbazole-11,16-dione	17.458

[1]Shown in Fig. 5

TLC fraction 5 was extracted with hexane/ethyl acetate (4 : 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 1′ -oxocannabinol (peak No. 1), 3β-acetoxy-17-methyl-5-18 (13-17) abeoardrost-13-ene (peak 2), 4-methoxy-2′ 6′ -dinitro-3,5-di-*t*-butylbiphenyl-(peak 3), 8,9-dihydro-5,6-dimethoxy-dibenz [c,h] isoquino [2,1,8-1ma] carbazole-11,16-dione (peak No. 4) (Table 4). Hydrocarbons (Fig. 5) were identified in this fraction, however these compounds are not considered as antimutagenic compounds and they may be simply carried over due to their high solubilities in hexane.

ACKNOWLEDGEMENT

This work was supported by the research grant (90-0500-03) from KOSEF.

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(Received April 13, 1993)

감잎 헥산획분의 항돌연변이 효과와 항돌연변이 물질의 GC-MS를 이용한 동정

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요 약

감잎의 메탄올 추출물이 *Salmonella typhimurium* TA100에서 aflatoxin B_1(AFB_1), DMAB, MNNG, 그리고 4-NQO의 돌연변이 유발성을 억제시키는 효과가 있었다. 메탄올 추출물을 다시 hexane, chloroform, ethyl acetate, butanol, 그리고 수용성층으로 분획하여 각 획분의 수득율과 항돌연변이 효과를 조사하였다. Hexane, butanol, 그리고 수용성 획분의 수득율이 높았으며, 이중 hexane 획분이 AFB_1, DMAB, MNNG, 그리고 4-NQO에 대한 *Salmonella typhimurium* TA100에서 항돌연변이효과가 가장 크게 나타났다. Hexane 획분을 silica gel column과 thin layer chromatography (TLC)법으로 연속분리하여 TLC상에서 8개의 bands로 분리하였다. 그중 항돌연변이 효과가 가장 컸던 band를 hexane/ethylacetate (1 : 1, v/v)로 추출한 다음 그중에 존재하는 화합물을 GC-MS를 이용하여 분리동정하였다. 활성획분에서는 1′-oxocannabinol, 3β-acetoxy-17-methyl-5α-18 (13-17) abeoard-rost-13-ene, 4-methoxy-2′ 6′-dinitro-3,5-di-*t*-butylbiphenyl, 8,9-dihydro-5,6-dimethoxy-dibenz [c,h] isoquino [2,1,8-1ma] carbazole-11,16-dione 등이 분리동정되었다.