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Studies on Cosmetic Whitening Effect and Antibacterial Activities of Dry Persimmon Peel (*Diospyros Kaki* THUNB.)

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SUMMARY

Persimmon peels (*Diospyros Kaki* THUNB.) are discarded during the production of dried fruit. The 2-methoxy-4-vinylphenol (8) which is component of persimmon peel had high antioxidant activity on the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay and SOD (superoxide dismutase) assay. And (8) had higher tyrosinase inhibiting activity than that of arbutin using both *L*-tyrosine and *L*-DOPA as substrates. In addition, tyrosinase inhibitting activity of synthesized 2-methoxy-4-vinylphenol glycoside (12a) was studied. (12a) had tyrosinase inhibiting activity, suggesting that (12a) has possibilities for ingredient of cosmetics that are possessed of whitening effect. Moreover, for the creation of high value added product, we composed glycoside of (8) and cell toxicity assay with homosapiens origin alveolus epithelium cell strain A549 was carried on toward for these two kinds of compounds. Under high density condition (100 μg/mL), both of the compounds were not found to present toxic liability. Furthermore, we discovered that extracted hexane of dried persimmon include antibacterial activity (*B. subtilis* NBRC 3972, *S. aureus* NBRC13276 and *E. coli* NBRC3972) and ascertained methyl tridecanoate, 9-methyl hexadecenoate, 9-methyl octadecenoate, methyl tetradecanoate as the main components. Consequently we examined these components as the factor of antiseptic effect.

Key words : *Disopyros Kaki* THUNB., whitening effect, antibacterial activity.

INTRODUCTION

Persimmon (*Diospyros kaki* THUNB.) is food that has been cultivated for a long time in Japan. Persimmon includes a number of compounds such as sugars, terpenoids, carotenoids, flavonoids, tannins, naphthoquinones, steroids, amino acids, minerals, and lipids[1-3]. The fruit of persimmon which is used as folk medicine[4] has pharmacological effects suchas blood pressure-lowering effect, diuretic effect and hangover remedy and prophyl-

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axis against the cold. though the various parts of persimmonare used as medicine, persimmon peel is usually discarded. Recently, persimmon peel has been reported to includes than fruit[5], and persimmon peel extracteffe higher concentration of carotenoids and polyphenols ctive for reducing oxidative stress, DNA and cellular oxidative damage[6-8]. Oxidation of DNA and cell are involved in free radical and active oxygen, and they are the actors to cause various illnesssuch as promotion of the aging allergy, multiple scleraosisand parkinson's. Thus possibility of compounds in persimmon peel for application to ingradient of cosmetics that are possessed of whitening effect was studied. Here in were port whitening activity of compounds in persimmon peel for fur ther utilization.

EXPERIMENTAL

Experimental Materials

Persimmon peel was supplied from a *cv. "Nagara"* tree which was growing in Minami-Echizen-cho (Takano), Nanjo-gun, Fukui, Japan. The peel were air-dried for a month and pulverized. 3.0kg of that was extracted with 10L of methanol for a month at room temperature (20±2°C).

The solvent was concentrated *in vacuo* and methanol extract (653.1g) was obtained.

Sample Preparation Methods

First, 0.5L of water was added to 20.0g of methanol extract and successively extracted with hexanes, benzene, diethyl ether, dichloromethane, and ethyl acetate. Each solvent was concentrated *in vacuo* and hexane extract (1.5g), benzene extract (0.4g), diethyl ether extract (0.3g), dichloromethane extract (0.1g), ethyl acetate extract (0.2g) and residue (16.7g) were obtained. Second, Ethyl acetate extract 0.2g was chromatographed on DIAION HP-20 column and successively eluted with water, water:methanol (1:1), methanol, acetoneand and chloroform. Each fraction was concentrated *in vacuo* and water fraction (120mg), water:methanol (1:1) fraction (17mg), methanol fraction (23mg), acetone fraction (10mg), chloroform fraction (3mg) were obtained.

Analysis

GC-MS (Hewlett Packerd 6890 GC, Hewlett Packerd HP 5972 MSD, column : TC-WAX, 0.25mm × 60m, 70°C (5min hold) ~ 240°C (3°C/min), injection temp : 240℃, library : NIST (National Institute of Standards and Technology) Web Book).

Synthesis of Glycoside (12a)

The 2-methoxy-4-vinylphenyl-β-D-glucopyranoside (12a) were prepared according to reported procures[9].

Biological Activity

1) DPPH radical scavenging assay : Each compound was dissolved in ethanol at 1mM, and radical scavenging activity was measured according to the method previously described [10].

2) SOD assay : Each compound was dissolved in DMSO at 1mM, and activity was measured with a SOD activity detection kit (Wako Pure Chemical Industries Ltd.) according to the method previously described [11].

3) Inhibition of tyrosinase activity assay : Each compound was dissolved in DMSO at 3mM, and tyrosinase activity was measured using tyrosinase (Sigma Chemicals Co.) as an enzyme standard with *L*-tyrosine and *L*-3,4-dihydroxyphenylalanine (*L*-DOPA) as substrates, according to a method previously described [11].

4) Cell toxicity assay : Human pulmonary epithelial cells A549 were obtained from the american type culture collection. dulbecco's modified eagle medium (DMEM) was also purchased from gibco (NY, USA.). Fetal calf serum (FCS) was obtained from gibco.

The FCS used was heat inactivated. culture were maintained in complete DMEM supplemented with 10% heat–inactivated FCS, 1mM *L*-glutamine, 50 units/ml of penicillin, 50 μg/ml of streptomycin and 50 μM of 2-mercaptoethanol at 37°C in a moist 5% CO_2 incubator. A549 cells were passaged at 80 to 100% confluency using 0.25 trypsin and 0.02% ethylene diamine tetraacetic acid (EDTA). Cell toxicity was described in detail previously[12].

5) Antibacterial activity assay : Test were conducted against bacteria (*Escherichia coli* NBRC 3972, *Staphylococcus aureus* NBRC 13276 and *Bacillus subtilis* NBRC 3134), which were supplied by the national institutebof technology and evaluation biological resource center (NBRC). In these experiment, we used a solution mixture comprising equivalent volumes of tryptone soya agar media in which each bacteria was cultures until their concentration was 10^8 cfu/mL (except for 10^7 cfu/mL for *B. subtilis*). In the case of bacteria, 200μL of the bacteria mixture was added to 30g of sterilized water. The liquid was then mixed and diluted 10^3 times and 10^5 times. In method after, the incubation 1mL of these solution were respectively inoculated into the culture media, then the bacteria was cultured at 30°C for 4d. Colonies were then counted in order to investigate their proliferative states[13].

RESULTS AND DISCUSSION

The DPPH radical scavenging assay measures radical capturing activity, and SOD activity determines the ability of a compound to scavenge oxygen free radicals (O_2^-, HO·, LOO·, RO·)[14].

The antioxidative activity of all fraction was assessed on the basis of DPPH radical scavenging

Table 1 Tyrosinase lnhibitiing Effect, DPPH Radical Scavenging Effect and Superoxide Dismutase Effect of Compounds in Water:Methanol (1:1) Extract.

Compound	Tyrosinase inhibition effect[a]		DPPH radical scavenging effect		Superoxide dismutase effect
	L-Tyrosine	*L*-DOPA	Scavenging rate(%)[b]	SC_{50}[c]	Activity rate(%)[d]
(1)	0.03	0.03	41.2	76	-
(2)	2.5	0.4	-	>400	7.7
(3)	>100	6.3	-	>400	-
(4)	3.6	1.1	-	>400	-
(5)	5.7	0.8	-	>400	-
(6)	>100	7.7	-	35	-
(7)	0.5	0.7	-	>400	-
(8)	12.5	6.8	96.8	16	6.5
(9)	>100	8.7	-	>400	-
(10)	3.1	0.7	-	>400	6.9
(11)	2.7	>100	-	>400	1.0
Arbutin	0.7	2.9	-	-	-
α-Tocopherol	-	-	97.6	10	17.8
Ascorbic acid	-	-	98.9	7	18.7

a) Concentration 3.0mM. b) Corrected concentration 0.1mM.
c) 50% Scavenging concentration (μM). d) Concentration 1.0 mM.

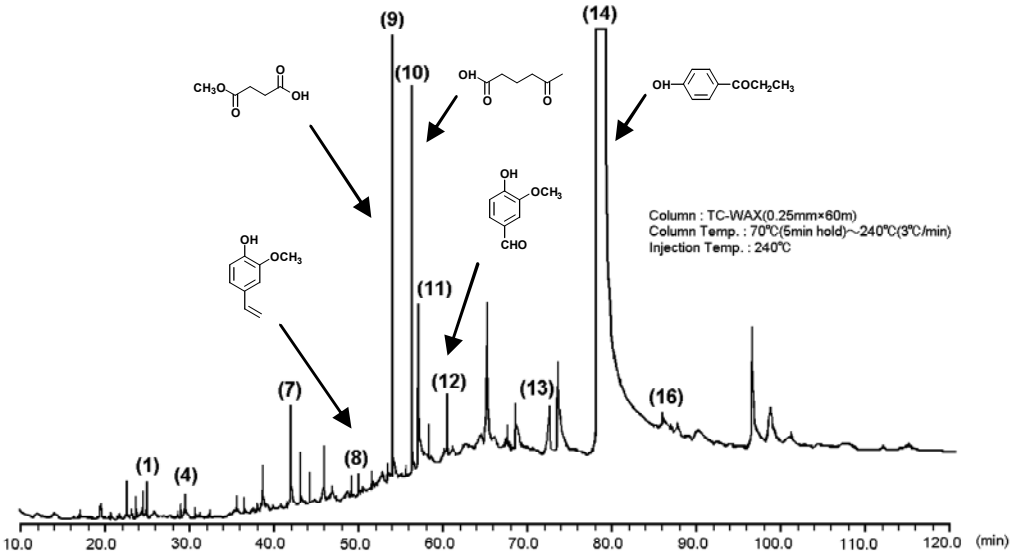

Fig. 1 GC-MS Chromatogram of Water:Methanol (1:1) Extract.

Table 2 Chemical Structure of Several Compounds in Water:Methanol (1:1) Extract.

P.N. [a]	R.T. [b]	Compound	Peak Area (%)
(1)	24.26	2-Mercaptoethanol	0.087
(2)	25.88	Benzaldehyde	trace
(3)	28.29	Dimethyl succinate	0.021
(4)	29.16	2-(2-Ethoxyethoxy)ethanol	0.061
(5)	30.94	Acetophenone	0.023
(6)	38.62	Benzyl alcohol	0.035
(7)	41.54	Diethylene glycol	0.272
(8)	49.29	2-Methoxy-4-vinylphenol	0.097
(9)	53.67	Monomethyl succinate	1.670
(10)	55.72	4-Acetylbutyric acid	0.913
(11)	57.08	Benzoic acid	1.207
(12)	59.60	Vanillin	0.161
(13)	71.46	Palmitic acid	0.297
(14)	77.47	4-Hydroxypropiophenone	84.768
(15)	83.35	Stearic acid	trace
(16)	85.05	Oleic acid	0.303
-	-	Others	10.085
		Total	100.000

a) Peak number. b) Retention time (min).

assay and SOD assay, that of water:methanol (1:1) fraction was the highest of all (**Table 1**). Then we identified the chemical structure of the compounds (1) to (16) as the components of main peaks in water:methanol (1:1) fraction by GC-MS (**Fig.1** and **Table 2**). Then the antioxidative activity of 11 compounds ((1) through (11)) was also studied and that results are shown in (**Table 1**). Relative antioxidative activity was determined using α-tocopherol as the reference compound. 2-mercaptoethanol (1) and 2-methoxy-4-vinyl-phenol (8) had potent radical scavenging activity. And compound (8) showed 96.8% DPPH radical scavenging activity that was comparable to

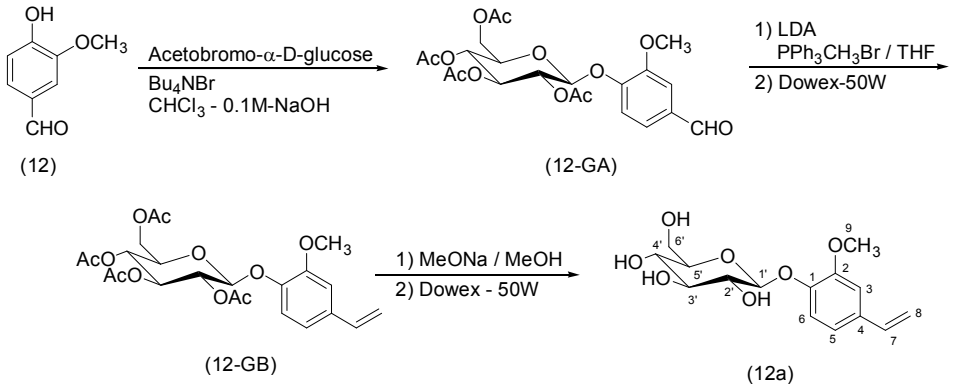

Fig.2 Synthsis of Glycoside (12a)

α-tocopherol. Melanin is a causative factor of spots and freckles, and their formation can be suppressed by the inhibition of melanin pathway enzymes in melanocytes, including tyrosinase[15].

Thus whitening activity of compounds in persimmon peel was assessed on the basis of tyrosinase inhibiting activity for application to ingredient of cosmetics that arepossessed of whitening effect (**Table 1**). Compound (8) which showed potent antioxidative activity in the DPPH radical scavenging assay also showed inhibiting activity against tyrosinase with satisfactory inhibitory values (*L*-tyrosine = 12.5, *L*-DOPA= 6.8).

Tyrosinase inhibiting activity of (8) was higher than that of arbutin as a reference compound (*L*- = 0.7, *L*-DOPA = 2.9). The (8) which has an OCH_3 group at the *ortho* position to a phenolic OH group has a vinyl group at the *para* position to a phenolic OH group. The importance of a vinyl group for tyrosinase inhibiting activity is supported by the fact that other compounds which have tyrosinase inhibiting activity also have

Table 3 Physiological Activity of (8) and (12a)

Compound	Tyrosinase inhibition effect[a]		DPPH radical scavenging effect		Superoxide dismutase effect	A549 Cell Toxicity[e]
	L-Tyrosine	*L*-DOPA	Scavenging rate(%)[b]	SC_{50}[c]	Activity rate(%)[d]	% of Control
(8)	12.5	6.8	96.8	16	-	94.8
(12a)	3.2	7.9	-	-	7.7	89.7
Eugenol	1.1	25.2	-	-	-	-
Citrusin C	3.3	20.1	-	-	-	-
Arbutin	0.7	2.9	-	-	-	-
α-Tocopherol	-	-	98.7	10	17.8	-
Ascorbic acid	-	-	97.6	8	18.7	-

a) Concentration 3.0mM. b) Corrected concentration 0.1mM.
c) 50% Scavenging concentration (μM). d) Concentration 1.0 mM. e) Concentration 100μM

vinyl groups[16]. The (1) which has SH-group had potent tyrosinase inhibiting activity. With regard to the action of sulfer atoms, SH compound do not inhibit the activity of tyrosinase by acting at its catalytic site, but melanocytes to form complexes such as cystinyldopa, thereby producing pheomelanin[17].

Phenylpropanoid is usually includeed as a glucoside in plant. And we have previously reported that tyrosinase inhibiting activity of glucoside of phenylpropanoids such as citrusin C is higher than that of lipophilic compounds such as eugenol[18].

Thus the glucoside of compound (8) which has a similar structure to that of eugenol was synthesized (**Fig. 2**).

However tyrosinase inhibiting activity of synthesized (12a) was not higher than that of (8) as the precursor ((8) → (12a) : *L*-tyrosine = 12.5 → 3.2, *L*-DOPA = 6.8 →7.9), and that was comparable to 1/3 ~ 1/5 activity of arbutin (**Table 3**). Then, cytotoxicity of (8) which had high activity at antioxidative assay [5] and whitening assay [6] (**Table 1**) was examined by homo sapiens origin alveolus epithelium cell strain A549 in consideration of really using as cosmetics.

Thus ATP detected by breathing of the mitochondria which is the life and death of the cell is measured to examine of the cytotoxicity [7]. As a result, (8) showed 94.8% survival rate of the cell in the density of 100μg/mL.

In addition, the glucoside (**Fig. 2**) of (12a) showed 89.7% survival rate of the cell. It is suggested that (8) and glucoside (12a) of that is available as cosmetics because of the density 100μg/mL is equal to combination density to real cosmetics (**Fig. 3**).

On the other hand, though persimmon peel was used for the purpose of prevention of decay such as fresh foods for a long time in Hokuriku region, persimmon peel has not been reported about the antibacterial activity and became not use as preservative recently. Thus as the application to product possessed of more value, persimmon peel examined the function as the preservative in food and cosmetics. As a result, hexane extract (**Fig. 4** and **Table 4**, main products : methyl tridecanote (16.27%), 9-methyl hexadecanoate (9.71%),9-methyl octadecenoate (9.35%) and methyl tetradcanoate (3.64%)) had higher antibacterial activity (*E. coli* , *S. aureus* and *B. subtilis*) than that of benzo-

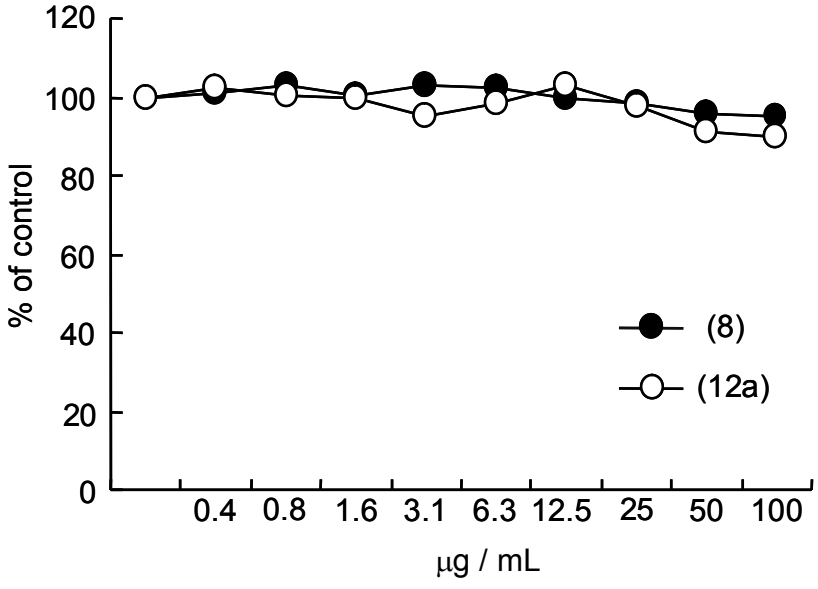

Fig.3 A549 Cell Toxicity Assay of (8) and (12a)

ic benzoic acid and methyl paraben which is really combined with food and cosmetics as preservative. Persimmon peel has possibility as replacement of the conventional preservative (**Table 5**). In conclusion, the compound that is possessed of higher antioxidative activity than that of α-tocopherol and higher tyrosinase inhibiting activity than that of arbutin and higher antibacterial activity than that of benzoic acid and methyl paraben were identified in persimmon peel which is really combined with food and cosmetics.

These results were suggested that discarded

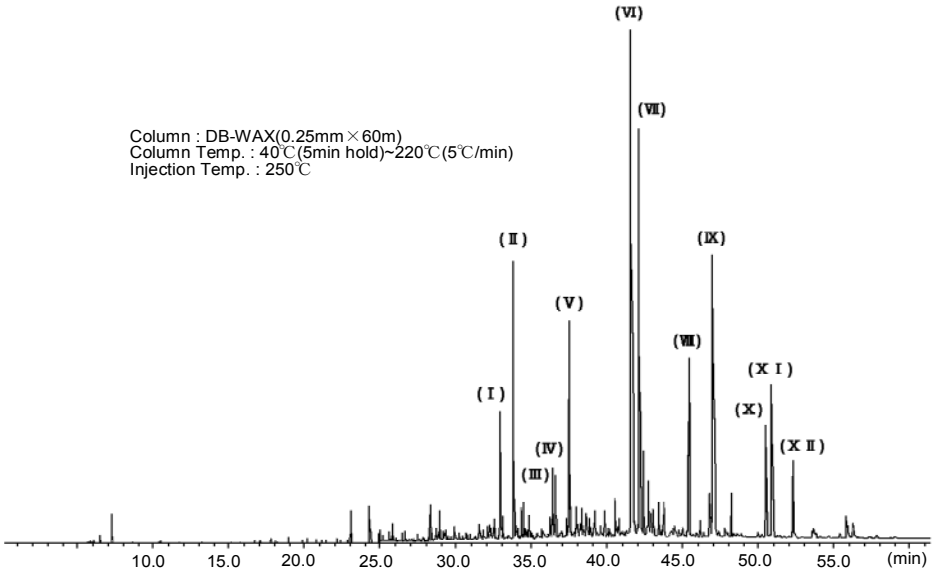

Fig.4 GC-MS Chromatogram of Hexane Extracion.

Table 4 Major Compounds of Hexane Extraction

P.N.[a)]	R.T.[b)]	Compound	Peak Area(%)
(Ⅰ)	32.86	Methyl nicotinate	1.71
(Ⅱ)	33.76	1-Methoxy-4-(1-propenyl) benzene	3.74
(Ⅲ)	36.28	4-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-3-buten-2-one	0.96
(Ⅳ)	36.43	2-Hexenoic acid	1.16
(Ⅴ)	37.43	Methyl tetradecanoate	3.64
(Ⅵ)	41.45	Methyl tridecanoate	16.27
(Ⅶ)	41.99	9-Methyl hexadecenoate	9.71
(Ⅷ)	45.12	5,6,7,7-Tetrahydro-4,4,7-trimethyl-2(4H)-benzofuranone	4.09
(Ⅸ)	46.8	9-Methyl octadecenoate	9.35
(Ⅹ)	50.17	9,12,15-Octadecatrien-1-ol	2.89
(Ⅺ)	50.53	Vanillin	4.20
(Ⅻ)	51.91	Methyl 4-hydroxy-3-methoxybenzoate	1.99
-	-	Others	40.29
		Total	100.00

a) Peak number. b) Retention time (min).

Table 5 Antibacterial Activity of *Persimmon Peel* Extracts.

Microorganism	Extract / Compound	Inhibition diameter				
		Concentration (μg / disc)				
		400	300	200	100	10
Escherichia coli	Methanol	1.0	0	0	0	0
	Hexane	2.5	1.5	1.0	0	0
	Ethyl acetate	2.0	0	0	0	0
	Water	2.0	0	0	0	0
	Benzoic acid	2.5	0	0	0	0
	Methylparaben	2.0	0	0	0	0
	Ampicillin sodium	>10	>10	>10	>10	>10
Staphylococcus aureus	Methanol	1.0	0	0	0	0
	Hexane	3.0	1.5	1.0	0.8	0
	Ethyl acetate	1.0	0.3	0	0	0
	Water	1.0	0	0	0	0
	Benzoic acid	2.5	1.5	0	0	0
	Methylparaben	1.0	0	0	0	0
	Ampicillin sodium	>10	>10	>10	>10	>10
Bacillus subtilis	Methanol	0	0	0	0	0
	Hexane	2.5	1.5	1.0	1.0	0
	Ethyl acetate	1.0	0.5	0	0	0
	Water	1.0	0	0	0	0
	Benzoic acid	2.0	1.0	0	0	0
	Methylparaben	1.0	0	0	0	0
	Ampicillin sodium	>10	>10	>10	>10	>10

persimmon peel has possibilities to utilize to food and cosmetics that possessed of physiological activity such as antioxidative activity and whitening effect and antibacterial activity for further utilization.

REFERENCES

1. Mallavadhani, U.; Panda, A.; Rao, Y. : Pharmacology and Chemotaxonomy of Diospyros. *Phytochemistry*, **49**, 901-951 (1998).
2. Inaba, A.; Sobajima, Y.; Ishida, M. : Seasonal Changes in the Major Components of Kaki Fruits. *Bull. Kyoto Prefecutual Univ.*, **23**, 24-28 (1971).
3. Niikawa, T.; Suzuki, T.; Ozeki, T.; Kato, M.; Ikoma, Y. : Characteristics of Carotenoid Accumulation During Maturation of the Japanese Persimmon 'Fuyu'. *Hort. Res.,* **6**, 251-256 (2007).
4. Taira, S.; Ohkawa, K.; Ikeda, K. : Distribution and Utilization of Local Varieties of Persimmon in the Shonai District of Yamagata Perfecture. *Bull. Yamagata Univ.*, Agr. Sci., **14**, 21-28 (2003).
5. Gorinstein, S.; Zemser, M.; Weitz, M.; Halevy, S.; Deutsch, J.; Tilus, K.; Feintuch, D.; Guerra, N.; Fishman, M.; Bartnikowska, E. : Fluorometric

Analysis of Phenolics in Persimmon. *Bioscience Biotechnology and Biochemistry,* **58**, 1087--1092 (1994).

6. Yokozawa, T.; Kim, K. Y.; Kim, H.Y.; Lee, Y. A; Nonaka,G. : Protective Effect of Persimmon Peel Polyphenol Against High Glucose-induced Oxidative Stress in LLC-PK$_1$ Cells. *Food and Chemical Toxicology*, **45**, 1979-1987 (2007).
7. So-Young, K.; Seok-Moon, J.; Sun-Jung, K.; Kyung-Im, J.; Eunji, P.; Hae-Ryong, P.; Seung-Cheol, L. : Effect of Heat Treatment on Antioxidative and Antigenotoxic Activity of Extracts from Persimmon (*Diospyros kaki L.*) Peel. *Bioscience Biotechnology and Biochemistry,* **70**, 999-1002 (2006).
8. Lee, Y. A.; Cho, E. Ju.; Yokozawa, T. : Protective Effect of Persimmon (*Diospyros kaki*) Peel Proanthocyanidin Against Oxidative Damage under H_2O_2-Induced Cellular Senescence. *Biol. Pharm. Bull.,* **31**, 1265-1269 (2008).
9. Fukai, S.; Tanimoto, S.; Maeda, A.; Fukuda, H.; Okada, Y.; Nomura, M. : Pharmacological Activity of Compounds Extracted from Persimmon Peel (*Diospyros kaki* THUNB.). *J. Oleo Sci.*, **58**, 213-219(2009).
10. Maeda, A.; Tanimoto, S.; Abe, T.; Hazama, S.; Tanizawa, H.; Nomura, M. : Physiological Activities of *Myristica fragrans Houttuyn. Yakugaku zasshi*, **128**, 129-133 (2008).
11. Nomura, M.; Nishimura, K.; Fujihara, Y.; Tada, T.; Hattori, F.; Shimomura, K. : Condensation Reaction of trans-Anethole Derivative with Several Monoterpenyl Compounds and Their Inhibiting Properties towards Tyrosinase, Superoxide Scavenging Activity and Hyaluronidase Activity. *Journal of Japan Oil Chemists' Society*, **49**, 143-149 (2000).
12. Bakand, S.; Winder, C. : Comparative in Vitro Cytotoxicity Assessment of Selected Gaseous Compounds in Human Alveolar Epithelial Cells. *Toxicology in Vitro*, **21**, 1341-1347 (2007).
13. Abe, T.; Hisama, M.; Tanimota, S.; Shibayama, H.; Mihara, Y.; Nomura, M. : Antioxidant Effects and Antimicrobial Activites of Phytoncide. *Bio. Sci.*, **13**, 23-27(2008).
14. Fujita, T. : Formation and Removal of Reactive Oxygen Species, Lipid Peroxides and Free Radicals, and Their Biological Effects. *Yakugaku Zasshi*, **122**, 203-218 (2002).
15. Kobayashi, S. : UVB-induced Skin Damage and Protection / Treatment-Effects of Novel, Hydrophilic α-Tocopherol Derivative. *Yakugaku Zasshi*, **126**, 677-693 (2006).
16. Sawabe, A.; Nomura, M. : Cosmetic substances for Skin Depigmentation from African Dietary Leaves. *Celosia argentea L*, *Fragrance Journal*, **9**, 54-58 (2000).
17. Giuseppe, P. : Recent Advances in the Chemistry of Melanogenesis in Mammals. *Journal of Investigative Dermatology*, **75**, 122-127 (1980).
18. Nomura, M.; Sawabe, A.; Fujiwara, Y. : Studies on Synthsis of the Glycoside and Whitening Effects as a Cosmetics Material Using the Chlorogenic Acid its Metabolite Contained in the Plants. *Co smetology Reserch*, **10**, 2-7 (2002).