

Secoiridoid Type of Antiallergic Substances in Olive Waste Materials of Three Japanese Varieties of *Olea europaea*

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ABSTRACT: 2-Hydroxy-3-ethylidene-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-4-acetic acid 2-(3,4-dihydroxyphenyl)ethyl ester (3,4-DHPEA-EA) is a kind of secoiridoid first found in three Japanese olive pomaces: Mission, Lucca, and Manzanillo. These varieties showed high activity of 3,4-DHPEA-EA as an antiallergic active substance with IC₅₀ at 33.5 ± 0.6 µg/mL. Because 3,4-DHPEA-EA was the most abundant among the active substances in the pomaces and the activity of 3,4-DHPEA-EA was greater than that of hydroxytyrosol and elenolic acid, 3,4-DHPEA-EA, which has the ester linkage of hydroxytyrosol and elenolic acid, should be essential for antiallergic activity. Although a trace amount (1.04 mg/kg) of luteolin in the pomace showed the highest antiallergic activity with IC₅₀ at 0.752 ± 0.1 µg/mL, we concluded that the entire antiallergic effect derives from the abundance of 3,4-DHPEA-EA, especially in the green olive pomace of the Mission variety in October, which showed the highest level of 3,4-DHPEA-EA (5033 ± 118 mg/kg). Therefore, the Mission variety had the most effective antiallergy property.

KEYWORDS: RBL-2H3, allergy, degranulation, olive pomace, waste materials, secoiridoid, 3,4-DHPEA-EA

INTRODUCTION

Food allergies are serious problems for many people around the world,¹ especially for the increasing number of patients who suffer from many kinds of allergies in urban areas.² Food additives, air pollution, and changes in food habits are believed to influence directly or indirectly the high incidence of food allergies. Therefore, nowadays, food ingredients that may cause immunological inflammatory diseases must be indicated on food packaging in Japan, the U.S.A., and other countries.

Histamine analogues that inhibit immune reactions of allergies as antagonists have, until now, been developed as new drugs. However, histamine analogues may have side effects in patients³ because of high similarity with chemical mediators that may have biological effects and higher affinity to the functions of human cells. Therefore, new drugs that protect against histamine release for the decrease in chemical mediators in the human body are being explored as a new phase in the development of medicinal drugs. Because degranulation of RBL-2H3 cells is linked to the release of hexosaminidase and histamine, many researchers observe the suppression of hexosaminidase release in developing antiallergic activity drugs.⁴ *Perilla* (shiso in Japanese) leaves are commonly used in Japan and China for folk medicine, and the antiallergy effect using β -hexosaminidase release inhibitory activity is one of the functions of *Perilla* leaf extract.⁵

Flavonoids, curcumin, phenylpropanoids, and other phytochemicals are well-known as antioxidant,^{6,7} anticancer,^{8,9} and antiallergic^{10–12} substances. Matsuda et al. compared the effects of various flavonoids as potent chemicals for antiallergy drugs. Various kinds of plant flavonoids and their related metabolites have been investigated as functional substances for antiallergy drugs. However, using the metabolites of those chemicals in humans has not been fully tested. The absorption rate of some flavonoid aglycones and their glycosides in the small intestine may be, for example, still low, unclear, or under discussion.¹³

Medicines and drugs should have an intermediate polarity (log *P* < 5) and hydrogen donors less than 5 (NH and OH groups) to have higher absorption rates.¹⁴

Olives and grapes are fruit that are used to make olive oils and wine, respectively, the processing of which typically results in a huge amount of waste material remaining. To improve the productivity of olive oil and wine processing, using the waste material is being promoted. These days, it is indispensable to find new ways of using olive and grape waste material to promote higher production of olive oils and wines from the same amount of fruit.

Olive leaves have been used for olive tea in Italy and other countries, including Japan, to add to the benefit of thinning leaf.¹⁵ Furthermore, many reports in scientific journals indicate that olive fruit has many substances with biological activities, such as anti-inflammatory,¹⁶ antioxidant,¹⁷ antiatherogenic,¹⁸ and anti-*Helicobacter pylori* activities,¹⁹ whitening of human skin,^{20,21} etc. Oleuropein, hydroxytyrosol, and verbascoside are specific and common chemicals in the pomace and leaves, and the biological activities shown above are partly attributed to those metabolites and have recently attracted attention. However, little detail on the antiallergic effects of olive waste material has been reported thus far.

In this paper, we demonstrate the usefulness of olive waste material and clarify the antiallergic effects of the metabolites.

MATERIALS AND METHODS

Olive Fruit. The Mission variety of green olive fruit (2.3 kg) was harvested from Shodoshima Olive Park in December 2009 and was used to determine the chemical structure of antiallergic substances.

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Three varieties of olive fruit (Mission, Manzanillo, and Lucca) were harvested (1–2 kg) from the Arai Olive Co., Ltd. in October, November, and December 2011 on Shodoshima Island, Kagawa Prefecture, Japan, for quantitatively measuring target chemicals during maturation.

Chemicals and Reagents. Dulbecco's modified Eagle's medium (D-MEM), phosphate-buffered saline (PBS) (–), and Triton X-100 were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was obtained from Equitech-Bio (Kerrville, TX). Antibiotic–antimycotic 100× was obtained from Invitrogen (Grand Island, NY). Mouse monoclonal anti-dinitrophenyl (DNP) antibody, albumin dinitrophenyl (HSA), Tyrode's salt solution, and *p*-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside (*p*-NAG) were obtained from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Verbascoside, hydroxytyrosol, oleuropein, and luteolin were purchased from Extrasynthèse (Genay, France) and used without further purification.

Preparation of the Media and Samples. DMEM including 10% FBS and 1% antibiotic–antimycotic 100× was used as a base medium. Modified Tyrode's (MT) buffer was prepared by dissolving *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (4.76 g/L) and BSA (1 g/L) in Tyrode's salt solution. Anti-DNP IgE solution (antibody) was prepared by diluting mouse monoclonal anti-DNP antibody with the base medium (50 ng/mL). HSA solution (antigen) was prepared by dissolving HSA in MT buffer (2.5 μ g/mL). The samples tested were dissolved in dimethyl sulfoxide (DMSO), and the solutions were added to MT buffer (final DMSO concentration was adjusted at 0.1%).

Extracts from Olive Fruit. Olive fruit (2.3 kg, Mission variety, December 2009) was crushed using a hand mixer. The crushed olives were then transferred to a 100% cotton bag, and the fruit juice and oil were separated from the pomace by compression. The pomace was then soaked in 100% methanol (700 mL) 3 times. The total methanol extracts (2.1 L) were obtained after the extraction. The oil separated from the methanol extracts was recovered again from hexane extracts by partition with 100% methanol and 100% hexane. Both solvents were removed under vacuum. Finally, 69 g of olive pomace extract was obtained from the methanol solution.

Extraction Using the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) Method. In quantitative comparison of olive metabolites in different varieties and seasons, the extraction was performed by the QuEChERS method that was usually used in residual agricultural chemicals analysis.^{22,23} Olive fruit (100 g) was chopped in a food processor. A total of 10 g of the chopped sample was placed into a polypropylene centrifuge tube (50 mL), and 10 mL of acetonitrile was then added. The mixture was homogenized for 3 min. A total of 1 g of sodium chloride, 1 g of trisodium citrate dihydrate, 0.5 g of disodium hydrogen citrate sesquihydrate, and 4 g of anhydrous magnesium sulfate were added to the solution, and the mixture was immediately hand-shaken for 1 min. Finally, the mixture was centrifuged at 3000 rpm for 5 min. Hexane (10 mL) was added to the recovered acetonitrile layer to remove the remaining oil and chlorophylls. The acetonitrile extract obtained was dried using an evaporator with a vacuum pump. Solid acetonitrile extract (205.35 mg) was obtained from 10 g of green Mission olive in October 2011.

Fractionation of the Methanol Extracts from the Olive Pomace. The fractionation was performed by an Amberlite XAD-7 column according to Goto et al.,²⁴ who applied for the separation of anthocyanins. The separation of the olive pomace extracts (34.5 g) using an Amberlite XAD-7 column was started from 20% methanol (500 mL), and then 2 L each of 40% methanol, 60% methanol, 80% methanol, and 100% methanol were applied to the column in this order, as shown in Figure 1. Eluents (950 mL each of fractions 1–9) were collected using column chromatography. Each extract was dried under vacuum.

Isolation of 2-Hydroxy-3-ethylidene-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-4-acetic Acid 2-(3,4-Dihydroxyphenyl)-ethyl Ester (3,4-DHPEA-EA) and Elenolic Acid from Fractions 7 and 4. To isolate 3,4-DHPEA-EA, fraction 7 (50 mg) was purified using a high-performance liquid chromatography (HPLC) 5 μ m

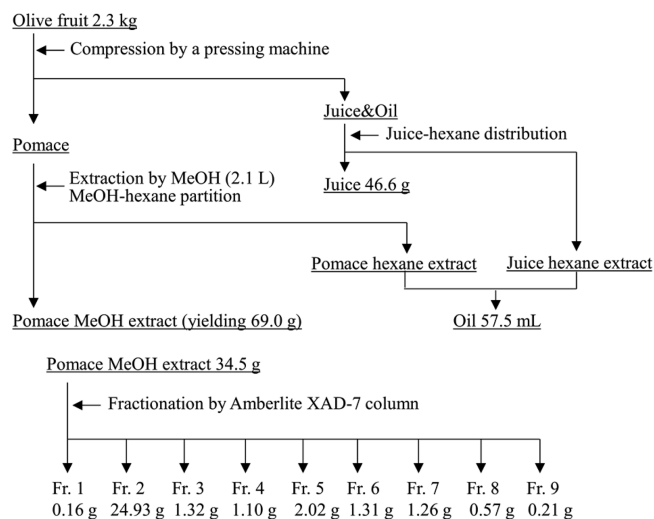


Figure 1. Extraction and fractionation from the Mission variety of green olive fruit harvested in December 2009.

octadecylsilane (ODS) column with a mixture of 16% acetic acid and 20% acetonitrile in water at a flow rate of 1 mL/min and an ultraviolet (UV) wavelength of 290 nm. Finally, 20 mg of pure 3,4-DHPEA-EA with 97% purity was isolated after drying under vacuum. To isolate elenolic acid, 50 mg of fraction 4 was purified using a HPLC 5 μ m ODS column with a mixture of 8% acetic acid and 10% acetonitrile in water at a flow rate of 1 mL/min and at an UV wavelength of 250 nm. Finally, 10 mg of pure elenolic acid with 90% purity was isolated after drying under vacuum.

Physical data of 3,4-DHPEA-EA: UV λ_{max} 243 and 279 nm; electrospray ionization–mass spectrometry (ESI–MS) (intensity of fragment ions), m/z 137 (100), 158 (68), 213 (57), 225 (30), 229 (12), 379 (72, $[M + H]^+$, $C_{19}H_{23}O_8$; found, 379.1373; calculated, 379.1393).

Physical data of elenolic acid: UV λ_{max} 243 nm; ESI–MS (intensity of fragment ions), m/z 95 (80), 127 (100), 139 (65), 165 (30), 209 (11), 241 (70, $[M-H]^-$, $C_{11}H_{13}O_6$; found, 241.0708; calculated, 241.0712).

Nuclear magnetic resonance (NMR) data of 3,4-DHPEA-EA and elenolic acid are shown in Table 1.

Analytical HPLC. The HPLC analysis was performed by the following conditions according to Asada et al.²⁵ The HPLC system was comprised of a 250 $\times$ 4.6 mm COSMOSIL 5C₁₈-AR II column, coupled to a JASCO MD 2010 Plus photodiode array (PDA) detector and double JASCO PU-980 pumps. Chromatograms were obtained from UV absorbance at 290 nm. The column temperature was set at 40 °C. The flow rate was 1 mL/min. The mobile phase used was a mixture of 2% acetic acid, 2.5% acetonitrile, and 1.5% phosphoric acid in water (A) and a mixture of 20% acetic acid, 25% acetonitrile, and 1.5% phosphoric acid in water (B). The total analytical time was 41 min, and the gradient was performed as follows: from 100 to 0% A for 35 min, and 0% A was kept for 5 min. For preparative HPLC, the HPLC system consisted of a 10 $\times$ 250 mm Develosil 5 μ m ODS column, coupled to a JASCO 875-UV detector (290 nm for 3,4-DHPEA-EA and 250 nm for elenolic acid) with a JASCO PU-980 pump at 1 mL/min. For the preparative HPLC, non-volatile phosphoric acid was removed from the solvents described above. Hydroxytyrosol, 3,4-DHPEA-EA, elenolic acid, luteolin, verbascoside, and oleuropein were dissolved in dimethyl sulfoxide and then diluted to prepare a standard solution (0.067–0.5 μ g/mL) for making the calibration curve of each chemical.

Hydrolysis of 3,4-DHPEA-EA. The hydrolysis of 3,4-DHPEA-EA was performed by hydrochloric acid according to the study by Davidek et al.²⁶ Fraction 7 extract (50 mg) containing 3,4-DHPEA-EA was dissolved in 1 mL of 1% HCl aqueous solution and left for 24 h at room temperature. The product and 3,4-DHPEA-EA were quantitatively monitored using a HPLC 5 μ m ODS column.

Table 1. Chemical Shifts (δ) of Compounds 3 and 6 and Literature Data of Elenolic Acid and 3,4-DHPEA-EA

atom	compound 3			compound 6			reference data ^a					
	δ ¹ H (ppm)	J (Hz)	δ ¹³ C (ppm)	δ ¹ H (ppm)	J (Hz)	δ ¹³ C (ppm)	elenolic acid ³¹		(5S,8R,9S)-3,4-DHPEA-EA ³³			
1	9.61	s	199.74	9.47	d, J = 1.38	200.36	9.60	s	199.40	9.45	d, J = 1.5	201.34
3	7.62	s	156.97	7.55	d, J = 0.72	155.92	7.63	s	156.66	7.51	d, J = 1.3	154.75
4			106.45			106.37			105.90			107.11
5	3.35	dd, J = 2.76, 11.04	27.90	3.36	m, J = 0.72, 3.42, 4.80, 8.94	26.97	3.35	m	28.90	3.29	m, J = 1.3, 3.7, 4.9, 9.7	26.70
6	a 2.27	dd, J = 11.04, 16.50	38.35	a 2.52	dd, J = 8.94, 15.78	37.17	a 2.32	dd	37.80	a 2.51	dd, J = 9.7, 15.9	37.28
	b 2.95	dd, J = 2.76, 16.50		b 2.86	dd, J = 3.42, 15.78		b 2.85	dd		b 2.76	dd, J = 3.7, 15.9	
7			176.09			172.03			170.20			172.49
8	4.20	q, J = 6.90	69.66	4.43	quin, J = 5.52, 6.84	70.81	4.20	q	69.62	4.53	quin, J = 5.0, 6.7	71.05
9	2.64	s	50.96	2.54	d, J = 1.38, 4.80, 5.52	54.29	2.69	s	50.96	2.54	dt, J = 1.5, 4.9, 5.0	54.33
10	1.56	d, J = 6.90	17.96	1.36	d, J = 6.84	19.36	1.49	d	17.88	1.34	d, J = 6.7	19.17
11			167.18			167.72			167.34			167.65
12	3.70	s	51.50	3.71	s	51.69	3.75	s	51.48	3.65	s	51.42
1'				4.25	m, J = 6.18	65.32				4.21	dt, J = 6.7, 14.6	65.50
2'				2.79	dt, J = 6.18	34.25				2.76	t, J = 6.7	34.39
3'						130.27						130.55
4'				6.74	d, J = 1.38	116.10				6.69	d, J = 2.1	116.38
5'						142.95						145.10
6'						143.65						144.20
7'				6.76	d, J = 8.28	115.26				6.72	d, J = 8.1	115.85
8'				6.58	dd, J = 1.38, 8.28	121.14				6.56	d, J = 2.1, 8.1	121.19

^aNMR data of compounds 3 and 6 with elenolic acid and (5S,8R,9S)-3,4-DHPEA-EA.

β -Hexosaminidase Release Assay of RBL-2H3 Cells. For the amount of β -hexosaminidase release by basophilic leukemia cells, RBL-2H3 was determined using a previous method with some modifications.^{4,27} RBL-2H3 were obtained from RIKEN BioResource Center Cell Bank (Ibaraki, Japan). The RBL-2H3 cells in the base medium (DMEM containing 10% FBS and 1% antibiotic–antimycotic 100X) were dispensed into a 24-well plate (2.5×10^5 cells/well) and were incubated overnight at 37 °C under a humidified 5% CO₂ atmosphere. After the cells were washed with 1 mL of PBS, they were incubated with 500 μ L of antibody solution (mouse monoclonal anti-DNP antibody, 50 ng/mL dissolved in base medium) for 2 h of sensitization. A test sample solution of 490 μ L was added to the well after washing twice with 500 μ L of MT buffer (Tyrode's salt solution containing 1 g/L BSA and 4.76 g/L HEPES). The MT buffer was used as a control instead of the sample. The test sample was dissolved in DMSO and diluted with MT buffer (the final concentration of DMSO was set at 0.1%). After 10 min of incubation, albumin dinitrophenyl (10 μ L, final concentration of 50 ng/mL) was added to each well and the cells were incubated for 30 min to evoke allergic reactions (degranulation). The reaction was stopped by cooling in an ice bath for 10 min. The supernatant (50 μ L) was transferred to a 96-well plate and incubated with 100 μ L of substrate (3.3 mM *p*-nitrophenyl-2-acetamide-2-deoxy- β -D-glucopyranoside) in 0.1 M citrate buffer (pH 4.5) at 37 °C for 25 min. The reaction was terminated by adding 100 μ L of stop solution (2 M glycine buffer at pH 10.0). The absorbance (OD) was measured at 405 nm using a microplate reader (Thermo Scientific Multiskan FC, Yokohama, Japan). The gaining OD reflects β -hexosaminidase release. The calculation was performed using eqs 1 and 2 shown below. To obtain a valid value, the factors that are not typically induced by samples need to be excluded. In “blank”, neither antibody solution nor sample was added to the cells to confirm spontaneous β -hexosaminidase release from the cells. In “control”, MT buffer instead of samples was added to the cells to confirm β -hexosaminidase release from the cells in conditions without a sample. In “total”, the cells were lysed in 0.1% Triton X-100 in MT

buffer to confirm the total amount of β -hexosaminidase contained in the cells. In “sample”, both antibody solution and samples were added to the cells to confirm β -hexosaminidase release from the cells in these conditions.

$$\text{ratio of } \beta\text{-hexosaminidase release (\%)} = \frac{(\text{OD}_{\text{control or sample}} - \text{OD}_{\text{blank}})}{(\text{OD}_{\text{total}} - \text{OD}_{\text{blank}})} \times 100 \quad (1)$$

$$\beta\text{-hexosaminidase release (\%)} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{(\text{OD}_{\text{control}} - \text{OD}_{\text{blank}})} \times 100 \quad (2)$$

The ratio of β -hexosaminidase release of “control” should be greater than 25%.

β -Hexosaminidase Inhibitory Activity Assay. β -Hexosaminidase inhibitory activity was measured according to previous methods, with some modifications.^{10–12} RBL-2H3 cells were grown in base medium. The cells were lysed in 0.1% Triton X-100 (5.0×10^5 cells/mL). The cell lysate (25 μ L) was transferred to a 96-well plate. The tested solutions were diluted in different concentrations with MT buffer and then added to the sample well (25 μ L); MT buffer was added to the control well (25 μ L). Following that, 100 μ L of 0.1 M citrate buffer (pH 4.5) containing 3.3 mM *p*-NAG (substrate) was added to each well and incubated for 25 min at 37 °C. The reaction was stopped by adding 100 μ L of 2 M glycine buffer (the stop buffer solution) at pH 10.0 to each well. The absorbance (OD) of each reaction was measured at 405 nm using a microplate reader (Thermo Scientific Multiskan FC, Yokohama, Japan). The inhibitory activity of β -hexosaminidase was calculated using the following equation:

$$\beta\text{-hexosaminidase inhibitory (\%)} = \left(1 - \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}}\right) \times 100 \quad (3)$$

NMR Analysis. ^1H , ^{13}C , distortionless enhancement by polarization transfer (DEPT)-45, DEPT-90, DEPT-135, correlated spectroscopy (COSY), heteronuclear correlation (HETCOR), and heteronuclear multiple-bond connectivity (HMBC) NMR spectra were recorded with a 600 MHz JNM-ECA spectrometer, dissolving samples in deuterated chloroform with tetramethylsilane (TMS, Isotec, Taiyo Nippon Sanso Corp., Ltd., Tokyo, Japan) as an internal standard.

Mass Spectrometry (MS) Analysis. Mass spectra of 3,4-DHPEA-EA and elenolic acid were measured using liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry (LC/ESI-Q-TOF-MS) analysis using an Acquity UPLC/Xevo QTOF MS system (Waters). Chromatographic separations were conducted using an Acquity UPLC BEH C_{18} (1.7 μm , 2.1×50 mm) column (Waters). The instrument was operated with ESI source in negative-ion mode (elenolic acid) and positive-ion mode (3,4-DHPEA-EA).

Statistical Analysis. Statistical analysis was carried out using EZR (version 1.23) with the Tukey test [one-way analysis of variation (ANOVA)]. The values represent the mean $\pm$ standard deviation (SD) in triplicates at least. Significant differences were set at $p < 0.05$.

RESULTS AND DISCUSSION

Isolation and Identification of Antiallergic Active Compounds. The pomace portion showed significant suppression of β -hexosaminidase release (Figure 2) compared to the oil and juice. Therefore, the pomace extracts were further separated into nine fractions with an Amberlite XAD-7 column

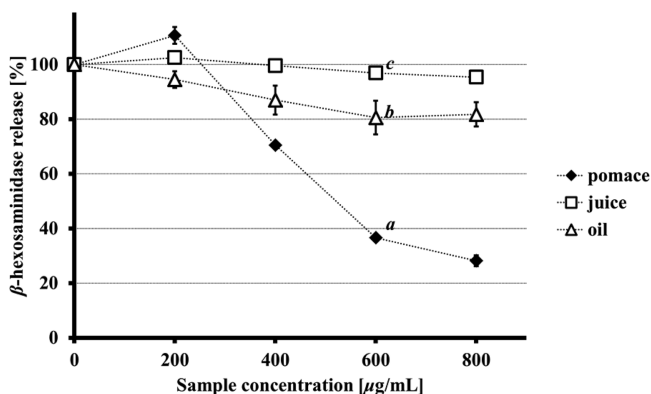


Figure 2. Antiallergic activity of olive extracts, such as pomace, oil, and juice, of Mission green olive fruit. Each value represents the mean $\pm$ SD ($n = 4$). Means with different letters are significantly different ($p < 0.05$).

chromatograph using solvents from 20% methanol to 100% methanol stepwise, as shown in Figure 1. The residues from pomace, oil, and juice were 69.0 g, 46.6 g, and 57.5 mL, respectively. Antiallergic activities of each fraction at the 200 $\mu\text{g/mL}$ concentration found that fraction 7 exhibited the lowest β -hexosaminidase release ($12.8 \pm 5.3\%$), followed by fraction 6 ($39.2 \pm 2.1\%$), with significant differences ($p < 0.05$), while other fractions exhibited β -hexosaminidase release over 65% (Figure 3). Thus, fractions 1–3 did not show any antiallergic activity. On the other hand, fractions 4, 5, 8, and 9 showed some activity.

The nine fractions of the pomace extracts, the oil, and the juice were analyzed using HPLC, and the retention times and UV spectra obtained from a PDA detector were compared to known authentic and commercially available chemicals, as shown in Figure 4. There were large numbers of major peaks of chemicals (compounds 1–6) in the pomace of the olive fruit (Figure 4A), but there were none of those substances in the oil

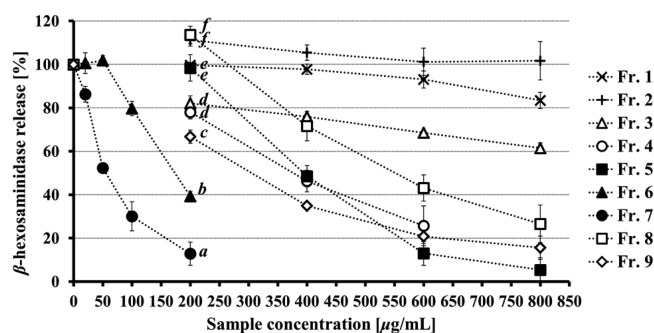


Figure 3. Antiallergic activity of each fraction of Amberlite XAD-7 column chromatography. Each value represents the mean $\pm$ SD ($n = 4$). Means with different letters are significantly different ($p < 0.05$).

and juice. Thus, hydroxytyrosol (1), which has significant antioxidant²⁸ and whitening^{20,21} activities, was found in fractions 2 and 3 as a main chemical (Figure 4B). Verbascoside (2) was found in fractions 4 and 5. Oleuropein (4), which is a major compound in olive leaves, was found in fractions 4 and 5. A small amount of luteolin (5), which has been reported in many papers^{10,29,30} as one of the most powerful antiallergic compounds, was detected in fractions 8 and 9.

Fractions 6 and 7, which had significant antiallergic activity, showed a marked peak as compound 6. The antiallergic active compound in these two fractions was newly determined by MS spectra and NMR analyses.

Determination of the Structure of the Antiallergic Active Substance, Compound 6. Compound 3 in fraction 4 was purified using a 5 μm ODS preparative HPLC column. Mass spectra data of compound 3 showed the molecular ion at m/z 241.0708 ($[\text{M} - \text{H}]^-$, negative-ion mode) using high-resolution HPLC–TOFMS, giving the molecular formula as $\text{C}_{11}\text{H}_{14}\text{O}_6$ for compound 3. ^{13}C NMR (DEPT) and ^1H NMR data made it possible to assign 11 carbon, 14 hydrogen, and 6 oxygen atoms in the molecule. ^{13}C NMR signals at 167.18, 176.09, and 199.74 ppm indicated two carboxylic acid or ester groups and carbonyl aldehyde. These data explain the existence of 5 oxygen and 3 carbon atoms in the molecule. ^{13}C NMR signals at 106.45 ppm (C) and 156.97 ppm (CH) indicated one double bond ($\text{C}=\text{CH}-$). Furthermore, DEPT analysis of signals at 69.66 and 51.50 ppm supported the existence of methoxy and methine groups with oxygen. Because two carboxylic acid or ester groups were found in the molecule, the methoxy group at position 12 should be part of the ester group ($-\text{COOMe}$). The methine group should not be the part of ester but part of the ether group. Other fragments of the molecule were one methyl group, one methylene group, and two methine groups.

The partial structure of fragment a in Figure 7 clarified the linkage of the partial structure of compound 3 from the ^1H – ^1H COSY spectrum. Thus, the $\text{CH}-\text{O}-$ group at position 8 was connected to one methyl group (at position 10 on the molecule), and one methine group (position 9) was coupled with one aldehyde group. The methine group at position 9 was related to the methine group at position 5 and then followed by the methylene group at position 6. Finally, one carboxylic acid at position 7 of the partial structure of fragment a was determined by HMBC data, as shown in Figure 7. Thus, HMBC measurement determined that the 176.09 ppm carbon signal ($\text{COO}-$) showed a strong cross-peak with the 2.27 and 2.95 ppm signals at H-6a and H-6b (C-6, 38.35 ppm ^{13}C NMR, methylene group).

For the partial structure of fragment b, HMBC data showed a strong correlation between the 167.18 ppm carbon signal

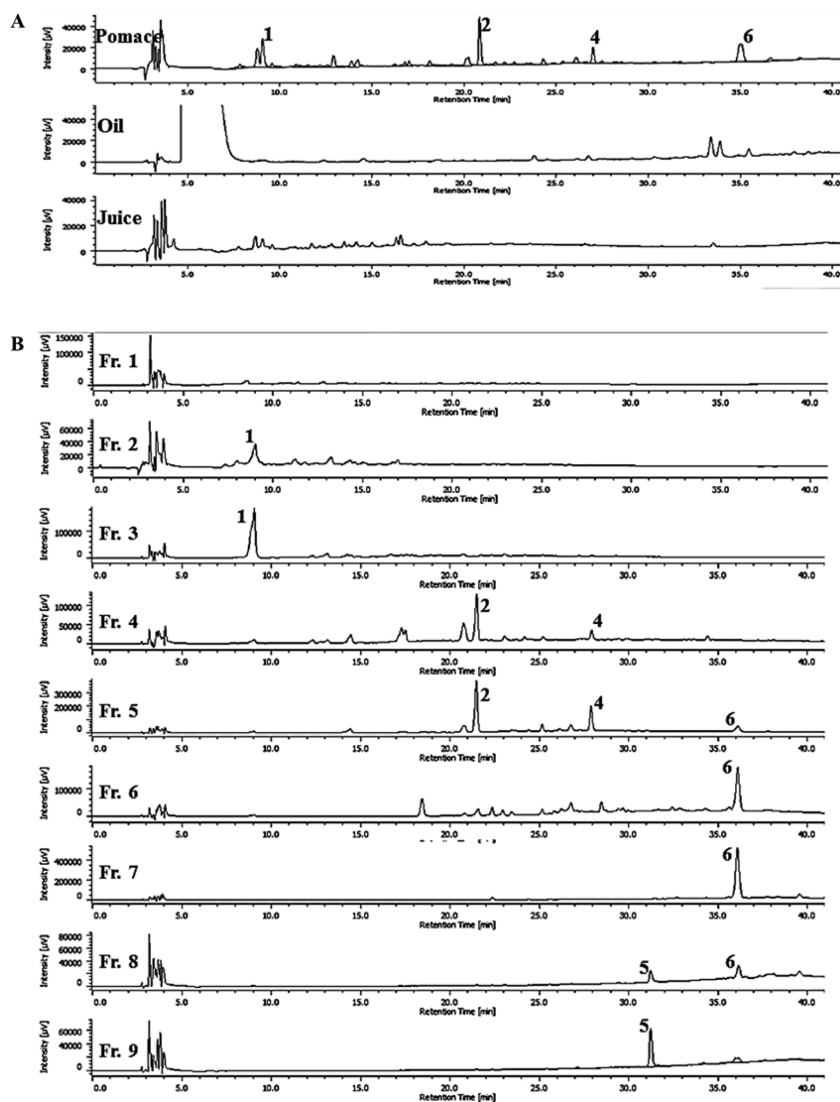


Figure 4. (A) HPLC chromatograms of extracts of pomace, oil, and juice. (B) HPLC chromatograms of nine fractions ($\lambda = 290$ nm). Compounds 1, 2, 4, and 5 were identified as hydroxytyrosol, verbascoside, oleuropein, and luteolin, respectively, by comparing their retention times and UV spectra of each peak to those of authentic compounds.

(COOMe) at position 11 and the 7.62 ppm signal at H-3 (C-3, 156.97 ppm ^{13}C NMR) and the 3.35 ppm signal at H-5 (C-5, 27.90 ppm ^{13}C NMR). Furthermore, the COOMe group (fragment b) at position 11 was connected with $\text{C}=\text{CH}-$ (Figure 7A) in the same manner. Finally, when fragments a and b are combined, the molecular formula of compound 3 was confirmed to be elenolic acid by comparing the MS spectra, spectra of ^1H and ^{13}C NMR,³¹ and UV spectra (λ_{max} of elenolic acid = 239 nm)³² (Figure 6).

Compound 6 (50% purity at 290 nm) in fraction 7 was hydrolyzed using 1% HCl for 24 h at room temperature to give elenolic acid and hydroxytyrosol (Figure 5A). The percentage of hydroxytyrosol calculated by standard curve was 24.3% in fraction 7 after hydrolysis. Elenolic acid was detected at 250 nm and found in a small amount in the hydrolysate and also in fractions 3–5 (Figure 5B).

Compound 6 should be an ester of hydroxytyrosol and elenolic acid because compound 3 and hydroxytyrosol were released by acid hydrolysis (Figure 5A). The chemical structure of compound 6 was determined by interpreting the mass spectra data and ^1H and ^{13}C NMR spectra, individually. The

mass spectra data of compound 6 showed the molecular ion at m/z 379.1373 ($[\text{M} + \text{H}]^+$, positive-ion mode) using high-resolution HPLC–TOFMS, giving the molecular formula as $\text{C}_{19}\text{H}_{22}\text{O}_8$ for compound 6. This molecular weight corresponds to the dehydrated compound of compound 3 ($\text{C}_{11}\text{H}_{14}\text{O}_6$) and hydroxytyrosol ($\text{C}_8\text{H}_{10}\text{O}_3$). Because the HMBC spectrum of compound 6 showed cross-peaks between C-7 and H-1' (Figure 7B), compound 6 should be 3,4-DHPEA-EA.^{32,33}

Antiallergic Active Compounds in Olive Pomace.

Table 2 shows the antiallergic activities among the metabolites identified in olive pomace. Luteolin showed the highest antiallergic activity and is expressed as IC_{50} at $0.752 \pm 0.1 \mu\text{g/mL}$. The second highest activity was observed with 3,4-DHPEA-EA, with IC_{50} at $33.5 \pm 0.6 \mu\text{g/mL}$. Because the activities of hydroxytyrosol and elenolic acid were weaker than 3,4-DHPEA-EA, the ester linkage of the two molecules should enhance the antiallergic activity several times.

Furthermore, the antiallergic activity of 3,4-DHPEA-EA was 30 times greater than that of oleuropein. It was reported that the glycoside linkage decreased the activity because many flavonoids show greater activity in the aglycone of flavonoids.

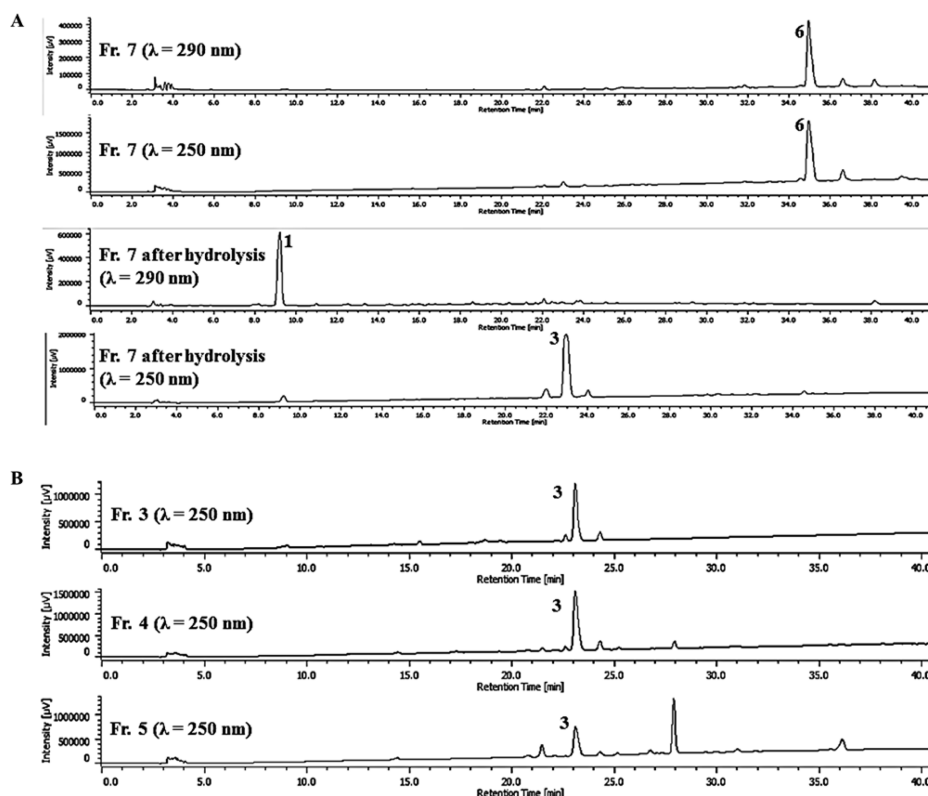


Figure 5. (A) HPLC chromatograms of fraction 7 before and after hydrolysis ($\lambda = 250$ and 290 nm). (B) HPLC chromatograms of fractions 3–5 ($\lambda = 250$ nm).

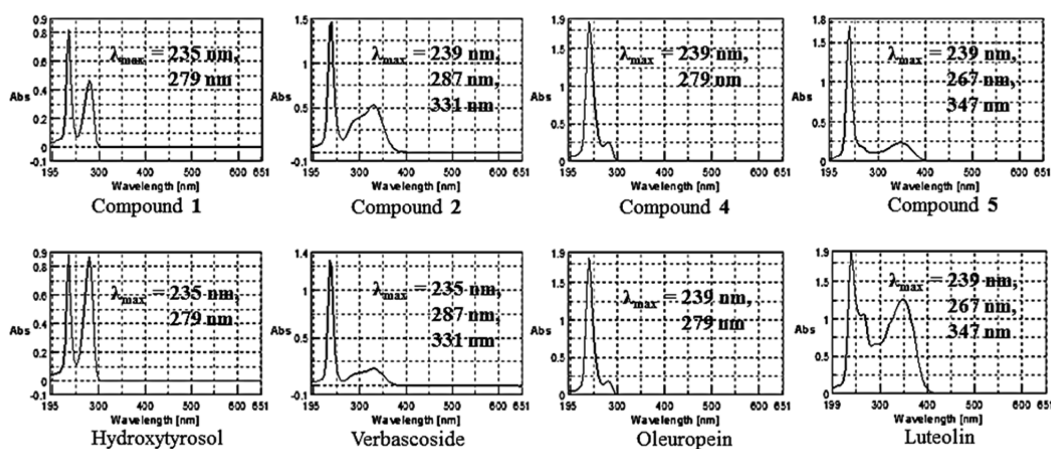


Figure 6. UV absorption spectra of the antiallergic substance in olive from the PDA detector of HPLC analyses. Sources of UV spectra: compound 1 (from fraction 3), compounds 2 and 4 (from fraction 5), and compound 5 (from fraction 9). λ_{max} of each compound is as follows: compound 1, 235 and 279 nm; hydroxytyrosol, 235 and 279 nm; compound 2, 239, 287, and 331 nm; verbascoside, 235, 287, and 331 nm; compound 4, 239 and 279 nm; oleuropein, 239 and 279 nm; compound 5, 239, 267, and 347 nm; and luteolin, 239, 267, and 347 nm.

The antiallergic activities of 3,4-DHPEA-EA (secoiridoid aglycone) and oleuropein (its glucoside) in olive pomace were consistent with those of flavonoids reported elsewhere.^{10,34}

Each metabolite was tested for the enzyme inhibition rate of hexosaminidase itself. No enzyme inhibition was observed. Therefore, each metabolite inhibits the release of hexosaminidase from RBL-2H3 cells but did not inhibit the activity of hexosaminidase.

Finally, the total antiallergic activities of the isolated compounds were compared among all metabolites, as shown in Table 2. 3,4-DHPEA-EA showed significant antiallergic activity because of its low IC_{50} and was more abundant in the olive pomace than in

luteolin. *p*-Hydroxyphenyl ethanol-decarboxymethyl elenolic acid ester dialdehyde [oleocanthal, *p*-HPEA-EDA, 3-(2-oxoethyl)-4-formyl-4-hexenoic acid 4-hydroxyphenethyl ester], one of the anti-inflammatory substances,¹⁶ and 3,4-dihydroxyphenyl ethanol-decarboxymethyl elenolic acid ester dialdehyde [3,4-DHPEA-EDA, 3-(2-oxoethyl)-4-formyl-4-hexenoic acid 3,4-dihydroxyphenethyl ester] are secoiridoid compounds in some olives. In further research, we identified oleocanthal and 3,4-DHPEA-EDA in only trace amounts in the pomace of Japanese Mission olive (data not shown; retention times of 3,4-DHPEA-EDA and oleocanthal were 25.6 and 31.2 min under the same analytical conditions shown in Figure 4, respectively).

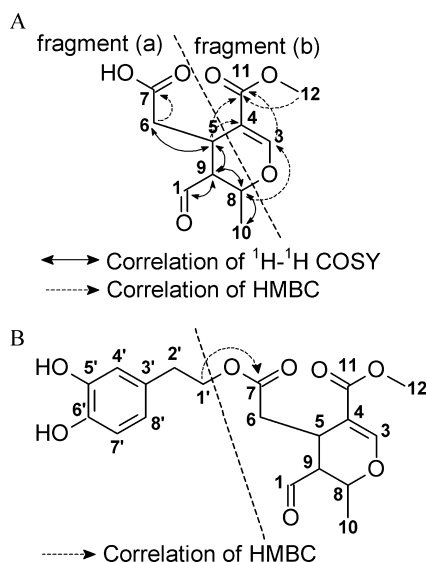


Figure 7. Chemical structure of (A) compound 3 and (B) compound 6 in olive.

The bioavailability of 3,4-DHPEA-EA and luteolin requires further study, especially in terms of antiallergic activity and the absorption rate of chemicals.¹³ 3,4-DHPEA-EA is soluble in ethanol, diethyl ether, or other organic solvents, but luteolin can be resolved only in ethanol or ethanol containing water but not in diethyl ether. There may be big differences in solubility, absorption efficiency, and affinity of 3,4-DHPEA-EA and

luteolin in neutral aqueous solution and physiological pH in the small intestine. Therefore, actual bioavailability in humans should be further studied.

Comparison of Active Chemicals in Three Kinds of Olive Fruit Using the QuEChERS Method. The QuEChERS method is commonly used to quantitatively analyze food residues and agrochemicals. The operation for solvent extraction, moisture removal, and recovery of agrochemicals can be performed simply and quickly. It is also suitable for a series of analyses of several samples.^{22,23} Therefore, the QuEChERS method to analyze the active chemicals in olive pomace was selected as an appropriate extraction procedure for samples (about 10 g) of three varieties of olive leaves in different seasons. The comparative analysis of the antiallergic substances in olive fruit is shown in Table 3. Of the three varieties used in this study, the green olive fruit of the Mission variety in October had the highest concentration of 3,4-DHPEA-EA (50.33 ± 1.18 mg/10 g of olive fruit) as determined by quantitative analysis using ODS-HPLC and then decreased with the change in seasons. On the other hand, the amounts of hydroxytyrosol and elenolic acid increased when the harvesting period was delayed to November or December. To obtain efficient amounts of antiallergic substances, early harvest in October at the green stage of maturation was found to be the most important factor. Indeed, the antiallergic activities (IC_{50} values) of the fruit extracts harvested in October, November, and December were 138.91 ± 8.53 $\mu\text{g/mL}$ for the October sample, 247.85 ± 3.34 $\mu\text{g/mL}$ for the green sample in November, 337.71 ± 10.03 $\mu\text{g/mL}$ for red sample in November,

Table 2. Comprehensive Antiallergic Activities of Substances Found in Olive Pomace Extract

compound	IC_{50} [$\mu\text{g/mL}$ (μM)]	enzyme inhibition (%) ^a	amount (mg/kg) ^b	total activity ^c	active unit ^d
3,4-DHPEA-EA (6)	33.5 ± 0.6 (88.7)	0.5 ± 1.7	1854	55343	350
elenolic acid (3)	377.9 ± 1.6 (1561.6)	18.4 ± 4.3	902	2386	15.1
luteolin (5)	0.752 ± 0.1 (2.6)	3.8 ± 0.8	1.04	1382	8.74
hydroxytyrosol (1)	305.3 ± 15.2 (1980.9)	0.9 ± 0.9	140	458	2.89
oleuropein (4)	891.2 ± 6.1 (1648.8)	0.7 ± 7.5	346	388	2.45
verbascoside (2)	788.0 ± 29.2 (1261.7)	15.7 ± 5.5	125	158	1.00

^aValues indicate enzyme inhibition (%) against β -hexosaminidase. Sample concentrations: hydroxytyrosol, 800 $\mu\text{g/mL}$; verbascoside, 800 $\mu\text{g/mL}$; luteolin, 2 $\mu\text{g/mL}$; 3,4-DHPEA-EA, 50 $\mu\text{g/mL}$; oleuropein, 800 $\mu\text{g/mL}$; and elenolic acid, 600 $\mu\text{g/mL}$. Each value represents the mean $\pm$ SD ($n = 4$). ^bThe amount was calculated and shown from green Mission olive harvested in December 2009. ^cTotal activity was estimated using the following formula: total activity = amount (mg/kg)/ IC_{50} (mg/mL). ^dActive unit was calculated on the basis of the relative intensity of total activity (total activity of verbascoside was defined as 1.00).

Table 3. Amount of Hydroxytyrosol Derivative and Elenolic Acid in QuEChERS Extract Olive Harvest in 2011^a

month	October	November	December
variety	Manzanillo	Mission	Mission
color	green	green	red
extract from 10 g of olives			
weight (mg)	181.33	205.35	116.33
yield (%)	1.81	2.05	1.16
hydroxytyrosol			
amount in 100 mg of extract (mg)	2.59 ± 0.02	2.45 ± 0.05	1.04 ± 0.02
amount in 10 g of fruits (mg)	4.71 ± 0.03	5.04 ± 0.09	1.21 ± 0.02
elenolic acid			
amount in 100 mg of extract (mg)	2.48 ± 0.05	3.05 ± 0.04	3.29 ± 0.01
amount in 10 g of fruits (mg)	4.51 ± 0.08	6.27 ± 0.07	3.83 ± 0.01
3,4-DHPEA-EA			
amount in 100 mg of extract (mg)	18.29 ± 0.09	24.50 ± 0.58	12.39 ± 0.08
amount in 10 g of fruits (mg)	33.18 ± 0.17	50.33 ± 1.18	14.42 ± 0.09

^aEach value represents the mean $\pm$ SD ($n = 3$).

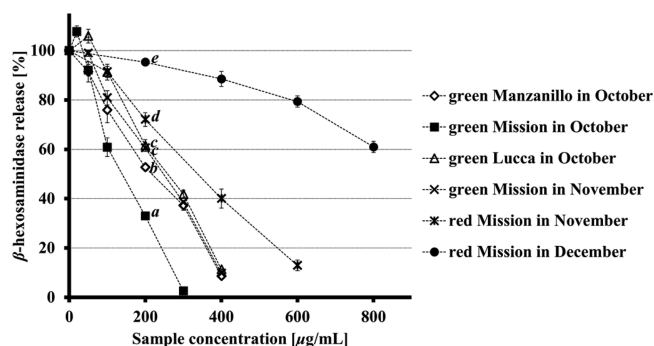


Figure 8. Difference in antiallergic activity of different olive varieties and harvesting seasons. Each value represents the mean $\pm$ SD ($n = 4$). Means with different letters are significantly different ($p < 0.05$).

and $919.83 \pm 23.25 \mu\text{g/mL}$ for the December sample, as shown in Figure 8. Antiallergic activities of methanol extracts at the $200 \mu\text{g/mL}$ concentration in different harvesting time were conducted and then concluded that green Mission in October exhibited the lowest β -hexosaminidase release ($32.9 \pm 0.5\%$), followed by green Manzanillo in October ($52.6 \pm 1.2\%$), with significant differences ($p < 0.05$) (Figure 8) in the earlier harvest time, the greater antiallergic activity, and the higher concentration of 3,4-DHPEA-EA. The antiallergic activities of the olive fruit extracts depended upon the amount of 3,4-DHPEA-EA.

In conclusion, 3,4-DHPEA-EA was found as an antiallergic active substance in olive pomace. To increase the consumption of olive oils, olive oil manufacturers need to consider the utility of olive juice and pomace. The Japanese Mission variety contained a higher amount of 3,4-DHPEA-EA as a major component of the pomace fraction and also as a major antiallergic substance.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

3,4-DHPEA-EA, 2-hydroxy-3-ethylidene-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-4-acetic acid 2-(3,4-dihydroxyphenyl)ethyl ester; oleocanthal, 3-(2-oxoethyl)-4-formyl-4-hexenoic acid 4-hydroxyphenethyl ester; 3,4-DHPEA-EDA, 3-(2-oxoethyl)-4-formyl-4-hexenoic acid 3,4-dihydroxyphenethyl ester; UV, ultraviolet; DEPT, distortionless enhancement by polarization transfer; COSY, correlated spectroscopy; HETCOR, heteronuclear correlation; HMBC, heteronuclear multiple-bond connectivity

REFERENCES

- (1) Hadley, C. Food allergies on the rise? Determining the prevalence of food allergies, and how quickly it is increasing, is the first step in tackling the problem. *EMBO Rep.* **2006**, *11*, 1080–1083.
- (2) Piftis, K. N.; Anthracopoulos, M. B.; Nikolaou-Papanagiotou, A.; Mantziou, V.; Paliatou, A. G.; Tzavelas, G.; Nicolaidou, P.; Mantzouranis, E. Increased sensitization in urban vs. rural environment—Rural protection or an urban living effect? *Pediatr. Allergy Immunol.* **2007**, *18*, 209–216.

(3) Morissette, G.; Lodge, R.; Bouthillier, J.; Marceau, F. Receptor-independent, vacuolar ATPase-mediated cellular uptake of histamine receptor-1 ligands: Possible origin of pharmacological distortions and side effects. *Toxicol. Appl. Pharmacol.* **2008**, *229*, 320–331.

(4) Shimoda, H.; Tanaka, J.; Yamada, E.; Morikawa, T.; Kasajima, N.; Yoshikawa, M. Anti type I allergic property of Japanese butterbur extract and its mast cell degranulation inhibitory ingredients. *J. Agric. Food Chem.* **2006**, *54*, 2915–2920.

(5) Zhu, F.; Asada, T.; Sato, A.; Koi, Y.; Nishiwaki, H.; Tamura, H. Rosmarinic acid extract for antioxidant, antiallergic, and α -glucosidase inhibitory activities, isolated by supramolecular technique and solvent extraction from *Perilla* leaves. *J. Agric. Food Chem.* **2014**, *62*, 885–892.

(6) Burda, S.; Oleszek, W. Antioxidant and antiradical activities of flavonoids. *J. Agric. Food Chem.* **2001**, *49*, 2774–2779.

(7) Ruby, A. J.; Kuttan, G.; Dinesh Babu, K.; Rajasekharan, K. N.; Kuttan, R. Anti-tumour and antioxidant activity of natural curcuminoids. *Cancer Lett.* **1995**, *94*, 79–83.

(8) Adams, B. K.; Ferstl, E. M.; Davis, M. C.; Herold, M.; Kurtkaya, S.; Camalier, R. F.; Hollingshead, M. G.; Kaur, G.; Sausville, E. A.; Rickles, F. R.; Snyder, J. P.; Liotta, D. C.; Shoji, M. Synthesis and biological evaluation of novel curcumin analogs as anti-cancer and anti-angiogenesis agents. *Bioorg. Med. Chem.* **2004**, *12*, 3871–3883.

(9) Vidya Priyadarsini, R.; Senthil Murugan, R.; Maitreyi, S.; Ramalingam, K.; Karunakaran, D.; Nagini, S. The flavonoid quercetin induces cell cycle arrest and mitochondria-mediated apoptosis in human cervical cancer (HeLa) cells through p53 induction and NF- κ B inhibition. *Eur. J. Pharmacol.* **2010**, *649*, 84–91.

(10) Matsuda, H.; Morikawa, T.; Ueda, K.; Managi, H.; Yoshikawa, M. Structural requirements of flavonoids for inhibition of antigen-induced degranulation, TNF- α and IL-4 production from RBL-2H3 cells. *Bioorg. Med. Chem.* **2002**, *10*, 3123–3128.

(11) Matsuda, H.; Morikawa, T.; Managi, H.; Yoshikawa, M. Antiallergic principles from *Alpinia galanga*: structural requirements of phenylpropanoids for inhibition of degranulation and release of TNF- α and IL-4 in RBL-2H3 cells. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3197–3202.

(12) Matsuda, H.; Tewtrakul, S.; Morikawa, T.; Nakamura, A.; Yoshikawa, M. Anti-allergic principles from Thai zedoary: structural requirements of curcuminoids for inhibition of degranulation and effect on the release of TNF- α and IL-4 in RBL-2H3 cells. *Bioorg. Med. Chem.* **2004**, *12*, S891–S898.

(13) Walle, T. Absorption and metabolism of flavonoids. *Free Radical Biol. Med.* **2004**, *36*, 829–837.

(14) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Delivery Rev.* **1997**, *23*, 3–25.

(15) Harai, N.; Takagi, M. Manufacturing of olive leaf tea. *Jpn. Kokai Tokkyo Koho*, JP 2006-191854A, 20060727, 2006.

(16) Beauchamp, G. K.; Keats, R. S. J.; Morel, D.; Lin, J.; Pika, J.; Han, Q.; Lee, C.; Smith, A. B.; Breslin, P. A. S. Ibuprofen-like activity in extra-virgin olive oil. *Nature* **2005**, *437*, 45–46.

(17) McDonald, S.; Prenzler, P. D.; Antolovich, M.; Robards, K. Phenolic content and antioxidant activity of olive extracts. *Food Chem.* **2001**, *73*–84.

(18) Gonzalez-Santiago, M.; Martín-Bautista, E.; Carrero, J. J.; Fonolla, J.; Baro, L.; Bartolome, M. V.; Gil-Loyzaga, P.; Lopez-Huertas, E. One-month administration of hydroxytyrosol, a phenolic antioxidant present in olive oil, to hyperlipemic rabbits improves blood lipid profile, antioxidant status and reduces atherosclerosis development. *Atherosclerosis* **2006**, *188*, 35–42.

(19) Romero, C.; Medina, E.; Vargas, J.; Brenes, M.; Castro, A. D. In vitro activity of olive oil polyphenols against *Helicobacter pylori*. *J. Agric. Food Chem.* **2007**, *55*, 680–686.

(20) Peak, M. J.; Peak, J. G. Hydroxy radical quenching agents protect against DNA breakage caused by both 365-nm UVA and by gamma radiation. *Photochem. Photobiol.* **1990**, *51*, 649–652.

(21) Tyrrell, R. M.; Pidoux, M. Singlet oxygen involvement in the inactivation of cultured human fibroblasts by UVA (334 nm, 365 nm)

and near-visible (405 nm) radiations. *Photochem. Photobiol.* **1989**, *49*, 407–412.

(22) Payá, P.; Anastassiades, M.; Mack, D.; Sigalova, I.; Tasdelen, B.; Oliva, J.; Barba, A. Analysis of pesticide residues using the quick, easy, cheap, effective, rugged, and safe (QuEChERS) pesticide multiresidue method in combination with gas and liquid chromatography and tandem mass spectrometric detection. *Anal. Bioanal. Chem.* **2007**, *389*, 1697–1714.

(23) Lehotay, S. J.; Son, K. A.; Kwon, H.; Koesukwiwata, U.; Fu, W.; Mastovska, K.; Hoh, E.; Leepipatpiboon, N. Comparison of QuEChERS sample preparation methods for the analysis of pesticide residues in fruits and vegetables. *J. Chromatogr., A* **2010**, *1217*, 2548–2560.

(24) Goto, T.; Kondo, T.; Tamura, H.; Kawahori, K.; Hattori, H. Structure of platyconin, a diacylated anthocyanin isolated from the Chinese bell-flower *Platycodon grandiflorum*. *Tetrahedron Lett.* **1983**, *24*, 2181–2184.

(25) Asada, T.; Tamura, H. Isolation of bilberry anthocyanidin 3-glycosides bearing *ortho*-dihydroxyl groups on the B ring by forming an aluminum complex and their antioxidant activity. *J. Agric. Food Chem.* **2012**, *60*, 10634–10640.

(26) Davidek, J.; Velisek, J.; Kubelka, V.; Janicek, G.; Simicova, Z. Glycerol chlorohydrins and their esters as products of the hydrolysis of tripalmitin, tristearin and triolein with hydrochloric acid. *Z. Lebensm.-Unters. Forsch.* **1980**, *171*, 14–17.

(27) Sugiyura, Y.; Torii, T.; Matsuda, K.; Yamada, Y. Anti-allergic effects of extracts from commercial products of cooked burdock. *Food Sci. Technol. Res.* **2009**, *15*, 423–426.

(28) Visioli, F.; Bellomo, G.; Galli, C. Free radical-scavenging properties of olive oil polyphenols. *Biochem. Biophys. Res. Commun.* **1998**, *247*, 60–64.

(29) Cheong, H.; Ryu, S. Y.; Oak, M. H.; Cheon, S. H.; Yoo, G. S.; Kim, K. M. Studies of structure activity relationship of flavonoids for the anti-allergic actions. *Arch. Pharm. Res.* **1998**, *21* (4), 478–480.

(30) Kimata, M.; Shichijo, M.; Miura, T.; Serizawa, I.; Inagaki, N.; Nagai, H. Effects of luteolin, quercetin and baicalein on immunoglobulin E-mediated mediator release from human cultured mast cells. *Clin. Exp. Allergy* **2000**, *30*, 501–508.

(31) Briante, R.; La Cara, F.; Pia Tonziello, M.; Febbraio, F.; Nucci, R. Antioxidant activity of the main bioactive derivatives from oleuropein hydrolysis by hyperthermophilic β -glycosidase. *J. Agric. Food Chem.* **2001**, *49*, 3198–3203.

(32) Inarejos-Garcia, A. M.; Androulaki, A.; Salvador, M. D.; Fregapane, G.; Tsimidou, M. Z. Discussion on the objective evaluation of virgin olive oil bitterness. *Food Res. Int.* **2009**, *42*, 279–284.

(33) Perez-Trujillo, M.; Gomez-Caravaca, A. M.; Segura-Carretero, A.; Fernandez-Gutierrez, A.; Parella, T. Separation and identification of phenolic compounds of extra virgin olive oil from *Olea europaea* L. by HPLC-DAD-SPE-NMR/MS. Identification of a new diastereoisomer of the aldehydic form of oleuropein aglycone. *J. Agric. Food Chem.* **2010**, *58*, 9129–9136.

(34) Itoh, T.; Ohguchi, K.; Nakajima, C.; Oyama, M.; Iinuma, M.; Nozawa, Y.; Akao, Y.; Ito, M. Inhibitory effects of flavonoid glycosides isolated from the peel of Japanese persimmon (*Diospyros kaki Fuyu*) on antigen-stimulated degranulation in rat basophilic leukemia RBL-2H3 cells. *Food Chem.* **2011**, *126*, 289–294.