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Antiallergic Effect of *Picholine* Olive Oil-in-Water Emulsions through β-Hexosaminidase Release Inhibition and Characterization of Their Physicochemical Properties

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ABSTRACT: The inhibitory effect of Picholine olive oil from Montpellier in Southern France on the chemical mediator release in type I allergy, using rat basophilic leukemia (RBL-2H3) cells, was investigated. Oil-in-water (O/W) emulsions prepared using Picholine olive oil showed an inhibitory effect on the chemical mediator release and decreased expressions of genes related to type I allergy in RBL-2H3 cells. We then measured the phenolic compounds present in Picholine olive oil using high-performance liquid chromatography and investigated some physical properties, such as droplet size, size distribution, viscosity, and surface tension of the resulting olive O/W emulsions. Our findings indicate that Picholine olive oil has high flavonoids content, especially apigenin, and the prepared emulsion of Picholine olive oil resulted in a considerable small size distribution, with an average droplet size of 170 nm.

KEYWORDS: *Southern France olive oil, β-hexosaminidase inhibitory effect, phenol content, physical property*

INTRODUCTION

Allergy is classified into type I to type IV allergy according to a pathogenic mechanism. The incidence of type I allergy, such as hay fever, atopy, allergic rhinitis, food allergy, and allergic asthma, has gradually increased in recent decades, particularly in developed countries. Current drugs that are used to treat type I allergy, such as chemical mediator release inhibitors, antihistamines, and even anti-IgE antibodies, aim to intervene in or suppress effecter phase responses. These drugs can suppress certain symptoms or part of the allergic inflammatory process but fail to regulate the complete allergic cascade.[1] For example, the prevalence of Japanese cedar hay fever in the general population in Japan was estimated to be 24.5% in 2004.[2]

On the other hand, the mortality rate of the Mediterranean population due to cardiovascular disease, cancer, Parkinson's disease, and Alzheimer's disease is lower than those of the European region and America. Furthermore, the morbidity rate in the Mediterranean region due to type I allergy, especially, hay fever and atopy, is lower than in Japan.[3] There are, however, no differences between the Japanese and the Mediterranean diets with regard to the intake of fish, bean, vegetables, and cereals, except for the use of olive oil in the Mediterranean region.

For centuries, olive oil produced in a traditional manner has been a key contributor to good health to many civilizations: Greeks, Romans, and Europeans. Olive oil is produced in a relatively small scale using the traditional method to maintain most of its health benefits, without damaging its natural nutrients (such as vitamins, essential fatty acids, and antioxidants). The phenolic compounds naturally present in olive oil and leaves are complex mixtures of constituents, including 3,4-dihydroxyphenylethanol (hydroxytyrosol), 4-hydroxyphenylethanol (tyrosol), 4-hydroxyphenylacetic acid, protocatechuic acid, caffeic acid, *p*-coumaric acid, apigenin, and luteolin, among others.[4,5] Hydroxytyrosol and oleuropein have been shown to possess anti-inflammatory, bactericidal, and bacteriostatic activities.[6,7] Moreover, hydroxytyrosol has been shown to have an anticancer effect on human colon adenocarcinoma HT-29 cells and human promyelocytic leukemia HL-60 cells,[8,9] besides having anti-melanogenesis activity, whereas oleuropein has been reported to inhibit the cell growth of LN-18, poorly differentiated glioblastoma; TF-1a, erythroleukemia; 786-O, renal cell adenocarcinoma; T-47D, infiltrating ductal carcinoma of the breast pleural effusion; RPMI-7951, malignant melanoma of the skin-lymph node metastasis; and LoVo, colorectal adenocarcinoma cells.[10] Seemingly, apigenin has been reported to have significant anti-inflammatory activity that involved blocking NO-mediated cyclooxygenase-2 expression and monocyte adherence.[11] Apigenin and luteolin also inhibited significantly TNFα-induced NF-kB transcriptional activation in KF-8 cells.[12] In our previous study, Tunisian olive oils, which showed an inhibitory effect on β-hexosaminidase release at the antigen−antibody binding stage on type I allergy by rat basophilic leukemia (RBL-2H3) cells, had higher apigenin and luteolin contents than other olive oils.[13] The health benefits associated with diets high in foods containing antioxidants have been studied for many

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decades. The bioavailability of those active substances plays a critical role in the development of their delivery systems. For instance, flavonoids are well-known for their antioxidant activity. Additionally, at high experimental concentrations that would not exist in vivo, the antioxidant activities of flavonoids in vitro are stronger than those of vitamins C and E.[14] The challenge for functional food manufacturers is to formulate easy to disperse emulsions that are suitable for various applications. The optimization of processing conditions, choice of emulsifier, and other ingredients are the most important variables to take into consideration to achieve the optimum droplet size and optimal color for each application. As differences in particle size may also give rise to variations in color shade, a good process control is essential during the manufacture of coloring emulsions.[15] In this study, we investigated the effect of the compounds present in Picholine olive oil from Southern France on the chemical mediator release from RBL-2H3 cells. To gain more information regarding the mechanism for the suppression of degranulation following Picholine olive oil-in-water (O/W) emulsion treatment, we studied the effect of Picholine olive O/W emulsion on type I allergic reaction in RBL-2H3 cells using microarray analysis. Liquid chromatography with mass spectrometry and high-performance liquid chromatography (HPLC) coupled with a diode array detector (DAD) were used for the identification of 14 phenolic compounds and, more specifically, the quantification of apigenin and luteolin. HPLC is still the most popular method of choice for the analyses of phenolic compounds in olive oil. To extract the polar fraction, a simple and fast method-based liquid–liquid extraction (LLE) was followed.[16] Moreover, we formulated an O/W emulsion of Picholine olive oil from Southern France, followed by the characterization of their physical properties, such as droplet size, size distribution, viscosity, and surface tension, of the resulting olive oil.

MATERIALS AND METHODS

Safety: The authors declare that there were no issues related to human hazard during the term of this research work, neither upon handling the chemicals nor while performing the protocols described herein.

Picholine Olives from Montpellier. Oils extracted from Picholine olive, of the Picholine variety, from Montpellier in Southern France, were used in this study.

Reagents, Cell Lines, and Culture Media. Eagle's minimum essential medium (MEM) was purchased from Nissui Pharmaceutical Co. (Japan). Fetal bovine serum (FBS) was purchased from Hyclone Co. (Japan), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) and 2-morpholinoethanesulphonic acid (MES) were purchased from Dojindo (Japan). Dinitrophenylated bovine serum albumin (DNP-BSA) was obtained from Cosmo Biotechnology Co. (Japan), and anti-DNP-IgE, ketotifen and L-glutamine were purchased from Sigma (Japan). RBL-2H3 cells were purchased from Riken Cell Bank, Japan, and cultivated in MEM supplemented with 10% FBS and 2 mM L-glutamine at 37 °C in a 5% CO_2 incubator.

Formic acid, acetonitrile, and methanol were purchased from Carlo Erba (Milan, Italy). Distilled water was deionized using the Milli-Q system (Millipore, Waters, Milford, MA). Ten selected phenolic compound standards were purchased from different companies as follows: apigenin from WAKO; luteolin, gallic acid, vanillic acid, acid 3,4-dihydroxybenzoïque (protocatechic acid), ferulic acid, cafeic acid, syringic acid, *p*-coumaric acid, and (±)-*α*-tocopherol from Sigma (St. Louis, MO); sinnapic acid, tyrosol, and kaempferol from Fluka (Steinheim, Germany); and hydroxyl-tyrosol and oleuropein from Extrasynthese (Genay, France).

Preparation of O/W Emulsions. Picholine olive oil was used as the disperse phase. The continuous phase consisted of a mixture containing the following culture media: Eagle's MEM (pH 7.4) and FBS, 9:1 (v/v), respectively. Premixtures containing an oil-to-water weight ratio of 1:9 were used in all experiments. Fine emulsions were prepared by sonication in a water bath type sonicator for 15 min (model US-102, power 100 W, frequency 38 kHz, SND Corp., Japan).

***β*-Hexosaminidase Inhibition Assay.** In type I allergy reactions in mast/basophil cells, the binding of antigens and antibodies is a direct cause of intracellular organelle flows such as histamine and *β*-hexosaminidase. Therefore, to determine the inhibitory effect of Picholine olive oil on the chemical mediator release, *β*-hexosaminidase inhibition assays were performed according to the method described by Kawasaki et al.[17] For the *β*-hexosaminidase inhibition assay at the antigen–antibody binding stage, RBL-2H3 cells were seeded onto a 96-well plate at 5.0×10^5 cells/mL in 100 μL of medium. The cells were incubated and sensitized for 24 h at 37 °C and 5% CO_2 with 0.3 μg/mL anti-DNP-IgE. The cells were then washed twice with PBS to eliminate free IgE. After the cells were incubated at 37 °C for 10 min in 60 μL/well of a releasing mixture (116.9 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 5.6 mM glucose, 25.0 mM HEPES, 2.0 mM $CaCl_2$, and 1.0 mg/mL BSA at pH 7.7) containing 5 μL/well of Picholine olive O/W emulsion (dilution of 1/1000, 1/100, and 1/50 in medium), the cells were exposed to 5 μL/well of 4 μg/mL DNP-BSA in PBS (−) and then incubated again at 37 °C for 1 h. As a positive control, 3 mM ketotifen was used. The plates were then put on ice for 10 min to terminate reactions before 20 μL of supernatant was transferred to another plate; 80 μL of substrate solution (5 mM 4-nitrophenyl *N*-acetyl-*β*-D-glucosaminide in a 50 mM $C_6H_8O_7$ buffer at pH 4.5) was added to the supernatant and incubated at 37 °C for 30 min. Then, 100 μL/well of a stop buffer (0.1 M $NaHCO_3/Na_2CO_3$, pH 10) was added, and the absorbance at 405 nm was obtained using the multidetection microplate reader to measure the total activity of *β*-hexosaminidase. The percentage inhibition rate of *β*-hexosaminidase release from RBL-2H3 cells by Picholine or Lucques olive oil was calculated using eq 1, as follows:

$$\text{inhibition rate (\%)} = \{1 - (T - B)/(C - B)\} \times 100 \quad (1)$$

where the test assay (T) contained cell (+), DNP-BSA (+), and the test sample (+); the blank assay (B) contained cell (−), DNP-BSA (+), and the test sample (+); and the control assay (C) contained cell (+), DNP-BSA (+), and the test sample (−).

MTT Assay. The MTT assay is a sensitive and quantitative colorimetric assay that is used to determine the cytotoxicity of potential medicinal agents and other toxic materials.[18] The RBL-2H3 cells were seeded onto 96-well plates at 5.0×10^5 cells/mL in 100 μL of medium. The cells were incubated (37 °C, 5% CO_2) overnight to allow them to attach to the wells. After incubation, cultured cells were washed with PBS (−), and 100 μL of Picholine olive O/W emulsion dissolved in the medium was added to obtain final dilutions of 1/1000, 1/100, and 1/50. The cells were incubated for 24 h after which 10 μL of 5 mg/mL MTT solution was added to each well. Cells were incubated for another 24 h to allow the MTT to be metabolized. Next, 100 μL of 10% sodium dodecyl sulfate (SDS) was added, followed by another 24 h of incubation to completely dissolve the formazan produced by the cells. The absorbance of the converted dye was measured at a wavelength of 570 nm with a multidetection microplate reader (Power Scan HT, Dainippon Pharmaceutical Co.). Blanks were prepared at the same time to correct for the absorbance caused by sample color and by the inherent ability of samples to reduce MTT in the absence of cells. The optical density of the formazan produced by the untreated control cells was considered to represent 100% viability.

Total RNA Isolation. The RBL-2H3 cells at a concentration of 5.0×10^5 cells/mL were incubated and sensitized for 24 h at 37 °C and 5% CO_2 with 0.3 μg/mL anti-DNP-IgE. The cells were then washed twice with PBS to eliminate free IgE and treated with Picholine olive O/W emulsion (1/10 dilution rate) for 10 min and incubated at 37 °C, after which DNP-BSA was added, and the cells were incubated further for 0.5 h. DNA-free total RNA was isolated from the cells using Isogen reagent (Nippon Gene Co., Tokyo, Japan) following the manufacturer's instruction.

DNA Microarray Analyses. Rat genome-wide gene expression was examined using the GeneChip system RG 230-PM microarray (Affymetrix Inc., Santa Clara, CA), which is comprised of over 31000

probe sets representing approximately 28700 well-characterized rat genes on a single array.[19] Double-stranded cDNA was synthesized from 100 ng of total RNA with the GeneAtlas 3′ IVT Express Kit (Affymetrix, Inc.). Biotin-labeled aRNA was then synthesized by in vitro transcription using the GeneChip 3′ IVT Express Kit (Affymetrix, Inc.). After fragmentation, 7.5 μg of labeled aRNA was hybridized to the oligonucleotide microarray. The chips were washed and stained in the GeneAtras Fluidics Station 400 (Affymetrix) and then scanned with the GeneAtras Imaging Station (Affymetrix). Data analysis was performed using the Partek Express software (Affymetrix). Using the Affymetrix-defined comparison mathematical algorithms, a fold change in expression between each of the infected samples in comparison to the control (IgE sensitization/DNP-BSA stimulation) was calculated and transformed to log2 data.

HPLC Analysis of Luteolin and Apigenin. The olive oil was prepared as a LLE procedure according to the method developed by Pirisi et al. with some modifications.[16] Oil (100 mL) was added to 100 mL of methanol in a separating funnel. The mixture was stirred for 10 min after which 20 mL of distilled water and 50 mL of *n*-hexane were added to separate the methanol layer. All of the extracts were combined and washed twice. The methanol extract was then evaporated to dryness on rotary evaporator at 30 °C under reduced pressure. The aqueous residue was lyophilized, prior to storage, and the dry residue was dissolved in 10 mL of methanol prior to injecting 10 μL into the chromatographic system.

The analysis method uses HPLC coupled with UV detector diode array followed by a mass spectrometer type trap LCQ Thermo advantage equipped with electrospray ionization, controlled with the software EXCALIBUR brand ThermoFisher. The HPLC system was equipped with a separation column ACE C18 250 mm × 4.6 mm, 5 μm. Gradient elution occurred with (A) acetonitrile and (B) 0.01% formic acid in water (pH 3.8) at a flow rate of 700 μL/min. The linear gradient is shown in Table 1. The total run cycle was therefore 75 min. An improved liquid chromatographic (LC) tandem mass spectrometry method has been developed.[20,21] The DAD scan (200−600 nm) was used for the identified phenolic and flavonoid compounds. The wavelength used for the quantification of luteolin and apigenin was set at 340 nm. The identity of phenolic and flavonoid compounds was confirmed by LC-mass spectrometry. Mass spectrometric data were acquired in single ion recording (SIR) mode. All of the compounds were characterized by the deprotonated molecules $(M - H)^-$ at m/z shown in Table 2. Mass spectrometric data were collected with optimized following optimized MS parameter settings for the five segments shown in Table 3.

Table 1. Gradient of Elution

time (min)	flow (mL/min)	A (%)	B (%)
0.0	0.7	95.0	5.0
4.0	0.7	95.0	5.0
45.0	0.7	65.0	35.0
50.0	0.7	0.0	100.0
60.0	0.7	0.0	100.0
61.0	0.7	95.0	5.0
72.0	0.7	95.0	5.0

Calibration curves of luteolin and apigenin were prepared to estimate their concentration in olive oils. Stock solutions of each compound were prepared at a concentration of 1 g/L in methanol and stored at 4 °C. Working solutions of luteolin and apigenin were extemporaneously diluted in methanol to obtain four working standard solutions with concentration ranges between 10 and 100 mg/L.

Droplet Size Measurement. Measurements of the mean droplet size of the O/W emulsions obtained were performed using a dynamic light scattering particle size analyzer that had a measurement range of 0.6 nm to 6 μm (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, United Kingdom). Refractive indices of 1.47 for olive oil and 1.33 for water were used to calculate the mean droplet size. The droplet concentration in the sample was diluted 10 times with the continuous phase prior to analysis to avoid multiple scattering effects during measurement. The measurements were conducted at a temperature of 25 °C. The mean droplet size was calculated from the average of at least three measurements. The droplet size distributions of all samples were expressed as number frequency.

Table 2. Molecular Masses (Deprotonated), Fragments Characteristic Wavelength, and Maximum Retention Times of Various Standard Compounds

	$[M - H]^-$ m/z			
name	precursor	fragment	λ_{max} (nm)	Tr (min)
gallic acid	169.1	125.1	214.0/270.0	7.9
protocatechuic acid	153.0	109.1	259.0/293.0	14.9
hydroxytyrosol	153.1	123.1	280.0	15.3
tyrosol	137.1	92.7	222.0/276.0	20.6
vanillic acid	167.0	123.0/152.0	260.0/292.0	23.4
caffeic acid	179.0	135.0	295.0/332.0	24.3
syringic acid	197.0			24.9
p-coumaric acid	163.0	119.0	309.0	30.1
sinapic acid	223.0	179.0/208.1	322.0	32.2
ferulic acid	193.0	149.1	322.0	32.4
oleuropein	539.0	376.9 (total fragmentation)	230.2/281.0	40.2
luteolin	285.2		348.0	45.8
apigenin	269.1		267.0/337.0	51.4
kaempferol	285.0		366.0	52.0

Table 3. MS Parameter of Acquisition

segment (run time, min)	m/z (MS/MS)	m/z scan
1 (0.0−17.0)	169.5, 153.0	50.0−2000.0
2 (17.0−28.3)	167.0, 137.1, 153.1, 179.0, 197.0	300.0−1000.0
3 (28.3−45.3)	163.0, 223.0, 193.0, 539.0	50.0−1500.0
4 (45.3−49.3)	285.0	50.0−2000.0
5 (49.3−60.0)	285.0, 269.0	50.0−2000.0

Surface Tension Measurement. Surface tension between the liquid phase (droplets formed at the tip of a syringe) and the surrounding air phase was measured using an automatic interfacial tensiometer (PD-W, Kyowa Interface Science Co., Saitama, Japan) adopting the pendant drop method.[22] In brief, the shape of a drop of oil formed at the tip of a syringe can be determined from the balance of forces, which includes the surface tension of the liquid measured. The surface tension can be related to the droplet shape by using the Young−Laplace equation (eq 2), as follows:

$$\gamma = \frac{\Delta\rho \cdot g \cdot {R_0}^2}{\beta} \tag{2}$$

where γ = interfacial tension, $\Delta\rho$ = the difference in density between fluids at interface, g = gravitational constant, R_0 = radius of the drop curvature at apex, and β = shape factor.

β can be determined empirically by using computational methods. The software FAMAS was used in this study. All surface tension measurements were conducted using the same apparatus and protocol for bulk oils and O/W emulsions; the experimental data expressed in mN/m are shown here as mean values ± SDs (n = 10 repetitions).

Viscosity Measurement. The viscosity of bulk oils was measured using a vibro-viscometer (SV-10, A&D Co. Ltd., Japan), which measures viscosity by controlling the amplitude of the sensor plates immersed in a sample and measuring the electric current to drive the sensor plates. The shear rate of the sensor plates keeps periodically circulating from zero to peak shear rate (42−130 s^{-1}) because sine-wave vibration with a frequency of 30 Hz and amplitude of approximately 0.2 mm is utilized. The viscosity measurement range of this equipment is between 0.3 and 10000 mPa·s. In fact, considering that vegetable oils in general, such as

olive oil, are intrinsically Newtonian fluids, with their shear rate proportional to the shear stress, viscosity measurements may be conducted without determining the value of the shear rate or shear stress. All measurements were performed at 25 °C, and the results are expressed as mean values ± SDs of 10 replicates.

Statistical Analysis. Statistical analysis was performed on the surface tensions data through the analysis of variance (ANOVA) test with Statistica for Windows release 5.1B software (StatSoft, Inc., Tulsa, OK). Experiments were designed using a single factor (surface tension) and fixed effects model with three replicates. Treatment means were compared using Duncan's multiple range test.[23]

RESULTS AND DISCUSSION

Inhibitory Effect of Picholine Olive O/W Emulsions on β-Hexosaminidase Release at the Antigen−Antibody Binding Stage. Type I allergy occurs due to environmental substances known as allergens present in foods, dust, medicine, cosmetics, and pollen. This class of antigens induces the production of antigen-specific IgE antibodies that bind to FcεRI receptors in mast cells or basophilic cells. Allergic reaction is generally divided into two components: the early phase reaction and the late phase reaction. The early phase reaction in type I allergy occurs within minutes, and then, mediators such as histamine and β-hexosaminidase are released from the cell.[24] These chemical mediators induce vasodilatation, mucous secretion, and bronchoconstriction. The RBL-2H3 cells are considered as a good cell line model for studying the effect of unknown compounds on β-hexosaminidase release activity because it displays characteristics of mucosal type mast cells and express several hundred thousand IgE receptors on the membrane surface.[25] In this study, we assayed β-hexosaminidase-release inhibitory effect of Picholine olive O/W emulsion using this cell line (RBL-2H3 cells). The inhibitory effects of the O/W emulsion on β-hexosaminidase release by RBL-2H3 cells are shown in Figure 1. The β-hexosaminidase release from IgE-sensitized RBL-2H3 cells was induced by DNP-BSA as a stimulatory antigen at the antigen−antibody's binding stage. Picholine olive oil had an inhibitory effect on β-hexosaminidase release from RBL-2H3 cells dose dependently (compare to stimulation by DNP-BSA). We compared the effect of our samples with the clinically available antiallergic drug, ketotifen fumarate (Keto.), which is known as a mast cell stabilizer, H1-receptor antagonist, and an eosinophil inhibitor.[26,27] The O/W emulsion based on Picholine olive was found to have a similar inhibitory effect on β-hexosaminidase release from RBL-2H3 cells at 1/50 dilution as compared to Keto. (final 214 μM, IC_{50} = 200−300 μM). Meanwhile, the Picholine olive O/W emulsion in the dilution rate of 1/1000−1/50 did not cause any cytotoxicity after treating the RBL-2H3 cells after 24 h of treatment (data not shown).

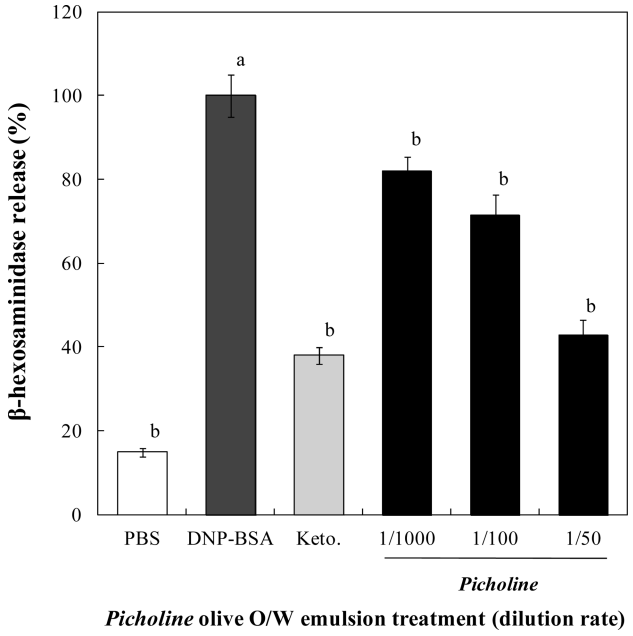

Figure 1. Inhibitory effect of Picholine olive O/W emulsions on the β-hexosaminidase release from RBL-2H3 cells. Results represent one trial (n = 4−6). Additional trials showed similar results. Different superscripts indicate statistical differences (P < 0.01).

Gene Expression Profile of Picholine Olive O/W Emulsion-Treated Cells. Microarray analysis is a technique that has been shown to be useful for the simultaneous profiling of global gene expression and uncovering new genes or new functions of known genes.[28] We used microarray analysis to further investigate the mechanism of suppression of degranulation in cells following Picholine olive O/W emulsion treatment and to determine which genes were differentially expressed in response to Picholine olive O/W emulsion in IgE-sensitized/BSA-stimulated RBL-2H3 cells. The late phase reaction occurs hours after the early phase in type I allergies, and mediators such as inflammatory cytokines IL13, IL4, IL5, IL6, and TNF-α are then secreted from the affected cells. These cytokines are involved in initiating and perpetuating the host immune response to allergens. The modulation of the secretion of cytokines from mast cell can therefore be used as a useful therapeutic strategy for allergic inflammatory disease.[29] A comparison of the up- with down-regulated cytokine genes and transcription factors in RBL-2H3 cells, treated with Picholine olive O/W emulsion with IgE/DNP-BSA, is shown in Table 4. Moreover, possible FcεRI-mediated signaling pathways in mast cells are indicated in Figure 2. The KEGG database was used as a reference for significant known pathway of type I allergy (http://www.genome.jp/kegg-bin/show_pathway?org_name=rno&mapno=04664&mapscale=1.0&show_description=show). The Picholine olive O/W emulsion inhibited type I allergy-related genes that coded for the synthesis of a specific protein in intracellular signaling transduction pathway, although the activation of pathways is regulated both positively and negatively by the interactions of numerous signaling molecules. Our data showed that Picholine olive O/W emulsion would inhibit the cytokine genes expressions through the inhibition of the FcεRI signaling pathway (Figure 2).

Quantification of Luteolin and Apigenin. The concentrations of apigenin and luteolin in the olive oil extract of Picholine were, respectively, 9.7 and 18.2 mg/L.

Physicochemical Properties of Picholine Olive Oil. The average viscosity of Picholine olive oil was 72.4 ± 6.3 mPa s (n = 10 repetitions), which is relatively higher, as compared to other vegetable oils. For reference, the viscosity of refined soybean oil is around 54 ± 5.1 mPa s. This viscosity difference could be attributed to the higher unsaturated fatty acids content of Picholine olive oil as compared to soybean oil. For instance, unsaturated fatty acids are frequently in *cis* conformation and have less linear chains as compared to saturated fatty acids. Further experiments may be needed to confirm such compositional differences for Picholine olive oil.

Changes on Physicochemical Properties of Picholine Olive Oil, upon Formulation into O/W Emulsions. As

Table 4. Comparison of Up- or Down-Regulated Genes in RBL-2H3 Cells Treated with Picholine Olive O/W Emulsion with IgE/DNP-BSA

functional category and gene name	gene symbol	accession no.	fold change in gene expression in response to Picholine olive O/W emulsion
cytokine			
interleukin 3 (colony-stimulating factor, multiple)	IL3	NM_000588	−1.21
interleukin 4	IL4	NM_000589 /// NM_172348	−1.1
interleukin 5 (colony-stimulating factor, eosinophil)	IL5	NM_000879	−1.2
interleukin 6 (interferon, β 2)	IL6	NM_000600	1.04
interleukin 8	IL8	NM_000584	−1.07
interleukin 13	IL13	NM_002188	−1.2
tumor necrosis factor	TNF	NM_000594	−1.12
transcription facter			
jun oncogene	JUN	NM_002228	−1.22
v-akt murine thymoma viral oncogene homologue 2	ATF-2	NM_001626	−1.27
FBJ murine osteosarcoma viral oncogene homologue	FOS	NM_005252	−1.31

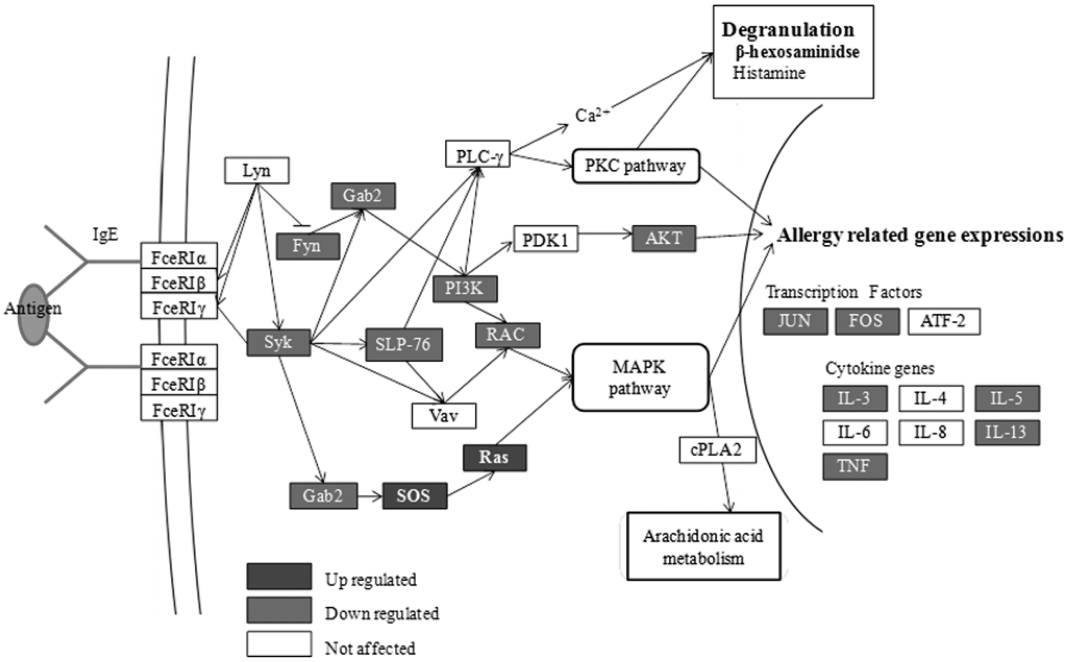

Figure 2. Intracellular signal transduction pathways activated by Picholine olive O/W emulsion (1/10 dilution rate) on IgE-sensitized/DNP-BSA-stimulated type I allergy in RBL-2H3 cells. The KEGG pathway was used as a reference for significant known pathway of type I allergy (http://www.genome.jp/kegg-bin/show_pathway?org_name=rno&mapno=04664&mapscale=1.0&show_description=show). Ten percent different expressed genes were regulated by Picholine olive O/W emulsion treatment and compared to the control (IgE-sensitized and DNP-BSA-stimulated) selected.

indicated in Figure 3, the surface tension of bulk Picholine olive oil is considerably lower, as compared to the O/W emulsion, upon emulsification. Whereas the surface tension values of both oils in bulk form are nearly the same, upon formulation, Picholine olive oil was revealed with a slightly higher surface tension value. This behavior might be related to specific components of Picholine olive oil, such as polyphenols, which may have more affinity for the oil−water interface, resulting in higher interfacial activity. The results of statistical analysis for surface tension measurements are indicated in Table 5. It is reasonable to assume that Picholine olive oil contains a slightly high concentration of surface active compounds, which may have caused the reduction of the interfacial tension between the two phases upon emulsification, finally leading to the formation of considerable small droplets (within the nanometer range). Among these compounds, apigenin especially may accumulate at the oil/water interface. Considering that this bioactive compound is a major flavonoid in olive oil that has been reported to have an antiallergenic effect, it is reasonable to assume that the olive from Southern France, Picholine olive oil may also have an antiallergenic activity.[13]

The droplet size distribution of O/W emulsion preparations of Picholine olive oil is shown in Figure 4. For the purpose of supporting the hypothesis that droplet size in the nanometer range (as opposed to larger droplets of around 1 μm) may have caused the inhibitory effect on the chemical mediator release, the droplet size distributions of all samples were expressed as number frequency rather than size distribution per volume. The

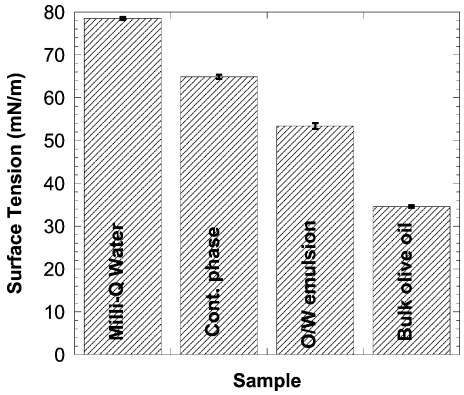

Figure 3. Changes on the surface tension of olive oil upon formulation into O/W emulsions, as compared to the water phase. Dispersed phase, Picholine olive oil; continuous phase, Eagle's MEM (pH 7.4) and FBS, 9:1 (v/v) respectively. The oil content in O/W emulsions was 10 wt %. Emulsified using sonicator for 15 min.

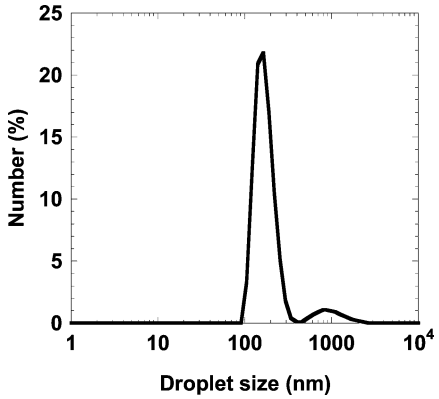

Figure 4. Droplet size distribution of O/W emulsions prepared using Picholine olive oil as the dispersed phase; continuous phase, Eagle's MEM (pH 7.4) and FBS, 9:1 (v/v), respectively. The oil content in O/W emulsions was 10 wt %. Emulsified using sonicator for 15 min.

emulsion prepared with Picholine olive oil consisted of droplets with average size of 170 nm, whereas a major peak at around 110 nm (93.3% of particles' number distribution) and a minor peak at 990 nm (6.7% of number distribution) were observed.

In this work, O/W emulsions with major droplet size around 110 nm were used. On the other hand, emulsions containing droplets within a certain size range could be used, and its effect on the β-hexosaminidase inhibitory effect was evaluated. In fact, the use of such emulsions has been reported by Ribeiro et al.[30] while formulating an O/W emulsion loaded with lycopene or astaxanthin, stabilized by different emulsifiers. According to those authors, the kind of emulsifiers used and the resulting droplet size played important roles on the cellular uptake of carotenoids by the colon carcinoma cells line HT-29 and Caco-2.

Regarding the use of a sonicator bath for emulsification, the increase in temperature during the process may be disregarded from the point of view of degradation of polyphenolic compounds present in Picholine olive oil, since these compounds may have considerable resistance to relatively mild temperatures between 50 and 70 °C.[31]

Stability of Olive O/W Emulsions upon Reaction with the Releasing Mixture and Bovine Serum Albumin. To investigate the stability of O/W emulsions prepared using either Picholine olive oils during the β-hexosaminidase inhibition assay (described above), both emulsions were treated as follows: 100 μL of emulsion was mixed with 1200 μL of releasing mixture (116.9 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 5.6 mM glucose, 25.0 mM HEPES, 2.0 mM $CaCl_2$, and 1.0 mg/mL BSA at pH 7.7) and incubated at 37 °C for 10 min. Upon reaction, samples for surface tension measurements were taken. Aliquots of 1000 μL volume of the previous reaction mixture were mixed with 1000 μL of BSA (diluted in PBS) and incubated at 37 °C for 60 min. The surface tension values were monitored during this reaction as well. As indicated in Figure 5, treating the O/W emulsions with the releasing mixture for 10 min did not have significant effect on the surface tensions values, as compared to the emulsion freshly prepared.

The reason why Picholine olive O/W emulsion showed an inhibitory effect on the chemical mediator release, whereas the bulk oil did not show such activity, may be related to the oil droplets floating velocity (v), which is a function of droplet size as indicated by Stoke's law (eq 3), as follows:

Table 5. ANOVA (Section A) and Duncan's Test (Section B) Summaries for Changes on the Surface Tension of Olive Oils during the β-Hexosaminidase Inhibition Assay[a]

Section A: ANOVA								
	effect			error				
parameter	SS	df	MS	SS	df	MS	F	P value
surface tension	5651.963	10	565.1963	58.349	99	0.589384	958.9613	0.0004[b]

Section B: Duncan's Test					
	{1}	{2}	{3}	{4}	{5}
treatment	M = 34.580	M = 53.37	M = 52.84	M = 55.26	M = 54.79
{1}		0.0000171[b]	0.000020[b]	0.0000171[b]	0.0000175[b]
{2}	0.0000171[b]		0.125961	0.0000468[b]	0.000155[b]
{3}	0.000020[b]	0.125961		0.0000291[b]	0.0000466[b]
{4}	0.0000171[b]	0.0000468[b]	0.0000291[b]		0.174215
{5}	0.0000175[b]	0.000155[b]	0.0000466[b]	0.174215	

[a]Experiments were designed using a single factor (surface tension) with six treatment levels and fixed effects model with 10 replicates for Picholine olive oil based O/W emulsions. Treatments: {1}, 100 bulk oil; {2}, O/W emulsion freshly prepared; {3}, 100 μL of emulsion and 1200 μL of releasing mixture incubated (37 °C, 10 min); {4}, 1000 μL of reaction mixture and 1000 μL of BSA incubated (37 °C, 10 min; {5} reaction mixture incubated (37 °C, 30 min); and {6} incubated (37 °C, 60 min). Dependent variable, surface tension. [b]Indicates significant differences among treatments at the $P \leq 0.05$ level.

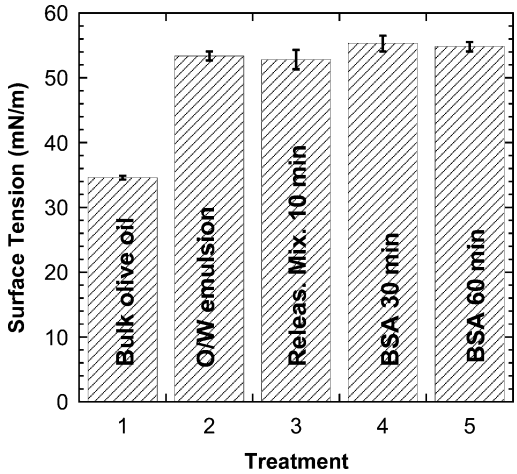

Figure 5. Changes on the surface tension of olive oils during the β-hexosaminidase inhibition assay. One hundred microliters of emulsion and 1200 μL of releasing mixture were incubated at 37 °C for 10 min. On the sequence, 1000 μL of the reaction mixture and 1000 μL of BSA (diluted in PBS) were incubated at 37 °C up to 60 min. Treatments marked with different indices (a−d) indicate significant differences ($P < 0.05$).

$$V = \frac{D^2 g(\rho_p - \rho_f)}{18\mu} \quad (3)$$

where v is the floating speed (m/s), D is the particle diameter (m), g is the gravity acceleration (m/s^2), ρ_p is the oil density (kg/m^3), ρf is the water density (kg/m^3), and μ is the water viscosity (Pa s). On the basis of this equation, we calculated the v for oil droplets in an O/W emulsion containing droplets of different sizes. For instance, in the case of oil droplets of around 100 nm, v is 2.3 μm/h, which is considerably lower as compared to oil droplets of around 1 μm, whose v is nearly 230 μm/h. Considering that the approximate depth of the 96-well plates used on β-hexosaminidase inhibition assay is around 3 mm and that this assay is carried over up to 60 min, larger droplets may have floated toward the liquid surface, reducing the interaction between the oil droplets and the releasing mixture.

Furthermore, the droplet size dependence on the surface area for emulsion droplets may be related to the inhibitory effect on the chemical mediator release, shown only by the smaller emulsion droplets. For instance, larger emulsion droplets prepared in this work (around 1 μm) have an approximate surface area of around 3 m^2/g oil, whereas smaller droplets (around 100 nm) have around 10 times higher surface area,[32] so that exposing more polyphenolic compounds from Picholine olive oil for interaction with the releasing mixture.

In a previous study, we investigated the antiallergic activities of O/W emulsions from five principal virgin olive oils.[13] First, we confirmed the inhibitory effect of bulk olive oil on chemical mediator release by RBL-2H3 cells. Our data showed that the bulk olive oil did not show the inhibitory effect of chemical mediator from the cells, although an assay using an O/W emulsion, which was prepared from same olive oil, showed an inhibitory effect of the chemical mediator. We observed the O/W emulsions by freeze fracture electron microscopy, and it was hypothesized that in the O/W emulsion, proteins in the FBS of the medium acted as surfactants from the electron micrograph. Our previous study showed that olive oil O/W emulsion sample that had the highest inhibitory activity on β-hexosaminidase release, at antigen−antibody binding stage in five samples, had higher apigenin and luteolin contents than other olive oil samples. From these backgrounds, physicochemical properties of olive oil O/W emulsion from Southern France were investigated in this study. As described in the previous section, Picholine olive oil contained apigenin and luteolin. From this result, it was considered that inhibitory effects of Picholine olive oil on β-hexosaminidase release in antigen−antibody binding stage using RBL-2H3 cells were attributable to the antiallergic activities of apigenin and luteolin. A tentative explanation for this behavior is that major polyphenolic compounds present in Picholine olive oil, such as apigenin, may have interfacial activity, besides their antiallergic effect. The effects of Picholine olive oil O/W emulsion on allergy-related gene expressions were investigated using microarray experiments. Picholine olive oil O/W emulsion inhibited β-hexosaminidase through the decrease in the expressions of genes associated with the FcεRI signaling pathway, which include transcription factors and cytokine genes. Moreover, we suggest that the reason for the inhibitory effect of Picholine olive oil on β-hexosaminidase release was the droplet size in the Picholine olive oil emulsion. Polyphenolic compounds may diffuse from the droplet core toward the oil−water interface, enhancing the inhibitory effect of chemical mediator. The contact area of an active substance with cellular surface is an important factor in the pathway on antigen−antibody's binding stage of type I allergy. Emulsions prepared using Picholine olive oil, with droplets in the nanometer size range, can interact with larger molecules in the cellular membrane, indicative of its high efficacy against β-hexosaminidase release.

It has been reported that polyphenols from tea, apple, and aromatic plants have an antiallergic effect on antigen−antibody's binding stage of type I allergy.[33−35] Hydroxytyrosol, luteolin, and apigenin are the major polyphenols from olives and olive oil, and they are well-known components, due to their potent antioxidant activity. For instance, hydroxytyrosol is a hydrophilic compound, partitioning preferable into water rather than being dispersed in an oil phase. Thus, such polyphenols may diffuse from the core of olive oil droplets toward the oil−water interface, as they may also react with antigen−antibody's binding stage on the surface of mast cells. Nevertheless, additional studies may be needed to confirm these hypotheses.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

RBL-2H3, rat basophilic leukemia; O/W, oil-in-water; DAD, diode array detector; LLE, liquid−liquid extraction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; DNP-BSA, dinitrophenylated bovine serum albumin

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