Anti-allergic triterpenes isolated from olive milled waste

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Abstract The degranulation of basophils stimulated by antigen is one of the allergic mechanisms of immediate hypersensitivity reactions. In a previous study, the β -hexosaminidase release of rat basophilic leukemia (RBL-2H3) cells stimulated by IgE cross-linking was specifically inhibited by ethanol extract of olive milled waste (OMW). OMW is a potential source of triterpenes, which show several biological activities. However, little is known about the effect of triterpenes in OMW on β -hexosaminidase release. Here we investigated anti-allergic triterpenes from the ethanol extract of OMW, and we discuss the structure–activity relationship. We subjected ethanol extract of OMW to activity-guided fractionation; this led to the isolation of five known triterpenes, i.e., maslinic acid (**1**), oleanolic acid (**2**), punicanolic acid (**3**), 2-*O*-acetylmastlinic acid (**4**), and epimaslinic acid (**5**) as well as one new triterpene, 11-oxo-mastlinic acid (**6**). An anti-allergic assay revealed that 2-*O*-acetylmastlinic acid (**4**) and 11-oxo-mastlinic acid (**6**) showed

anti-allergic activity. Considering the structure–activity relationship of these triterpenes, it appears that the existence of a ketone group at C-11 or an acetyl group at C-2 would influence the anti-allergic activity.

Keywords β -Hexosaminidase release · RBL-2H3 · Olive milled waste · Triterpene · 11-oxo-mastlinic acid · 2-*O*-acetylmastlinic acid

Abbreviations

RBL-2H3	Rat basophilic leukemia cell line
OMW	Olive milled waste
MPLC	Medium pressure liquid chromatography
HPLC	High-performance liquid chromatography
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
DNP-BSA	Albumin from bovine serum, 2,4-Dinitrophenylated
DMSO	Dimethyl sulfoxide
Hex	<i>n</i> -Hexane
EtOAc	Ethyl acetate
MeOH	Methanol
CD ₃ OD	Deuterated methanol
DMSO- <i>d</i> ₆	Deuterated dimethyl sulfoxide
HR-ESI-MS	High-resolution electrospray ionization mass spectrometry
NMR	Nuclear magnetic resonance
HSQC	Heteronuclear single quantum coherence

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HMBC	Heteronuclear multiple bond correlation
NOESY	Nuclear Overhauser effect spectroscopy

Introduction

Although basophils compose less than 1% of peripheral blood leucocytes, recent findings indicate that basophils play important roles in a wide variety of immune regulation functions and disorders such as immediate hypersensitivity reactions, late-phase hypersensitivity reactions, autoimmunity, and cancers (Cromheecke et al. 2014; Harvima et al. 2014; Siracusa et al. 2013). Basophils are involved in the orchestration of immune responses by releasing histamine or several cytokines mediated by IgE or other cytokines. The degranulation of basophils mediated by IgE cross-linking is one of the main triggers of immediate hypersensitivity reactions such as anaphylaxis and allergy to foods, drugs, insects, pollens and so on.

In our previous examinations of the biological active potentials of ethanol extract and water extract of each part of the olive (*Olea europaea* L.), we observed that the degranulation of a basophilic model, i.e., rat basophil leukemia (RBL-2H3), was inhibited only by the ethanol extract of olive milled waste (OMW) whereas the ethanol extract of fruit pulp or that of seeds showed very weak activity (Kishikawa et al. 2015). OMW, which is the agricultural by-product from two-phase olive oil extraction, is composed of the fruit juice and the solid part of the olive fruit. Approximately six million tons of OMW is generated within a short period of the harvesting season in a single olive oil-producing country, Spain, and the OMW causes a serious waste-disposal problem for two reasons: the components of OMW are phytotoxic, and the moisture of OMW decreases its heat efficiency.

However, recent progress also revealed that depending on the extraction methods, OMW can provide large amounts of several useful compounds stored in olive fruits: phenolic compounds, oligosaccharides, triterpenes and more (Fernandez-Hernandez et al. 2015). Moreover, the fruit paste malaxation (i.e., churning or mixing) performed to obtain higher olive oil yields sets off various enzymatic and/or chemical

reactions such as oxidation, glycosidation and hydrolysis of the glycosidic bonds, and as a result, versatile modified compounds are naturally produced (Cardoso et al. 2011; Klen and Vodopivec 2012). OMW is thus now regarded as a potential source of useful natural compounds.

Olive oil and methanol extract of olive waste materials were also reported to have inhibitory activity on degranulation of RBL-2H3. The active compounds of those olive materials were mainly phenolic compounds. For example, flavonoids such as apigenin and luteolin were shown to be the active compounds for olive oil, and a secoiridoid connected to hydroxytyrosol like 2-hydroxy-3-ethylidene-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-4-acetic acid 2-(3,4-dihydroxyphenyl)ethyl ester (3,4-DHPEA-EA) are reported to be the active compound for olive waste materials (Isoda et al. 2012; Sato et al. 2014). The main triterpenes of OMW were reported to have antitumoral activity, cardioprotective activity, anti-inflammatory activity and antioxidant activity (Sanchez-Quesada et al. 2013).

As a result of the increasing interest in OMW, we chromatographed the ethanol extract of OMW for the present investigation of its anti-allergic compounds. Here, we report a new triterpene from OMW with anti-allergic activity, isolated together with five known triterpenes. We discuss some possible substructures of the olive triterpenes that may be involved in the anti-allergic activity.

Materials and methods

Cell line and reagents

The cell line of rat basophilic leukemia (RBL-2H3, Riken Bioresource Center, Ibaraki, Japan) was maintained in 10% FBS (Thermo Fisher Science, Gibco BRL, Tokyo, Japan) in Eagle's minimal essential medium (EMEM, Nissui, Tokyo, Japan) in an incubation at 37 °C, 5.0% CO₂. Monoclonal anti-dinitrophenyl antibody produced in mouse (IgE anti-DNP) and *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide were purchased from Sigma–Aldrich (St. Louis, MO, USA) and dinitrophenyl-bovine serum albumin (DNP-BSA) was purchased from Thermo Fisher Scientific, Invitrogen (Tokyo, Japan). 3-(4,5-Dimethylthiazol-2-yl)-

2,5-Diphenyltetrazolium Bromide (MTT) reagent was purchased from Tokyo Chemical Industry (Tokyo, Japan). TLC silica gel 60 F₂₅₄, methanol-*d*₄ (CD₃OD) and dimethyl sulfoxide-*d*₆ (DMSO-*d*₆) were purchased from Merck (Darmstadt, Germany), Cambridge Isotope Laboratories (Andover, MA, USA) and Sigma–Aldrich, respectively. Wakogel C 200 (silica gel, pore size 7 nm, particle diameter 75–15 µm), *n*-hexane (Hex), ethyl acetate (EtOAc) and methanol (MeOH) were purchased from Wako Pure Chemical Industries (Osaka, Japan) and used for open column chromatography.

Extraction and chromatography

OMW was collected from an olive farm in Nakagawa in Japan's Fukuoka prefecture in October 2014. The fresh OMW was freeze-dried, and then 30 g of the freeze-dried OMW was extracted with 300 mL of ethanol with shaking at 200 rpm in a 500-mL flask for 72 h. This extraction step was repeated until 360.1 g of ethanol extract was obtained from 1.92 kg of freeze-dried OMW in total. The extraction yield was 18.8%.

The ethanol extract of OMW was chromatographed roughly with a silica gel open column (15 cm dia., with 2 kg of silica gel) using the gradient solvent system of Hex, EtOAc and MeOH. The effluent was classified into fractions by monitoring with a thin-layer chromatography (TLC) analysis, detected by irradiating UV light at 254 nm and by spot visualization, in which the TLC plate was sprayed with 5% sulfuric acid/methanol and burned at 100–180 °C. A medium pressure liquid chromatography (MPLC) system (EPCLC, Yamazen, Osaka, Japan) connected with columns of silica gel (particle size 50 µm, 3.0 × 16.5 cm, 37 g) and ODS-SM (particle size 30 µm, 3.0 × 16.5 cm, 40 g) was the second step of fractionation. A preparative high-performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA) connected with an Inertsil ODS column (particle size 5 µm, 20 × 250 mm, GL Science, Tokyo, Japan) was used for further purification.

Identification of isolated compounds

We identified the isolated compounds by measuring high-resolution electrospray ionization mass spectrometry (HR-ESI–MS) and nuclear magnetic

resonance (NMR) spectroscopy, and the optical rotation of the compounds was evaluated. The HR-ESI–MS results were obtained using a liquid chromatography-ion trap-time of flight-mass spectrometry (LC-IT-TOF–MS) system (Shimadzu, Kyoto, Japan) equipped with an Inertsil ODS-3 column (particle size 3 µm, 1.5 × 150 mm, GL Science). The solvent system was isocratic, composed of 90% LC grade MeOH containing 0.1% formic acid. The flow rate was set at 0.2 mL/min. The ¹H-, ¹³C-, heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC) and nuclear Overhauser effect spectroscopy (NOESY) NMR spectra were obtained on an NMR spectrophotometer (Bruker DRX 600; Bruker Daltonics, Billerica, MA, USA) at room temperature with tetramethylsilane (TMS) as an internal standard of chemical shifts and CD₃OD or DMSO-*d*₆ as solvents. Optical rotations in MeOH were determined with a polarimeter (DIP-370; Jasco, Tokyo, Japan).

Anti-allergic assay

We subjected the triterpenes to an anti-allergic assay to determine their cytotoxicity (CC₅₀, half-minimum cytotoxic concentration and CC₁₀, 10%-minimum cytotoxic concentration) and their effect on the degranulation of RBL-2H3 (IC₅₀, half-minimum inhibitory concentration and IC₄₀, 40%-minimum inhibitory concentration). In this study, we defined 'cytotoxicity' as the characteristic causing a reduction in the cell viability to 90% or lower versus the control, and we defined 'anti-allergic activity' as an inhibitory effect on β-hexosaminidase release from RBL-2H3 cells without showing cytotoxicity, because a reduction of β-hexosaminidase release by killing the cell cannot be accepted as anti-allergic activity. We also defined an 'anti-allergic compound' as a compound that shows 50 or 40% inhibition of β-hexosaminidase release at a concentration lower than 100 µM without cytotoxicity. The experiment was done as described (Kishikawa et al. 2015) with some modifications.

Cytotoxicity was evaluated by an MTT assay. The RBL-2H3 cells were cultured on a 96-well plate at a density of 5 × 10⁴ cells/well for 36 h, and treated with a dimethyl sulfoxide (DMSO) solution of either one of the test samples at various concentrations or with only DMSO as a control (0.5 µL/well, *n* = 3). The cytotoxicity was evaluated using MTT assay after 1-hr

treatment. Each of the values of CC_{50} and CC_{10} was calculated from the concentrations of two data points interposing cytotoxicity of 50 or 10%, respectively.

For the determination of the IC_{50} and IC_{40} values, the RBL-2H3 cells were cultured on a 96-well plate at 5×10^4 cells/well for 24 h and with IgE anti-DNP (0.5 μ g/mL) for an additional 12 h. The cells were treated with DMSO solution of the samples at several concentrations lower than the CC_{10} or only DMSO as a control for 1 h (0.5 μ L/well, $n = 3$). The cells were then stimulated in 100 μ L of Tyrode's buffer (pH 7.2) containing 5 μ g/mL of DNP-BSA for 40 min. Then 50 μ L of supernatant was carefully transferred to a new 96-well plate to be mixed with 50 μ L of citric acid buffer (pH 4.5) containing 2.0 mM of *p*-nitro-phenyl *N*-acetyl- β -D-glucosaminide. The β -hexosaminidase reaction occurred in a dark room with shaking at 40 rpm at room temperature for 3 h.

For the determination of the IC_{50} and IC_{40} values, we calculated the inhibition of β -hexosaminidase release from the absorbance at 405 nm after stopping the reaction with 100 μ L of sodium bicarbonate (100 mM, pH 10):

$$\text{Inhibition}(\%) = \left(1 - \frac{\text{Abs}_{405\text{comp.}} - \text{Abs}_{405\text{blank}}}{\text{Abs}_{405\text{cont.}} - \text{Abs}_{405\text{blank}}}\right) \times 100$$

where $\text{Abs}_{405\text{comp.}}$ is the absorbance at 405 nm of the test well (DMSO solution: +, DNP-BSA: +), $\text{Abs}_{405\text{cont.}}$ is the absorbance at 405 nm of a control well (DMSO: +, DNP-BSA: +), and $\text{Abs}_{405\text{blank}}$ is the absorbance at 405 nm of a blank well (DMSO: +, DNP-BSA: -). Each of the values of IC_{50} and IC_{40} was calculated from the concentrations of two data points interposing the inhibition value of 50 or 40%, respectively. Ursolic acid was used as a positive control, because ursolic acid was reported to inhibit the degranulation of RBL-2H3 cells (Murata et al. 2014), and it has a structure similar to that of the isolated compounds.

Results

Isolation of triterpenes from ethanol extract of OMW

As a result of silica gel open column chromatography, the ethanol extract of OMW was fractionated into 10

fractions with detection of effluents by TLC: (SiO_2 , Hex/EtOAc 8:2, spot visualization) Fr. 1, R_f 0.63–0.83; Fr. 2, R_f 0.45–0.55; Fr. 3, R_f 0.28–0.35; Fr. 4, R_f 0.13–0.17; (SiO_2 , Hex/EtOAc 5:5, spot visualization) Fr. 5, R_f 0.33–0.60; Fr. 6, R_f 0.10–0.29; (SiO_2 , EtOAc/MeOH 7.5:2.5, spot visualization) Fr. 7, R_f 0.36–0.67; Fr. 8, R_f 0.11–0.40; Fr. 9, R_f 0.11–0.22; Fr. 10, $R_f < 0.02$. Fr. 5 and Fr. 6 were subjected to further fractionations, because Fr. 6 showed the highest anti-allergic activity and Fr. 5 showed similar TLC profile with Fr. 6. Fr. 5 (3.5 g) was the fraction eluted out from the open column with the gradient mobile phase Hex:EtOAc = 70:30 to 30:70 and Fr. 6 (15.5 g) was the fraction eluted with the solvent Hex:EtOAc = 30:70 to 10:90.

Fr. 5 was chromatographed using MPLC system and preparative HPLC system (Waters Corporation). As a result, Fr. 5 provided compound **1** (15 mg), **2** (20 mg) and **3** (1.5 mg). Fr. 6 was fractionated again with a silica gel open column (ϕ 6 cm, with 0.8 kg of silica gel) by gradient solvent system of Hex, EtOAc and MeOH. In order to obtain better separation, acetic acid was added to the chromatographic solvent at 0.25% (v/v) and the solvent and acetic acid were evaporated soon after collected from the open column. By monitoring with TLC, the effluents of Fr. 6 were fractionated into 10 sub fractions: (SiO_2 , Hex/EtOAc 5:5, spot visualization) Fr. 6–1, R_f 0.63–0.86; Fr. 6–2, R_f 0.31–0.70; Fr. 6–3, R_f 0.23–0.63; Fr. 6–4 to 6–9 R_f 0.08–0.55; Fr. 6–10, $R_f < 0.08$. Compound **1** was distributed in the sub fractions from Fr. 6–3 to Fr. 6–8 in large amount, some other spots in Fr. 6–3, 6–4 and 6–8 were the target for the further isolation. Fr. 6–3 was the fraction collected from the open column with the mobile phase *n*-hexane:ethyl acetate = 80:20 to 75:25, Fr. 6–4 was collected with *n*-hexane:ethyl acetate = 70:30 and Fr. 6–8 was collected with *n*-hexane:ethyl acetate = 55:45 to 50:50. With the several times of purification with MPLC and preparative HPLC systems, compound **1** (4.0 mg), **2** (0.8 mg), **4** (0.6 mg) were isolated from Fr. 6–3 (453 mg), compound **5** (0.4 mg) was obtained from Fr. 6–4 (187 mg), and compound **6** (1.8 mg) was successfully isolated from Fr. 6–8 (2.3 g).

Identification of the isolated triterpenes

Totally six triterpenes including one new triterpene, 2 α ,3 β -dihydroxy-11-oxo-18 β -olean-12-en-28-oic acid (11-oxo-maslinic acid, **6**), and five known triterpenes were identified (Fig. 1).

Compound **6** was obtained as a yellowish amorphous powder with $[\alpha]_D^{26} +49.0$ (*c* 1.0, MeOH). HR-ESI-MS exhibited a protonated molecular ion peak at m/z 487.2911 $[M + H]^+$ and a deprotonated molecular ion peak at m/z 485.3356 $[M - H]^-$ in positive and negative ion modes, respectively, indicating a molecular formula of $C_{30}H_{46}O_5$. It showed UV λ_{max} 252 nm (0.1% formic acid in MeOH). The 1H -NMR

spectral data were indicative of seven tertiary methyl groups at δ_H 1.46 (H-27), 1.17 (H-25), 1.02 (H-23), 0.98 (H-26), 0.96 (H-30), 0.95 (H-29) and δ_H 0.82 (H-24) together with an olefinic proton at δ_H 5.55 (br s). The methyl protons were correlated through HSQC spectrum to δ_C 24.0, 18.0, 29.3, 19.9, 23.8, 33.0 and δ_C 17.5, respectively. Furthermore, the HSQC experiment showed the correlation of the broad singlet at δ_H 5.55 with δ_C 128.3. These NMR characteristic features suggested compound **3** to be a triterpene possessing an olean-12-ene skeleton (Mahato and Kundu, 1994). The carbon signal at δ_C 128.3 was then placed to C-12 of the skeleton. The ^{13}C -NMR spectrum furthermore revealed carbon signals at δ_C 202.7, 180.9, 128.3,

Fig. 1 The structure of ursolic acid and the isolated triterpenes: maslinic acid (**1**), oleanolic acid (**2**), punicanolic acid (**3**), 2-*O*-acetylmazlinic acid (**4**), epimaslinic acid (**5**) and 11-oxo-maslinic acid (**6**)

172.9, 84.2 and δ_C 69.2. With the help of HMBC correlations, the carbon signal at δ_C 202.7 was unambiguously assigned to C-11 of the skeleton based on the correlation observed from δ_H 5.55 (H-12) to δ_C 202.7 and further confirmed from the correlations of δ_H 2.48 (1H, s), assigned to H-9 with δ_C 202.7. The carbon signal at δ_C 180.9 was assigned to a carboxylic group attached to C-28. The quaternary carbon signal at δ_C 172.9 was placed to C-13 of the skeleton. The assignment of δ_C 180.9 to C-28 and δ_C 172.9 to C-13, was evidenced from the long-range correlations observed from δ_H 3.00 (dd, $J = 3.6$, 13.8 Hz, H-18) with the carbon signals at δ_C 180.9, 172.9 together with its correlation to δ_C 128.3 (C-12) and δ_C 43.4 (C-18). The two oxygen-bearing carbons at δ_C 84.2 and δ_C 69.2 were carefully placed to C-3 and C-2 of the olean-12-ene skeleton based on long-range correlations observed from δ_H 3.08 (dd, $J = 4.2$, 12.6 Hz, H-1) with δ_C 84.2 and δ_C 69.2 and by comparison with Martinez et al. (2013), Rudiyanasyah and Garson (2006), and Tanaka et al. (2003) which justified 2 α ,3 β -diol. On the basis of the above discussion, the structure of **6** was established as 2 α ,3 β -dihydroxy-11-oxo-18 β -olean-12-en-28-oic acid, which is a new natural product.

We identified the other compounds as maslinic acid (**1**), oleanolic acid (**2**) (Martinez et al. 2013), punicanolic acid (**3**) (Xie et al. 2008), 2-*O*-acetylmaslinic acid (**4**) (Thoison et al. 2004) and epimaslinic acid (**5**) (Wen et al. 2008) by comparing their spectral data with those reported in the literature. Maslinic acid (**1**) and oleanolic acid (**2**) are known as the main triterpenes in OMW, olive fruits, olive oil and olive leaves. These triterpenes are reported to have a wide variety of biological effects such as anticancer, cardioprotective, anti-inflammatory, antioxidant, anti-HIV, antimicrobial, and hepatoprotective activities (Sanchez-Quesada et al. 2013; Fernandez-Hernandez et al. 2015). Punicanolic acid (**3**), 2-*O*-acetylmaslinic acid (**4**) and epimaslinic acid (**5**) were isolated from the olive plant for the first time in this study. As for 2-*O*-acetylmaslinic acid (**4**), considering the earlier finding that pectin extracted from the olive mill wastewater was highly acetylated to increase its oil holding capacity (Rubio-Senent et al. 2015), it is apparent that maslinic acid can also exist in OMW as an acetylated form. Maslinic acid (**1**), oleanolic acid (**2**) and epimaslinic acid (**5**) are present in many plants, whereas punicanolic acid (**3**) was isolated from

flowers of *Punica granatum* (pomegranate) (Xie et al. 2008) and 2-*O*-acetylmaslinic acid (**4**) was isolated from leaves of the trees *Nothofagus dombeyi* and *N. pumilio* (Thoison et al. 2004). The data used for identification are follows:

Maslinic acid (2 α ,3 β -Dihydroxyolean-12-en-28-oic acid, **1)** White amorphous solid: $[\alpha]_D^{26} +52.2$ (c 1.0, MeOH): TLC (SiO₂, Hex/EtOAc/MeOH 5:5:0.1, spot

Table 1 ¹³C NMR data for isolated triterpenes **1–6**

Position	1 ^a	2 ^a	3 ^b	4 ^a	5 ^a	6 ^a
1	48.2	39.9	38.3	45.1	42.5	49.1
2	69.5	27.9	27	74	67.2	69.2
3	84.5	79.8	76.7	81	80.1	84.2
4	40.5	39.9	38.2	40.6	39.5	40.6
5	56.7	56.8	54.7	56.6	49	56.2
6	19.6	19.6	17.9	19.6	19.2	18.7
7	33.9	33.9	37.1	33.6	33.9	32.3
8	40.6	40.6	40.6	41	40.8	45.1
9	49	49.1	48.5	49	49	63
10	39.3	38.2	37.2	39.5	39.5	39.4
11	24.7	24.6	21.1	24.6	24.6	202.7
12	123.5	123.7	28.5	123.4	123.5	128.3
13	145.4	145.3	40	145.4	145.3	172.9
14	43	42.9	42.1	43	43.1	46.5
15	28.8	28.9	28.8	28.8	28.9	28.9
16	24	24.1	34.7	24	24.1	23.9
17	47.7	47.7	49.3	47.3	47.7	47.2
18	42.8	42.8	46.3	42.8	42.8	43.4
19	47.3	47.3	38.5	47.7	47.4	45.6
20	31.7	31.7	71.1	31.7	31.7	31.7
21	34.9	35	36.4	34.9	35	34.8
22	34	34.1	32.4	33.6	33.9	34
23	29.3	28.8	28	29.2	29.3	29.3
24	17.1	16.4	15.7	17.7	22.5	17.5
25	17.5	15.9	15.9	16.9	16.9	18
26	17.8	17.8	16.1	17.4	17.8	19.9
27	26.5	26.4	14.6	26.4	26.5	24
28	181.9	181.9	177.6	181.9	182.1	180.9
29	33.6	33.6	18.1	33.9	33.6	33
30	24.1	24	29.9	24.1	24	23.8
COCH ₃				173.1		
COCH ₃				21.4		

^a ¹³C NMR measured in CD₃OD

^b ¹³C NMR measured in DMSO-*d*₆

visualization) Rf 0.29: ^1H NMR (600 MHz, CD_3OD) δ_{H} 5.25 (1H, dd, $J = \text{Hz}$, H-12), 3.61 (1H, ddd, $J = 4.8, 9.6, 11.4$ Hz, H-2 β), 2.90 (1H, d, $J = 9.6$ Hz, H-3 α), 2.85 (1H, dd, $J = 4.2, 14.4$ Hz, H-18), 2.00 (1H, ddd, $J = \text{Hz}$, H-16), 1.16 (3H, s, H-27), 1.01 (3H, s, H-23), 1.00 (3H, s, H-25), 0.94 (3H, s, H-30), 0.91 (3H, s, H-29), 0.81 (3H, s, H-26), 0.81 (3H, s, H-24): for ^{13}C NMR (150 MHz, CD_3OD), see Table 1: HR-ESI-MS m/z 471.3504 $[\text{M} - \text{H}]^-$.

Oleanolic acid (3 β -hydroxyolean-12-en-28-oic acid, 2) White amorphous solid: $[\alpha]_{\text{D}}^{26} +65.6$ (c 1.0, MeOH): TLC (SiO_2 , Hex/EtOAc/MeOH 5:5:0.1, spot visualization) Rf 0.52: ^1H NMR (600 MHz, CD_3OD) δ_{H} 5.24 (1H, dd, $J = \text{Hz}$, H-12), 3.14 (1H, dd, $J = 4.2, 9.6, 11.4$ Hz, H-2 β), 2.84 (1H, dd, $J = 4.2, 13.8$ Hz, H-18), 2.01 (1H, ddd, $J = 4.2, 13.8, 13.8$ Hz, H-16), 1.16 (3H, s, H-27), 0.97 (3H, s, H-23), 0.94 (3H, s, H-25), 0.94 (3H, s, H-30), 0.90 (3H, s, H-29), 0.81 (3H, s, H-26), 0.77 (3H, s, H-24), 0.75 (1H, d, $J = 12.0$ Hz, H-5): for ^{13}C NMR (150 MHz, CD_3OD), see Table 1: HR-ESI-MS m/z 471.3504 $[\text{M} - \text{H}]^-$.

Punicanolic acid (3 β , 20 β -Dihydroxytaraxastane-28-oic acid, 3) White amorphous powder: $[\alpha]_{\text{D}}^{26} +5.8$ (c 1.0, MeOH): TLC (SiO_2 , Hex/EtOAc/MeOH 5:5:0.1, spot visualization) Rf 0.57: ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ_{H} 2.95 (1H, m, H-3), 1.01 (3H, s, H-30), 0.92 (3H, d, $J = 5.0$ Hz, H-29), 0.92 (3H, s, H-27), 0.87 (3H, s, H-23), 0.86 (3H, s, H-26), 0.78 (3H, s, H-25), 0.65 (3H, s, H-24): for ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$), see Table 1: HR-ESI-MS m/z 473.3684 $[\text{M} - \text{H}]^-$.

2-O-Acetylmassic acid (4) White amorphous powder: $[\alpha]_{\text{D}}^{26} +25.0$ (c 1.0, MeOH): TLC (SiO_2 , Hex/EtOAc/MeOH 5:5:0.1, spot visualization) Rf 0.48: ^1H NMR (600 MHz, CD_3OD) δ_{H} 5.24 (1H, dd, $J = 3.5, 3.5$ Hz, H-12), 4.94 (1H, m, H-2), 3.15 (1H, d, $J = 9.6$ Hz, H-3), 2.85 (1H, dd, $J = 4.2, 13.9$, H-18), 2.03 (3H, s, COCH_3), 1.16 (3H, s, H-27), 1.05 (3H, s, H-25), 1.04 (3H, s, H-23), 0.94 (3H, s, H-29), 0.90 (3H, s, H-30), 0.85 (3H, s, H-24), 0.81 (3H, s, H-26): for ^{13}C NMR (150 MHz, CD_3OD), see Table 1: HR-ESI-MS m/z 513.3591 $[\text{M} - \text{H}]^-$.

Epimaslinic acid (2 α , 3 α -Dihydroxyolean-12-en-28-oic acid, 5) White amorphous solid: $[\alpha]_{\text{D}}^{26} +108.8$ (c 1.0, MeOH): TLC (SiO_2 , Hex/EtOAc/MeOH

5:5:0.1, spot visualization) Rf 0.41: ^1H NMR (600 MHz, CD_3OD) δ_{H} 5.25 (1H, dd, $J = 3.6, 3.6$ Hz, H-12), 3.92 (1H, ddd, $J = 3.0, 3.0, 12.0$ Hz, H-2), 3.31 (over rapped with the solvent peak, H-3), 2.85 (1H, dd, $J = 6.3, 13.2$ Hz, H-18), 2.00 (1H, ddd, $J = 3.9, 13.5, 13.5$ Hz, H-16), 1.18 (3H, s, H-27), 0.99 (3H, s, H-23), 0.98 (3H, s, H-25), 0.94 (3H, s, H-29), 0.91 (3H, s, H-30), 0.86 (3H, s, H-25), 0.81 (3H, s, H-25): for ^{13}C NMR (150 MHz, CD_3OD), see Table 1: HR-ESI-MS m/z 471.3574 $[\text{M} - \text{H}]^-$.

11-Oxo-massic acid (2 α , 3 β -Dihydroxy-11-oxo-18 β -olean-12-en-28-oic acid, 6) Yellowish amorphous powder: $[\alpha]_{\text{D}}^{26} +49.2$ (c 1.0, MeOH): TLC (SiO_2 , Hex/EtOAc/MeOH 5: 5: 0.1, spot visualization) Rf 0.15: ^1H -NMR (600 MHz, CD_3OD) δ_{H} 5.55 (1H, s, H-12), 3.66 (1H, ddd, $J = 4.2, 9.6, 9.6$ Hz, H-2 β), 3.08 (1H, dd, $J = 4.2, 12.6$ Hz, H-1), 3.00 (1H, dd, $J = 3.6, 13.8$ Hz, H-18), 2.92 (1H, d, $J = 9.6$ Hz, H-3 α), 2.48 (1H, s, H-9), 2.14 (1H, ddd, $J = 4.2, 13.4, 13.4$ Hz, H-25), 1.46 (3H, s, H-27), 1.17 (3H, s, H-25), 1.02 (3H, s, H-23), 0.98 (3H, s, H-26), 0.96 (3H, s, H-30), 0.95 (3H, s, H-29) 0.84 (1H, dd, $J = 1.6, 11.7$ Hz, H-5), 0.82 (3H, s, H-24): ^{13}C -NMR (150 MHz, CD_3OD), see Table 1: HR-ESI-MS m/z 487.2911 $[\text{M} + \text{H}]^+$, m/z 485.3356 $[\text{M} - \text{H}]^-$.

Anti-allergic activity

The anti-allergic activity of the six isolated triterpenes was evaluated (Table 2). First, the CC_{50} and CC_{10} were tested at the final concentration of 100 μM or lower. The anti-allergic activity was then tested at the final concentrations lower than the CC_{10} . Ursolic acid as a positive control showed high anti-allergic activity with the IC_{50} value of 39.4 ± 9.9 μM and a CC_{10} value >100 μM .

Among the isolated compounds, the new compound, 11-oxo-massic acid (6), showed anti-allergic activity with the IC_{50} value 80.6 ± 10.6 μM and the IC_{40} value 71.5 ± 30.0 μM , which was approx. one-half strength of the activity of ursolic acid, and with a CC_{50} value >100 μM . 2-O-Acetylmassic acid (4) affected the anti-allergic reaction of the cells to some degree, with the IC_{40} value 5.9 ± 2.4 μM , the CC_{10} value 17.6 ± 3.4 μM and the CC_{50} value 71.3 ± 7.4 μM . For the other compounds, anti-allergic activity was not detected by epimaslinic acid (5) or maslinic acid (1) at the concentrations lower than the

Table 2 The anti-allergic activity (IC₅₀ and IC₄₀) and cytotoxicity (CC₅₀ and CC₁₀) of the isolated compounds

Compound	IC ₅₀ (μM)	IC ₄₀ (μM)	CC ₅₀ (μM)	CC ₁₀ (μM)
Maslinic acid (1)	>28.3 ^{Tox}	>28.3 ^{Tox}	>100	28.3 ± 2.8
Oleanolic acid (2)	>100	>100	>100	>100
Punicanolic acid (3)	>51.6 ^{Pre}	>51.6 ^{Pre}	>51.6 ^{Pre}	>51.6 ^{Pre}
2- <i>O</i> -Acetylmasic acid (4)	>17.6 ^{Tox}	5.9 ± 2.4	71.3 ± 7.4	17.6 ± 3.4
Epimaslinic acid (5)	>9.2 ^{Tox}	>9.2 ^{Tox}	>100	9.2 ± 1.0
11-Oxo-maslinic acid (6)	80.6 ± 10.6	71.5 ± 30.0	>100	>100
Ursolic acid	39.4 ± 9.9	25.7 ± 1.5	>100	>100

^{Pre} The exact value was not determined because the compound precipitated in the medium

^{Tox} The exact value was not determined because of cytotoxicity

CC₁₀ (IC₄₀, IC₅₀ > 9.2 μM and IC₄₀, IC₅₀ > 28.3 μM, respectively). Oleanolic acid (**2**) had no effect on the anti-allergic activity (IC₄₀, IC₅₀ > 100 μM, CC₁₀, CC₅₀ > 100 μM). Punicanolic acid (**3**) precipitated in the medium at higher concentration, and we thus evaluated the IC₅₀ and CC₅₀ values at the soluble concentration, but it showed no activity (IC₄₀, IC₅₀ > 51.6 μM, CC₁₀, CC₅₀ > 51.6 μM).

Discussion

We isolated six triterpenes including one new compound from the ethanol extract of OMW for our investigation of anti-allergic triterpenes, and the new triterpene, 11-oxo-maslinic acid (**6**), was evaluated as an anti-allergic triterpene. In our comparison of the structures of the tested triterpenes, we suspected that there were some important substructures for the anti-allergic activity.

First, among the isolated compounds, 11-oxo-maslinic acid (**6**) and 2-*O*-acetylmasic acid (**4**) showed anti-allergic activity. In comparison with the structure of maslinic acid (**1**), the ketone at C-11 of 11-oxo-maslinic acid (**6**) and the acetyl group at C-2 of 2-*O*-acetylmasic acid (**4**) would be effective for the anti-allergic activity. As the next step, it should be determined whether the ketone at C-11 and the ketone of acetyl at C-2 play the same role in the anti-allergic activity, that is, in the target protein of these active triterpenes and with the mechanism of action.

This paper is the first report of anti-allergic triterpenes in OMW, and our present findings revealed that not only the phenolic compounds which were

reported to date but also some triterpenes in OMW relate to the anti-allergic activity of OMW extract. Until now, the focus has been on polyphenols such as luteolin, apigenin and 3,4-DHPEA-EA as anti-allergic agents of olive oil or OMW, but triterpenes in OMW are also worth investigating, and they have the potential to be useful in functional ways such as food and cosmetic additives or drug development.

To the best of our knowledge, there are only a few reports about the effect of triterpenes on β-hexosaminidase release and its structure–activity relationship, whereas the structure–activity relationship of flavonoids, stilbenes, and curcuminoids have been summarized (Matsuda et al. 2016). Triterpenes are also widely distributed in natural products, plants and mushrooms, and different types of triterpenes have been found in these materials. More research should be conducted to obtain a comprehensive understanding of the anti-allergic effect of natural triterpenes as well as olive triterpenes, in part because humans consume triterpenes from their daily food. Our present findings of a new anti-allergic triterpene from OMW and the steric structures that are effective for the anti-allergic activity help clarify the useful characteristics of triterpenes and OMW for anti-allergic treatment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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