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# Multiple Biological Effects of Olive Oil By-products such as Leaves, Stems, Flowers, Olive Milled Waste, Fruit Pulp, and Seeds of the Olive Plant on Skin

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**As olive oil production increases, so does the amount of olive oil by-products, which can cause environmental problems. Thus, new ways to utilize the by-products are needed. In the present study, five bioactive characteristics of olive oil by-products were assessed, namely their antioxidant, anti-bacterial, anti-melanogenesis, anti-allergic, and collagen-production-promoting activities. First, the extracts of leaves (May and October), stems (May and October), flowers, olive milled waste, fruit pulp and seeds were prepared using two safe solvents, ethanol and water. According to HPLC and LC/MS analysis and Folin–Ciocalteu assay, the ethanol extracts of the leaves (May and October), stems (May and October) and flowers contained oleuropein, and the ethanol extract of the stems showed the highest total phenol content. Oleuropein may contribute to the antioxidant and anti-melanogenesis activities of the leaves, stems, and flowers. However, other active compounds or synergistic effects present in the ethanol extracts are also likely to contribute to the anti-bacterial activity of the leaves and flowers, the anti-melanogenesis activity of some parts, the anti-allergic activity of olive milled waste, and the collagen-production-promoting activity of the leaves, stems, olive milled waste and fruit pulp. This study provides evidence that the by-products of olive oil have the potential to be further developed and used in the skin care industry. Copyright © 2015 John Wiley & Sons, Ltd.**

*Keywords:* Olive (*Olea europaea* L.) by-products; antioxidant activity; antibacterial activity; anti-melanogenesis activity; anti-allergic activity; collagen-production-promoting activity.

## INTRODUCTION

Olive (*Olea europaea* L.) cultivation and olive oil production have recently become more popular in Japan. This means that the amount of waste by-products of olive oil has also been increasing in Japan. Olive oil by-products are produced in much greater amounts than olive oil, and the by-products have caused environmental problems in the Mediterranean area. For example, the amount of olive milled waste (OMW) produced is more than four times larger than the olive oil produced. Most OMW is now discarded; however, OMW was not degraded easily because phenolic compounds that remain in OMW inhibit its biodegradation. Also, the phenolic compounds in OMW pollute the soil and inhibit the growth of plants (Ghanbari *et al.,* 2012). Thus, the utilization of olive oil by-products could help solve these environmental problems and create a new agricultural demand.

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The phenolic compounds contained in olive oil, which include both unsaponifiable and polar substances in olive oil, show several biological activities. For example, oleuropein inhibited the skin thickness and DNA damage induced by UV when it was administered orally to mice (Sumiyoshi *et al*., 2010). Also, oleuropein inhibited inflammatory cell recruitment and the release of inflammatory cytokines (Giner *et al.,* 2013). Hydroxytyrosol upregulated heme oxygenase-1, which is an antioxidant enzyme, in keratinocyte, indicating that it could have a radio-protective effect on human skin (Rafehi *et al.,* 2012). Because of the skin treatment effects of these compounds, olive oil is used not only for cooking but also for topical external folk treatments, e.g. as a remedy for dermatitis, eczema, xerosis, other types of inflammation, and photo aging of the skin (Aburjai and Natsheh, 2003). In recent studies, those compounds have been found not only in olive oil but also in olive oil by-products such as olive leaves and OMW (Klen and Vodopivec, 2012). However, the biological activities of the crude extracts of these by-products have not been fully elucidated.

In this research, in order to evaluate the potential of olive oil by-products for use as skin treatments, the biological activities of the ethanol (EtOH) extracts and water extracts of olive oil by-products were evaluated. For the olive samples, olive leaves, stems, flowers, OMW, fruit pulp, and seeds were collected. Popular

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uses for skin treatments include helping individuals avoid folliculitis and aging spots, as an allergy remedy, and removing skin wrinkles. In order to estimate the ability of the extracts to fulfill these functions, several *in vitro* assays were conducted. These screening data will elucidate the potential of olive oil by-products for use in skin treatment applications to undergo further research.

## MATERIALS AND METHODS

**Microorganisms and cell lines.** *Escherichia coli* (NBRC, 3301) and *Staphylococcus aureus* (NBRC, 12732) were purchased from the NITE Biological Resource Center (NBRC, Chiba, Japan).

B16 melanoma cells and rat basophilic leukemia (RBL-2H3) cells (Riken Bioresource Center, Japan) were routinely maintained in 10% FBS in Eagle's minimal essential medium (EMEM). Adult normal human dermal fibroblasts (NHDF-Ad, Lonza, Tokyo, Japan) were routinely maintained in 10% FBS in Dulbecco's modified Eagle's medium (DMEM). All of the cultivation was conducted at 37 °C, 5.0% $CO_2$.

**Extraction from olive oil by-products.** Fresh leaves, stems, flowers, OMW, fruit pulp, and seeds were collected on Kyushu Island in 2013. Leaves and stems (Mission cultivar) were collected at two different seasonal stages, May (flowering time) and October (fruit harvesting time). Flowers (Mission cultivar) were collected in May. Olive fruits were collected in October (mixture of Mission, Manzanillo, Nevadillo Blanco, and Lucca cultivars). Fruits were squeezed freshly using a two-phase centrifuge, and OMW was collected soon after the separation from the oil. Fruits were divided into two parts to make fruit pulp and seeds. Soon after sample collection, each part was freeze-dried and milled into powder, and extracted with 99.5% ethanol (EtOH) or water on a shaker at 200 rpm at room temperature for 24 h. Then the extraction yield was calculated after EtOH extracts were concentrated and dried using a rotary evaporator, and the water extracts were freeze-dried.

**HPLC and LC/MS analysis.** For HPLC and LC/MS analysis, the EtOH extracts, oleuropein, and hydroxytyrosol were dissolved in EtOH, and the water extracts were dissolved in water. The concentration of the extracts was set at 1 μg/mL. HPLC analysis was performed on a CAPCELL PAK $C_{18}$ UG120 (5 μm, 4.6 mm I.D. × 250 mm, Shiseido, Japan) under the following conditions: column temperature, 40 °C; flow rate, 1.0 mL/min; injection volume, 10 μL; elution solvents, (A) acetonitrile and (B) formic acid (0.1%) in water; mobile phase program, a linear gradient elution of 5%–30% (A) for the first 30 min followed by an isocratic elution of 30% (A) for 10 min; detection wave lengths, 280 nm and 380 nm. LC/MS (ESI) analysis was conducted using a CAPCELL PAK C18 UG120 (3 μm, 2.0 mm I.D. × 150 mm, Shiseido, Japan). The flow rate was 0.2 mL/min, the injection volume was 5 μL, the ion polarity was in negative mode, and the other analytical conditions were the same as in the HPLC analysis. The chromatogram of each extract was compared with that of oleuropein or hydroxytyrosol.

**Folin–Ciocalteu assay.** Total phenol (TP) content was determined using the Folin–Ciocalteu assay. Fifty microliters of sample solution (2 mg/mL or 4 mg/mL) was mixed with 100 μL of Folin–Ciocalteu reagent. After 3-min mixing, 400 μL of 7.5% sodium carbonate solution was added to the mixture. After a 2-h reaction, the absorbance was measured at 765 nm. Finally, the TP content was calculated as a gallic acid equivalent (mg GAE/g) ($n = 3$).

**Biological assays.** The antioxidant, antibacterial, antimelanogenesis, anti-allergic, and collagen-production-promoting activities of the samples were estimated. To prepare the samples for the following biological assays, EtOH extracts and oleuropein were dissolved in DMSO, and water extracts were dissolved in distilled water.

**Antioxidant assay.** The antioxidant activities of the samples were estimated by ABTS (3-ethyl-benzothiazoline-6-sulfonic acid) assay, DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, oxygen radical absorption capacity (ORAC) assay, and superoxide anion scavenging activity (SOSA) assay.

The ABTS assay described previously (Tanaka *et al.,* 2014) was performed to measure the ABTS radical scavenging activity of the samples. In order to make a standard curve, 1 mL of the working solution was mixed with 100 mL of Trolox solution, and the mixture was incubated at 30 °C for 4 min. Instead of Trolox, each sample was added to the working solution by the same method. The results are expressed in terms of Trolox equivalent antioxidant capacity (TEAC) (μg/mg).

The DPPH assay described previously (Thaipong *et al.,* 2006) was performed to measure the DPPH radical scavenging activity of the samples. Trolox was used for a standard curve. One milliliter of Trolox solution in Tris–HCl Buffer (0.1 M) was allowed to react with 1 mL of DPPH solution (0.2 mM) for 30 min. Instead of Trolox, each sample was added to the DPPH solution. The results were expressed in terms of TEAC (μg/mg).

The ORAC assay described previously (Mira *et al.,* 2013) was performed to measure the peroxyl radical scavenging activity of the samples. Data were expressed as micromoles of TEAC (μmol TE/mg).

These three TEAC values were determined using the following mathematical expression: TEAC = [$IC_{50}$ of Trolox] / [$IC_{50}$ of sample].

SOSA was performed as described previously (Tanaka *et al.,* 2014). Kit-WST (Dojindo, JAPAN, S311) was used in this assay. Data were expressed in terms of SOD equivalent antioxidant capacity (U/μg extract).

**Antibacterial assay.** The assay described previously (Tanaka *et al.,* 2014) was performed with slight modification. *E. coli* and *S. aureus* ($10^4$ CFU/mL) suspended in NB medium were mixed with the samples at the maximum concentration. Sorbic acid (final concentration: 400 μg/mL, 3.57 mM) was used as a positive control. After incubation

at 37 °C ± 1 °C for 18 h, bacterial growth was measured using a microplate reader at 630 nm (Biotek-ELX800, BioTek), and the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were measured.

**Non-cytotoxic concentration.** In order to determine the concentration of the extracts used in further cell experiments, the cell viability (CV) of cells (B16 melanoma, RBL-2H3, and NHDF-Ad cell) treated with the samples was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Tanaka *et al.,* 2014). The cells were treated with the extracts at the maximum solubility of the sample and its several diluted concentrations. The methods by which the cells were treated with samples are described in each assay's section. After the treatment, CV was measured using MTT assay. Then the maximum concentration of the sample at which CV was higher than 90% was defined as the non-cytotoxic concentration. Extracts were added to the cells at the non-cytotoxic concentration in further cell experiments.

**Anti-melanogenesis assay.** The assay described previously (Arung *et al.,* 2007) was performed to screen for inhibitors of melanin synthesis in melanocytes. Melanin produced by melanocytes is the cause of aging spots and darkness of the skin. B16 cells, which are a tumor analog of melanocytes, were seeded on 24-well plates ($1 \times 10^5$ cells/well) with 1 mL of EMEM containing 90 μg/mL theophylline and 10% FBS. The medium was replaced with a mixture of 998 μL of fresh EMEM and 2 μL of the sample in 24-h and 72-h incubation periods. The absorbance of melanin in 1 M NaOH cell lysate was measured at 405 nm in a 96-h incubation period for calculating the melanin content (MC). CV was measured using MTT assay. The solvent (water or DMSO) used for dissolving the sample was used as a corresponding control, and arbutin (final concentration: 100 μg/mL, 367 μM) was used as a positive control.

**Anti-allergic assay.** The assay described previously (Mira *et al.,* 2013) was performed with some modifications to estimate the effect of the samples on granule release from basophil. A colorimetric assay using ß-hexosaminidase activity was performed to determine the amount of granules released from basophil. To measure the effect of the samples on the granule release, RBL-2H3 cells, which are a tumor analog of basophil, were seeded on 96-well plates ($5 \times 10^4$ cells/well), 24 h before the treatments. First, the cells were treated with 0.5 μg/mL anti-DNP (dinitrophenol) mouse IgE. After 24-h incubation, the medium was replaced with 100 μL of Tyrode's buffer (130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2 \cdot 6H_2O$, 10 mM HEPES, 5.6 mM glucose, 0.1% (g/v) BSA, pH 7.2), and 0.5 μL of the sample was added to Tyrode's buffer. After 1-h incubation, 50 μL of the supernatant (S1) was transported to the first new 96-well plate. Tyrode's buffer containing sample was replaced with 100 μL of fresh Tyrode's buffer containing 5 μg/mL DNP-BSA, which is an antigen of IgE. After 40-min incubation to stimulate the cells to release granules, 50 μL of the supernatant (S2) was transported to the second new 96-well plate. The amount of ß-hexosaminidase in S1 and S2 was measured by colorimetric assay (the absorbance at 405 nm of enzyme degradation product was measured), and the rates of granule release were estimated as G1 and G2, correspondently. The equations are as follows.

$$G1 = \{Abs_{405}[S1(Sample)] - Abs_{405}[S1(Blank)]\}/\{Abs_{405}[S2(Control)] - Abs_{405}[S2(Blank)]\} \times 100$$

$$G2 = \{Abs_{405}[S2(Sample)] - Abs_{405}[S2(Blank)]\}/\{Abs_{405}[S2(Control)] - Abs_{405}[S2(Blank)]\} \times 100$$

S1(Sample): supernatant taken after EMEM media was replaced with Tyrode's buffer and the cells were treated with 0.5 μL of the sample solution for 1 h. S2 (Sample): after S1(Sample) was taken, Tyrode's buffer containing sample was replaced with Tyrode's buffer containing DNP-BSA. S2(Sample) is supernatant taken after the cells were stimulated with DNP-BSA for 40 min. S1(Blank): supernatant taken after the media was replaced with Tyrode's buffer and the cells were treated with 0.5 μL of the solvent used for dissolving the sample (DMSO or distilled water) for 1 h. S2(Blank): after S1(Blank) was taken, Tyrode's buffer containing the solvent was replaced with fresh Tyrode's buffer (DNP-BSA was not added). S2(Blank) is supernatant taken after the cells were just incubated in fresh Tyrode's buffer for 40 min. S2(Control): first, the media was replaced with Tyrode's buffer, and the cells were treated with 0.5 μL of the solvent used for dissolving the sample for 1 h. Second, Tyrode's buffer containing the solvent was replaced with Tyrode's buffer containing DNP-BSA. S2(Control) is supernatant taken after the cells were stimulated with DNP-BSA for 40 min.

The CV was measured using an MTT assay after 1-h sample treatment after 48-h cultivation period. The solvent of the sample was used as a control, and quercetin dehydrate (final concentration: 3.4 μg/mL, 10 μM) was used as a positive control.

**Collagen-production-promoting assay.** The assay was performed to screen for promoters of collagen production of dermal fibroblasts. NHDF-Ad was seeded on 96-well plates ($2 \times 10^4$ cells/well) 24 h before the treatment. The medium was replaced with a mixture of 0.5 μL of DMSO solution of EtOH extract and 100 μL of DMEM supplemented with 0.5% FBS. After 72-h cultivation, the amount of collagen in the medium was measured using a human collagen type I ELISA kit (ACEL, Japan). The cells remaining in the 96-well plate were subjected to an MTT assay. The solvent used for dissolving the sample was used as a control, and ascorbic acid (17.6 μg/mL, 100 μM) was used as a positive control.

**Statistical analysis.** The results are expressed as means ± standard deviation ($n = 3$). Significant differences between each tested group and the control group

were determined using Dunnett's multiple post hoc test (* $p < 0.05$, ** $p < 0.01$), when the one-way analysis of variance (ANOVA) was significant ([a] $p < 0.01$).

## RESULTS AND DISCUSSION

### Chemical properties of EtOH extracts and water extracts of olive oil by-products

Table 1 shows four parameters related to the extracts: the ratio of extraction solvent (EtOH and water) versus dried raw material, the extraction yield, and the oleuropein content and TP content in the extracts and dried raw materials, respectively.

The extraction yields from OMW, fruit pulp, and seeds were relatively higher than those from leaves, stems, and flowers because the former parts contained oil.

The oleuropein and hydroxytyrosol content in the extract was analyzed by HPLC and LC/MS. Standards of oleuropein and hydroxytyrosol were detected by HPLC at 280 nm with retention times of 30.4 min and 8.7 min, respectively. A peak at 8.7 min appeared in one of the chromatograms. A peak at 30.4 min appeared in the chromatogram of EtOH extract from the leaves (May and Oct.), stems (May and Oct.), and flowers (chromatograms are shown in Supplementary Data). The relative amounts of oleuropein were then determined to be 56.2, 174, 189, 246, and 33.4 mg/g in the extracts, respectively. The peaks at 30.4 min were confirmed to be oleuropein by further LC/MS analysis ($m/z = 539$) (data not shown).On the other hand, oleuropein was not detected in the EtOH extracts of OMW, fruit pulp, or seeds nor in any of the water extracts. Oleuropein in drupe is converted gradually to dimethyloleuropein or elenolic acid glucoside after the drupe enters the mature stage (Romero *et al.,* 2002). October is the last maturation stage for olive fruits in Japan, so oleuropein was not detected in the fruit samples. The oleuropein content of the EtOH extract of the stems (Oct.) was the highest (246 mg/g) among all the extracts. However, the oleuropein content of raw dried leaves (Oct.) was the highest (22.1 mg/g) because it had the highest extraction yield.

The TP content of the extracts was estimated using the Folin–Ciocalteu assay. All the extracts were estimated to contain phenolic compounds. Of the EtOH extracts, the stems (May and Oct.) showed the highest TP content (169.7 and 165.3 mg GAE/g, respectively, in the extract) followed by the leaves (May and Oct.), flowers, seeds, fruit pulp, and OMW. Of the water extracts, the seeds showed the highest TP content (46.8 mg GAE/g in the extract) followed by the fruit pulp, stems (Oct.), OMW, stems (May), leaves (May and Oct.), and flowers. The EtOH extracts of stems (May and Oct.), leaves (May and Oct.), and flowers were the only extracts to contain a high TP content over 50 mg GAE/g. EtOH is more hydrophobic than water, so EtOH can extract more hydrophobic compounds like polyphenol and water can extract more hydrophilic compounds like sugar. Each activity exhibited by the EtOH and water extracts of olive oil by-products is discussed in the sections below.

**Table 1. Sample preparation condition, oleuropein content, and total phenol (TP) content of (a) EtOH extracts and (b) water extracts of olive oil by-products**

(a)

| | Extraction | | Oleuropein content (mg/g) | | TP content (mg GAE/g) | |
|---|---|---|---|---|---|---|
| EtOH extract | Solvent (mL/g of dry weight) | Yield (g/g of dry weight × 100) | In extract | In dry weight | In extract | In dry weight |
| Leaves (May) | 6.7 | 4.8 | 56.2 | 2.7 | 100.7 | 4.8 |
| Leaves (Oct.) | 10 | 12.7 | 174 | 22.1 | 117.6 | 14.9 |
| Stems (May) | 4.8 | 3.2 | 189 | 6.0 | 169.7 | 5.4 |
| Stems (Oct.) | 10 | 5.5 | 246 | 13.5 | 165.3 | 9.1 |
| Flowers | 14 | 6.9 | 33.4 | 2.3 | 81.6 | 5.6 |
| OMW | 10 | 18.3 | — | — | 22.8 | 4.2 |
| Fruit pulp | 10 | 37.2 | — | — | 23.9 | 8.9 |
| Seeds | 10 | 9.2 | — | — | 27.5 | 2.5 |

(b)

| | Extraction | | Oleuropein content (mg/g) | | TP content (mg GAE/g) | |
|---|---|---|---|---|---|---|
| Water extract | Solvent (mL/g of dry weight) | Yield (g/g of dry weight × 100) | In extract | In dry weight | In extract | In dry weight |
| Leaves (May) | 6.7 | 12.7 | — | — | 22.6 | 2.9 |
| Leaves (Oct.) | 10.0 | 7.5 | — | — | 21.0 | 1.6 |
| Stems (May) | 3.3 | 6.9 | — | — | 23.5 | 1.6 |
| Stems (Oct.) | 10.0 | 10.6 | — | — | 39.2 | 4.2 |
| Flowers | 11.1 | 17.9 | — | — | 21.0 | 3.8 |
| OMW | 10.0 | 18.4 | — | — | 34.9 | 6.4 |
| Fruit pulp | 10.0 | 25.3 | — | — | 43.8 | 11.1 |
| Seeds | 10.0 | 4.0 | — | — | 46.8 | 1.9 |

TP content: total phenol content equivalent to gallic acid.

—: not detected.

## Antioxidant activity

Table 2 shows the antioxidant activity of EtOH extracts, oleuropein, and water extracts. The antioxidant activity of the sample was considered 'not detectable' when its $IC_{50}$ was higher than the maximum solubility. In EtOH extracts, leaves, stems, and flowers showed relatively higher antioxidant activities than OMW, fruit pulp, and seeds. In particular, stems (Oct.) showed the highest antioxidant activity in the ABTS (271.0 μg/mg) and SOSA assays (1.12 U/μg), and stems (May) showed the highest antioxidant activity in the DPPH (302.2 μg/mg) and ORAC assays (2.94 μmol TE/mg). Oleuropein, one of the polyphenols in olives, showed antioxidant activity (ABTS, 424.5 μg/mg; DPPH, 612.9 μg/mg; ORAC, 3.69 μmol TE/mg; SOSA, 2.00 U/μg). Generally, the catechol moiety in the polyphenol structure has strong reducing power and shows antioxidant activity. The oleuropein and TP contents of the EtOH extracts of leaves, stems, and flowers were higher than those of OMW, fruit pulp, and seeds. This could be one of the reasons for the high antioxidant activities of the leaves, stems, and flowers. Some antioxidant activity, however, had no correlation with the oleuropein content or TP content. For example, the antioxidant activity of the leaves (May) was lower than that of the leaves (Oct.) in the ABTS and DPPH assays. On the other hand, the antioxidant activity of the leaves (May) was higher than that of the leaves (Oct.) in the ORAC and SOSA assays, even though the TP content of the leaves (May) was lower than that of the leaves (Oct.). These uncorrelated results were caused by seasonal variation of the components in the extracts and differences in principal among the four antioxidant assays. In the previous report, which compared the antioxidant activity of immature and mature olive leaves, DPPH radical scavenging activity correlated with the TP content of the olive leaf extract; however, ABTS radical scavenging activity did not correlate with the TP content in this way (Brahmi *et al.,* 2013).

The water extracts of seeds, stems (Oct.), and fruit pulp showed higher ABTS radical scavenging activity than the other water extracts (Table 2 (b)). According to the Folin–Ciocalteu assay, the TP content of the water extracts of seeds, stems (Oct.), and fruit pulp was higher than that of the other water extracts (Table 1 (b)). It is likely that the three water extracts showed high ABTS radical scavenging activity because this activity reflects TP content.

Oxidative stress in the skin induced by UV irradiation results in conditions associated with photo aging, such as damaged DNA and skin cancer (Budiyanto *et al.,* 2000) and inflammation activation of melanin synthesis (Ha *et al.,* 2009). These antioxidants have the potential for use in the treatment of aged skin.

## Antibacterial activity on *E. coli* and *S. aureus*

Table 3 shows the effects of EtOH extracts, oleuropein, and water extracts on antibacterial activity against *E. coli* and *S. aureus*. None of the extracts inhibited the growth of *E. coli*; however, EtOH extracts of leaves (May and Oct.) and flowers inhibited the growth of *S. aureus.* The MICs

**Table 2. Antioxidant activity of (a) EtOH extracts and (b) water extracts of olive oil by-products**

(a)

| EtOH extract | ABTS TEAC (μg/mg) | DPPH TEAC (μg/mg) | ORAC TEAC (μmol TE/mg) | WST-1 SOSA (U/μg) |
|---|---|---|---|---|
| Leaves (May) | 55.0 ± 1.9 | 189.2 ± 0.9 | 2.12 ± 0.08 | 0.70 ± 35 |
| Leaves (Oct.) | 168.9 ± 2.2 | 289.6±25 | 0.47 ± 0.01 | 0.25 ± 0.1 |
| Stems (May) | 101.1 ± 1.9 | 302.2 ± 21 | 2.94 ± 0.08 | 0.45 ± 16 |
| Stems (Oct.) | 271.0 ± 4.4 | 239.1 ± 6.6 | 1.25 ± 0.00 | 1.12 ± 0.3 |
| Flowers | 51.8 ± 1.2 | 185.2 ± 2.8 | 1.72 ± 0.08 | 0.69 ± 85 |
| OMW | — | — | 0.08 ± 0.00 | 0.20 ± 0.0 |
| Fruit pulp | — | — | 0.03 ± 0.00 | 0.14 ± 0.0 |
| Seeds | — | — | 0.11 ± 0.00 | 0.48 ± 0.1 |
| Oleuropein | 424.5 ± 3.1 | 612.9 ± 31 | 3.69 ± 0.01 | 2.00 ± 0.2 |

(b)

| Water extract | ABTS TEAC (μg/mg) | DPPH TEAC (μg/mg) | ORAC TEAC (μmol TE/mg) | WST-1 SOSA (U/μg) |
|---|---|---|---|---|
| Leaves (May) | — | — | 0.42 ± 0.02 | 0.04 ± 12 |
| Leaves (Oct.) | — | — | 0.12 ± 0.01 | 0.05 ± 0.0 |
| Stems (May) | — | — | 0.75 ± 0.01 | 0.08 ± 5.8 |
| Stems (Oct.) | 88.5 ± 1.9 | — | 0.08 ± 0.00 | 0.04 ± 0.0 |
| Flowers | — | — | 0.54 ± 0.04 | — |
| OMW | — | — | 0.19 ± 0.01 | 0.34 ± 0.1 |
| Fruit pulp | 75.5 ± 2.0 | — | 0.57 ± 0.01 | 0.17 ± 0.0 |
| Seeds | 133.4 ± 18 | — | 0.17 ± 0.00 | 0.16 ± 0.1 |

The antioxidant values are presented as means ± SD ($n$ = 3).

—: not detectable.

TEAC: Trolox equivalent antioxidant capacity.

SOSA: superoxide anion-scavenging activity.

**Table 3. The effect of (a) EtOH extracts and (b) water extracts of olive oil by-products on growth of *E. coli* and *S. aureus***

(a)

| | | *E. coli* | *S. aureus* | | |
|---|---|---|---|---|---|
| EtOH extract | (μg/mL) | OD630 (%) | OD630 (%) | MIC (μg/mL) | MBC (μg/mL) |
| Control | | 100 ± 1.3 | 100 ± 3.6 | | |
| Sorbic acid | 400 | 0.35 ± 0.1 | 0.00 ± 0.0 | | |
| Leaves (May) | 800 | 64.7 ± 8.3 | 0.00 ± 0.0 | 800 | — |
| Leaves (Oct.) | 800 | 86.5 ± 3.4 | 0.00 ± 2.7 | 800 | — |
| Stems (May) | 1600 | 82.7 ± 6.8 | 112 ± 1.9 | — | — |
| Stems (Oct.) | 800 | 88.8 ± 18 | 88.4 ± 4.6 | — | — |
| Flowers | 800 | 74.1 ± 2.9 | 0.00 ± 0.0 | 800 | 1600 |
| OMW | 900 | 94.3 ± 3.3 | 116 ± 6.7 | — | — |
| Fruit pulp | 900 | 111 ± 1.9 | 97.1 ± 19 | — | — |
| Seeds | 900 | 66.0 ± 8.2 | 99.0 ± 7.0 | — | — |
| Oleuropein | 700 | 81.0 ± 3.2 | 98.9 ± 0.3 | — | — |

(b)

| | | *E. coli* | *S. aureus* |
|---|---|---|---|
| Water extract | (μg/mL) | OD630 (%) | OD630 (%) |
| Control | | 100 ± 1.2 | 100 ± 4.4 |
| Sorbic acid | 400 | 0.35 ± 0.1 | 0.00 ± 0.0 |
| Leaves (May) | 1600 | 92.5 ± 18 | 116 ± 4.6 |
| Leaves (Oct.) | 400 | 123 ± 1.1 | 118 ± 21 |
| Stems (May) | 800 | 129 ± 2.1 | 152 ± 1.6 |
| Stems (Oct.) | 800 | 128 ± 0.8 | 130 ± 21 |
| Flowers | 800 | 114 ± 19 | 180 ± 5.5 |
| OMW | 400 | 98.8 ± 4.5 | 140 ± 46 |
| Fruit pulp | 400 | 96.0 ± 5.6 | 106 ± 21 |
| Seeds | 400 | 91.1 ± 2.9 | 166 ± 17 |

Sorbic acid: positive control. MIC: minimum inhibitory concentration, MBC: minimum bactericidal concentration.
The relative activity values (mean ± SD) were normalized to the control ($n$ = 3).

of the active samples were 800 μg/mL, and the MBC of the EtOH extract of flowers was 1600 μg/mL. Oleuropein, which was contained in the EtOH extracts of leaves and flowers, showed no inhibition effect on the bacterial growth, so the active compound for the antibacterial activity of the extracts might not be oleuropein.

*S. aureus* is a cause of skin infections such as folliculitis and atopic dermatitis in both developed and developing countries (Miller and Cho, 2011). To develop anti-staphylococcal agents as remedies for skin infections, further studies are needed.

## Cell experiments

Cell experiments were conducted at the highest non-cytotoxic concentration of each sample (the maximum soluble concentration without cytotoxicity). The concentrations of the samples are given in Table 4. A superscript '+' was attached to any non-cytotoxic concentrations that were equal to the maximum solubility.

## Anti-melanogenesis effect on B16 cells

Table 4 (a) shows the effects of EtOH extracts, oleuropein, and water extracts on MC in B16 cells and CV. The MC and CV levels of the controls were set at 100%. A low MC value means that the sample has anti-melanogenesis activity. As shown in Table 4 (a), oleuropein and all the EtOH extracts except for those from seeds showed significant differences from the control group. In particular, the EtOH extracts of leaves (May) strongly inhibited melanogenesis (the MC was 31.8%). Oleuropein also showed this same activity (the MC was 58.4%). None of the water extracts showed a significant difference from the control in terms of their anti-melanogenesis effects. As for the effect of oleuropein, it is possible for oleuropein to partially contribute to the activity of some EtOH extracts. However, there was little correlation between the activity strength and oleuropein content of these extracts. For example, comparing the MC value of each EtOH extract at the same concentration, 20 μg/mL, which showed no cytotoxicity, leaves (May), leaves (Oct.), stems (Oct.), and flowers gave the MC value of 58.4 ± 6.7%, 80.7 ± 2.9%, 86.2 ± 5.8%, and 83.4 ± 2.0%, respectively. The other EtOH extracts gave the MC value over 90% at 20 μg/mL. From these results, we can see that in spite of lower amount of oleuropein content shown in Table 1 (a), EtOH extract of leaves (May) has the strongest anti-melanogenesis activity. There are several possibilities regarding the mechanism of anti-melanogenesis activity. One possible reason for this was that the compounds had a synergistic effect. The synergistic effect of oleuropein and flavonoids was described previously, and olive leaf extract showed anti-cancer activity in B16 cells at concentrations higher than 150 μg/mL (Mijatovic *et al.,* 2010). In our experiment, synergistic effects were also expected to result in strong anti-melanogenesis

**Table 4. Biological activities of extracts of olive oil by-products on (a) B16 melanoma cells, (b) RBL-2H3 cells, and (c) NHDF-Ad cells**

(a)

| | B16 | | | | | |
|---|---|---|---|---|---|---|
| | EtOH extract | | | Water extract | | |
| | Sample concentration (μg/mL) | Melanin content (MC) (%)[a] | Cell viability (CV) (%) | Sample concentration (μg/mL) | Melanin content (MC) (%) | Cell viability (CV) (%)[a] |
| Control | — | 100 ± 5.3 | 100 ± 2.7 | — | 100 ± 6.1 | 100 ± 3.1 |
| Arbutin | 100 | 55.6 ± 3.1 ** | 95.0 ± 3.2 | 100 | 59.2 ± 2.3 | 89.5 ± 2.9 ** |
| Leaves (May) | 80 | 31.8 ± 1.9 ** | 98.6 ± 1.4 | 160 [1] | 98.9 ± 4.1 | 92.9 ± 4.4 |
| Leaves (Oct.) | 20 | 80.7 ± 2.9 ** | 101 ± 2.3 | 160 [1] | 95.0 ± 2.1 | 101 ± 1.8 |
| Stems (May) | 80 | 61.2 ± 2.4 ** | 103 ± 0.5 | 160 [1] | 106 ± 6.3 | 103 ± 2.3 ** |
| Stems (Oct.) | 40 | 67.5 ± 2.8 ** | 97.2 ± 0.8 | 320 [1] | 98.0 ± 5.1 | 105 ± 3.7 |
| Flowers | 80 | 67.0 ± 1.2 ** | 97.8 ± 3.8 | 40 [1] | 105 ± 14 | 90.0 ± 1.8 |
| OMW | 80 [1] | 84.1 ± 4.6 ** | 95.0 ± 3.9 | 160 [1] | 98.2 ± 7.6 | 105 ± 1.9 |
| Fruit pulp | 80 | 84.7 ± 2.0** | 93.9 ± 2.1 | 160 [1] | 101 ± 3.8 | 101 ± 4.8 |
| Seeds | 160 [1] | 89.4 ± 1.7 | 92.7 ± 3.8 | 160 [1] | 81.8 ± 0.4 | 95.4 ± 3.3 |
| Oleuropein | 350 | 58.4 ± 0.3 ** | 97.8 ± 0.3 | | | |

(b)

| | RBL-2H3 cell | | | |
|---|---|---|---|---|
| | EtOH extract | | | |
| | Sample concentration (μg/mL) | Granule release (%) | | Cell viability |
| | | G1[a] | G2[a] | (CV) (%)[a] |
| Control | — | 23.8 ± 3.5 | 100 ± 7.6 | 100 ± 9.4 |
| Quercetin dihydrate | 3.4 | 60.4 ± 6.4 ** | 64.6 ± 19 * | 106 ± 6.6 |
| Leaves (May) | 50 | 36.9 ± 2.4 | 115 ± 15 | 106 ± 6.3 |
| Leaves (Oct.) | 50 | 46.2 ± 10 | 100 ± 2.9 | 115 ± 1.8 ** |
| Stems (May) | 100 | 51.9 ± 8.3 * | 149 ± 22 * | 103 ± 4.7 |
| Stems (Oct.) | 100 | 55.8 ± 2.4 ** | 113 ± 17 | 109 ± 2.0 |
| Flowers | 50 | 45.4 ± 10 | 137 ± 12 * | 107 ± 1.9 |
| OMW | 400 | 60.4 ± 5.9 ** | 56.5 ± 12 ** | 100 ± 1.6 |
| Fruit pulp | 400 | 56.5 ± 5.2 ** | 83.1 ± 9.7 | 108 ± 1.6 |
| Seeds | 400 | 63.8 ± 25 ** | 83.5 ± 15 | 110 ± 0.5 |
| Oleuropein | 350 | 5.6 ± 2.0 | 97.2 ± 7.2 | 100 ± 2.7 |

| | RBL-2H3 cell | | | |
|---|---|---|---|---|
| | Water extract | | | |
| | Sample concentration (μg/mL) | Granule release (%) | | Cell viability |
| | | G1[a] | G2[a] | (CV) (%)[a] |
| Control | — | 51.0 ± 7.0 | 100 ± 16 | 100 ± 4.4 |
| Quercetin dihydrate | 3.4 | 47.8 ± 8.4 | 79.0 ± 7.4 | 105 ± 2.2 |
| Leaves (May) | 400 [1] | 183 ± 36 ** | 29.3 ± 23 ** | 112 ± 2.2 ** |
| Leaves (Oct.) | 400 [1] | 139 ± 14 ** | 37.4 ± 4.3 ** | 111 ± 2.3 ** |
| Stems (May) | 800 [1] | 175 ± 17 ** | 21.2 ± 5.6 ** | 108 ± 1.0 * |
| Stems (Oct.) | 800 [1] | 52.3 ± 1.7 | 60.7 ± 38 | 109 ± 0.9 ** |
| Flowers | 200 [1] | 89.7 ± 4.7 | 61.1 ± 10 ** | 109 ± 5.5 ** |
| OMW | 800 [1] | 74.7 ± 4.8 | 62.4 ± 2.2 ** | 111 ± 2.2 ** |
| Fruit pulp | 800 [1] | 84.5 ± 33 | 73.7 ± 15 | 108 ± 3.3 * |
| Seeds | 400 | 60.3 ± 7.5 | 95.6 ± 8.1 | 109 ± 2.6 * |

(Continues)

**Table 4.** (continued)

(c)

| | NHDF-Ad cell | | |
|---|---|---|---|
| | EtOH extract | | |
| | Sample concentration (μg/mL) | Collagen production (CP) (%)[a] | Cell viability (CV) (%)[a] |
| Control | — | 100 ± 36 | 100 ± 15 |
| Ascorbic acid | 17.6 | 483 ± 38 ** | 107 ± 0.52 |
| Leaves (May) | 100 [1] | 364 ± 51 ** | 145 ± 6.4 ** |
| Leaves (Oct.) | 100 [1] | 279 ± 27 ** | 122 ± 3.7 |
| Stems (May) | 100 [1] | 352 ± 27 ** | 97.3 ± 3.6 |
| Stems (Oct.) | 100 [1] | 275 ± 39 ** | 130 ± 4.8 * |
| Flowers | 50 [1] | 124 ± 28 | 152 ± 6.8 ** |
| OMW | 400 [1] | 324 ± 60 ** | 122 ± 3.5 |
| Fruit pulp | 400 [1] | 427 ± 11 ** | 137 ± 1.3 * |
| Seeds | 400 [1] | 163 ± 0 * | 93.2 ± 7.6 |
| Oleuropein | 432 | 118 ± 13 | 104 ± 0.7 |

G1, G2: the relative amounts of released granules versus control of G2.
The relative activity values (mean ± SD) were normalized to the control ($n = 3$).
Significant difference between each tested group and the control group was determined using
Dunnett's multiple post hoc test (*: $p < 0.05$, **: $p < 0.01$) when the one-way ANOVA was significant ([a]: $p < 0.01$).
+: The non-cytotoxic concentration was equal to the maximum solubility.

activity of EtOH extract of leaves (May) at non-cytotoxic concentrations. Another reason for this was that there existed some other effective compounds than oleuropein.

### Effect on granule release of RBL-2H3 cells

A basophil releases granules when IgEs on the surface of the basophil recognize an antigen. The granules contain histamines and cytokines, which are inflammatory mediators. Released granules induce the immediate inflammatory reaction of type I allergies such as hay fever and atopy (Ikawati *et al.,* 2001). Therefore, substances that inhibit the release of granules induced by antigens have the potential to be effective treatments for type I allergies.

Table 4 (b) shows the effects of EtOH extracts, oleuropein, and water extracts on G1, G2, and CV in RBL-2H3 cells. The results show that the EtOH extract of OMW and the water extracts of flowers and OMW were inhibitors and the water extracts of leaves (May and Oct.) and stems (May) were stimulators of granule release from the cells.

RBL-2H3 cells were treated with the sample for 1 h, and after the Tyrode's buffer was changed, the cells were stimulated by DNP-BSA for 40 min. In this experimental procedure, the amount of granules released from RBL-2H3 cells was measured at two different stages: first, at the end point of the sample treatment (value G1); and second, at the end point of the treatment with DNP-BSA (value G2). G1 and G2 are the relative rate (%) of released granules versus the G2 value of the control. The G1 values of the water extracts of leaves (May and Oct.) and stems (May) increased significantly (183%, 139%, and 175%, respectively). These results indicate that the water extracts stimulated the cells to release granules. At the second stage, the cells were stimulated by DNP-BSA to release granules following the sample treatment. Quercetin dihydrate, a positive control, successfully inhibited the release of granules and reduced the G2 value (to 64.6% and 79.0%, respectively). Also, the G2 values of the EtOH extract of OMW and the water extracts of flowers, OMW, leaves (May and Oct.), and stems (May) decreased significantly (to 56.5%, 61.1%, 62.4%, 29.3%, 37.4%, and 21.2%, respectively). However, oleuropein showed no activity. On the other hand, according to the G2 values, the water extracts of leaves (May and Oct.) and stems (May) inhibited granule release at the second stage; however, these water extracts stimulated granule release at the first stage. The mechanism of this contradictory phenomenon has not been clarified; however, we speculate that those water extracts were stimulators of granule release and let RBL-2H3 cells run out of ready-to-release granules at the first stage, and that is why RBL-2H3 cells did not release many granules at the second stage. Based on these results, the EtOH extract of OMW and the water extracts of flowers and OMW were screened for anti-allergic substances. In the previous report, a secoiridoid and luteolin were isolated as anti-allergy active compounds from olive pomace that was prepared using a hand mixer from fruits of the Mission cultivar grown in Japan (Sato *et al.,* 2014). These kinds of compounds may be the active compounds in the EtOH extract of OMW.

The results suggest that EtOH extract of OMW can be used as an anti-allergy substance. Further studies are needed to determine the active compounds and the mechanisms responsible for this activity and to investigate the ways in which it can be utilized.

### Collagen-production-promoting effect and proliferation-promoting effect on human dermal fibroblast

Table 4 (c) shows the effects of EtOH extract and oleuropein on the collagen production of NHDF-Ad

(CP) and CV. To express CP and CV, each control value was set at 100%. Ascorbic acid, a positive control, increased CP (487%) and had no effect on CV (107%). Of the samples, fruit pulp increased CP the most (427%), followed by leaves (May), stems (May), OMW, leaves (Oct.), stems (Oct.), and seeds. Oleuropein showed no effect on CP (118%). Of the effective samples, leaves (May), fruit pulp, and stems (Oct.) increased CV (145%, 137%, and 130%, respectively), although ascorbic acid did not. Ascorbic acid is a cofactor of procollagen-proline 4-dioxygenase, which is an enzyme necessary for collagen production. Fe (III) ion, which is also a cofactor of the enzyme, is reduced by ascorbic acid, and the enzyme is activated to produce collagen (Dao *et al.,* 2009). Given these results, some compounds other than oleuropein or the synergistic effect of these three extracts should affect substances other than this enzyme.

Wrinkles or sagging of aged skin is a result of a loss of elasticity and stiffness in the skin due to the reduction of the dermal extracellular matrix (ECM), e.g. collagen, elastin, and hyaluronic acid (Decorps *et al.,* 2014). Collagen production by dermal fibroblasts decreases as fibroblasts age (McGrath *et al.,* 2012). Also, fibroblast proliferation decreases as they age. In the present results, EtOH extracts of olive by-products promoted not only the CP but also the CV of aged fibroblasts. Further studies on EtOH extracts of fruit pulp and leaves are needed to determine the mechanism.

## CONCLUSIONS

This study provides evidence that olive oil by-products have the potential for multiple applications in skin treatment. In this screening study, some biological activities of olive oil by-products were estimated. For example, first, the EtOH extract of stems showed high radical-scavenging activity. EtOH extracts of leaves and flowers inhibited the growth of *S. aureus*. Also, the EtOH extract of leaves reduced the melanin biosynthesis in B16 melanoma cells, the EtOH extract of OMW inhibited granule release from RBL-2H3 cells, and the water extract of leaves and stems stimulated granule release. Finally, the EtOH extracts of leaves and fruit pulp activated collagen production and fibroblast proliferation at each specific concentration for each assay without any cytotoxicity.

Oleuropein showed antioxidant and anti-melanogenesis activities, but there was little correlation between these activities and the oleuropein content of each extract. One reason for this is that a complicated synergistic mechanism exists behind the activity, because crude extract may include various compounds with different activities. For example, *Helminthostachys zeylanica* root contains quercetin and its glycoside derivatives. Quercetin was reported to be a melanogenesis inhibitor (Arung *et al.,* 2010), and the glycoside derivative was reported to be a melanogenesis promoter (Yamauchi *et al.,* 2013). In such cases, it is difficult to estimate the activity of the total extract.

Methods for the utilization of olive oil by-products are badly needed, but few characteristics of olive oil by-products have been elucidated previously. This research is the first to evaluate their multiple potential activities, and further detailed research is needed to investigate effective ways to utilize olive oil by-products.

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### Conflict of Interest

The authors have declared that there is no conflict of interest.

## REFERENCES

Aburjai T, Natsheh FM. 2003. Plants used in cosmetics. *Phytother Res* **17**: 987–1000.

Arung TE, Shimizu K, Kondo R. 2007. Structure activity relationship of prenylsubstituted polyphenols from *Artocarpus heterophyllusas* inhibitors of melanin biosynthesis in cultured melanoma cells. *Chem Biodivers* **4**: 2166–2171.

Arung TE, Furuta S, Ishikawa H, Kusuma IW, Shimizu K, Kondo R. 2010. Anti-melanogenesis properties of quercetin- and its derivative-rich extract from *Allium cepa*. *Food Chem* **124**: 1024–1028.

Brahmi F, Mechri B, Dhibi M, Hammami M. 2013. Variations in phenolic compounds and antiradical scavenging activity of *Olea europaea* leaves and fruits extracts collected in two different seasons. *Ind Crops Prod* **49**: 256–264.

Budiyanto A, Ahmed NU, Wu A, *et al.* 2000. Protective effect of topically applied olive oil against photocarcinogenesis following UVB exposure of mice. *Carcinogenesis* **21**: 2085–2090.

Dao JH, Kurzeja RJM, Morachis JM, *et al.* 2009. Kinetic characterization and identification of a novel inhibitor of hypoxia-inducible factor prolyl hydroxylase 2 using a time-resolved fluorescence resonance energy transfer-based assay technology. *Anal Biochem* **384**: 213–223.

Decorps J, Saumet JL, Sommer P, Sigaudo-Roussel D, Fromy B. 2014. Effect of ageing on tactile transduction processes. *Aging Res Rev* **13**: 90–99.

Ghanbari R, Anwar F, Alkharfy KM, Gilani AH, Saari N. 2012. Valuable nutrients and functional bioactives in different parts of olive (*Olea europaea* L.)—a review. *Int J Mol Sci* **13**: 3291–3340.

Giner E, Recio MC, Rios JL, Giner RM. 2013. Oleuropein protects against dextran sodium sulfate-induced chronic colitis in mice. *J Nat Prod* **76**: 1113–1120.

Ha JY, Choi HK, Oh MJ, Choi HY, Park CS, Shin HS. 2009. Photoprotective and anti-melanogenic effect from phenolic compound of olive leaf (*Olea europaea* L. var. Kalamata) extracts on the immortalized human keratinocytes and B16F1 melanoma cells. *Food Sci Biotechnol* **18**: 1193–1198.

Ikawati Z, Wahyuono S, Maeyama K. 2001. Screening of several Indonesian medicinal plants for their inhibitory effect on histamine release from RBL-2H3 cells. *J Ethnopharmacol* **75**: 249–256.

Klen TJ, Vodopivec BM. 2012. The fate of olive fruit phenols during commercial olive oil processing: traditional press versus continuous two- and three-phase centrifuge. *Food Sci Technol* **49**: 267–274.

McGrath JA, Robinson MK, Binder RL. 2012. Skin differences based on age and chronicity of ultraviolet exposure: results from a gene expression profiling study. *Br J Dermatol* **166**: 9–15.

Mijatovic SA, Timotijevic GS, Milijkovic DM, *et al.* 2010. Multiple antimelanoma potential of dry olive leaf extract. *Int J Cancer* **128**: 1955–1965.

Miller LS, Cho JS. 2011. Immunity against *Staphylococcus aureus* cutaneous infections. *Nat Rev Immunol* **11**: 505–518.

Mira A, Tanaka A, Tateyama Y, Kondo R, Shimizu K. 2013. Comparative biological study of roots, stem, leaves, and seeds of *Angelica shikokiana* Makino. *J Ethnopharmacol* **148**: 980–987.

Rafehi H, Smith AJ, Balcerczyk A, *et al*. 2012. Investigation into the biological properties of the olive polyphenol, hydroxytyrosol: mechanistic insights by genome-wide mRNA-Seq analysis. *Genes Nutr* **7**: 343–355.

Romero C, Garcia P, Brenes M, Garcia A, Garrido A. 2002. Phenolic compounds in natural black Spanish olive varieties. *Eur Food Res Technol* **215**: 489–496.

Sato A, Shinozaki N, Tamura H. 2014. Secoiridoid type of antiallergic substances in olive waste materials of three Japanese vatieties of *Olea europaea*. *J Agric Food Chem* **62**: 7787–7795.

Sumiyoshi M., Kimura Y. 2010. Effects of olive leaf extract and its main component oleuropeinon acute ultraviolet B irradiation-induced skin changes in C57BL/6J mice. *Phytother Res* **24**: 995–1003.

Tanaka A, Zhu Q, Tan H, *et al*. 2014. Biological activities and phytochemical profiles of extracts from different parts of bamboo (*Phyllostachys pubescens*). *Molecules* **19**: 8238–8260.

Thaipong K, Boonprakob U, Crosby K, Cisneros-Zevallos L, Byrne DH. 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *J Food Compost Anal* **19**: 669–675.

Yamauchi K, Mitsunaga T, Batubara I. 2013. Novel quercetin glucosides from *Helminthostachys zeylanica* root and acceleratory activity of melanin biosynthesis. *J Nat Med* **67**: 369–374.

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