A Comparative Study of the Antioxidant Profiles of Olive Fruit and Leaf Extracts against Five Reactive Oxygen Species as Measured with a Multiple Free-Radical Scavenging Method

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Running Head: Antioxidant Profiles of Olives

Abstract: Olive fruits and leaves are recognized to have great potential as natural sources of antioxidants. The major phenolic antioxidant component in these plant tissues is oleuropein. The antioxidant activity of olive fruits and leaves was evaluated in this study using multiple free-radical scavenging (MULTIS) methods, wherein we determined the scavenging abilities of different extracts against five reactive oxygen species (ROS: $HO^{\cdot}$, $O_2^{-\cdot}$, $RO^{\cdot}$, *t*-$BuOO^{\cdot}$, and $^1O_2$). Raw olive fruits taste bitter and are inedible without undergoing a debittering treatment. Following the NaOH-debittering process, the radical scavenging activity of olives decreased by 90%. The MULTIS measurements indicated that oleuropein and hydroxytyrosol are responsible for the radical scavenging activity of olive fruits. Furthermore, we evaluated the radical scavenging profiles of olive leaf extracts against five ROS and found significant seasonal variations in their antioxidant activities. Leaves picked in August possessed greater radical scavenging abilities (180% to 410% for different ROS) than those picked in the cold season (December and February). In roasted olive leaves, we found marked increases (230% to 300% and 180% to 220%) in the antioxidant activities of Maillard reaction products against $RO^{\cdot}$ and *t*-$BuOO^{\cdot}$, respectively. This study presented a useful comparative analysis of the antioxidant capacities of food against various types of ROS.

Keywords: olive, fruit extract, leaf extract, antioxidant capacity, MULTIS

Practical Application: In this study, we evaluated the natural antioxidant activity of olive fruits and leaves against five reactive oxygen species (ROS). We found characteristic differences in the antioxidant profiles of different olive tissues, which varied after different treatments (debittering (fruit), drying (leaf), and roasting (leaf)). Comparative studies of the antioxidant capacities of foods against various ROS are useful to improve the functionality of food products.

Introduction

Olive (*Olea europaea* L.) fruits and leaves have traditionally been used as food additives, functional foods, and pharmaceutical purposes (Bouaziz, Grayer, Simmonds, Damak, & Sayadi, 2005; Granato, Nunes, & Barba, 2017; Soussi, Hfaiedh, Sakly, & Rhouma, 2019). Olives contain considerably high amounts of phenolic antioxidant compounds, including oleuropein and hydroxytyrosol. Antioxidant compounds are not only important due to their nutritional properties but also because of their abilities to scavenge reactive oxygen species (ROS). Oleuropein, the major antioxidant in fresh olive fruits, is very bitter, and must be removed to make olive pulp edible (Marsilio, Campestre, & Lanza, 2001). The removal of the bitter taste (debittering) from olive fruits is generally achieved through alkaline hydrolysis, resulting in the decomposition of the phenolic antioxidant compounds in the olive fruit (Brenes & Castro, 1998; Charoenprasert & Mitchell, 2012). Further, olive leaves have received much attention from researchers owing to their distinctive phenolic compounds related to various biological activities (Goulas et al., 2009). Olive leaves have been widely used for health purposes in the form of extracts, herbal teas, and powders.

In many previous studies, the ability of a substance to quench the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) has been used as an index of its antioxidant capacity. The antioxidant activities of olive fruit and leaf extracts have been evaluated by the DPPH method (Kuo, Liu, Hsu, Lin, & Chen, 2015; Orak, Karamac, Amarowicz, Orak, & Penkacik, 2019; Nicoli et al., 2019). However, the reactivity of antioxidant compounds with the stable radical DPPH is thought to differ from their reactivity with real biological ROS, and the scavenging abilities of antioxidants vary among different ROS and/or other reactive species. Therefore, it is important to determine the antioxidant activities of a substance of interest against various types of ROS. However, direct and quantitative determination of the radical scavenging abilities of foods against various ROS has been hampered by experimental difficulties.

Olive fruits and leaves have scavenging abilities against multiple ROS. A number of studies have been conducted on the antioxidant activities of olive fruits, leaves, and oil phenolic components against several types of ROS. The multiple free-radical scavenging (MULTIS)

method for the determination of antioxidant activities utilizes photochemically generated ROS and the antioxidant activities of substances of interest against multiple ROS were quantified (Oowada, Endo, Kameya, Shimmei, & Kotake, 2012). Recently, we expanded the MULTIS assay for application in analyses of food samples, such as herbs (Sueishi, Sue, & Masamoto, 2018) and ginger root (Sueishi, Masamoto, & Kotake, 2019), and demonstrated its usefulness for studying the comparative antioxidant profiles of foods against various ROS. Analysis of the antioxidant profiles of olives against various ROS is important for the development and promotion of healthy olive food products.

In this study, we determined the radical scavenging abilities of olive fruits and leaves against five ROS using the MULTIS method. The MULTIS method was used to address the following questions: 1) What is the effect of debittering on the antioxidant activity of olive fruit? 2) Is there a seasonal dependence of the antioxidant capacity of olive leaves against five ROS? 3) How does roasting and drying impact the antioxidant activity of olive leaves?

Materials and methods

Reagents

The detection of hydroxyl ($HO^{\cdot}$) and alkoxyl ($RO^{\cdot}$) radicals utilized spin-trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 5-Diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO; Focus Biomolecules, Plymouth Meeting, PA. USA) was used for detection of superoxide ($O_2^{-\cdot}$) and alkylperoxyl radical (*t*-$BuOO^{\cdot}$). Spin-trap DEPMPO has a better ability to trap $O_2^{-\cdot}$ and *t*-$BuOO^{\cdot}$ than DMPO (Kamibayashi et al., 2006). High-purity 2,2,6,6-tetramethyl-4-piperidone (TMPD) was generously supplied by Mikuni Pharmaceutical Industrial Co., Ltd. (Osaka Japan) and used to quantify singlet oxygen ($^1O_2$) levels. The commercial precursors and sensitizers used for the formation of reactive species are listed in Table 1 (Oowada, Endo, Kameya, Shimmei, & Kotake, 2012; Sueishi, Sue, & Masamoto, 2018; Sueishi et al., 2014). Olive-related antioxidant compounds (oleuropein, hydroxytyrosol, luteolin 7-*O*-glucoside, and caffeic acid (purity > 98%)) were purchased from Cayman Chemical Company (Ann Arbor,

MI, USA), Carbosynth (Berkshire, UK), Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), and Tokyo Chemical Industry Co., Ltd., respectively. Acetonitrile (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and distilled water were combined and used as the mixture solvent (1:1, v/v) in electron spin resonance (ESR) spin-trapping analysis because of the solubility of reagents for MULTIS measurements (free radical precursors/photosensitizers and olive-related antioxidant compounds (hydrophilic and lipophilic)) and the formation of various reactive species.

Preparations of olive fruit and leaf extracts

Olive fruit extract samples

Experiments were carried out using Spanish-style Nevadillo Blanco olives. The olive fruits were harvested at the ripening stage, corresponding to when the fruit surface was green, purple, or black in color, in Okayama City, Japan. Green, purple, and black olives were harvested in mid-September, late-September, and mid-October, respectively, in 2017. Olive fruits were immediately packed and sealed in polyethylene bags and stored at −12 ℃. The antioxidant capacity of olives was maintained for 6 weeks. The olive pulp (3 g) was chopped into small pieces and agitated in 30 mL of acetonitrile at room temperature (25 ℃) for 1 h. After filtration, the extract solution was stored at 5 ℃ for analysis. In the NaOH-debittering process, cut olive fruits were immersed in a 2% NaOH solution for 12 h and in water for 3 days at 5 ℃. The treated olive fruits were washed with water to remove the debittering solution, and then subjected to the same procedures for extraction as outlined above.

Olive leaf extract samples

Olive leaves were collected from branches with mature leaves from December 2018 to December 2019 in Okayama City, Japan. The olive leaves (3 g) were chopped into small pieces, suspended in 30 mL of acetonitrile, and heated at 80 ℃ for 1 h. The olive leaf extract solutions, which included antioxidant compounds, were then transferred to brown glass bottles. The sample solutions were cooled with ice water and brought to room temperature.

The extract sample was then kept at 5 ℃. To examine the influence of drying and roasting on the antioxidant activities of olive leaves, leaf samples were dried at room temperature for 14 days, and then the olive leaf powder was roasted at 200 ℃ for 90 s. The dried and roasted olive leaves (3 g) were agitated in 30 mL of acetonitrile at 80 ℃ for 1 h. After filtration, the extract samples were stored at 5 ℃.

ESR measurements of spin-trapping adducts

The concentrations of five ROS in the presence and absence of antioxidants (olive fruit and leaf extracts) were quantified using the ESR spin-trapping method. The olive extract sample was added to the spin-trap and precursors/sensitizers solution, and the resulting solution was loaded into an ESR flat cell. Five ROS were independently generated with ultraviolet/visible (UV/Vis) light illumination (UV illuminator: RUVF-203S, Radical Research Inc., Tokyo, Japan). The experimental conditions used for the evaluation of antioxidant abilities herein are listed in Table 1. The detailed procedures used for ESR measurements have been previously described by Sueishi, Sue, and Masamoto (2018). The reactive species $HO^{\cdot}$ and $RO^{\cdot}$ were generated from the photo-decomposition of H_2O_2 and AAPH, respectively, and $^1O_2$ and $O_2^{-\cdot}$ were formed from the photosensitizers rose bengal (Wako Pure Chemical Industries, Ltd.) and riboflavin (Tokyo Chemical Industry Co., Ltd.), respectively. Peroxide *t*-$BuOO^{\cdot}$ was generated from the photolysis of *t*-butylhydroperoxide (Tokyo Chemical Industry Co., Ltd.) (Bors, Michel, & Stettmaier, 1992). A JEOL FA200 X-band ESR spectrometer (JEOL Ltd., Akishima, Japan) was used to record the ESR spectra of ROS adducts, and the ESR signal intensity was used to obtain measurements of reactive species' concentrations. Typical ESR spectrometer settings were as follows: a center magnetic field of 337 mT, sweep time of 1 min, modulation width of 0.06 mT, time constant of 0.1 s, and microwave power of 5 mW.

Determination of ROS scavenging rates (antioxidant activities)

Radical scavenging rates and rate constants were determined from the ESR signal

intensities of spin adducts measured with or without the addition of antioxidants. The ROS scavenging activities of foods result from the activities of multiple antioxidants. The antioxidants (AOx(1), ··· AOx(n)) in the olive extracts and spin trap (ST) were assumed to undergo competitive scavenging reactions against reactive species. The total scavenging activities can be calculated as the sum of the scavenging rates of all antioxidant components in a sample. The relative ROS scavenging rate ($v_{\text{olive extract}}/v_{\text{ST}}$) can then be determined according to the following equation (Sueishi, Sue, & Masamoto, 2018) :

$$\frac{v_{\text{olive extract}}}{v_{\text{ST}}} = \frac{\mathrm{I}_0 - \mathrm{I}}{\mathrm{I}} = \frac{\sum_i^n k_i[\mathrm{AOx}(i)][\mathrm{RO}\cdot]}{k_{\text{ST}}[\mathrm{ST}][\mathrm{RO}\cdot]} = \frac{\sum_i^n k_i \alpha_i [\mathrm{AOx\%}]_0}{k_{\text{ST}}[\mathrm{ST}]_0} \quad (1)$$

where I and I_0 denote the ESR signal heights in the presence and absence of antioxidant compounds, respectively; k_i and k_{ST} denote the rate constants of the ROS scavenging reactions of the antioxidant AOx(i) and ST, respectively; α_i is a constant; and the [ ]$_0$ and [%]$_0$ symbols express the initial concentration (M) of the spin trap and the concentration of olive extract in a given volume (%), respectively. The relative scavenging rates were determined from the slope of a plot of $(\mathrm{I}_0 - \mathrm{I})/\mathrm{I}$ against $[\mathrm{AOx\%}]_0/[\mathrm{ST}]_0$, which was generated using Eq. (1) and the relative antioxidant activities of the olive pulp and leaf extracts (100%) for a 1 mM ST solution, calculated as: $v_{\text{olive extract (100\%)}}/v_{\text{ST(1 mM)}}$ (where $v_{\text{olive extract (100\%)}}$ denotes the scavenging rate of the extract).

To evaluate the relative scavenging rate constants of olive-related components, the following competitive relationship between the antioxidant compounds and the spin trap was assumed (Oowada, Endo, Kameya, Shimmei, & Kotake, 2012; Kohri et al., 2009):

$$\mathrm{AOx} + \mathrm{RO}\cdot \rightarrow \text{Product}, \quad \text{rate constant } k_{\text{AOx}}$$

$$\mathrm{ST} + \mathrm{RO}\cdot \rightarrow \text{ST-OR}, \quad \text{rate constant } k_{\text{ST}}$$

$$\frac{\mathrm{I}_0 - \mathrm{I}}{\mathrm{I}} = \frac{k_{\text{AOx}}}{k_{\text{ST}}} \frac{[\mathrm{AOx}]_0}{[\mathrm{ST}]_0} \quad (2)$$

The relative scavenging rate constant was determined from the slope ($k_{\text{AOx}}/k_{\text{ST}}$) of a plot of $(\mathrm{I}_0 - \mathrm{I})/\mathrm{I}$ against $[\mathrm{AOx}]_0/[\mathrm{ST}]_0$. A straight-line relationship passing through the origin was

obtained for the tested antioxidant components, suggesting that the above competitive mechanism was reasonable. Trolox was selected as the standard scavenger, and the relative radical scavenging rate constant of each component was expressed as its antioxidant capacity value in trolox equivalent units (TEU) (Kohri et al., 2009; Prior et al., 2003).

Total phenols content

The content of total phenols (TPC) in the extract samples was determined using Folin-Ciocalteu assay (Skerget et al., 2005). The extract solution (0.5 mL) was mixed with 2.5 mL of the Folin-Ciocalteu reagent (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan), and the reaction was terminated using 2.0 mL of 7.5% sodium carbonate. After 1 h of incubation at room temperature, the absorbance at 760 nm was measured on a Hitachi U-3900 spectrophotometer (Hitachi High-Tech Corp., Tokyo, Japan). The results were expressed in units of milligrams of gallic acid equivalents per milliliter of sample (mg GA mL^{-1}).

Statistical analyses

The same series of measurements was repeated five times for the three independent extracts obtained from the same treatment. The variation in the reactive species scavenging rates measured in various dilutions of olive extracts was always within 5%, and thus these measurements had high reproducibility. All data are expressed as means ± standard deviation (SD). The statistical significance of differences among groups was evaluated using one-way analysis of variance (ANOVA). $P \leq 0.05$ was defined as the threshold for statistical significance.

Results and discussion

ROS scavenging ability of olive fruits

In raw olive fruit extracts, the relative scavenging rates ($v_{\text{olive extract (100\%)}}/v_{\text{ST(1 mM)}}$, with olive extract (100%) denoting the olive extract solution) were determined using the MULTIS method. The results obtained for green, purple, and black olive fruits are listed in Table 2. As

the color of the olive fruit changed from green to purple and black, the scavenging activities of the fruit extracts against all five ROS decreased (Fig. 1). The scavenging activity diminished as a function of fruit maturity, as was expected from the observed decrease in TPC values of fruit with maturity (Table 2). In our extraction conditions, the decrease in the fruit extracts' radical scavenging activities with olive fruit maturity was similar for all five tested ROS, suggesting that similar antioxidant components were present throughout fruit maturation. Compared with the radical scavenging ability of green fruits, that of purple fruits decreased by 35%, and that of black fruits by 59% on average. Mature olive fruits are less bitter than young green ones (Charoenprasert & Mitchell, 2012). Therefore, it is reasonable to assume that the bitter component, oleuropein, possesses high ROS scavenging activity. Trolox has been customarily used as a standard radical scavenger. In Table 2, the MULTIS values found relative to that of 10 mM trolox solution (acetonitrile-water mixture) are expressed as 10 mM trolox equivalent units (TEU10).

Antioxidant activities of NaOH-debittered olive fruits

Green olive fruits were treated by immersing them in a NaOH solution. Using the MULTIS measurements of treated and non-treated olive fruits, antioxidant activities of olive fruits were quantified and could be compared before and after debittering (Table 2). The TEU10 values of fruit extracts in Table 2 were calculated relative to the MULTIS value found for 10 mM trolox solution. The relative scavenging rates (antioxidant activities $v_{(\text{treated olive})}/v_{(\text{green olive})}$) found are further illustrated in Fig. 1. Radical scavenging abilities were significantly decreased by the debittering treatment. The reduction in the radical scavenging abilities of the extracts resulting from the NaOH-debittering process averaged 90% for all five ROS compared to those of fresh green olive fruits. This was consistent with the dramatic decrease in TPC values observed after debittering. This effect of debittering by NaOH (i.e., a decrease in ROS scavenging activity) suggests that the bitter components in raw olive fruits play significant roles in their ROS scavenging activities.

Scavenging abilities of antioxidant components in olive fruits

Oleuropein and hydroxytyrosol are the major antioxidant components in olive fruit pulp (Marsilio, Campestre, & Lanza, 2001). The structures of oleuropein and hydroxytyrosol are shown in Fig. 2. Oleuropein is responsible for the bitter taste of raw olive fruits, and the oleuropein levels in fruits are decreased by the debittering process. This decrease occurs because the alkaline treatment promotes the hydrolytic cleavage of oleuropein's ester bond, forming hydroxytyrosol and oleoside-11-methyl ester (Charoenprasert & Mitchell, 2012).

The relative ROS scavenging rate constants of extracts against those of spin traps were determined using the MULTIS method, and the resulting TEU values are listed in Table 3. Oleuropein and hydroxytyrosol were found to be effective scavengers of $O_2^{-\cdot}$ and *t*-BuOO$^{\cdot}$. We generated radar charts (Fig. 2) to visualize the antioxidant profiles of the antioxidant components of olives against various ROS (Oowada, Endo, Kameya, Shimmei, & Kotake, 2012). The resulting pentagonal MULTIS profiles (relative MULTIS values of green olive extract, oleuropein, and hydroxytyrosol) are illustrated in Fig. 2. The hydroxyl radical scavenging abilities of oleuropein and hydroxytyrosol were adjusted to values of 1.0 because of their lack of specificity for this ROS (Sueishi et al., 2014). Notably, there was marked similarity between the pentagonal antioxidant profiles of the fruit extract and oleuropein. The profile of hydroxytyrosol also showed a moderate resemblance to the other profiles produced. This suggests that the antioxidant capacity of olive fruits when consumed as food has an oleuropein-like antioxidant profile, although other antioxidant components have also been reported in olive fruits (Marsilio, Campestre, & Lanza, 2001; Charoenprasert & Mitchell, 2012). The similarity of the radar chart shapes found provides strong support for the conclusion that oleuropein is primarily responsible for the radical scavenging activity of raw olive fruits. This is supported by the fact that oleuropein was previously identified as a major antioxidant component of Intosso olives (Marsilio, Campestre, & Lanza, 2001).

The antioxidant profiles of the debittered olive fruit extracts are displayed as additional radar charts, together with those of different olive antioxidant components (oleuropein and hydroxytyrosol), in Fig. 2b. The radical scavenging ability profiles found for the extracts of

NaOH-treated olives against all five ROS resembled the profile found for hydroxytyrosol, indicating that hydroxytyrosol is likely responsible for the radical scavenging activity in debittered fruit. The treatment of olive fruits with NaOH causes the formation of hydroxytyrosol from the decomposition of oleuropein, as was suggested by Charoenprasert and Mitchell (2012). However, the low MULTIS values found for debittered olive fruits indicated that the products of oleuropein hydrolysis diffuse into the surrounding medium during the debittering process, and thus their activities are largely lost from the treated fruit.

Antioxidant activities of olive leaf extracts

Using the ESR signal heights I0 and I, the relative ROS scavenging rates ($v_{\text{leaf extracts (100\%)}}$ / $v_{\text{ST(1 mM)}}$) of olive leaf extracts against 1 mM ST were determined according to Eq. (1), and the results of these calculations are listed in Table 4. Further, the radical scavenging rates of olive leaf extracts relative to those of 10 mM trolox were calculated and expressed in TEU10 (Table 4). The relative scavenging rates (antioxidant activities) for the two extracts could be expressed using Eq. (1), as follows:

$$\frac{v_{\text{leaf extract}}}{v_{\text{leaf extract (Dec. 2018)}}} = \frac{(I_0 / I) - I}{(I_0 / I_{\text{(Dec. 2018)}}) - 1} \quad (3)$$

Figure 3a shows the relative scavenging rates (antioxidant abilities) measured over the period of 1 year, taking the December 2018 value as a unit, and in relation to the hours of sunlight per month across seasons in Okayama, Japan (Fig. 3b).

We found seasonal variations in the ROS scavenging abilities of olive leaf extracts. The olive leaves harvested in August 2018 showed higher scavenging abilities against all tested ROS than those of olives harvested in other months. The scavenging abilities calculated against ROS increased by 1.4 to 2.1 fold from December to August, which was consistent with the observed increases in TPC values of olive fruits. Using the DPPH scavenging method, Blasi et al. (2016) examined seasonal variations in the antioxidant activities of olive leaves and reported that olive leaves collected in March exhibited the highest antioxidant activity.

The difference between the findings of that study and our current study may be due to differences in the characteristics of the olive-growing area.

Oleuropein, hydroxytyrosol, luteolin 7-*O*-glucoside, verbascoside, and caffeic acid are the major antioxidant components of olive leaves (Pereira et al., 2007; Xie et al., 2015). Hydroxytyrosol is a component of oleuropein and verbascoside, and caffeic acid is a component of verbascoside. The MULTIS values found showed that luteolin 7-*O*-glucoside has low antioxidant ability against $HO^{\cdot}$, and caffeic acid has remarkably high antioxidant abilities against $HO^{\cdot}$, $O_2^{-\cdot}$, and $^1O_2$ (Table 3). Therefore, the increase in the ROS scavenging abilities of olive leaves collected in August against $RO^{\cdot}$, *t*-$BuOO^{\cdot}$, and $^1O_2$ suggests that oleuropein undergoes thermal- and/or photo-decomposition reactions leading to the production of hydroxytyrosol, which has high antioxidant abilities against these ROS. The scavenging activity of olive leaves against various ROS was correlated with the hours of sunlight received in a season (Fig. 3b). Therefore, it is possible that the amounts of antioxidant compounds in olive leaves change and/or their antioxidant components decompose due to irradiation by sunlight. This is supported by the photochemical reaction results reported by Longo, Morozova, and Scampicchio (2017).

Antioxidant activities of dried and roasted olive leaves

For the dried and roasted olive leaves collected in December 2018 and August 2019, the radar charts of their antioxidant profiles (Figs. 4a and 4b) demonstrated that the drying treatment decreased the antioxidant ability of the leaf extract depending on the month, in which the leaves were collected, while the roasting treatment increased the leaf extract's radical scavenging abilities against *t*-$BuOO^{\cdot}$ and $RO^{\cdot}$. It is likely that the drying treatment leads to the oxidation of some phenolic compounds to form their corresponding quinones, resulting in a decrease in the total phenols content (Bahloul et al., 2009). We also observed a decrease in TPC values of dried olive leaves (Table 4). In contrast, in the roasted treatment, various antioxidant compounds were generated in the Maillard reaction, which is a chemical reaction occurring between amino and carbonyl compounds. Roasting has been reported to

enhance the antioxidant activities of almonds and coffee (Lin et al., 2016; Perrone, Farah, & Donangelo, 2012). In roasted olive leaves, a considerable increase in TPC values was also observed, and the products of the Maillard reaction may have helped to enhance the antioxidant abilities of olive components against *t*-BuOO· and RO· (Table 4 and Fig. 4).

Conclusions

We evaluated the radical scavenging abilities of fresh and debittered olive fruits against five ROS using the MULTIS method. The NaOH-treatment (debittering) of olive fruits markedly decreased their overall ROS scavenging abilities. By comparing the scavenging activities of fresh and debittered olive extracts with those of the antioxidant components of olives, such as oleuropein and hydroxytyrosol, we revealed the influence of debittering on these components.

In olive leaves, we found significant seasonal variation in the antioxidant activities against various types of ROS. Furthermore, we showed that there are differences in the antioxidant activities of olive leaves following different treatments (drying and roasting). In the roasting treatment, the increase in phenol content generated from the Maillard reaction enhanced the radical scavenging abilities of olive leaf extracts against *t*-BuOO· and RO·. The use of the MULTIS method facilitates detailed analysis of antioxidant activities of foods against various types of ROS.

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Author Contributions

Y. Sueishi and R. Nii designed and performed experiments. R. Nii performed data analysis. Y. Sueishi and R. Nii interpreted results and wrote the manuscript. Y. Sueishi revised the manuscript.

Conflict of Interest No potential conflicts of interest were disclosed.

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438 Figure captions

439

440 Figure 1 - Bar graph of relative scavenging rates (v(green, purple, black, NaOH-treated
441 olive)/v(green olive)) for five ROS scavenging: (▒) green, (▨) purple, (■) black olive,
442 and (□) NaOH-treated olive extracts. Broken lines show the average values. Significant
443 differences $p \leq 0.05$.

444

445 Figure 2 - Radar chart for relative scavenging rates of olive antioxidant components
446 (oleuropein and hydroxytyrosol) against (a) fresh green olive fruit and (b) NaOH-debittered
447 olive fruit extracts, together with the structures of antioxidant oleuropein and hydroxytyrosol.
448 The HO· scavenging ability is adjusted to 1.0.

449

450 Figure 3 - (a) Antioxidant activities of olive leaf extracts against five ROS for 1 year and (b)
451 hours of sunlight in Okayama, Japan.

452

453 Figure 4 - Radar charts for relative scavenging rates (MULTIS values) of dried and roasted
454 olive leaves collected in (a) December 2018 and (b) August 2019. The MULTIS values of
455 fresh olive leaves are set to 1.0.

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