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博士論文

Generation of reactive oxygen species from winemaking wastes by photoirradiation

ワイン生産における廃棄物抽出物への光照射による活性酸素種の生成

平成 28 年

塚田　愛

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Chapter I

General introduction

I-1 Introduction

Plant biomass such as grains, vegetables, fruits, and timber have been produced for food, architectural materials, paper manufacture, and so on. However, residual materials in production districts, pomace in food processing, and unnecessary products have been discharged as untapped biomass. Although some of them have been recycled for fertilizers, feeding stuffs, wood charcoal, and regenerated paper, the rest of them have been disposed because of limited demand for them and lack of advanced utilization methods. The trials to reduce industrial discharge to a maximum extent (*e.g.*, zero) with sustainable industrial activity have been made, which is termed as "zero emission". In the field of agriculture, zero waste agriculture is a type of sustainable agriculture optimizing use of the five natural resources *i.e.*, plants, animals, bacteria, fungi and algae for the production of biodiverse-food, energy and nutrients. For instance, a trial of zero discharge performance of an industrial pilot-scale plant has been made in treating palm oil mill effluent in Malaysia because huge quantities of palm oil mill effluent pose a great threat to aqueous environment due to its very high chemical oxygen demand [1].

Grape is the largest fruit crop in the world. The annual production worldwide amounts to almost 70 million tons [2], and around 80% is used to make wine. Thus,

waste materials or byproducts obtained from winemaking process could be a valuable resource to be recycled. The waste from winemaking process can be divided into three categories, *i.e.*, pomace, clarification sediment such as lees, and yeast sediment. The generated amount of waste depends on the condition of the grapes at the time of harvest, as well as on what processing methods are used, and in extreme cases, this can result in levels of waste being up to 20% of the harvested mass of the grapes [3]. Thus, waste materials or byproducts obtained from winemaking process could be a valuable resource to be recycled.

In this chapter, overview of revalorization of waste materials and byproducts from winemaking process as recyclable resources and of their antioxidative and prooxidative potentials in relation to polyphenolic compounds is described.

I-2 Revalorization of waste materials and byproducts from winemaking process

There have been many studies on revalorization of waste materials and byproducts from winemaking process. One of the typical examples is composting, which is defined as the aerobic biological decomposition and stabilization of organic substrates, under conditions allowing development of thermophilic temperatures to obtain a final product to be beneficially applied to land [4, 5]. The composting of

winery waste has been an alternative to the traditional disposal of residues, and also involves a commitment to reducing the production of waste products as reported by Bertran et al. [6]. Interestingly, it was also studied that the ability of the earthworm *Eisenia andrei* to compost different winery wastes (spent grape marc, vinasse biosolids, lees cakes, and vine shoots) into valuable agricultural products [7], showing that winery wastes have potential as raw substrates in vermicomposting, although further research is needed to evaluate the feasibility of such wastes in large-scale vermicomposting systems. The other approach is utilization of bioactive compounds from the winery industry residue. For instance, grape seed was revalorized for the production of an oil and defatted meal with nutraceutical properties [8]. More in detail, oil extracted was increased at the pressing stage, when an enzymatic pre-treatment is incorporated into the conventional process, and the defatted meal by enzymatic assisted process increased its content of phenolic compounds by two to four times, depending on the conditions of phenolics extraction in comparison to the control samples. In addition, solid byproducts from white and red wine industry were evaluated as potential sources of antioxidant phytochemicals on the basis of their content in phenolics and *in vitro* antioxidative activity [9], showing that wine industry byproducts, including not only grape seeds but also grape pomace and stems, were very rich sources of antioxidant polyphenols

compared with other agri-food solid wastes, and therefore their exploitation as a source of added-value products might be more cost-effective and merits a profounder investigation. Regarding the recovery of phenolic compounds from grape and wine byproducts, it was reported that a powdered yellow-light brown product with 50% phenolic content, expressed as gallic acid equivalents, with the radical scavenging capacity of one gram of product was equivalent to two–three g of Trolox was obtained under the optimized desorption conditions (using Sepabeads SP207 or Diaion HP20 as adsorbents and eluting with 96% ethanol at 50 °C) [10]. As for the phenolic compounds contained in winery waste, it was reported that gallic acid, catechin and epicatechin were the major phenolic compounds in the waste from red winemaking with variety Agiorgitiko, according to a high performance liquid chromatography determination of the extracts obtained under various conditions using different solvents [11]. In addition, hydroxytyrosol, tyrosol, cyanidin glycosides and various phenolic acids such as caffeic, syringic, vanillic, p-coumaric and o-coumaric acids were also identified. Chemical structures of the phenolic compounds described here are shown in Fig. I-1.

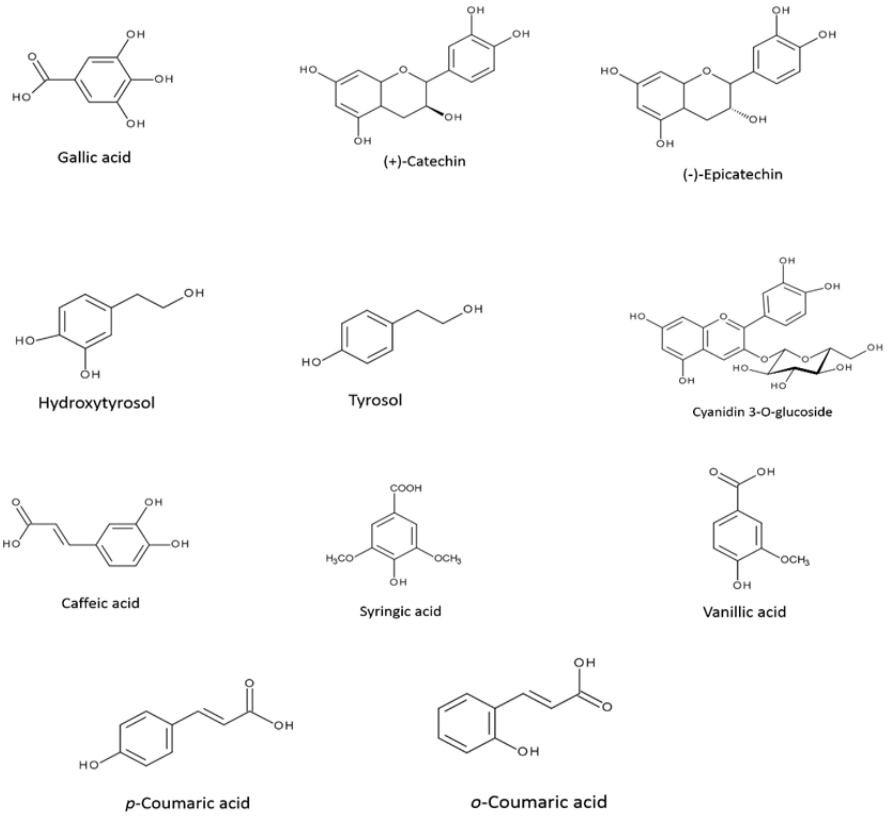

Figure I-1
Chemical structures of the phenolic compounds detected in winery waster (from red winemaking, variety Agiorgitiko)*

*Lafka, T., Sinanoglou, V. & Lazos, E.S. (2007): On the extraction and antioxidant activity of phenolic compounds from winery wastes. *Food Chem* 104, 1206-1214.

As described above, many phenolic compounds with antioxidative potential have been identified, which tempted many researchers to search for natural bioactive compounds from winery byproducts as health promoters as reviewed by Teixeira et al. [12], concomitantly with the increasing demand for not only environment-friendly industrial production but the challenge for gaining operational efficiency and

minimizing treatment cost for byproduct in the wine industry. Thus, the revalorization of these wastes would offer a way to reduce the environmental impact of winery activity.

I-3 Prooxidative properties of phenolic compounds

Polyphenolic compounds naturally occurring in fruits, nuts, vegetables and flowers have antioxidative activity [13-15] since the phenolic hydroxyl group in their structures acts as a hydrogen donor, and they have an ability to scavenge free radicals effectively [16, 17]. As such, the beneficial antioxidative activity of polyphenols has been well studied and applied to health promotion [18-20].

Besides antioxidative potential of polyphenolic compounds, their prooxidative potential has been also discussed. For instance, tea catechins with cupric ion were reported to show a prooxidative activity to DNA cleavage reaction and linoleic acid peroxidation [21]. Proposed mechanism for DNA cleavage induced by catechins with cupric ion is shown below. That is, polyphenols probably function to reduce Cu^{2+} to Cu^{+} expressed as Eq. i. Then the generated Cu^{+} produces reactive oxygen species (ROS) which attack the DNA (Eqs. ii-iv), in which ROS are abbreviated as follows: superoxide anion radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot$OH). And the Eq iv

is well known as a Fenton reaction in which transition metal catalyzes H_2O_2 to generate ·OH.

polyphenol + Cu^{2+} → oxidized polyphenol + Cu^{+} (i)

Cu+ + O_2 → Cu^{2+} + $\cdot O_2^{-}$ (ii)

$2Cu^{+} + O_2 + 2H^{+} \rightarrow 2Cu^{2+} + H_2O_2$ (iii)

Cu+ + H_2O_2 → Cu^{2+} + ·OH + OH^{-} (iv)

Similarly, it was reported that an important anticancer mechanism of plant polyphenols is mediated through intracellular copper mobilization and ROS generation, which is a characteristic feature of prooxidative properties of polyphenolic compounds, leading to cancer cell death [22]. Through multiple assays, they showed that polyphenols luteolin, apigenin, epigallocatechin-3-gallate, and resveratrol are able to inhibit cell proliferation and induce apoptosis in different cancer cell lines. Such cell death was prevented to a significant extent by cuprous chelator neocuproine and ROS scavengers, and normal breast epithelial cells become sensitized to polyphenol-induced growth inhibition when cultured in a medium supplemented with copper. This was supported by the idea that the concentration of copper is significantly elevated in cancer cells. From these, such prooxidant chemopreventive mechanism appears to be a

common mechanism to several polyphenols with diverse chemical structures to explain the preferential cytotoxicity of these compounds toward cancer cells.

It was also proposed that the mixed mechanism of polyphenol-mediated lipid oxidation and polyphenol radical scavenging antioxidative activity as shown in Fig. I-2 [23], suggesting that a possible explanation for prooxidative activity may arise from the nonenzymatic, metal-catalyzed oxidation of polyphenols, which results in H_2O_2 generation.

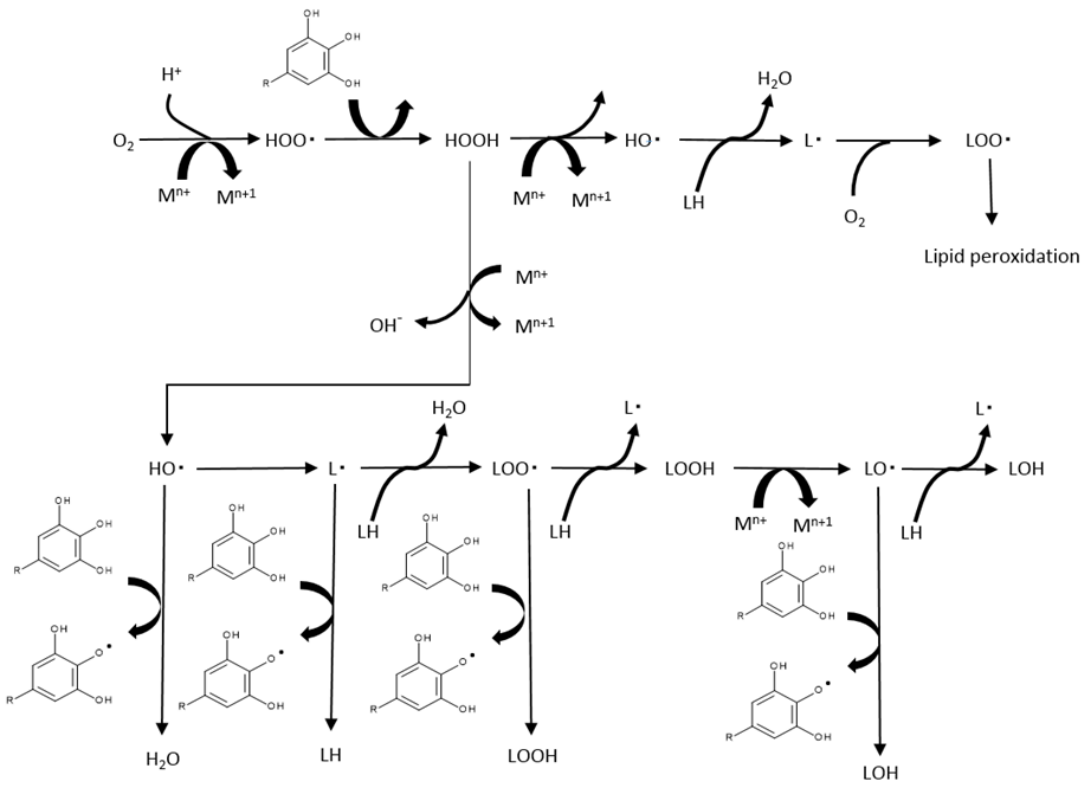

Figure I-2

Mixed mechanism of polyphenol-mediated lipid oxidation and polyphenol radical scavenging antioxidant activity proposed by Zhou et al. (2012).

Abbreviations: transition metal such as Fe and Cu (M), hydroperoxyl radical (HOO·), hydroxyl radical (HO·), reduced lipid (LH), lipid alkyl radical (L·), lipid hydroperoxyl radical (LOO·), lipid hydroperoxide (LOOH), lipid alkoxyl radical (LO·). Cited from Zhou et al, *J Agric Food Chem*, 60, 2906-2915, 2012, with a slight modification.

From the similar point of view, Arakawa et al. reported that catechins (epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate) possess strong bactericidal action due to ROS such as H_2O_2 generated from the catechins as the active

mechanism [24]. Based on such prooxidative property of polyphenols, Nakamura et al. tried to enhance the polyphenols' bactericidal activity by utilizing photolysis of H_2O_2 [25-27]. That is, the polyphenolic hydroxyl group would be oxidized by the photoirradiation, leading to a proton coupled electron transfer to dissolved oxygen, which in turn would result in H_2O_2 generation. The resultant H_2O_2 is phtotolyzed to ·OH [28]. The ·OH, one of ROS, has one unpaired electron in its structure, so that it is apt to deprive other substance of an electron; *e.g*. it easily oxidizes other substances [29]. It is also known that the ·OH is generated in the immunological response in the body to kill invading bacteria as illustrated in Fig. I-3 [30, 31]. Therefore, it is considered that artificially generated ·OH can be used for a disinfection system. A possible scheme for the bactericidal action of photoirradiated polyphenols is illustrated in Fig. I-4.

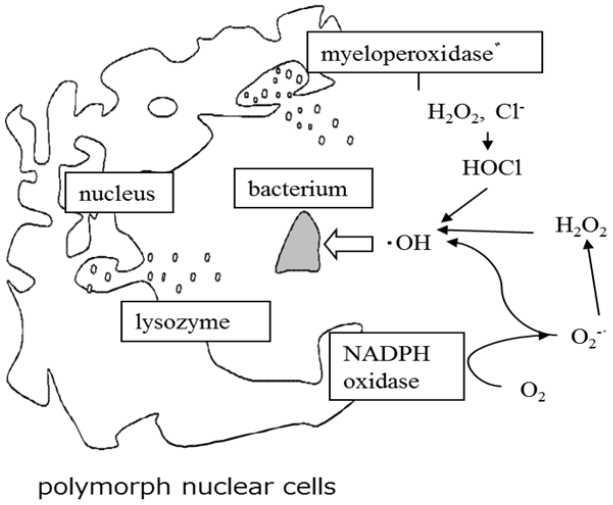

Figure I-3
Schematic illustration of killing bacterium by polymorph nuclear cells via ROS production

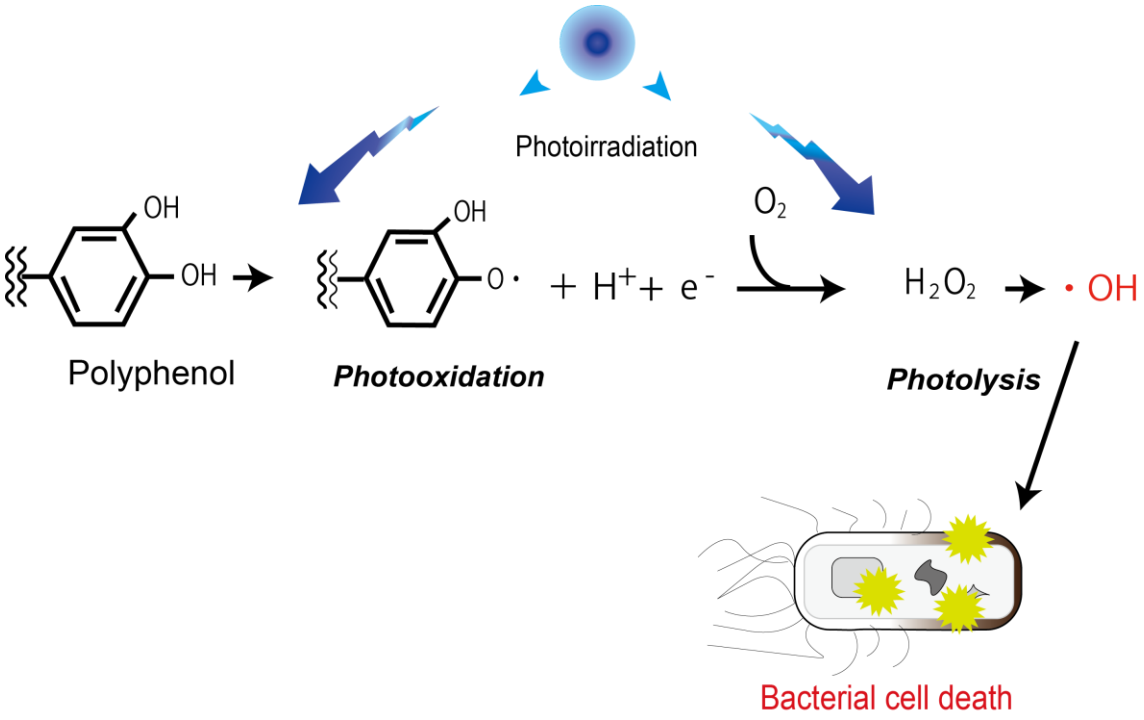

Figure I-4
A proposed mode of bactericidal action of photoirradiated polyphenols

I-4 Objective

It was reported that the grape residue extracts (mixture of seeds and peels) from the Isabel and Niagara varieties showed considerable amounts of total phenolic compounds, containing the flavonoids catechin and epicatechin as major compounds [32]. As such, the study showed that extracts from both varieties had high *in vitro* antioxidative activity as assessed by methods measuring of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and the inhibition of lipid peroxidation. Since Niagara variety is one of the representative green grapes for

wine production in Hokkaido, Japan, and potent anti-oxidative activity could be reflected well in prooxidative activity because both activities are mediated via oxidation of phenolic hydroxyl groups of phenolic compounds as described above. Thus, Niagara variety was chosen as a source of residue from winemaking process in the present study. Figure I-5 shows a typical Niagara vineyard in Hokkaido.

Figure I-5
A typical example of Niagara vineyard in Hokkaido
Photos were taken in Otaru, Hokkaido, Japan

In the white winemaking process using Niagara variety, which consists of preparation of the juice and addition of the yeast for fermentation, maturation, and bottling for aging, two types of wastes are disposed as shown in Fig. I-6. One is the remnant from crashed and pressed grape, and the other is the lees that refer to deposits of dead yeast or residual yeast and other particles precipitating to the bottom of a vat of wine after fermentation. Especially, lees must be appropriately treated to avoid uncontrolled dumping causing environmental problems due to their high content of

phenols, pesticides, heavy metals, and considerable concentrations of nitrogen, phosphate and potassium in addition to high organic content [33]. Therefore, the companies involved must promote more effective use of lees; *e.g.*, utilization of alternative environmental and economic physicochemical and biological treatments for their revalorization consisting in the recovery or transformation of the components of the lees into high value-added compounds.

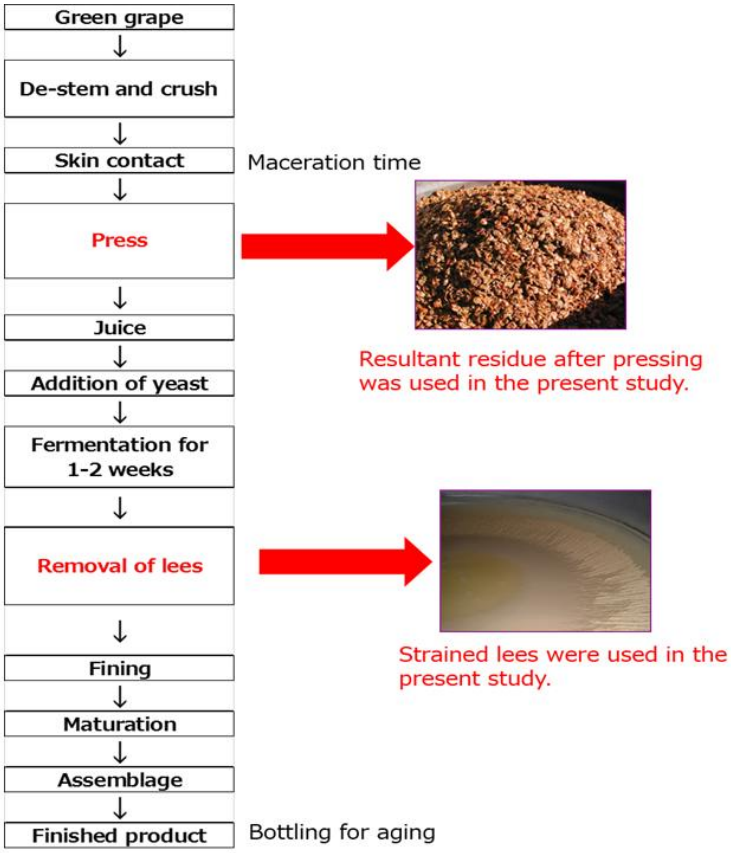

Figure I-6
Summary for the white winemaking process and samples used in the present study

The aim of the study presented in this thesis was to seek for a novel disinfection technique by utilizing two types of wastes disposed from the white winemaking process using Niagara variety as described above. To be more precise, since photoirradiated polyphenols could exert potent bactericidal activity via their prooxidative potential as

illustrated in Fig. I-4, the bactericidal activity of the two wastes upon photoirradiation was evaluated in relation to their abilities to generate ROS, and their prooxidative potentialities were compared to each other and to those of commercially available polyphenols.

In chapters II and III, photoirradiation induced bactericidal activity of the aqueous extracts from residue of crushed grapes (termed as grape pomace) and lees in wine preparation was investigated in relation to ROS formation. Furthermore, effect of ·OH scavengers on the bactericidal activity of these photoirradiated extracts was examined based on the hypothesis that ·OH would be a pivotal active ingredient for the activity. In Chapter IV, prooxidative and antioxidative potentials of the grape pomace extract were compared to those of authentic polyphenols.

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Chapter Ⅱ

Microbicidal action of photoirradiated aqueous extracts from the residue of crushed grapes (grape pomace) from winemaking

II-1 Introduction

Polyphenolic compounds are noteworthy for their antioxidative activity [1-3]. Besides their antioxidative activity, recent advances in polyphenol biochemistry have made it possible to apply their prooxidant potential to various fields such as anticancer treatment. For instance, it was reported that an important anticancer mechanism of plant polyphenols is mediated through intracellular copper mobilization and ROS generation, which is a characteristic feature of prooxidant properties of polyphenolic compounds, leading to cancer cell death [4]. As described in the Chapter I, the prooxidant potential of polyphenols was applied to the development of a novel disinfection technique [5-7]. That is, exposing an aqueous solution of polyphenols to blue light led to photo-oxidation of the polyphenolic hydroxyl group, resulting in the generation of ROS in the presence of dissolved oxygen. H_2O_2 is produced via electron transfer from photo-oxidized polyphenols to the dissolved oxygen. The H_2O_2 is subsequently photolyzed by the blue light, resulting in the generation of ·OH, which would be a main contributor of the bactericidal activity.

Food processing environments offer recyclable food resources. The residue of crushed and pressed grapes obtained in the winemaking process could be a candidate for such resources because it would contain a lot of polyphenols. For instance, a

high-resolution mass spectrometry coupled to suspect screening analysis with positive and negative ionization modes identified several hundred grape polyphenols, four flavonols, and some grape resveratrol trimers and tetramers which were found in grapes for the first time [8]. Thus, I focused on the residue of crushed and pressed grapes in the winemaking process as an untapped natural resource with antioxidant and prooxidant potential.

The purpose of the present study was to evaluate the bactericidal activity of the photoirradiated aqueous extract of crushed and pressed grape residue obtained from the process of making white wine in relation to ROS formation as a potential prooxidative activity. Regarding the light source, an LED with a wavelength at 400 nm was used to avoid the possible adverse effect by ultraviolet (UV) light as in the previous studies [5, 7], since UV is defined as an electromagnetic wave with a wavelength of <400 nm.

II-2 Materials and Methods

II-2-1 Reagents

Reagents were purchased from the following sources: 5,5-dimethyl-1-pyrroline N-oxide (DMPO) from Labotec (Tokyo, Japan); catalase from bovine liver, dimethyl sulfoxide (DMSO), and thiourea from Wako Pure Chemical Industries (Osaka, Japan);

4-hydroxy-2,2,6,6-tetramethylpiperidine N-oxyl (TEMPOL) from Sigma Aldrich (St. Louis, MO). All other reagents used were of analytical grade.

II-2-2 Preparation of aqueous extract

Fruitage (including the peel and seeds) of the white wine grape variety Niagara harvested at Hokkaido in Japan was crushed and pressed to obtain a juice in the vinification process of white wine. The remnant of crushed and pressed grapes (grape pomace) that was kindly provided by Hokkaido Wine Co., Ltd. (Otaru, Japan) was freeze-dried. Three times the volume of pure water (at the ratio of 3 mL pure water per 1 g powder) was added to the dried residue powder, and the resultant mixture was agitated at 150 rpm overnight at room temperature. The upper layer was taken and centrifuged at 3,000 rpm for 20 min to obtain a supernatant. Filtrate obtained through membrane filtration (φ0.22 μm), was subjected to total polyphenol determination by the Folin-Denis method in which gallic acid was used as a standard [9]. The aqueous extract solution (termed as grape pomace extract, GPE) was adjusted to contain 0.5 mg total polyphenol/mL with pure water and stored at -80°C until assayed.

II-2-3 Light source

An experimental device equipped with a light emitting diode with a wavelength of 400 nm (NHH105UV, Lustrous Technology, Shiji, Taiwan) was used according to the previous studies [5-7]. The output power of the LED, measured using a power meter (FieldMate, Coherent, Santa Clara, CA), was set to be 400 mW per LED corresponding to an irradiance of 130 mW/cm^2 at a distance of 15 mm from the LED. A four-clear-sided methacrylate plastic cuvette (Fisherbrand Disposable Cuvette, size: 12 x 12 x 45(H) mm, light transmission rate at 400 nm: 90%, Thermo Fisher Scientific K.K., Yokohama, Japan) containing the sample was placed in the experimental device. LED-light irradiation was performed toward both sides of the plastic cuvette (total irradiance: 260 mW/cm^2).

For analyses of wavelength dependence of H_2O_2 generation and of bactericidal activity, an LED spot curing device (OmniCure LX400+, Lumen Dynamics Group, Ontario, Canada) with the specific heads emitting the light at wavelengths of 365, 385 and 400 nm, and a dental LED light curing unit (G-Light Prima-II, GC, Tokyo, Japan) with a wavelength of 465 nm were used.

II-2-4 Microbicidal assay

Staphylococcus aureus JCM 2413, *Pseudomonas aeruginosa* JCM 6119, and *Candida albicans* JCM 153 purchased from the Japan Collection of Microorganisms, RIKEN BioResource Center (Wako, Japan) were used. Each suspension of *S. aureus* and *P. aeruginosa* was prepared in sterile physiological saline from a culture grown on brain heart infusion (BHI) agar (Becton Dickinson Labware, Franklin Lakes, NJ, USA) aerobically at 37°C overnight. A suspension of *C. albicans* was prepared in sterile physiological saline from a culture grown on Sabouraud dextrose agar (SDA) at 37°C overnight. In a plastic cuvette, 450 µL of GPE or pure water was mixed with 50 µL of the bacterial or fungal suspension to reach a final concentration of approximately 10^7 colony forming units (CFU)/mL for the two bacterial strains and 10^7 cells/mL for *C. albicans*. Then, the samples were exposed to LED light for 10, 20, or 40 min. After irradiation, 50 µL of the sample was mixed with an equal volume of sterile catalase solution (5,000 U/mL phosphate buffer [pH 7.4]) to terminate the bactericidal effect of H_2O_2 generated by photo-oxidation of polyphenols in GPE. A 10-fold serial dilution of the mixture was prepared using sterile physiological saline, and 10 µL of the diluted solution were seeded onto a BHI agar plate for bacteria or a SDA plate for *C. albicans*. The agar plates were cultured as described above for 2 days, and the CFU/mL or

cells/mL was determined. In addition, as controls, samples were kept for 10, 20, or 40 min in a light-shielding box, instead of being exposed to LED light, and subjected to the same procedures. The initial bacterial count (inoculum size) was evaluated using the viable counting method, and the initial count of *C. albicans* was microscopically determined.

To examine if the bactericidal effect of the photoirradiated GPE could be attributable to ·OH, DMSO and thiourea, which are well-known ·OH scavengers [10, 11], were added to the reaction mixture. The reaction mixture consisting of 425 μL of GPE, 50 μL of the bacterial suspension and 25 μL of DMSO or thiourea was prepared to reach final concentrations of approximately 10^7 CFU/mL for the bacteria and 700 mM for DMSO or 150 mM for thiourea. Then, the sample was irradiated with the LED light for 10 or 20 min. The CFU was determined after each treatment as described above. All tests were performed in triplicate.

II-2-5 Electron spin resonance (ESR) analysis of ·OH and colorimetric determination of H_2O_2

Qualitative and quantitative analyses of ·OH generated by photoirradiation of GPE were performed using an ESR spin trapping technique as described in the previous

study [12]. An aliquot (483 μL) of GPE was mixed with 17 μL of DMPO in a plastic cuvette to reach a final concentration of 300 mM for DMPO. Then, the sample was irradiated with the LED light for 0, 10, 20, and 60 s. After irradiation, the sample was transferred to a quartz cell for ESR spectrometry, and the ESR spectrum was recorded on an X-band ESR spectrometer (JES-FA-100; JEOL, Tokyo, Japan). The measurement conditions for ESR were as follows: field sweep, 331.89–341.89 mT; field modulation frequency, 100 kHz; field modulation width, 0.1 mT; amplitude, 200; sweep time, 2 min; time constant, 0.03 s; microwave frequency, 9.420 GHz; and microwave power, 4 mW. TEMPOL (2 μM) was used as a standard to calculate the concentration of the spin-trapped radicals, and the ESR spectrum of manganese (Mn^{2+}) held in the ESR cavity was used as an internal standard.

To examine if ·OH is continuously generated during LED-light irradiation for 20 min, after 20 min of irradiation DMPO was added to the photoirradiated GPE to a final concentration of 300 mM. Immediately after addition of DMPO, the sample was further irradiated with LED-light for 10 s. Then ESR analysis was performed as described above.

DMPO-OH, a spin adduct of ·OH, can be formed even in the absence of ·OH under certain conditions [13]. Consequently, additional ESR analysis was conducted to

confirm if the DMPO-OH was derived from the reaction between ·OH and DMPO. If ·OH is generated, the intensity of DMPO-OH signal would decrease and a signal for a spin adduct of the methyl radical (DMPO-CH_3) would appear when an ·OH scavenger containing a methyl group is added to the reaction system [14]. The reaction mixture consisting of 433 μL of GPE, 17 μL of DMPO, and 50 μL of pure water or 14 M DMSO was prepared to reach final concentrations of 300 mM for DMPO and 1.4 M for DMSO. Then, the sample was irradiated with the LED light for 20 s. ESR analysis was performed as described above.

For H_2O_2 determination, the samples were treated similarly to the case with the ESR analysis. That is, 483 μL of GPE was mixed with 17 μL of pure water instead of DMPO in a plastic cuvette followed by LED-light irradiation for 10 min. Immediately after the irradiation, the H_2O_2 concentration was determined by a colorimetric method based on the peroxide-mediated oxidation of Fe^{2+} followed by the reaction of Fe^{3+} with xylenol orange [15]. To confirm if the generated H_2O_2 responds to the catalytic action of catalase, 250 μL of GPE was mixed with 250 μL of 0.1 M Na-K phosphate buffer (PB, pH7.4) or catalase solution (5,000 U/mL in 0.1 M PB) followed by LED-light irradiation for 10 min. As a negative control, 500 μL of the mixture of GPE and 0.1 M PB was kept in a light-shielding box for 10 min. Then the H_2O_2 concentration was

similarly determined. All tests were performed in triplicate.

II-2-6 Analyses of wavelength dependence of H_2O_2 generation and of bactericidal activity

According to the ultraviolet (UV)-visible absorption spectrum of different dilutions of GPE (Fig. II-1), the value of absorbance decreased with the increase of wavelength in the range between 300-600 nm. Thus, it was hypothesized that UV-light irradiation (<400 nm) generated H_2O_2 more efficiently than visible light irradiation if the reaction is caused by the absorbed light energy. To examine the wavelength dependence of H_2O_2 generation, GPE put in a black microplate well (96-well) was irradiated with each LED light in a vertical direction. An output power was measured using the laser power meter (FieldMate, Coherent) and set at 230 mW. The diameter of the irradiation field was set to equal that of the well (6.4 mm) so that almost all of the light could pass through the test solution. Thus, the irradiance corresponding to the output power of 230 mW was calculated to be 715 mW/cm^2. In a microplate well, 200 μL of GPE was irradiated with LED light for 3 min. After irradiation, the H_2O_2 determination was performed as described above. All tests were performed in triplicate.

To examine the wavelength dependence of the bactericidal activity, in each well of

the black microplate well (96-well), 180 µL of GPE or pure water was mixed with 20 µL of the bacterial suspension to reach final concentration of approximately 10^6 colony forming units (CFU)/mL for the bacteria. Then, the samples were exposed to LED light for 5 min as described in the analysis of H_2O_2 generation. After irradiation, the CFU was determined after each treatment as described above.

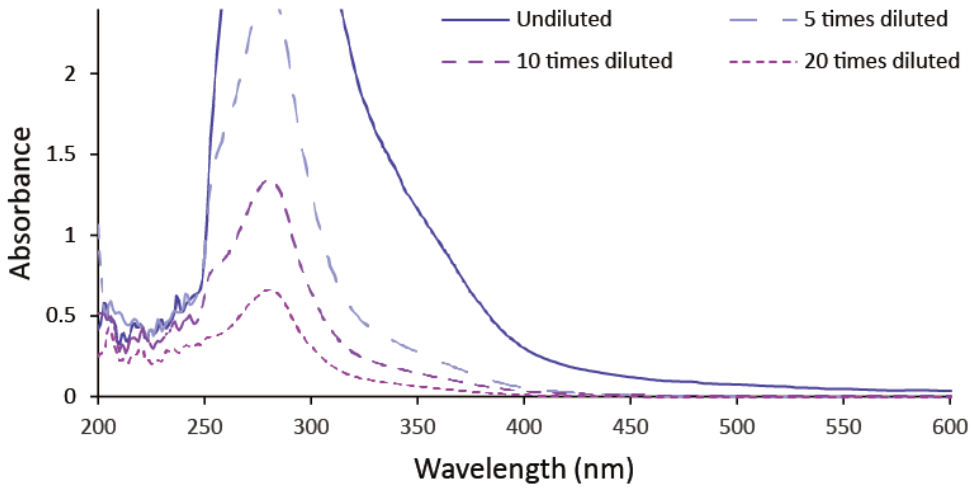

Figure II-1

Ultraviolet-visible spectra of GPE.

II-2-7 Liquid chromatography/mass spectrometry (LC/MS) analyses of GPE

For LC/MS analyses, GPE was diluted to a concentration of 0.04 mg total polyphenol equivalent/mL with pure water followed by passage through a filter (pore size, 0.2 μm). The resultant sample was injected into the electrospray ion source (Ultraspray 2^{TM} Dual ESI source), PerkinElmer, Inc., Waltham, MA, USA) of a time-of-flight mass spectrometer (AxION 2 TOF MS, PerkinElmer Inc.) coupled to the PerkinElmer Flexar^{TM} FX-15LC (PerkinElmer, Inc.). Chromatographic separation was undertaken on the C18 column (2.1×150 mm Brownlee SPP 2.7, PerkinElmer) at 40°C. With regard to gradient elution, solvent A was 0.1% formic acid, and B was acetonitrile containing 0.1% formic acid. Gradient elution was 0–10 min and 0–100% B. The flow rate was 0.4 mL/min, and the injection volume was 10 μL. Electrospray ionization-mass spectrometry was recorded for 10 min in the m/z region from 100 to 1000Da with the following instrument parameters: drying gas heater; 400°C, nebulizing gas; 80 PSI, capillary exit voltage; 120 V. LC/MS analyses were undertaken by high-resolution electrospray ionization-mass spectrometry ($R \geq 12,000$; tolerance for mass accuracy = 5 ppm). As standards, (+)-catechin (Tokyo Chemical Industry, Tokyo, Japan) was used.

II-2-8 Statistical analyses

Statistical significance in the CFU/mL obtained in the bactericidal assay was assessed by Tukey-Kramer HSD multi-comparison test. The analysis for the bactericidal assay was performed following logarithmic conversion. When colonies were not detected, the value of the detection limit (10^2 CFU/mL) was used for the statistical analysis. Regarding the yield of H_2O_2 in pure water and GPE with or without LED-light irradiation, since H_2O_2 was not detected in the two pure water groups, statistical significance for the remaining two groups was assessed by Student's t-test. Statistical significances in the other experiments were assessed by Tukey-Kramer HSD multi-comparison test. $P < 0.05$ was considered significant.

II-3 Results

II-3-1 Microbicidal assay

The results of the bactericidal assay are summarized in Fig. II-2. Under the conditions without the LED-light irradiation, GPE kept in a light-shielding box for 10 and 20 min showed almost no bactericidal activity in comparison with that of the corresponding pure water groups. LED-light irradiation alone showed somewhat bactericidal activity. That is, the LED-light irradiation of pure water for 10 and 20 min

showed approximately a 1.5 log and 3 log reduction of viable bacterial counts (CFU/mL), respectively, when compared with the corresponding pure water groups without the LED-light irradiation. Furthermore, the LED-light irradiation of GPE for 10 min killed the bacteria effectively with an approximately 4 log reduction, and the LED-light irradiation for 20 min achieved a >5 log reduction. The results of the bactericidal assay against *P. aeruginosa* and the fungicidal assay against *C. albicans* are summarized in Fig. II-3. Similar to *S. aureus*, although LED-light irradiation of *P. aeruginosa* in pure water for 20 min showed an approximate 3.5-log reduction of viable bacterial counts, LED-light irradiation of the bacteria in GPE for 20 min effectively killed the bacteria with a >5-log reduction. Unlike the two bacterial species tested, LED-light irradiation of *C. albicans* in pure water for 40 min showed almost no fungicidal effect. When *C. albicans* in GPE were irradiated with LED light for 40 min, the fungi were killed with an approximately 1-log reduction (90% reduction). In the experiment for examining the effect of ·OH scavengers, DMSO and thiourea significantly attenuated the bactericidal effect of the photoirradiated GPE (Fig. II-4).

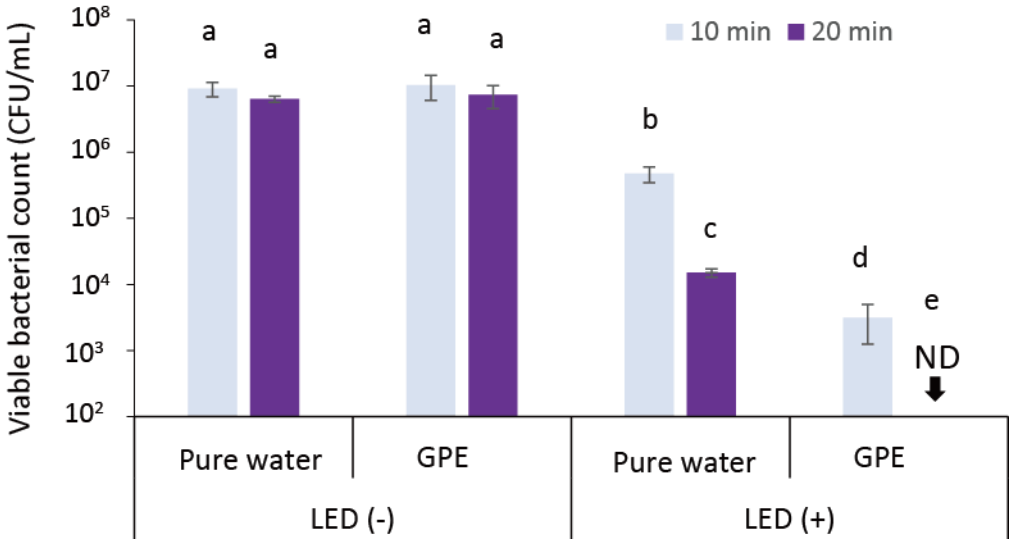

Figure II-2

The number of viable *Staphylococcus aureus* in the suspension after each treatment.

Each value is the mean with the standard deviation (n=3). Significant differences ($p < 0.01$) within each group are denoted by different alphabetical letters. ND: Not detected.

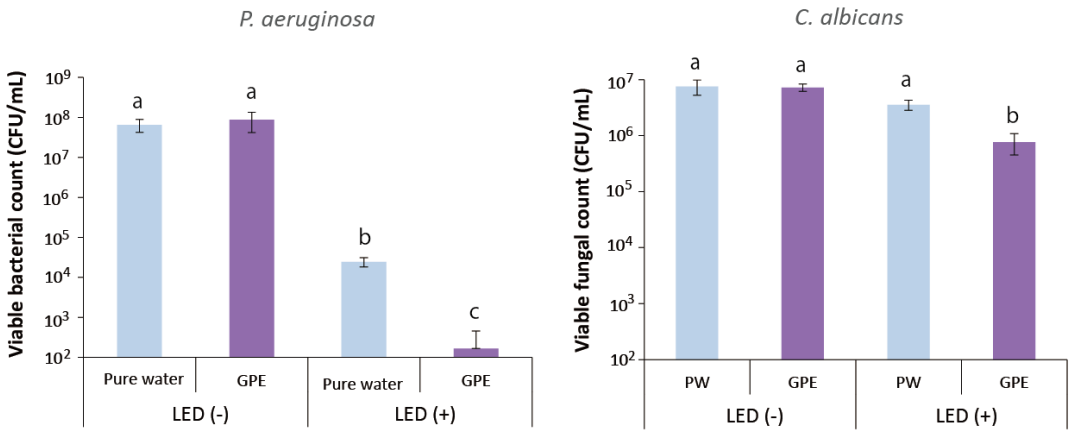

Figure II-3

The number of viable *Pseudomonas aeruginosa* and *Candida albicans* cells in suspension after each treatment (suspended in pure water or GPE with or without LED-light irradiation for 20 min for *P. aeruginosa* and 40 min for *C. albicans*).

Each value is the mean with the standard deviation (n=3). Significant differences ($p < 0.01$) are denoted by different alphabetical letters. ND: not detected

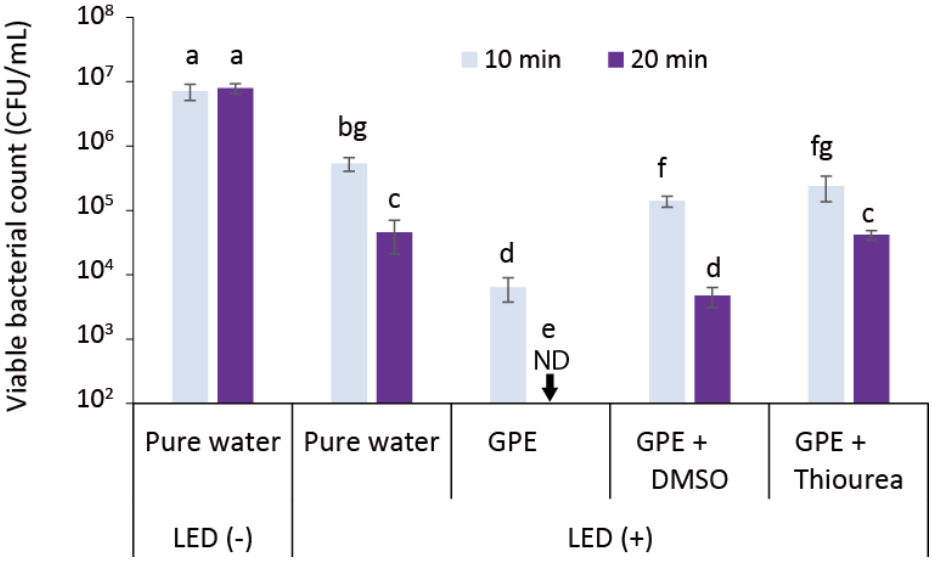

Figure II-4

Influence of ·OH scavengers on the bactericidal effect of photoirradiated GPE. The LED-light irradiation was performed for 10 min.

Each value is the mean with the standard deviation (n=3). Significant differences ($p < 0.01$) within each group are denoted by different alphabetical letters.

II-3-2 ESR analysis of ·OH and colorimetric determination of H_2O_2

When GPE with 300 mM DMPO was irradiated with the LED-light, the ESR signal of the ·OH spin adduct (DMPO-OH) was detected (Fig. II-5). The presence of the spin adduct was confirmed by hyper fine coupling constants of $a_N=a_H=1.49$ mT for DMPO-OH [16]. Addition of DMSO to the reaction mixture decreased the signal of DMPO-OH, and a signal for DMPO-CH_3 was observed, which was identified by its

hyper fine coupling constants of a_N=1.64 mT and a_H=2.35 mT [13, 16]. This suggests that the DMPO-OH was generated by the reaction between free ·OH and DMPO.

As shown in Fig. II-5, the yields of DMPO-OH after LED-light irradiation of GPE increased linearly with time up to 20 s, but the yield seemed to have reached a saturation level after that since LED-light irradiation for 60 s resulted in a similar DMPO-OH yield to that in LED-light irradiation for 20 s. This was possibly due to DMPO-OH would be degraded by newly formed ·OH and/or be reduced to a cyclic hydroxyl amine by electrons and protons derived from photo-oxidized polyphenols. In the latter case, the cyclic hydroxylamine is ESR silent [17]. To examine if ·OH was continuously being generated during LED-light irradiation, the photoirradiated GPE for 20 min was furthered irradiated with LED light for 10 s in the presence of 300 mM DMPO, and results showed a similar yield of DMPO-OH (around 0.9 μM) to that after 10 s irradiation of GPE without prior irradiation. ESR signals other than that of DMPO-OH, such as DMPO-OOH, were not clearly observed under the conditions used in the present study (data not shown).

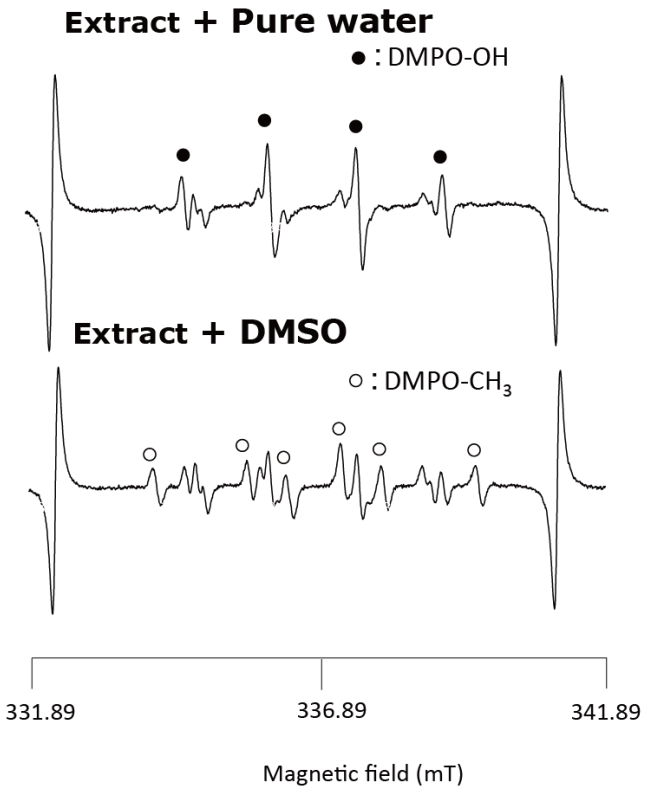

Figure II-5
Representative ESR spectra obtained by the LED-light irradiation of GPE in the absence or presence of 1.4 M DMSO.
The LED-light irradiation was performed for 10 min.

As shown in Fig. II-6, the yields of DMPO-OH after LED-light irradiation of GPE increased lineally with time up to 20 s, but the yield seemed to be saturated after that since LED-light irradiation for 60 s resulted in similar DMPO-OH yield to that in LED-light irradiation for 20 s possibly due to the decay of DMPO-OH [12]. To examine, if ·OH is continuously generated during LED-light irradiation, the photoirradiated GPE for 20 min was furthered irradiated with LED light for 10 s in the presence of 300 mM

DMPO, resulted in the similar yield of DMPO-OH (around 0.9 μM) to that in 10 s irradiation of GPE without prior irradiation. ESR signals other than that of DMPO-OH, such as DMPO-OOH, were not clearly observed under the conditions used in the present study.

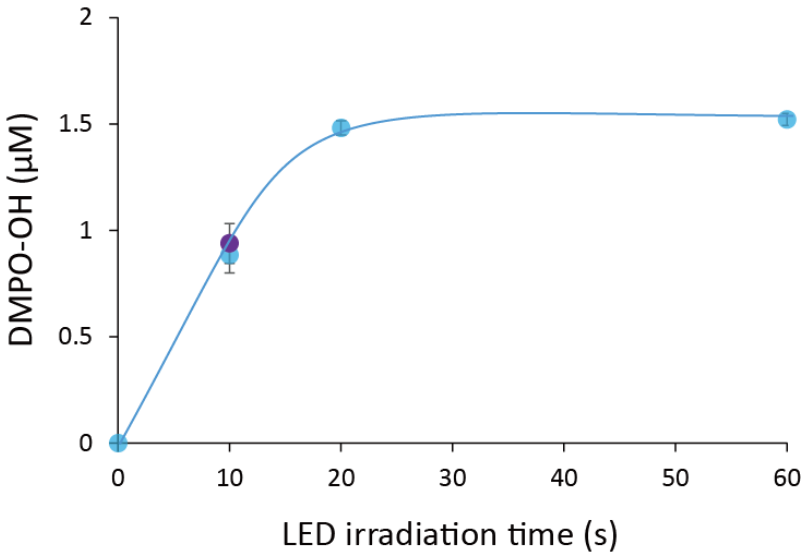

Figure II-6
Yield of ·OH (●) generated by the LED-light irradiation of GPE for 0, 10, 20, and 60 s, and of ·OH (●) generated by the 10 s irradiation of GPE that was subjected to prior photoirradiation for 20 min without DMPO. Each value is the mean with the standard deviation (n=3).

When GPE was irradiated with the LED light, H_2O_2 was prominently generated (Fig. II-7A). By contrast, only a small amount of H_2O_2 was found in GPE without the irradiation, and H_2O_2 was not detected in the pure water irrespective of whether it was

irradiated or not. The average yield of H_2O_2 generated in GPE with the LED-light irradiation for 10 min was approximately 300 µM. As shown in Fig. II-7B, the H_2O_2 generated by LED-light irradiation was almost completely scavenged by catalase.

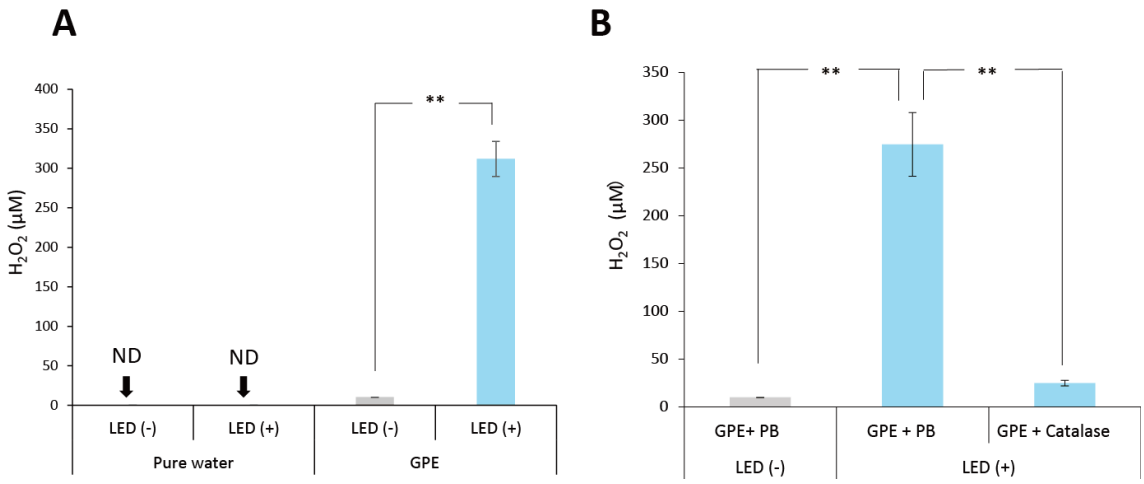

Figure II-7

Yield of H_2O_2 generated by the LED-light irradiation of samples for 10 min (A), of H_2O_2 generated in the absence or presence of catalase (B). PB stands for 0.1 M Na-K phosphate buffer (pH7.6) that was used for dissolving catalase.

Each value is the mean with the standard deviation (n=3). Significant differences between the two groups are shown as **p<0.01. ND: Not detected.

II-3-3 Analysis of wavelength dependence of H_2O_2 generation and of bactericidal activity

There were significant differences in the absorbance of GPE at 365, 385, 400, and 465 nm, and the absorbance increased as the wavelength of the light was shorter (Fig. II-8A). In addition, the yield of H_2O_2 in the photoirradiated GPE was significantly affected by the wavelength of light (Fig. II-8B). When GPE was irradiated with the light at the four wavelengths, the shorter wavelength of light gives higher concentration of H_2O_2.

Similar to H_2O_2 generation, potent bactericidal activity was obtained at the wavelengths at 365 and 385 nm whilst almost no bactericidal activity was found at the wavelengths at 400 and 465 nm under the experimental conditions with an inoculum size of 1×10^6 CFU/mL and irradiation time of 3 min (Fig. II-9). As for the wavelengths at 365 and 385 nm, LED-light irradiation alone had some bactericidal activity with a 2-log and a 1-log reduction of viable bacterial count, respectively.

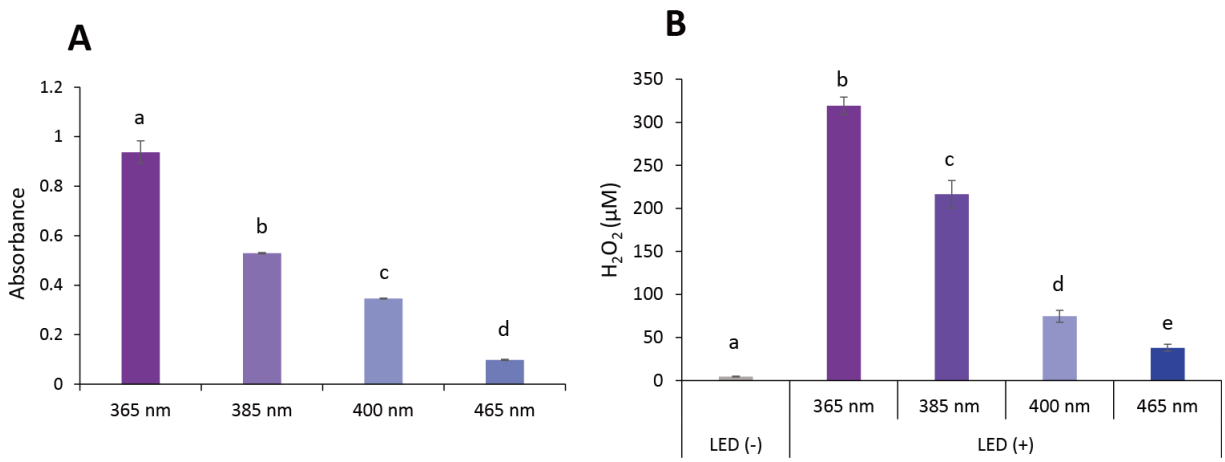

Figure II-8

Absorbance of GPE at each wavelength (A) and quantification of H_2O_2 generated in photoirradiated GPE at each wavelength (B).

Each value is the mean with the standard deviation (n=3). Significant differences ($p<0.01$) within each group are denoted by different alphabetical letters.

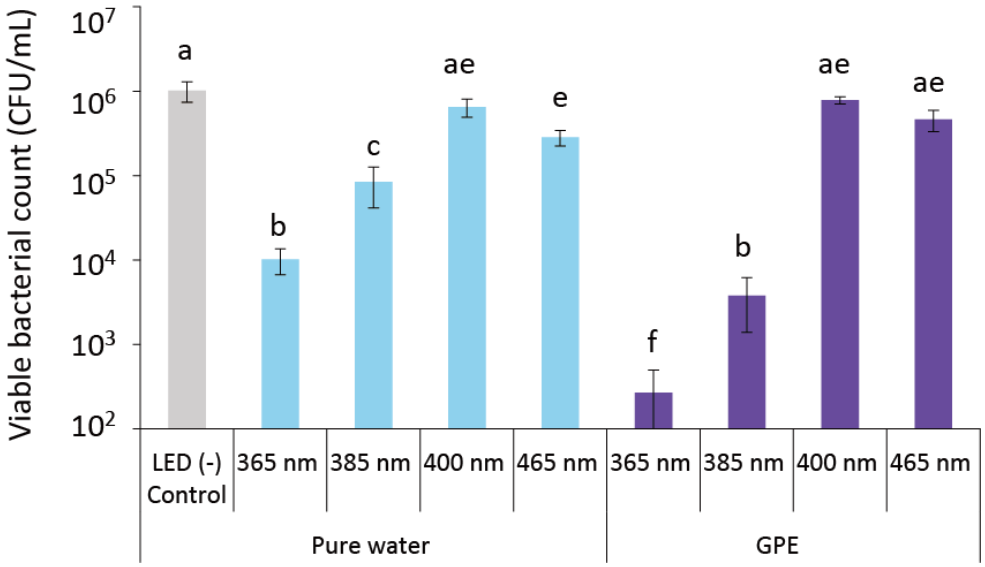

Figure II-9

Influence of wavelength on the bactericidal effect of photoirradiated GPE.

Each value is the mean with the standard deviation (n=3). Significant differences ($p<0.05$) within are denoted by different alphabetical letters.

II-3-4 Liquid chromatography/mass spectrometry (LC/MS) analyses of GPE

A representative mass spectrum of the three peaks with m/z of 291.0867, 292.0902, and 293.0930 which correspond to the main ingredient and its two isotopic ingredients, respectively, is shown in Fig. II-10. The ESI mass spectrum clearly showed that (+)-catechin (calcd. for $C_{15}H_{15}O_6$, 291.0863) was contained in GPE.

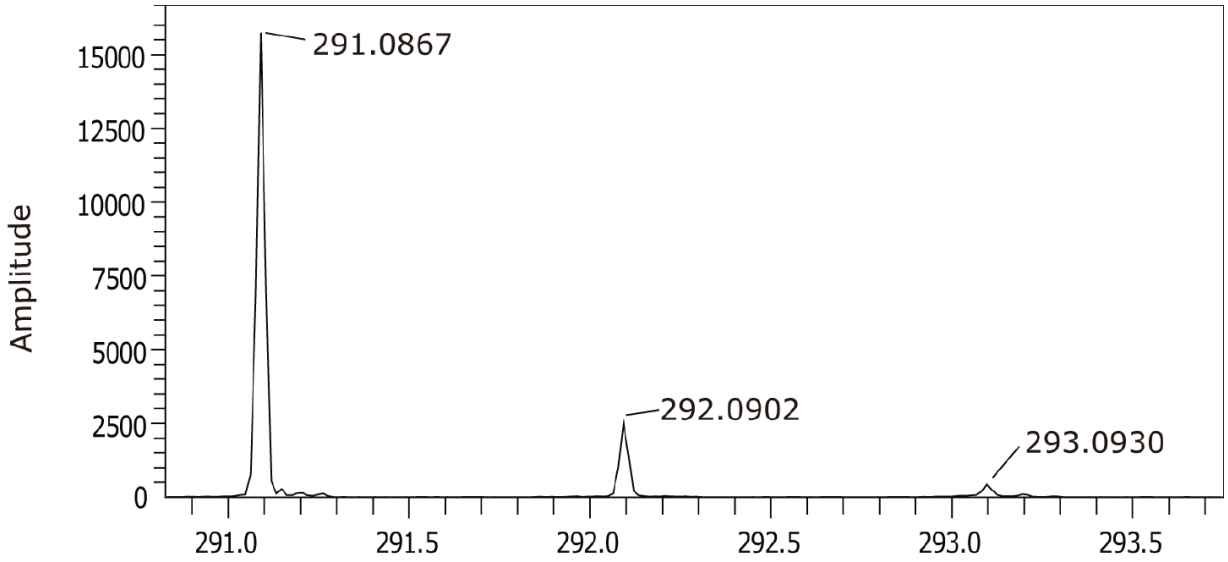

Figure II-10
A representative mass spectrum of the three peaks obtained from GPE with m/z of 291.0867, 292.0902, and 293.0930 which correspond to main ingredient of (+)-catechin and its two isotopic ingredients.
The spectrum was confirmed to be identical to (+)-catechin by an isomer simulation method.

II-4 Discussion

The present study demonstrated that after the LED-light irradiation at 400 nm, GPE obtained from the process of making white wine exerted bactericidal effect against *S. aureu*s and *P. aeruginosa*, and reduced the CFU by >5-log in the both bacterial species within 20 min. Unlike the bactericidal activity, fungicidal activity of photoirradiated GPE against *C. albicans* seemed to be weak because the number of viable *C. albicans* cells was reduced by only around 90% even by LED-light irradiation

for 40 min. It was reported that the catalase activity of *C. albicans* cells was comparable to that of aerobes [18], which would result in resistance to oxidative stress [19].

Regarding the involvement of ROS, because ·OH was detected by the ESR analysis and the bactericidal effect against *S. aureu*s was attenuated by ·OH scavengers, it was suggested that the major contributor to the bactericidal effect of the photoirradiated GPE was ·OH. As for the mild bactericidal potential of the LED-light irradiation alone, although the underlying mechanism is not clear at the present time, this finding agrees with an earlier study showing that irradiation with ultraviolet or visible blue light could exert bactericidal action depending on the level of irradiation [20].

According to the ESR analysis, the average amount of ·OH generated by LED-light irradiation of GPE for 10 s was 0.88 μM. Prior photoirradiation of GPE for 20 min did not affect the DMPO-OH yield produced by the subsequent irradiation for 10 s, suggesting that ·OH was continuously generated at least during the LED irradiation for 20 min. Thus, total amount of ·OH produced by LED-light irradiation for 20 min would be around 100 μM. Result of the previous study using photolysis of H_2O_2 as a ·OH generation system suggested that 200-300 μM ·OH yielded in 3 min would be needed to produce a >5 log reduction in *S. aureus* [21]. Thus, the amount of ·OH

obtained in the present study would be enough to kill bacteria in 10 to 20 min. In contrast, the amount of H_2O_2 determined after 10 min irradiation was around 300 μM, and the level seemed to be relatively low compared to the estimated amount of ·OH (around 50 μM for 10 min). However, H_2O_2 generated by LED-light irradiation possibly due to photo-oxidation of hydroxyl groups of polyphenolic compounds in GPE would be continuously photolyzed to generate ·OH. Thus, the net amount of H_2O_2 would be much higher than 300 μM.

Although GPE does not have an absorption peak around 365-465 nm, it absorbs the light somehow in the range of those wavelengths. Indeed, there were significant differences in the absorbance at 365, 385, 400, and 465 nm, and absorption of the light by GPE increased as the wavelength became shorter. Based on this finding, further analyses were conducted to examine if the yield of H_2O_2 and bactericidal activity depended on the wavelength of light. The results showed that H_2O_2 generation and bactericidal activity under the LED-light irradiation increased inversely with the wavelength. In other words, the yield of H_2O_2 correlated well with bactericidal activity.

In addition to the absorption of the light, the photon energy of light would also be responsible for the increased H_2O_2 formation since the photon energy is inversely proportion to the wavelength as shown by the following equation. That is, $E = hc/\lambda$

where E is energy of a photon, h is Planck's constant, c is the speed of light, and λ is the wavelength of the light. These findings suggest that the reaction of H_2O_2 generation via photoirradiation of GPE is originally derived from photo-oxidation in which the light energy absorbed by GPE triggers the oxidation of hydroxyl groups of polyphenolic compounds in GPE. Regarding the application of the photoirradiated GPE, a light source emitting wavelengths less than 400 nm could make it possible not only to enhance the bactericidal action but to shorten the treatment time. This means that the technique could be applied to disinfection processes in various fields once the safety of the technique is verified.

A possible scheme for the bactericidal action via ·OH formation is illustrated in Fig. I-4. Firstly, the hydroxyl group of the polyphenolic compounds contained in GPE would be oxidized by the photoirradiation (λ=400 nm). Secondly, because of the oxidation, a proton-coupled electron transfer to dissolved oxygen would result in H_2O_2 generation as shown in Fig. II-7 [22], followed by photolysis of H_2O_2 [21]. Finally, the ·OH generated by photolysis would cause oxidative damage to the bacterial cells. In the series of reactions, the photoirradiation would probably function in two different ways, that is, photo-oxidation of polyphenolic compounds and photolysis of H_2O_2 as reported in the previous study [5]. Regarding the polyphenolic compounds in GPE, (+)-catechin

was detected by the LC/MS analysis. Since it is speculated that relatively water soluble polyphenolic compounds would be contained in GPE, further study is required to search for such compounds.

The present study suggests that photoirradiated GPE from winemaking could be a recyclable food resource for a novel disinfection technique. GPE is regarded as relatively safe because it is obtained from residues of crushed grapes. The other advantageous point is that the ·OH is only generated in GPE during photoirradiation because of the extremely short life of ·OH as $<10^{9}$ s [23, 24], suggesting that its generation is controllable through termination of the light irradiation. In other words, the residual toxicity would probably be negligible.

II-5 Summary

Previous studies showed that photoirradiation of polyphenols could exert bactericidal action via ROS. In the present study, the photoirradiation-induced microbicidal activity of the aqueous extract from the residue of crushed grapes from winemaking was investigated in relation to ROS formation. *S. aureus*, *P. aeruginosa*, and *C. albicans* suspended in the extract were irradiated with LED light at 400 nm. Although 20 min of photoirradiation alone exerted bactericidal activity with a 2- to

3-log reduction, photoirradiated GPE for 20 min achieved a 5-log or greater reduction in viable *S. aureus* and *P. aeruginosa* cells. Regarding *C. albicans*, a 1-log reduction (90% reduction) of viable cells was achieved by photoirradiated GPE for 40 min, whereas photoirradiation alone did not show any fungicidal effect. Almost no change occurred in the suspensions without LED irradiation. When ·OH scavengers were added to the suspension of *S. aureus*, the bactericidal effect of the photoirradiated extract was attenuated. Furthermore, electron spin resonance analysis demonstrated that ·OH was generated by the photoirradiation of the extract. The present study suggests that polyphenolic compounds in the extract exert bactericidal activity via ·OH formation upon photoirradiation.

II-6 References

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Chapter Ⅲ

Microbicidal action of photoirradiated aqueous extract from wine lees

III-1 Introduction

As described in Chapter I, grape is the largest fruit crop in the world and waste materials or byproducts obtained from winemaking process could be a valuable resource to be recycled. The waste from winemaking process can be divided into three categories, *i.e.*, pomace, clarification sediment such as lees, and yeast sediment, and polyphenolic compounds included in these byproducts might possess not only antioxidant but prooxidant potential.

In Chapter II, I focused on grape pomace, and I discussed on bactericidal activity of photoirradiated GPE in relation to ROS generation.

One sphere of waste materials or byproducts obtained from winemaking process is wine lees that could also be a good resource to be recycled such as a sustainable source for economic nutrients [10]. Wine lees are the wastes generated during fermentation and aging process. The solid fraction of lees primary consist of yeast biomass, insoluble carbohydrates such as cellulosic or hemi-cellulosic materials, phenolic compounds, lignin, proteins, inorganic salts, organic acid salts (mainly tartrates), and other materials, and the liquid phase of lees is rich in ethanol and organic acids [10]. Since it was reported that wine lees can be applied for the recovery of value-added phytochemicals owing to the ability of yeast to form molecular interactions with phenolic compounds

[11], it is expected that the profile of phenolic compounds in lees might be different from that in the grape pomace.

In this Chapter, I focused on wine lees, and evaluated the microbicidal activity of photoirradiated aqueous extract of lees from making processes of wine in relation to ROS formation. Recent studies suggested that natural substances such as naturally occurring phenolic compounds, which possess antibacterial and anti-oxidative activities are required for food preservatives and sanitizers [12, 13]. Thus, in the present study, 2,2-diphenyl -1-picrylhydrazyl (DPPH), a stable free radical, scavenging activity of wine lees was also examined.

III-2 Materials and Methods

III-2-1 Reagents

Reagents were purchased from the following sources: DMPO from Labotec; catalase from bovine liver, DMSO, thiourea, and L-ascorbic acid from Wako Pure Chemical Industries; TEMPOL from Sigma Aldrich; DPPH from Tokyo Chemical Industry. All other reagents used were of analytical grade.

III-2-2 Preparation of aqueous extract

Strained wine lees after fermentation for 1-2 weeks obtained from a white wine grape variety Niagara harvested in Hokkaido, Japan was freeze-dried. Three times volume of pure water (at the ratio of 3 mL pure water per 1 g powder) was added to the dried powder of lees and the resultant mixture was agitated at 150 rpm overnight at room temperature. The upper layer was taken and centrifuged at 3,000 rpm for 20 min to obtain supernatant. Filtrate through membrane filtration (φ 0.22 μm) the supernatant was subjected to total polyphenol determination by Folin-Denis method in which gallic acid was used as a standard [14]. The aqueous extract solution (hereafter termed as WLE that stands for wine lees extract) was adjusted to contain 0.2 mg total polyphenol/mL with pure water and stored at -20℃ until assayed. In a DPPH scavenging assay, WLE was further freeze-dried and tested. One gram of freeze-dried WLE was obtained from 58.7 mL of WLE.

III-2-3 Light source

The experimental device equipped with LED with a wavelength of 400 nm (NHH105UV, Lustrous Technology) was identical to that described in Chapter II. The output power of the LED measured using the power meter (Field Mate) was set to be

400 mW per LED corresponding to an irradiance of 130 mW/cm^2 at a distance of 15 mm from the LED. The four-clear-sided methacrylate plastic cuvette (Fisherbrand Disposable Cuvette, size: 12 x 12 x 45(H) mm, light transmission rate at 400 nm: 90%, Thermo Fisher Scientific K.K.) containing the sample was placed in the experimental device. LED-light irradiation was performed toward both sides of the plastic cuvette (total irradiance: 260 mW/cm^2).

III-2-4 Microbicidal assay

S. aureus JCM 2413, *P. aeruginosa* JCM 6119, and *C. albicans* JCM 153purchased from the Japan Collection of Microorganisms, RIKEN Bio Resource Center was used. Each suspension of *S. aureus* and *P. aeruginosa* was prepared in sterile physiological saline from a culture grown on BHI agar (Becton Dickinson Labware) aerobically at 37°C overnight. A suspension of *C. albicans* was prepared in sterile physiological saline from a culture grown on SDA at 37°C overnight. The procedures for bactericidal and fungicidal assays were essentially identical to those described in Chapter II except that WLE was used instead of GPE.

III-2-5 Electron spin resonance (ESR) analysis of ·OH and colorimetric determination of H_2O_2

Qualitative and quantitative analyses of ·OH generated by photoirradiation of LED was essentially identical to that described in Chapter II. That is, 483 μL of the undiluted or 2-8 times diluted WLE was mixed with 17 μL of DMPO in a plastic cuvette to reach a final concentration of 300 mM for DMPO. Then, the sample was irradiated with the LED-light for 0, 10, 20, and 60 s. After irradiation, the sample was transferred to a quartz cell for ESR spectrometry, and the ESR spectrum was recorded to determine DMPO-OH concentrations.

Since linearity of the increase in DMPO-OH was confirmed in all of the undiluted and diluted groups within 10 s of irradiation, the following two experiments were conducted. One was to examine if ·OH was continuously generated during LED-light irradiation for 20 min. In this experiment, undiluted and 8 times diluted WLE samples were irradiated with LED light for 20 min, and then DMPO was added to each photoirradiated sample to be a final concentration of 300 mM. Immediately after addition of DMPO, the sample was further irradiated with LED light for 10 s. Then ESR analysis was performed. The other was to estimate the amount of ·OH generated in the photoirradiated undiluted WLE sample for 20 min. An aliquot (483 μL) of the sample in

the plastic cuvette was irradiated with LED-light for 0, 0.5, 1, 2, 3, 4, 5, 10, and 20 min in the absence of DMPO. Then 17 μL of DMPO was added to each cuvette to reach the final concentration of 300 mM for DMPO, and the cuvette was again irradiated with LED light for 10 s followed by ESR determination of DMPO-OH. Since the slope of each linear line for the DMPO-OH yield from 0 to 10 s indicates the velocity of ·OH generation (μM/s), the area under the curve (AUC) which is a curve represented as a function of the velocity of ·OH generation and the LED-irradiation time without DMPO was calculated to estimate the amount of ·OH generated for 20 min of LED-light irradiation.

For H_2O_2 determination, 500 μL of undiluted WLE sample in a plastic cuvette was irradiated with LED light for 0, 5, 10, and 20 min. Then the H_2O_2 concentration was measured as described in Chapter II.

III-2-6 Scavenging effect on the stable radical DPPH

Freeze-dried WLE and L-ascorbic acid were dissolved in pure water to be designated concentrations followed by filtration (pore size, 0.22 μm). An aliquot (80 μL) of each aqueous solution was mixed with 16 μL of 100 mM Tris-HCl buffer (pH 7.5), 64 μL of 100% ethanol, and 40 μL of 1 mM DPPH dissolved in 100% ethanol in a

well of 96-well microplate. The plate was then left in a light-shielding place for 20 min. Absorbance at 520 nm was read by a microplate reader (Filter Max F5). Rate of DPPH scavenging was calculated according to the following equation: ((A520 in the solvent control － A520 in specimen)/A520 in the solvent control) × 100, where A520 is absorbance at 520 nm. All tests were performed in duplicate.

III-2-7 Statistical analyses

Statistical significance in the CFU/mL obtained in the bactericidal assay was assessed by Tukey-Kramer HSD multi-comparison test. The analysis for the bactericidal assay was performed following logarithmic conversion. When colony was not detected, the value of the detection limit (10^2 CFU/mL) was used for the statistical analysis. Regarding the yield of H_2O_2, since H_2O_2 was not detected in the pure water group, statistical significance for the remaining four groups was assessed by Tukey-Kramer HSD multi-comparison test. $P < 0.05$ was considered significant.

III-3 Results

III-3-1 Microbicidal assay

The result of the bactericidal assay against *S. aureus* is summarized in Fig. III-1. Under the condition without LED-light irradiation, WLE kept in a light-shielding box for 10 and 20 min showed almost no bactericidal activity in comparison with that of the corresponding pure water groups. LED-light irradiation alone showed slight bactericidal activity. Namely, LED-light irradiation of pure water for 10 and 20 min showed an approximate 1.5- and 2.5-log reduction of viable bacterial counts, respectively, compared with the corresponding pure water groups without LED-light irradiation. Furthermore, LED-light irradiation of WLE for 10 min effectively killed the bacteria with an approximate 3-log reduction, and LED-light irradiation for 20 min achieved a 5-log reduction. The results of the bactericidal assay against *P. aeruginosa* and the fungicidal assay against *C. albicans* are summarized in Fig. III-2. Similar to *S. aureus*, although LED-light irradiation of *P. aeruginosa* in pure water for 20 min showed an approximate 3-log reduction of viable bacterial counts, LED-light irradiation of the bacteria in WLE for 20 min effectively killed the bacteria with a >5-log reduction. Unlike the two bacterial species tested, LED-light irradiation of *C. albicans* in pure water for 40 min showed almost no fungicidal effect. When *C. albicans* in WLE were

irradiated with LED light for 40 min, the fungi were killed with an approximately 1-log reduction (90% reduction).

I next examined the effect of 3% H_2O_2, used as a reference disinfectant (Fig. III-3), and revealed that treatment of *S. aureus* showed a time-dependent bactericidal activity, and 20 min-treatment achieved a 5-log reduction of viable cells. In contrast, treatment of *C. albicans* with 3% H_2O_2 showed almost no fungicidal effect, and even 40-min treatment resulted in only a slight reduction of viable cells.

I further examined the effect of ·OH scavengers, and revealed that LED-light irradiation of WLE for 20 min resulted in a >5-log reduction of viable *S. aureus* cells. This bactericidal activity of photoirradiated WLE was completely abrogated in the presence of 700 mM DMSO and 150 mM thiourea, and the viable bacterial counts in both cases were similar or even superior to that in the photoirradiated pure water group (Fig. III-4).

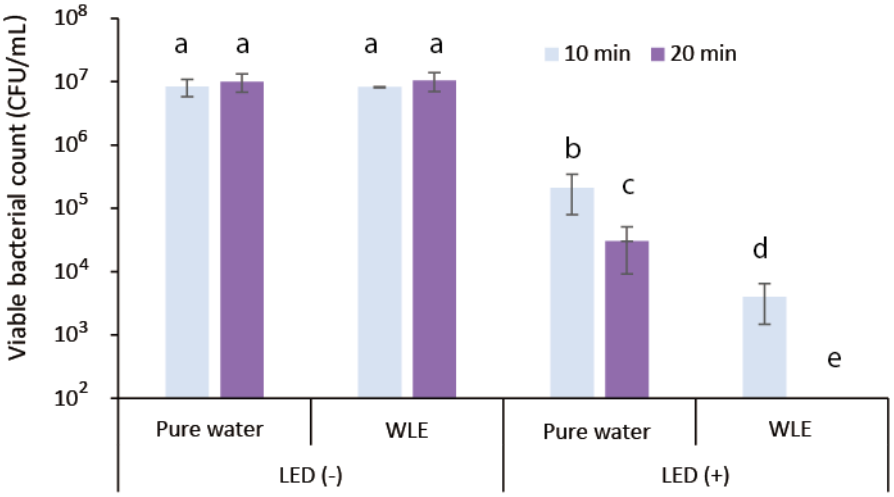

Figure III-1

The number of viable *Staphylococcus aureus* in the suspension after each treatment (suspended in pure water or WLE with or without LED-light irradiation for 10 and 20 min).

Each value is the mean with the standard deviation (n=3). Significant differences ($p<0.01$) are denoted by different alphabetical letters.

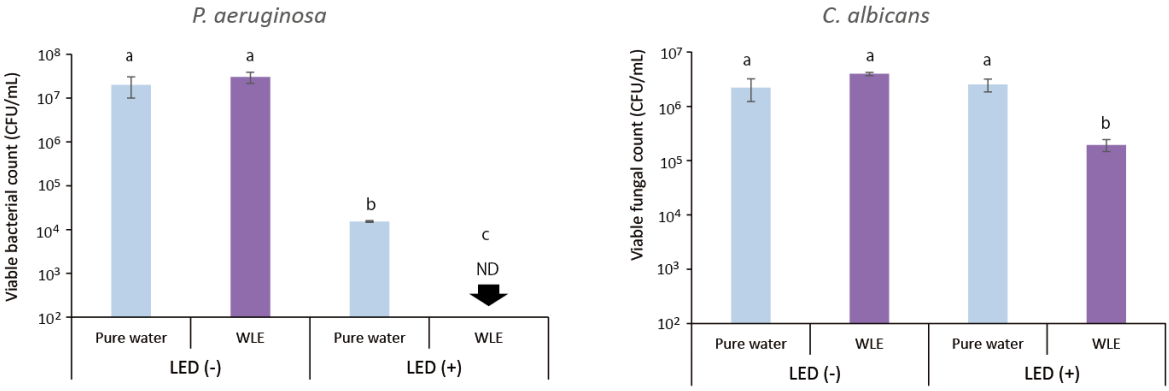

Figure III-2

The number of viable *Pseudomonas aeruginosa* and *Candida albicans* cells in suspension after each treatment (suspended in pure water or WLE with or without LED-light irradiation for 20 min for *P. aeruginosa* and 40 min for *C. albicans*).

Each value is the mean with the standard deviation (n=3). Significant differences ($p<0.01$) are denoted by different alphabetical letters. ND: not detected.

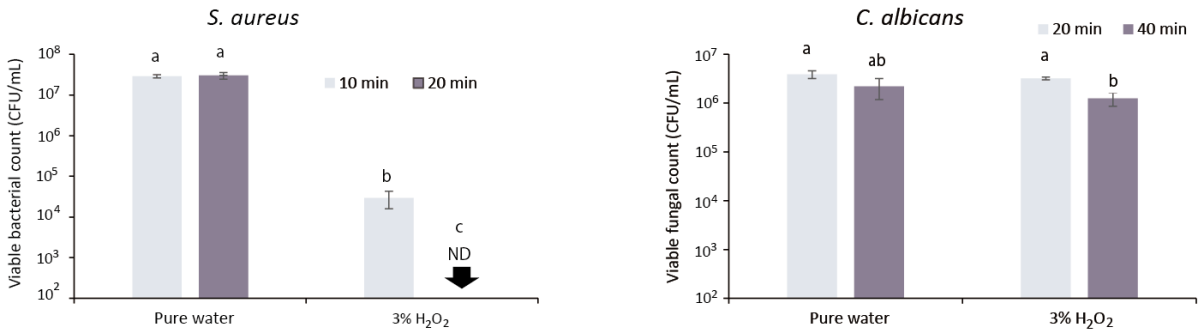

Figure III-3

The number of viable *Staphylococcus aureus* and *Candida albicans* cells suspended in 3% H_2O_2 for 10, 20, or 40 min.

Each value is the mean with the standard deviation (n=3). Significant differences ($P<0.01$ for *S. aureus* and $P < 0.05$ for *C. albicans*) within each group are denoted by different alphabetical letters. ND: not detected.

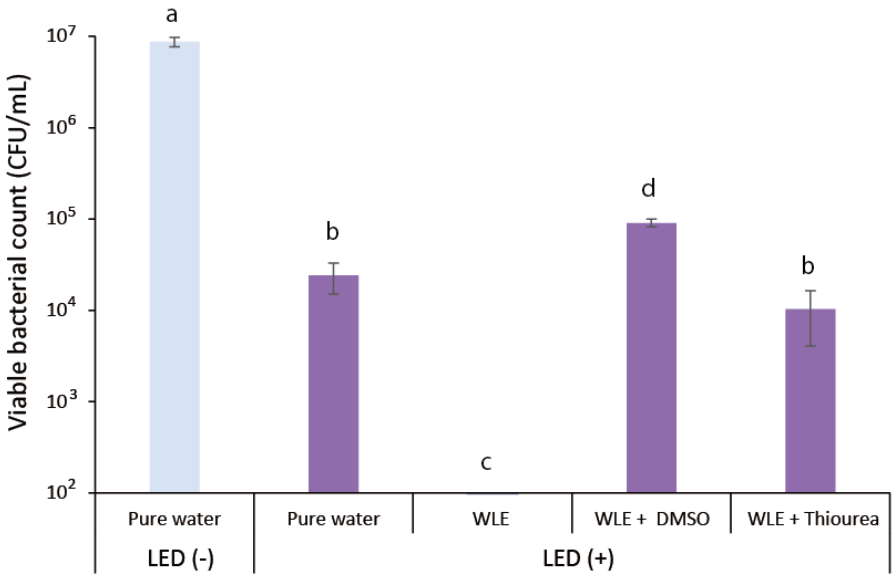

Figure III-4
Influence of ·OH scavengers on the bactericidal effect of photoirradiated WLE. The LED-light irradiation was performed for 20 min.
Each value is the mean with the standard deviation (n=3). Significant differences ($p<0.01$) within each group are denoted by different alphabetical letters.

III-3-2 ESR analysis of ·OH and colorimetric determination of H_2O_2

When WLE was irradiated with the LED light in the presence of 300 mM DMPO, the ESR signal of the ·OH spin adduct (DMPO-OH) was detected. The presence of the spin adduct was confirmed by hyper fine coupling constants of $a_N = a_H = 1.49$ mT for DMPO-OH [20]. Figure III-5 summarizes the yields of DMPO-OH after LED-light irradiation of undiluted and diluted WLE. The yield increased dependently on not only irradiation time but dilution rate of WLE. Within 10 s of irradiation, linearity of the

increase in DMPO-OH was confirmed in all of the undiluted and diluted WLE as shown in Fig.III-5. To examine, if ·OH is continuously generated during LED-light irradiation, undiluted and 8 times diluted WLE irradiated with LED light for 20 min in the absence of DMPO was furthered irradiated with LED light for 10 s in the presence of 300 mM DMPO, resulted in about 75% reduction of the DMPO-OH yield in both cases as compared to the yield without prior LED-light irradiation for 20 min (Fig. III-5).

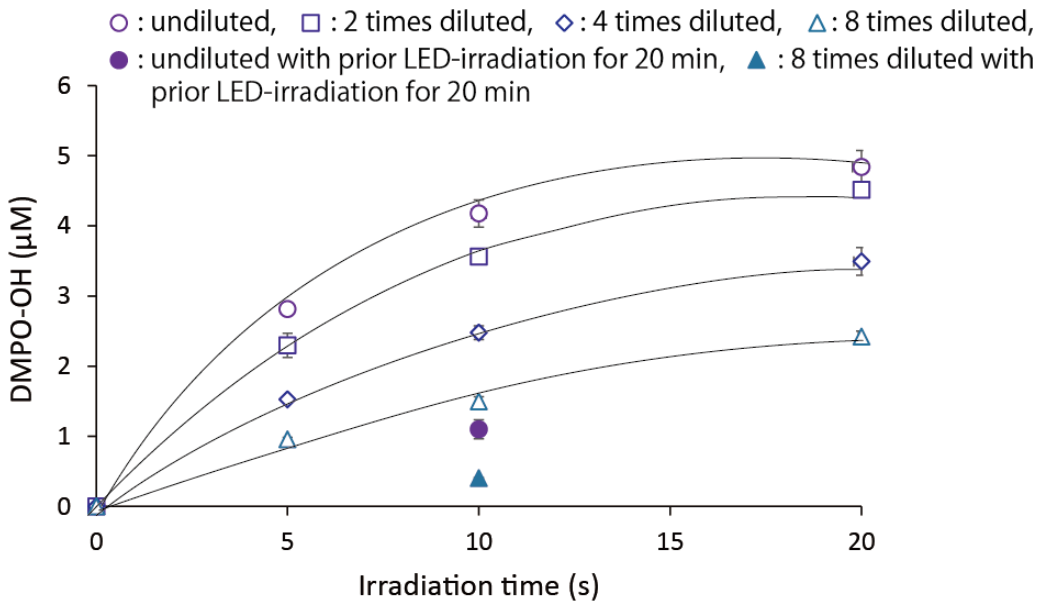

Figure III-5
Yield of ·OH (○, □, ◇, △) generated by the LED-light irradiation of the undiluted and 2–8 times diluted WLE for 0, 10, and 20 s, and of ·OH (●, ▲) generated by the 10 s irradiation of the undiluted and 8 time diluted WLE that were subjected to prior LED-light irradiation for 20 min without DMPO.
Each value is the mean with the standard deviation (n=3).

Furthermore, to estimate the amount of ·OH generated in the photoirradiated WLE (undiluted) for 20 min, WLE was irradiated with LED light for 0, 0.5, 1, 2, 3, 4, 5, 10, and 20 min in the absence of DMPO. Then ESR determination of DMPO-OH generated in 10 s of additional LED-light irradiation with DMPO was conducted. The curve of the function of velocity of ·OH and LED-light irradiation time was shown in Fig. III-6, and the AUC calculated was 169, indicating that the estimated total amount of ·OH generated in the photoirradiated WLE (undiluted) for 20 min was 169 μM.

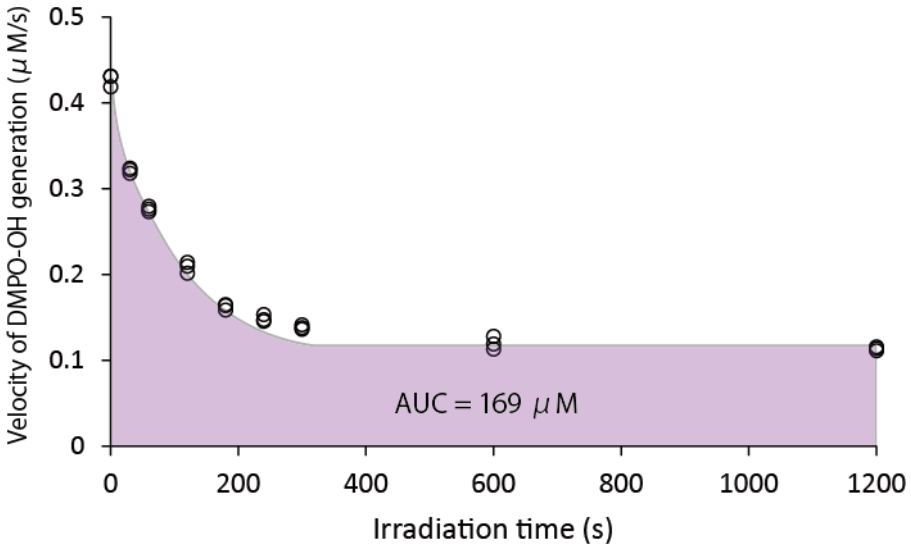

Figure III-6
Relationship between the velocity of DMPO-OH generation and LED-light irradiation time.
The area under the curve indicates the estimated amount of ·OH generated during LED-light irradiation for 20 min. Open circle (○) indicates individual data.

When WLE was irradiated with the LED light, H_2O_2 was generated in an irradiation time dependent manner (Fig. III-7). By contrast, only a small amount of H_2O_2 was found in WLE without the irradiation, and H_2O_2 was not detected in the pure water irradiated with LED light for 20 min. The average yields of H_2O_2 generated in WLE with the LED-light irradiation for 5, 10, and 20 min were approximately 280, 370, and 620 μM, respectively.

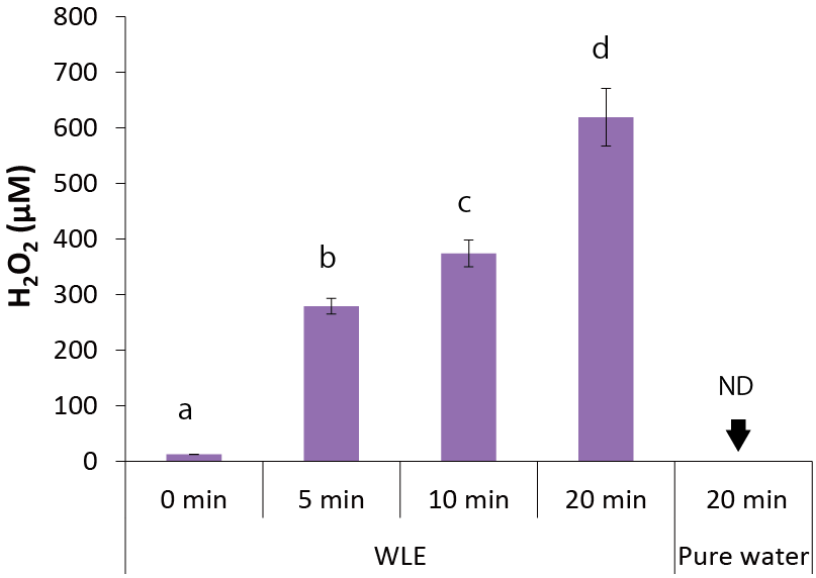

Figure III-7
Yield of H_2O_2 generated by the LED-light irradiation of WLE for 0, 5, 10, and 20 min.
Each value is the mean with the standard deviation (n=3). Significant differences between the two groups are shown as $p<0.01$. ND: Not detected.

III-3-3 Scavenging effect on the stable radical DPPH

Freeze-dried WLE and L-ascorbic acid scavenged DPPH in a concentration-dependent manner (Fig. III-8). On the weight base, the effect of freeze-dried WLE was much less potent than that of L-ascorbic acid because EC_{50}s (effective concentration showing 50% scavenging) of freeze-dried WLE and L-ascorbic acid were 7.85 mg/mL and 0.02 mg/mL, respectively.

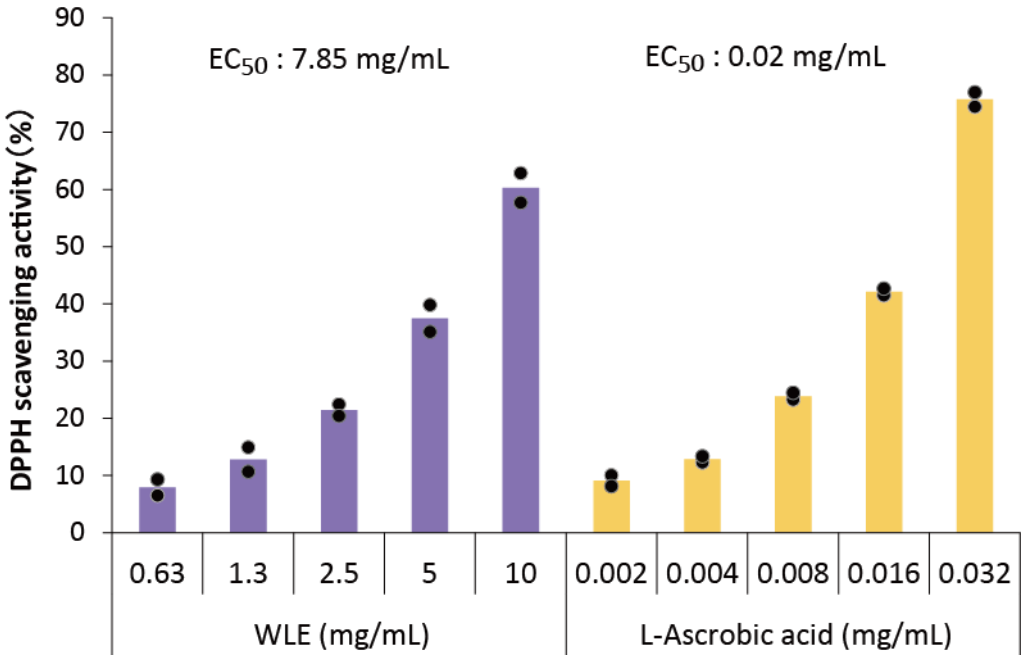

Figure III-8
Scavenging activity of freeze-dried WLE upon DPPH.
Each value is the mean with individual data (●). EC_{50} indicates the effective concentration showing 50% scavenging.

III-4 Discussion

The present study demonstrated that LED-light irradiation of WLE at 400 nm had the ability to elicit bactericidal activity comparable to that of 3% H_2O_2, which is within the range of commonly-used concentrations for food sanitary research. Regarding the fungicidal activity, photoirradiated WLE against *C. albicans* appears to be more potent than 3% H_2O_2. One of the reasons that *C. albicans* is resistant to 3% H_2O_2 could be the catalase activity of *C. albicans* cells. As I described in Chapter II, it was reported that the catalase activity of *C. albicans* cells was comparable to that of aerobes [21], which would result in resistance to oxidative stress [22].

Since ·OH was detected by the ESR analysis and the bactericidal effect of the photoirradiated WLE was cancelled by ·OH scavengers (DMSO and thiourea), the major contributor to the bactericidal effect of the photoirradiated WLE is ·OH. Since H_2O_2 was also detected in the photoirradiated WLE in an irradiation time dependent manner, ·OH would be generated via photolysis of H_2O_2.

According to the ESR analysis, ·OH was generated dependently on the dilution rate and the LED-light irradiation time at least up to 20 s of irradiation. However, once photoirradiation time was prolonged to 20 min without DMPO, the yield of DMPO-OH generated for 10 s of additional photoirradiation decreased to approximately one fourth

of that obtained by 10 s of photoirradiation without prior irradiation, indicating that the velocity of ·OH generation decreased gradually with irradiation time (0-20 min). Based on this finding, the curve of the function of velocity of ·OH and LED-light irradiation time was illustrated to estimate the total amount of ·OH generated from photoirradiated WLE for 20 min, showing that the estimated total amount of ·OH was 169 μM. The previous study using photolysis of H_2O_2 as a ·OH generation system suggested that 200-300 μM ·OH yielded in 3 min would be needed to produce a >5-log reduction in *S. aureus* [23]. Thus, the amount of ·OH obtained in the present study would be enough to kill bacteria in 20 min.

In addition to the bactericidal activity of photoirradiated WLE, freeze-dried WLE exerted DPPH scavenging activity, showing that WLE possesses anti-oxidant potential. The EC_{50}s of freeze dried WLE and L-ascorbic acid for the DPPH scavenging activity were 7.85 mg/mL and 0.02 mg/mL, respectively, indicating that the activity of 1 g freeze-dried WLE corresponds to that of 0.0025 g L-ascorbic acid (0.02/7.85 = 0.0025). In other words, 1 L of WLE would possess antioxidant potential equivalent to approximately 40 mg L-ascorbic acid (0.0025×(1,000/58.5) ≒0.04 g = 40 mg).There were studies showing that 0.05% (500 mg/L) ascorbic acid was effective not only to prevent the degradation of phenolic compounds in fresh lettuce [24] but lipid oxidation

of ground beef [25]. Therefore, WLE would be applicable as a food preservative and sanitizer in terms anti-oxidative potential if WLE is concentrated (*e.g.* 10-fold concentrated).

From these, WLE with photoirradiation could be a novel preservative and sanitizer for food processing in terms of antibacterial and anti-oxidative activities. In addition, photoirradiated WLE showed comparable microbicidal activity to the bactericidal and fungicidal effects of 3% H_2O_2, which is within the range of commonly used concentrations for food sanitary research. Therefore, photoirradiated WLE could be a novel alternative to H_2O_2 for use in the food industry.

III-5 Summary

Wine lees, a major waste product of winemaking, may be a source of polyphenolic compounds. LED-light irradiation at 400 nm elicited microbicidal activity of aqueous extract from WLE against Staphylococcus aureus, Pseudomonas aeruginosa, and Candida albicans, in addition to ROS formation, including ·OH and H_2O_2. Although 20 min of photoirradiation alone exerted bactericidal activity with a 2- to 3-log reduction, photoirradiated WLE for 20 min achieved a 5-log or greater reduction in viable *S. aureus* and *P. aeruginosa* cells. Regarding *C. albicans*, a 1-log reduction (90%

reduction) of viable cells was achieved by photoirradiated WLE for 40 min, whereas photoirradiation alone did not show any fungicidal effect. ROS analyses revealed that approximately 170 μM ·OH and 600 μM H_2O_2 were generated in photoirradiated WLE for 20 min. Because the bactericidal activity of photoirradiated WLE was abolished by ·OH scavengers, ROS, especially highly oxidative ·OH, may be responsible for the microbicidal activity of photoirradiated WLE. In addition to its microbicidal activity, WLE may act as an antioxidant as it exerted radical scavenging activity against 2,2-diphenyl-1-picrylhydrazyl, a stable free radical.

III-6 References

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Chapter IV

Prooxidative potential of photoirradiated aqueous extracts of grape pomace, a recyclable resource from winemaking process

IV-1 Introduction

In Chapters II and III, I revealed that photoirradiated GPE and WLE could exert potent bactericidal activity against *S. aureus*, and the both activities were attributable to ·OH. However, it was found that sustainability of ·OH generation in photoirradiated WLE seemed to be poor compared with that in photoirradiated GPE. Furthermore, GPE could be obtained in larger quantity than could WLE. These two points seem to be drawbacks of WLE when applied to a novel disinfection technology, and this tempted me to focus on GPE rather than WLE as a good recyclable resource for developing the technology.

The purpose of the present study was to assess the chemical composition in the aqueous extracts of grape pomace by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). In addition, prooxidative profile and potential indicated by ·OH generation induced by photoirradiation were compared to those of commercially available grape seed extract as an authentic polyphenol product and (+)-catechin as a pure polyphenolic compound

IV-2 Materials and Methods

IV-2-1 Reagents

Reagents were purchased from the following sources: DMPO from Labotec; catalase from bovine liver from Wako Pure Chemical Industries; TEMPOL from Sigma Aldrich, (+)-catechin from Tokyo Chemical Industry (Tokyo, Japan). GSE (Leucoselect®) was obtained from Indena (Milan, Italy). According to the manufacture, Leucoselect® is a grape seed extract with a well-defined chemical composition such as catechin-monomers and -oligomers, which was completely elucidated by instrumental analyses such as high performance LC-MS. This was also confirmed in a previous study in which Leucoselect® was subjected to a LC-MS analysis [1]. All other reagents used were of analytical grade.

IV-2-2 Preparation of aqueous extract

The preparation procedure of GPE from the fruitage of the white wine grape variety Niagara was identical to that described in Chapter II.

IV-2-3 Light source

The experimental device equipped with LED with a wavelength of 400 nm (NHH105UV, Lustrous Technology) and the conditions for LED-light irradiation were identical to those described in Chapters II and III. In brief, LED-light irradiation was performed toward both sides of the plastic cuvette (total irradiance: 260 mW/cm^2).

IV-2-4 LC-ESI-MS analysis for chemical composition of GPE

For LC-ESI-MS analysis, 100 mL of GPE was concentrated to dryness *in vacuo*, and extracted twice with 10 mL of methanol (MeOH) followed by centrifugation at 8,000rpm for 10 min. The supernatant was further concentrated to dryness *in vacuo*, and the resultant dried residue was dissolved in MeOH to be 100 mg/mL followed by passage through a filter (polyvinylidene difluoride; pore size, 0.2 μm). The resultant sample was injected into the electrospray ion source of a QSTAR Elite ESI quadruple time-of-flight mass spectrometer (AB Sciex; Framingham, MA, USA) coupled to Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was undertaken on an Inertsil ODS-4 (3.0 × 250 mm, GL Sciences, Tokyo, Japan) at 40°C. With regard to gradient elution, solvent A was water with 2 mM ammonium acetate, and B was methanol with 2 mM ammonium acetate. Gradient

elution was 0–30 min and 5–100% B. Flow rate was 0.5 mL/min, the injection volume was 5 μL, and UV detection was carried out by a photodiode array detector. ESI-MS was recorded for 30 min in the *m/z* region from 100 to 2,000 Da with the following instrument parameters: ion spray voltage = 5,500 V, source gas = 50 L/min, curtain gas = 30 L/min, declustering potential = 50 V, focusing potential = 250 V, temperature = 450°C, and detector voltage = 2,300 V. LC-MS analysis was undertaken by high-resolution ESI-MS ($R \geq 10,000$; tolerance for mass accuracy = 5 ppm).

IV-2-5 Total polyphenol determination of MeOH soluble and insoluble fractions of GPE

Since LC-ESI-MS analysis as described above revealed that phenolic compounds were contained in MeOH soluble fraction, total polyphenol concentrations of MeOH soluble and insoluble fractions of GPE were compared. An aliquot (50 mL) of GPE was concentrated to dryness *in vacuo,* and extracted twice with 10 mL of MeOH followed by centrifugation at 8,000 rpm for 10 min to obtain supernatant and precipitate. The supernatant was further concentrated to dryness *in vacuo,* and the resultant dried residue was dissolved in pure water to be 20 mL as a MeOH soluble fraction. The precipitate obtained after centrifugation was dissolved in pure water to be 20 mL as a MeOH

insoluble fraction. Total polyphenol concentrations in the two fractions were determined by Folin-Denis method as described above.

IV-2-6 ROS generation of photoirradiated MeOH soluble and insoluble fractions of GPE

H_2O_2 and ·OH generation of photoirradiated MeOH soluble and insoluble fractions of GPE were examined as described in Chapters II and III. That is, H_2O_2 and ·OH determinations were performed by the xylenol orange method [2] and the ESR spin trapping technique, respectively. For H_2O_2 determination, 500 μL each of the MeOH soluble and insoluble fractions placed in a plastic cuvette was irradiated with LED light for 0, 5, and 10 min. Immediately after the irradiation, H_2O_2 concentration was determined. Regarding the ·OH determination, 483 μL of the sample was mixed with 17 μL of DMPO in a plastic cuvette to reach a final concentration of 300 mM for DMPO. Then, the mixed sample was irradiated with LED light for 15, 30, and 60s. For a negative control, each mixed sample kept in a light-shielding box for 60 s was subjected to ESR determination of ·OH. After irradiation, the sample was transferred to the quartz cell for ESR spectrometry, and the ESR spectrum was recorded. All tests were performed in triplicate.

IV-2-7 Comparison of ·OH generation in photoirradiated GPE and polyphenols

Firstly, concentration effect of GPE, GSE, and (+)-catechin on DMPO-OH generation was examined. GPE was diluted with pure water to contain designated concentrations of total polyphenol. Both GSE and (+)-catechin were dissolved in pure water to make designated concentrations. An aliquot (483 μL) of the sample was mixed with 17 μL of DMPO in a plastic cuvette to reach a final concentration of 300 mM for DMPO. Then, the mixed sample was irradiated with LED light for 1 min followed by the ESR analysis as described above.

Secondly, effect of the photoirradiation time on DMPO-OH formation was examined. GPE, GSE, and (+)-catechin samples were mixed with DMPO as described above (final concentrations of GPE, GSE, (+)-catechin, and DMPO were 0.25 mg total polyphenol, 1mg, 1mg, and 300 mM, respectively), and was subjected to LED-light irradiation for designated time period followed by the ESR analysis as described above.

Thirdly, since irradiation time effect experiment as described above revealed that DMPO-OH level in all tested samples reached a plateau at irradiation time of around 1 min, it was examined if ·OH was continuously generated during LED-light irradiation for longer than 1 min. In this experiment, DMPO was added to GPE, GSE, and (+)-catechin samples irradiated in advance with LED light for up to 2 hr (final

concentrations of GPE, GSE, (+)-catechin, and DMPO were 0.25 mg total polyphenol, 1 mg, 1 mg, and 300 mM, respectively). Immediately after addition of DMPO, the sample was further irradiated with LED light for 1 min. Then ESR analysis was performed as described above.

Finally, to examine why DMPO-OH level reached a plateau, the following two experiments were conducted. One was examination of decay of DMPO-OH after LED-light irradiation for 1 min. GPE, GSE and (+)-catechin samples were irradiated with LED light for 1 min in the presence of DMPO as described above, and then time course changes in DMPO-OH level were monitored under a light shielding condition for up to 10 min. The other was examination of DMPO-OH generation under following these conditions; 1) each sample with 300 mM DMPO was irradiated with LED light for 1 min, and kept under the light shielding condition for 1 min, 2) each sample with 300 mM DMPO was irradiated with LED light for 2 min, and 3) each sample without DMPO was irradiated with LED light for 1 min, and further irradiated in the presence of 300 mM DMPO for 1 min. Then ESR analysis was performed as described above. In the two experiments, final concentrations of GPE, GSE, and (+)-catechin were 0.25 mg total polyphenol, 1 mg, and 1 mg, respectively. All tests were performed in triplicate.

IV-2-8 Bactericidal assay

S. aureus JCM 2413 (the Japan Collection of Microorganisms, RIKEN Bio Resource Center) was used. A bacterial suspension was prepared in sterile physiological saline from a culture grown on BHI agar (Becton Dickinson Labware) aerobically at 37°C overnight. The procedure for bactericidal assay was essentially identical to that described in Chapter II. In brief, the mixture of the sample and the bacterial suspension was exposed to LED light for 10min followed by the culture study for the determination of CFU/mL.

IV-2-9 Scavenging activity against ·OH generated by a Fenton reaction

The assay used in this study was essentially identical to that described in a previous paper [3]. In brief, 50 μL of 2 mM H_2O_2, 50 μL of 89 mM DMPO, 50 μL of sample, and 50 μL of 0.2 mM $FeSO_4$ were placed in a test tube and mixed. Each mixture was transferred to an ESR spectrometry cell and the DMPO-OH spin adduct was quantified 60 s after the addition of $FeSO_4$. The measurement conditions for ESR were essentially identical to those described above.

IV-2-10 Statistical analyses

Statistical analyses were undertaken using Tukey–Kramer multiple comparison test and Student's *t* test for pairwise comparisons between multiple groups and comparison between two groups, respectively. Regarding the statistical significance in the CFU/mL obtained in the bactericidal assay assessed by Tukey-Kramer multi-comparison test, the analysis was performed following logarithmic conversion. Two-way analysis of variance (ANOVA) was also performed to determine if prior-irradiation time and post-irradiation time were significantly affected the DMPO-OH level. $P<0.05$ was considered significant.

IV-3 Results

IV-3-1 LC-ESI-MS analysis for chemical composition of GPE

The MS and UV spectra of each peak were compared with those of known compounds using existing databases, the Dictionary of Natural Products (http://dnp.chemnetbase. com/). A representative LC chromatogram with estimated chemical structural formulas obtained from MeOH soluble fraction of GPE is shown in Fig. IV-1. The fraction contained phenolic compounds including (+)-catechin, (-)-epicatechin, catechin dimers, and polyphenolic glucosides. Figure IV-2 shows

extracted ion chromatograms of *m/z* 291, 579, and 867, obtained from MeOH fraction of GPE, indicating that the fraction contained catechin monomers, dimers, and trimmers.

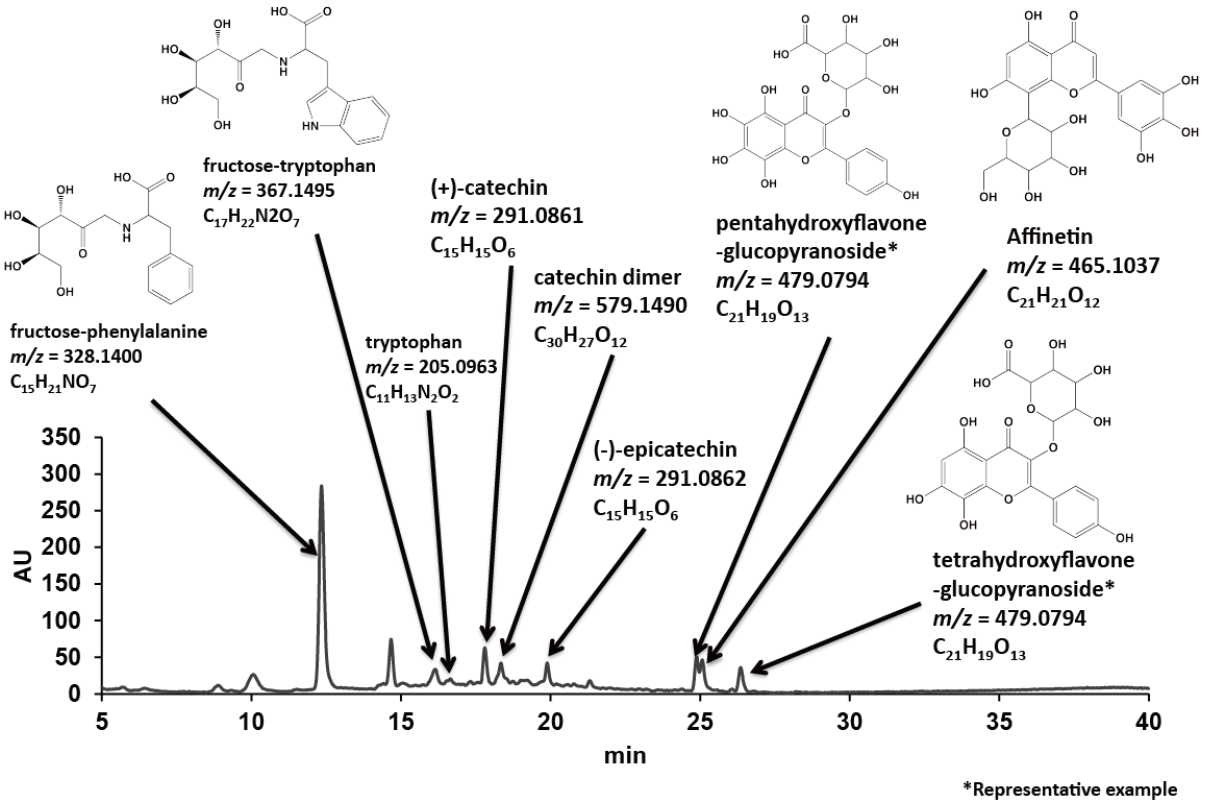

Figure IV-1
Representative LC chromatogram with estimated chemical structural formulas obtained from MeOH soluble fraction of GPE.

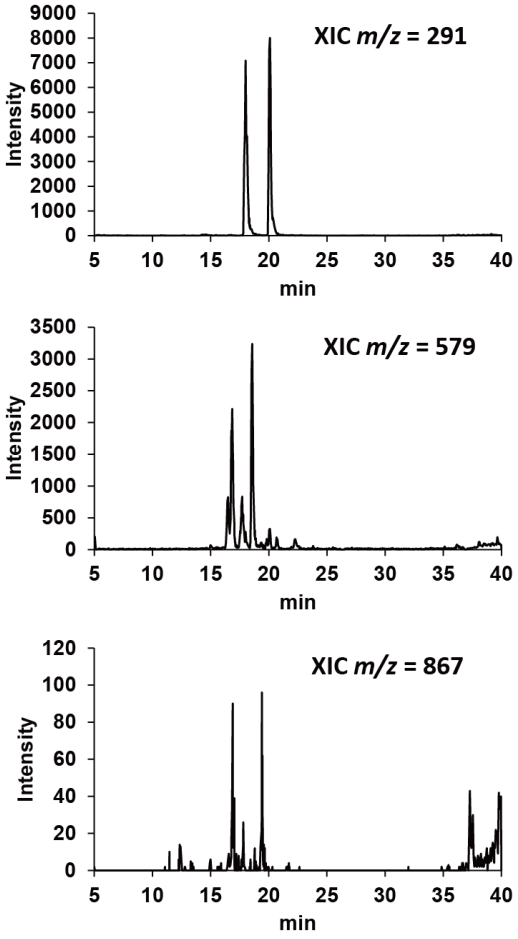

Figure IV-2
Extracted-ion chromatograms (XICs) with m/z corresponding to catechin monomers, dimers, and trimers.

IV-3-2 Total polyphenol content of MeOH soluble and insoluble fractions of GPE, and ROS generation upon photoirradiation

Total polyphenol contents of MeOH soluble and insoluble fractions are summarized in Fig. IV-3. Total polyphenol content of MeOH soluble fraction was approximately 3.5 times higher than that of MeOH insoluble fraction (p<0.01). Figure

IV-4 shows H_2O_2 concentrations in photoirradiated MeOH soluble and insoluble fractions. Although H_2O_2 formation increased with irradiation time in the both fractions, the resultant H_2O_2 concentrations in MeOH fraction were much higher than those in corresponding MeOH insoluble fractions (p<0.05). Figure IV-5 summarizes the effect of photoirradiation time on ·OH generation in MeOH soluble and insoluble fractions. As was the case with H_2O_2, ·OH generation as expressed as DMPO-OH concentration increased with irradiation time in the both fractions, and the resultant DMPO-OH concentrations in MeOH soluble fraction were much higher than those in corresponding MeOH insoluble fractions (p<0.05). DMPO-OH levels in the negative controls (kept under a light-shielding condition for 60 s) were trace levels in the both fractions.

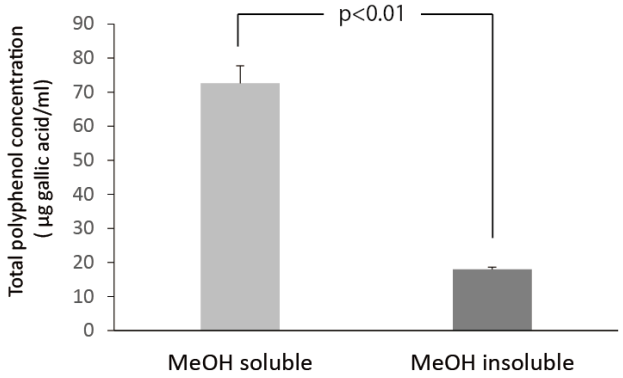

Figure IV-3
Total polyphenol concentrations in MeOH soluble and insoluble fractions.
Each value represents the mean with standard deviation (n=3).

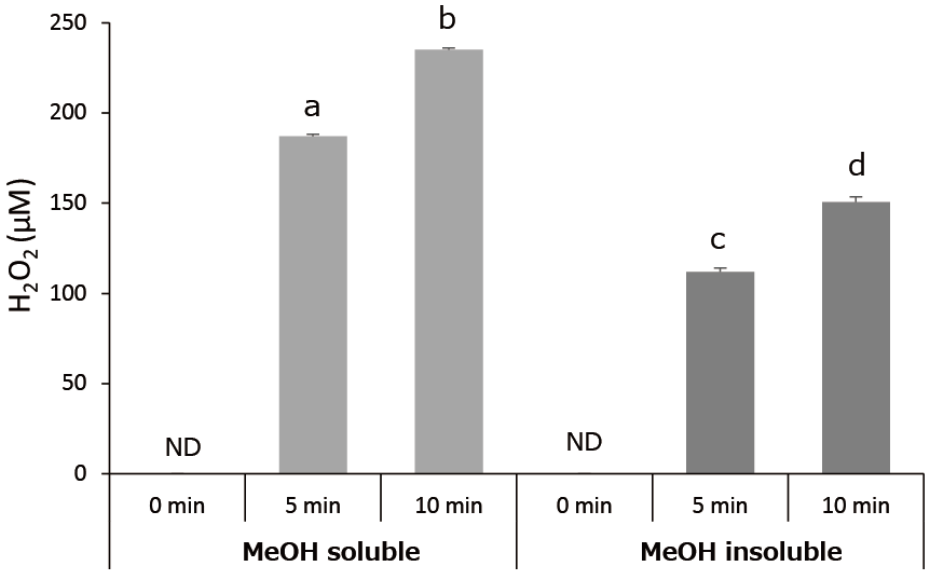

Figure IV-4

Effect of photoirradiation time on the yield of H_2O_2 in MeOH soluble and insoluble fractions.

Each value represents the mean with standard deviation (n=3).

Significant differences (p<0.05) are denoted by different alphabetical letters. ND: not detected.

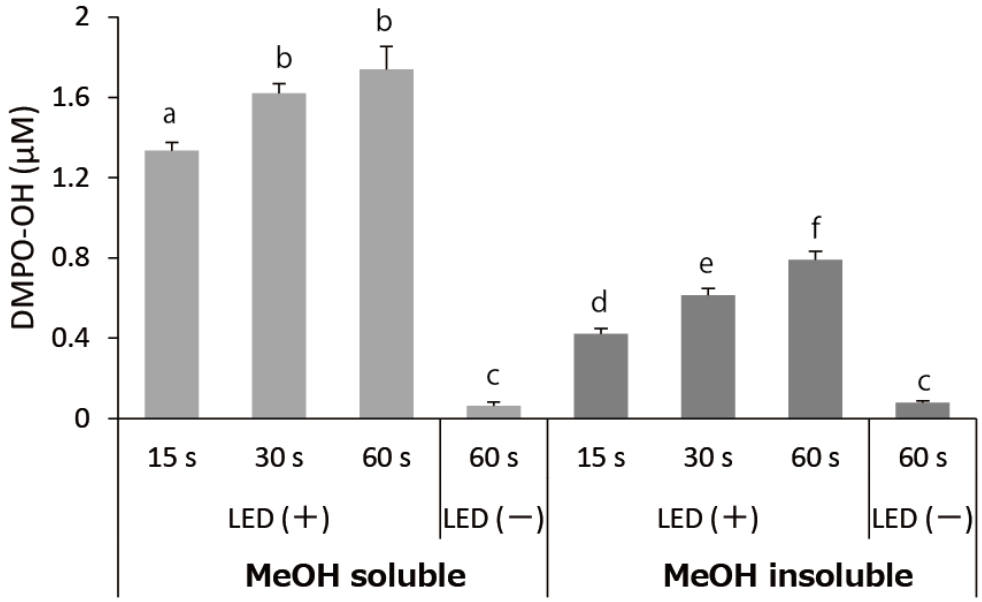

Figure IV-5
Effect of photoirradiation time on the yield of ·OH (expressed as DMPO-OH) in MeOH soluble and insoluble fractions.
Each value represents the mean with standard deviation (n=3). Significant differences ($p<0.05$) are denoted by different alphabetical letters. LED(-): Kept under a light shielding condition.

IV-3-3 Comparison of ·OH generation in photoirradiated GPE and polyphenols

Concentration effect of GPE, GSE, and (+)-catechin on ·OH generation during LED-light irradiation for 1 min is summarized in Fig. IV-6. Very slight concentration dependency in DMPO-OH concentration was observed in GPE up to 0.25 mg/mL of total polyphenol, but no increase in the concentration was found in 0.5 mg/mL of total

polyphenol. Similarly, DMPO-OH levels in photoirradiated GSE and (+)-catechin samples increased slightly with concentrations of GSE and (+)-catechin up to 1.0 mg/mL, and no increases in DMPO-OH concentrations were found in 2.0 mg/mL of both GSE and (+)-catechin. In whole, DMPO-OH generation was in the order of GPE > GSE ≥ (+)-catechin.

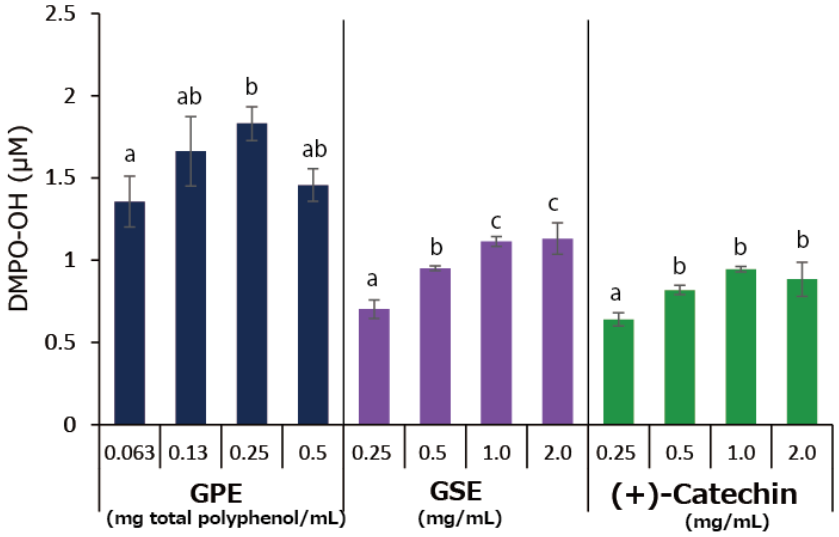

Figure IV-6
Concentration effect of GPE, GSE, and (+)-catechin on ·OH（expressed as DMO-OH) generation during LED-light irradiation for 1 min.
Each value represents the mean with standard deviation (n=3). Significant differences (p<0.05) in each group are denoted by different alphabetical letters.

Figure IV-7 summarizes the photoirradiation time effect on DMPO-OH generation. DMPO-OH levels in all the samples reached a plateau at around 1 min of irradiation. As

was the case with Fig. IV-5, DMPO-OH generation in whole was in the order of GPE>GSE≥(+)-catechin.

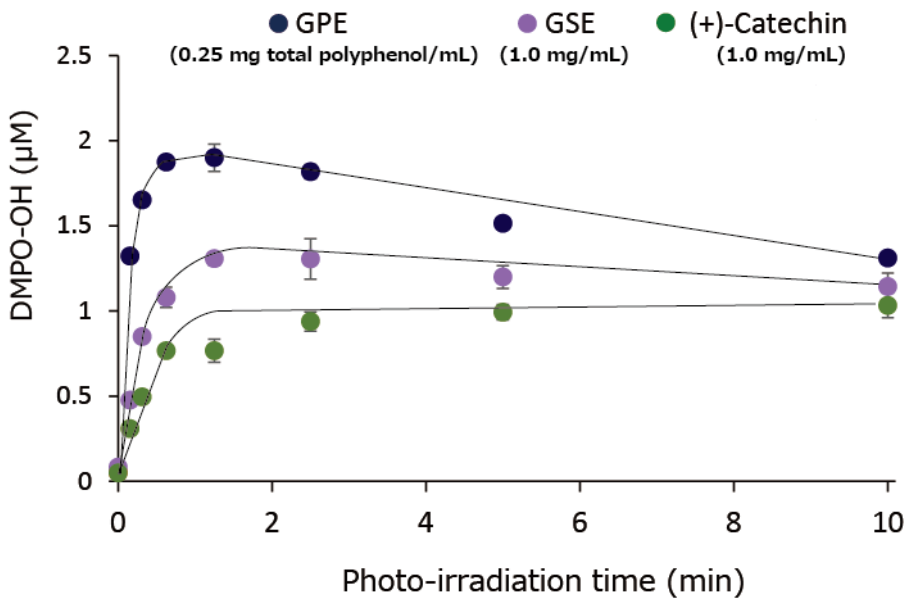

Figure IV-7
Effect of photoirradiation time on DMPO-OH yields.
Each value represents the mean with standard deviation (n=3).
TP: total polyphenol

Figure IV-8 shows the effect of time for prior-irradiation of each sample without DMPO on the DMPO-OH generation after additional LED-light irradiation of each sample with DMPO for 1 min, and the result of the two-way ANOVA is summarized in Table IV-1. The two-way ANOVA showed a significant prior-irradiation time effect on DMPO-OH generation. Indeed, in GPE (0.25 mg/mL of total polyphenol) and GSE

(1.0 mg/mL), slight decreases in DMPO-OH levels were observed with prior-irradiation time, and the levels reduced by around 30% in the both samples irradiated in advance for 2 hr as compared with those without prior-irradiation. DMPO-OH levels in (+)-catechin (1.0 mg/mL) did not significantly change, and almost the same level was kept even after irradiated in advance for 2 hr.

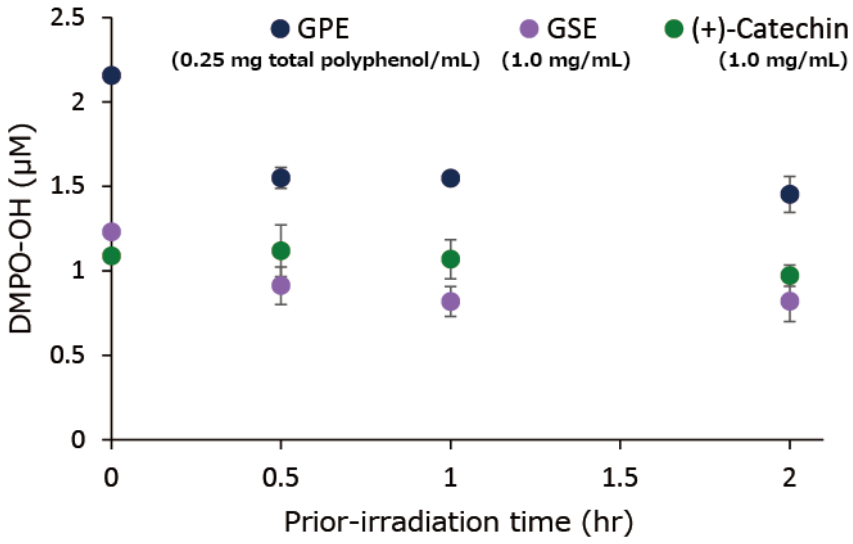

Figure IV-8
Effect of prior-irradiation time in the absence of DMPO on DMPO-OH yielded after additional LED-light irradiation for 1 min in the presence of DMPO.
Each value represents the mean with standard deviation (n=3).

Table IV-1
Summary table of two-way ANOVA for the prior-irradiation time effect on DMPO-OH generation

	df	Sum of squares	Mean square	F value	P value
Sample*	2	2.017	1.008	115.450	<0.0001
Time	3	0.892	0.297	34.036	<0.0001
Sample x Time	6	0.426	0.071	8.138	<0.0001
Error	24	0.210	0.009		

*GPE, GSE, and (+)-catechin
df: degree of freedom

Figure IV-9 summarizes time course changes in DMPO-OH level after termination of photoirradiation, and the result of the two-way ANOVA is summarized in Table IV-2. The two-way ANOVA showed no significant post-irradiation time effect on DMPO-OH levels. Indeed, DMPO-OH generated by photoirradiation was relatively stable at least for 10 min regardless of the initial levels.

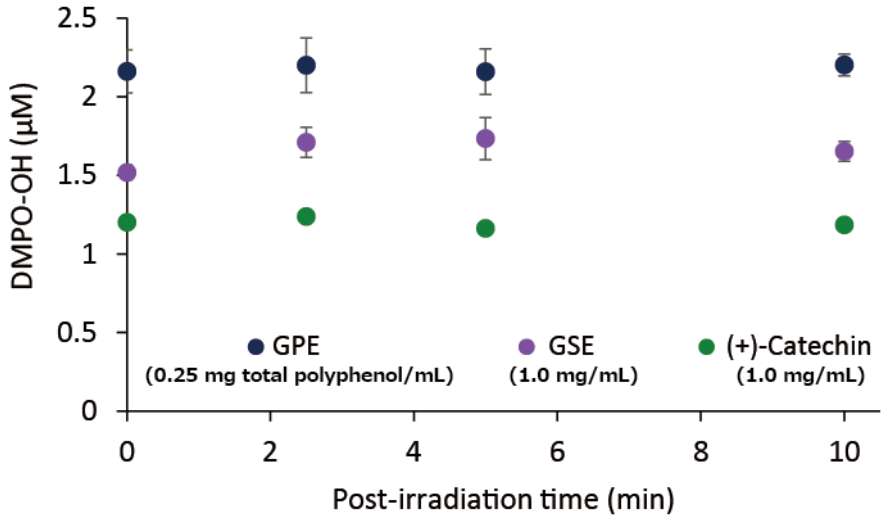

Figure IV-9

Time course changes in DMPO-OH level after termination of photoirradiation.

Each value represents the mean with standard deviation (n=3).

Table IV-2

Summary table of two-way ANOVA for the post-irradiation time effect on DMO-OH generation

	df	Sum of squares	Mean square	F value	P value
Sample*	2	1.430	0.715	77.7652	<0.0001
Time	3	0.036	0.0125	1.3205	0.2909
Sample x Time	6	0.061	0.010	1.0992	0.3915
Error	24	0.221	0.009		

*GPE, GSE, and (+)-catechin

df: degree of freedom

Figure IV-10 shows DMPO-OH generation under following there conditions: 1) each sample with 300 mM DMPO was irradiated with LED light for 1 min, and kept under a light shielding condition for 1 min, expressed as [D(+)L(+)][D(+)L(-)], 2) each sample with 300 mM DMPO was irradiated with LED light for 2 min, expressed as [D(+)L(+)][D(+)L(+)], and 3) each sample without DMPO was irradiated with LED light for 1 min, and further irradiated in the presence of 300 mM DMPO for 1 min, expressed as [D(-)L(+)][D(+)L(+)], where D and L indicate DMPO and LED light, respectively. DMPO-OH levels in each sample were almost the same regardless of the L and D treatment conditions.

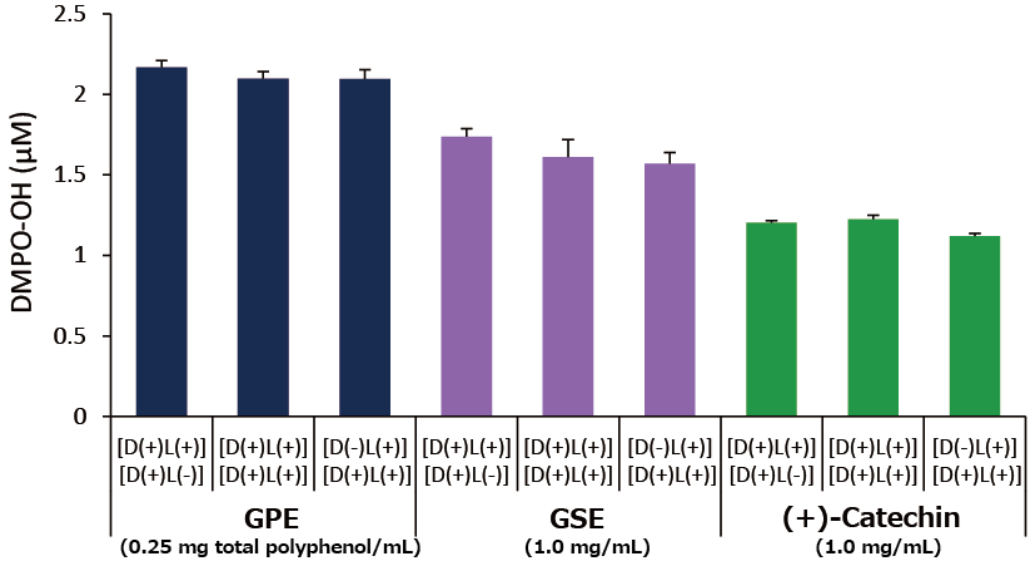

Figure IV-10

Effect of different treatment conditions on DMPO-OH yields.

Each value represents the mean with standard deviation (n=3).

[D(+)L(+)][D(+)L(-)]: sample with DMPO was photoirradiated for 1 min, and kept under a light shielding condition for 1 min, [D(+)L(+)][D(+)L(+)]: sample with DMPO was photoirradiated for 2 min, and [D(-)L(+)][D(+)L(+)]: sample without DMPO was photoirradiated for 1 min, and further irradiated with DMPO for 1 min.

IV-3-4 Bactericidal assay

Figure IV-11 summarizes the bactericidal activity of photoirradiated samples. Under the condition without the LED-light irradiation, 0.25 mg total polyphenol/mL of GPE, 0.25 mg/mL of GSE, and 0.25 mg/mL of (+)-catechin kept under a light-shielding

condition for 10 min showed almost no bactericidal activity in comparison with that of the corresponding pure water group. LED-light irradiation alone showed somewhat bactericidal activity. That is, LED-light irradiation of pure water for 10 min showed approximately 1 log reduction of viable bacterial count (CFU/mL) when compared with the pure water group without LED-light irradiation. LED-light irradiation of GPE for 10 min significantly killed the bacteria in a concentration dependent manner, and 0.063 and 0.25 mg total polyphenol/mL of GPE achieved 1 and 2 log reduction of viable bacterial count, respectively, compared with that of photoirradiated pure water group. Photoirradiated GPE and (+)-catechin also showed bactericidal effect in a concentration dependent manner, but their effects did not exceed that of GPE.

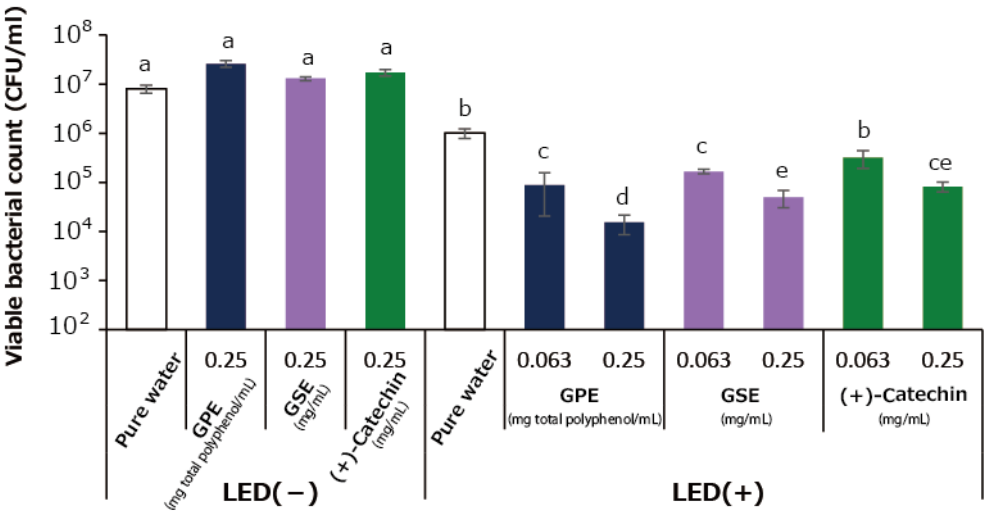

Figure IV-11

Bactericidal activity of photoirradiated GPE, GSE, and (+)-catechin on *Staphylococcus aureus*.

LED light was irradiated for 10 min. Each value represents the mean with standard deviation (n=3). Significant differences (p<0.05) in each group are denoted by different alphabetical letters.

IV-3-5 Scavenging activity against ·OH generated by a Fenton reaction

Figure IV-12 summarizes the scavenging activity of each sample against ·OH generated by a Fenton reaction. GPE showed a concentration dependent scavenging activity, and 58, 36, and 16% of ·OH were scavenged by 0.5, 0.25, and 0.13 mg total

polyphenol/mL of GPE, respectively. Although GSE and (+)-catechin also showed scavenging activity, only around 30% of ·OH was scavenged by the two even at the concentration of 0.5 mg/mL.

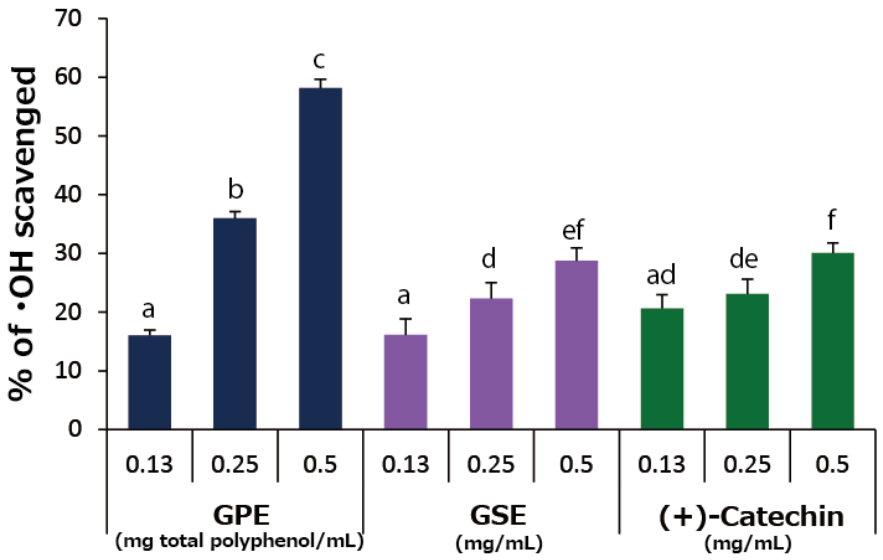

Figure IV-12
Scavenging activity of GPE, GSE, and (+)-catechin against ·OH generated by a Fenton reaction.
Each value represents the mean with standard deviation (n=3). Significant differences ($p<0.05$) in each group are denoted by different alphabetical letters.

IV-4 Discussion

LC-ESI-MS analysis revealed that polyphenolic compounds including catechin monomers, dimers, trimers, and polyphenolic glucosides were contained in MeOH

soluble fraction of GPE (Figs. IV-1 and IV-2). Since MeOH insoluble fraction was not suitable for MS analysis because of apprehensiveness of contamination in the ionization chamber, chemical composition of the fraction could not be analyzed. Instead, a spectrophotometric analysis showed that total polyphenol content of MeOH soluble fraction was much higher than that of MeOH insoluble fraction (Fig. IV-3), suggesting that MeOH soluble fraction would be a major contributor for prooxidant potential of GPE. Indeed, both H_2O_2 and ·OH generation in photoirradiated MeOH soluble fraction were much higher than those in photoirradiated MeOH insoluble fraction (Figs. IV-4 and IV-5). These results suggest that polyphenolic compounds in GPE would be pivotal players as I hypothesized.

In comparison with GSE that is an authentic polyphenol product and (+)-catechin, prooxidant profiles of GPE were similar to those of GSE and (+)-catechin. That is, 1) ·OH generation expressed as DMPO-OH concentration during 1 min of photoirradiation reached a plateau at a certain concentration in all three samples tested (Fig. IV-6), 2) in the experiment where the effect of photoirradiation time on DMPO-OH generation was examined, DMPO-OH levels in all the samples reached a plateau at around 1 min of irradiation (Fig. IV-7), 3) in the experiment where the effect of prior-irradiation time on the DMPO-OH generation was examined, the DMPO-OH levels reduced by only 30%

in GPE and GSE after 2 hr of prior-irradiation, and the level unchanged in (+)-catechin after 2 hr (Fig. IV-8). These results indicate that although ·OH was continuously generated in all the samples at least up to 2 hr, resultant DMPO-OH did not increase after around 1 min of photoirradiation. To explain the discrepancy between ·OH and DMPO-OH generation, the decay of DMPO-OH was examined (Fig. IV-9). Since DMPO-OH generated by photoirradiation was relatively stable at least for 10 min regardless of the initial levels, the decay of DMPO-OH was not involved in the mechanism by which DMPO-OH level reached a plateau at around 1 min of photoirradiation. Under the three conditions expressed as [D(+)L(+)][D(+)L(-)], [D(+)L(+)][D(+)L(+)], and [D(-)L(+)][D(+)L(+)] in which [D(+)L(+)], [D(+)L(-)], [D(-)L(+)] indicate LED-light irradiation with DMPO for 1 min, and no LED-light irradiation with DMPO for 1 min, and LED-light irradiation without DMPO for 1 min, respectively, DMPO-levels in all the three samples almost unchanged (Fig. IV-10). As discussed in Chapter II, it was suggested that DMPO-OH would be degraded by newly formed ·OH and/or be reduced to a cyclic hydroxyl amine by electrons and protons derived from photo-oxidized polyphenols. In the latter case, the cyclic hydroxylamine is ESR silent [4].

Bactericidal activity of photoirradiated GPE, GSE, and (+)-catechin seemed to well reflect their prooxidant potentials in terms of ·OH generation. That is, bactericidal effect of photoirradiated samples was in the order of GPE>GSE≥(+)-catechin (Fig. IV-11), which was in accordance with ·OH generation by photoirradiated samples as shown in Fig. IV- 6.

Although polyphenols are noteworthy for their antioxidative activity, attention has recently been paid to their prooxidant potential. For instance, it was reported that an important anticancer and antibacterial mechanisms of polyphenols is mediated through ROS generation, which is a characteristic feature of prooxidant properties of polyphenoliccompounds, leading to cancer and bacterial cell death [5, 6]. Based on these findings, I hypothesized that photo-oxidation of phenolic hydroxyl group of polyphenolic compounds results in reduction of oxygen molecule to form H_2O_2 which in turn is photolyzed to generate ·OH [7-9]. Since antioxidative activity of polyphenolic compounds is also mediated by the auto-oxidation of phenolic hydroxyl group [10, 11], prooxidant potential and antioxidant potential of polyphenolic compounds seem to like two sides of a coin. Indeed, as was the case with ·OH generation by photoirradiated samples, scavenging activity of GPE against ·OH generated by the Fenton reaction was higher than that of GSE and (+)-catechin (Fig. IV-12). This would be attributable to

their anti-oxidative activity. Getting back to prooxidant potential, as shown in Fig. IV-6, no increases in the DMPO-OH concentration were found in GPE of 0.5 mg/mL of total polyphenol, 2.0 mg/mL of GSE, and 2.0 mg/mL of (+)-catechin. When the concentration is over 0.25 mg/mL total polyphenol in GPE, for instance, the anti-oxidative activity might prevail against ·OH generation of photoirradiated GPE resulting in no increase in DMPO-OH yield.

From these, photoirradiated GPE, one of the representative waste materials or byproducts obtained from winemaking process, could be used for a novel disinfection technique, and its prooxidative potential upon photoirradiation would be attributable to polyphenolic compounds contained in GPE. In addition, the prooxidative activity in terms of ·OH generation upon photoirradiation could be retained for at least a couple of hours, and more potent than that of an authentic polyphenol product, GSE, and a pure polyphenolic compound, (+)-catechin.

IV-5 Summary

As described in Chapter II, I revealed that aqueous extract of grape pomace obtained from a winemaking process could exert bactericidal action upon photoirradiation via reactive oxygen species (ROS) formation. In the present study, I

focused on chemical composition and prooxidant profile of the extract. Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis showed that polyphenolic compounds including catechin monomers, dimers, trimers, and polyphenolic glucosides were contained. The polyphenol rich fraction used for the LC-ESI-MS analysis generated H_2O_2 upon photoirradiation possibly initiated by photo-oxidation of phenolic hydroxyl group. That is, reduction of dissolved oxygen by proton-coupled electron transferred from the photo-oxidized phenolic hydroxyl group would form H_2O_2. The resultant H_2O_2 was then photolyzed to generate ·OH. The prooxidative profile of the extract in terms of ·OH generation pattern upon photoirradiation was similar to that of grape seed extract (GSE) as an authentic polyphenol product and (+)-catechin as a pure polyphenolic compound, and in all the three samples ·OH generation could be retained during photoirradiation for at least a couple of hours. The prooxidative activity of the photoirradiated extract indicated by ·OH yield was more potent than that of the photoirradiated GSE and (+)-catechin, and this was well reflected in their bactericidal activity in which the photoirradiated extract could kill the bacteria more efficiently than did the photoirradiated GSE and (+)-catechin.

IV-6 References

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Chapter V

General conclusion

Regarding recent disinfection techniques utilizing ·OH, attention has been paid to advanced oxidation process (AOP). AOP is a technique generating ·OH by a combination of UV and H_2O_2, and is used for not only disinfection but removal of organic contamination [1]. Although AOP is an effective and potent technique for disinfection, direct use of H_2O_2 might be hazardous to human health and not be desirable to environments [2, 3]. Instead of using H_2O_2 directly, Nakamura *et al*. proposed a method using photoirradiated polyphenols that have an ability to generate ·OH [4-6]. As I described in Chapter I, byproducts or waste materials obtained from winemaking process would be polyphenol-rich resources. Therefore, I focused on wine pomace and lees both of which could be used for a novel disinfection technique. This would be beneficial in terms of not only effective utilization of plant biomass but also cost performance because pomace and lees are almost free of charge. The present study was conducted to investigate if byproducts obtained from winemaking process such as grape pomace and lees could be used for a novel disinfection technique with prooxidative activity induced by photoirradiation.

In chapter II, I examined bactericidal activity of GPE (the aqueous extracts from grape pomace) upon photoirradiation in relation to ROS formation. The followings are summaries of Chapter II.

1) LED-light irradiation to *Staphylococcus aureus* suspension in the presence of GPE for 10 and 20 min showed potent bactericidal activity.

2) DMSO and thiourea both of which are well known ·OH scavengers diminished the bactericidal activity of photoirradiated GPE, suggesting that ·OH is a pivotal player of the activity.

3) Polyphenolic compounds in GPE supposed to exert bactericidal activity via ·OH formation upon photoirradiation.

4) An ability of GPE to generate ·OH induced by photoirradiation was sustainable at least up to 20 min, suggesting that photoirradiated GPE could be a novel disinfectant.

5) H_2O_2 was generated by the photoirradiation of GPE was inversely proportional to the wavelength of light. Consequently, the shorter wavelength showed higher bactericidal activity. However, in terms of safety aspect, the wavelengths below 400 nm might be avoided the direct effect to the human skin in the assumed applications.

In chapter III, I examined bactericidal activity of photoirradiated WLE (the aqueous extract from wine lees), one of the major wastes of winemaking process, in relation to ROS formation. The followings are summaries of Chapter III.

1) LED-light irradiation to *Staphylococcus aureus* suspension in the presence of WLE for 20 min showed bactericidal activity.

2) The bactericidal action of WLE induced by photoirradiation was cancelled by ·OH scavengers such as DMSO and thiourea, suggesting that the major contributor of photoirradiated WLE to the action was most likely ·OH.

3) It was found by the ESR analysis that ·OH yield by photoirradiation of WLE increased with irradiation time at least up to 20 s as well as total polyphenol concentration. However, the prior photoirradiation experiment revealed that the ·OH generation rate gradually decreased with time. Despite the decreased generation rate, total yield reached more than 150 μM that could be enough to kill the bacteria.

4) H_2O_2 was generated dependently on photoirradiation time, and the resultant H_2O_2 would be photolyzed to ·OH.

From the results of Chapters II and III, sustainability of ·OH generation by GPE during photoirradiation seemed to be better than that in WLE. Furthermore, GPE could

be obtained in larger quantity than could WLE. In Chapter IV, therefore, I further examined the chemical composition of GPE. In addition, I compared the ·OH generation by photoirradiated GPE with GSE or commercially available polyphenol product. The followings are summaries of Chapter IV.

1) LC-ESI-MS analysis showed that polyphenolic compounds including catechin monomers, dimers, trimers, and polyphenolic glucosides were contained in MeOH soluble fraction of GPE.

2) Total polyphenol content of MeOH soluble fraction was much higher than that of MeOH insoluble fraction. Photoirradiation induced ·OH generation was much higher in MeOH soluble fraction than in MeOH insoluble fraction.

3) ·OH generation by the photoirradiation of GPE was much higher than that of GSE and (+)-catechin. This prooxidative activity could be sustained at least up to 2 hr in all three test samples.

4) Bactericidal activity of photoirradiated GPE was more potent than that of photoirradiated GSE and (+)-catechin, and likewise, ·OH scavenging activity of GPE was. The former and the latter would be categorized to prooxidative and antioxidative activity, respectively.

These results suggest that both photoirradiated GPE and WLE could be novel disinfection techniques. Considering actual application, however, GPE seemed to be better than WLE in terms of sustainability of photoinduced ·OH generation. Furthermore, according to the information from Hokkaido Wine Co., Ltd. that kindly provided me freeze dried grape pomace and lees, the amount of grape pomace discharged from winemaking would be apparently larger than that of lees. Thus, I focused on GPE rather than WLE, and further examined the photoinduced prooxidant potential of GPE in comparison with that of a commercially available polyphenol product, GSE, and (+)-catechin. The results clearly showed that the potential of GPE was superior to that of GSE and (+)-catechin. Regarding the cost–benefit performance, GPE has an advantage as compared with such polyphenol products because GPE is just a waste or a byproduct of winemaking.

Since the generation of ·OH from GPE is only observed under light irradiation, the prooxidative effect does not continue after the disinfection treatment. Even if excessive amount of ·OH would be generated, the radicals cannot exist for long time because of its extremely short life time [7, 8]. Thus, the residual toxicity is practically negligible. In addition, GPE could be edible because it is derived from grape, indicating that GPE is a safe material to be handled. As such, it is expected that this

disinfection technique with photoirradiated GPE is applicable to wide range fields as described below. In addition, since the other characteristic feature of GPE seems to be comparable or superior to that of commercially available polyphenols, applications of GPE for its antioxidative potential are also desired.

Since total amount of grape pomace discharged from winemaking was globally estimated to be approximately 10 million tons, there would be a plenty of rooms for the byproduct to be effectively utilized. As far as I am concerned, the following applications would be proposed. In a point of view of potent antioxidative activity of GPE, lyophilized GPE could be applied to law materials for cosmetics, functional foods, and deodorants. Meanwhile, prooxidative property of GPE could be applied to not only manufacturing line disinfection but also a beauty device for prevention of acne. In addition, sediment-associated substances obtained after GPE preparation would be applied to feedstuffs in the livestock industry and a coke alternative in the steel industry in terms of their antioxidative activity, and to UV-disinfection booster in terms of their prooxidative activity.

V-1 References

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