Scientific Research Publishing

American Journal of Analytical Chemistry, 2020, 11, 309-321
https://www.scirp.org/journal/ajac
ISSN Online: 2156-8278
ISSN Print: 2156-8251

Analysis of Bactericidal Components in Japanese Honeys

Koji Karasawa*, Masaru Kato, Hidetoshi Arakawa

Department of Bioanalytical Chemistry, School of Pharmacy, Showa University, Tokyo, Japan
Email: *koji180@pharm.showa-u.ac.jp

How to cite this paper: Karasawa, K., Kato, M. and Arakawa, H. (2020) Analysis of Bactericidal Components in Japanese Honeys. *American Journal of Analytical Chemistry*, **11**, 309-321.
https://doi.org/10.4236/ajac.2020.118025

Received: July 22, 2020
Accepted: August 23, 2020
Published: August 26, 2020

Abstract

It is known that the factors identified as contributing to the bactericidal activity of honeys are the high sugar concentration, hydrogen peroxide (H_2O_2) and low pH, and its bactericidal components depend on honey plant and natural environment. Manuka honey has been studied a lot about bactericidal effect. However, since Japanese honeys are mainly used as food, detailed analyzes of bactericidal components and its actions have not been reported. Therefore, we analyzed bactericidal components contained in nine Japanese honeys using Lucigenin-CL-HPLC. As our results, four species components of H_2O_2, glucose, fructose and methylglyoxal were detected from nine Japanese honeys. The H_2O_2 concentrations were 4.1×10^{-5} - 1.8×10^{-4} mol/L, the glucose concentrations were 1.4 - 2.8 mol/L, the fructose concentrations were 1.9 - 2.4 mol/L, the methylglyoxal concentrations were 4.0×10^{-3} - 1.6×10^{-2} mol/L. In this study, we confirmed that methylglyoxal is also contained in Japanese honeys, although it is a bactericidal component which is reported to be characteristic of manuka honey. It is considered that the further study of Japanese honeys is useful for more safe use, quality control, and clinical application.

Keywords

Japanese Honey, Bactericidal Components, Hydrogen Peroxide, Methylglyoxal, Lucigenin-CL-HPLC

1. Introduction

Honey has been known to possess bactericidal activity, and has been used in the treatment of injuries as well as against bacteria associated with disorders of the digestive system [1] [2] [3]. The bactericidal action of honey is ascribed to its high sugar concentration, presence of hydrogen peroxide (H_2O_2) and antibac-

terial peptides such as bee defensin-1, or its pH. Further, the composition of the honey is known to depend on the type of honey plant, from which bees gather nectar for its conversion to honey [4]. This nectar, stored in the flowers of honey plants, contains a wide variety of nutrients such as sucrose, fructose, and glucose. After the nectar is extracted by honey bees, decomposed by their internal enzymes, stored in hives, and acted upon by elements such as yeast, pollen, and royal jelly, it matures into honey. Therefore, honey constitutes of many components due to its biosynthetic route characterized by multiple steps and numerous molecular effectors. In addition to bactericidal action, honey exhibits keratin removal and moisturizing effects; and hence, is also used in foods, cosmetics, and medicines.

There has been much research on honey, such as manuka honey [4] [5] [6] [7], including the analysis of its bactericidal components, its effect on microorganisms, and its clinical applications. However, at present, not much is known about the bactericidal action of or distribution of components in honey derived from Japanese plants. Among bacteria that have been studied, anti-bacterial activity of manuka honey has been reported in many, including *Escherichia coli*, *Staphylococcus aureus*, multidrug resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecium*, vancomycin-resistant *Enterococci* (VRE), *Pseudomonas aeruginosa*, and *Helicobacter pylori* [4] [8] [9]. In addition, chemicals such as methylglyoxal (MGO) and leptosin have been studied in their capacity as bactericidal components [5] [10] [11] [12] [13], and several methods of analyzing the components of manuka honey have been reported [7] [13] [14] [15] [16]. We have earlier utilized lucigenin chemiluminescence high-performance liquid chromatography (lucigenin-CL-HPLC) for the development of simple analysis methods with high precision for MGO, dihydroxyacetone (DHA), fructose, glucose, and H_2O_2, which are five bactericidal components found in manuka honey. We have reported that components that are capable of generating active oxygen play an important role in the reported bactericidal action [17].

The bactericidal effect of Japanese honey has been ascribed to the presence of H_2O_2 generated by glucose oxidase, low pH, and high sugar concentration. The differential effect observed for other bactericidal components could be rationalized by the fact that the components depend on the type of honey plant. As mentioned above, since honey is considered a food in Japan, its antibiotic content [18], amino acid composition [19], residual chemicals [20], and pollen [21] has been extensively analyzed, along with research on honey quality control [22]. However, research on clinical applications of honey is mostly limited to studies on its suitability in treating injuries in mice [23]. At present, detailed analysis of the bactericidal components and insights into the bactericidal action of Japanese honey is not available.

Therefore, we have analyzed the bactericidal components in nine types of Japanese honey, utilizing methods including *bis*-(2,4,6-trichlorophenyl)oxalate chemiluminescence (TCPO/ANS-CL) [24], electron spin resonance (ESR), and

lucigenin-CL-HPLC [17]. In addition, we used isoluminol chemiluminescence to measure the antioxidative potential of Japanese honey and to study the relationship between the bactericidal components of honey and their antioxidative potential.

2. Materials and Methods

2.1. Honeys

Mr. Ogihara of Ogihara yohoen, Inc. (Nagano Prefecture) kindly donated nine types of Japanese honey, as well as chestnut leaves and chestnuts, to us. Honeys obtained from some plants (horse chestnut, blueberry, bush clover, clover, apple, mixed flowers, cherry, acacia, and chestnut) were used for this study.

2.2. Reagents

Bis-(2,4,6-trichlorophenyl)oxalate (TCPO), *bis*-(N-methyl acridinium) (lucigenin), and isoluminol manufactured by Tokyo Chemical Industry Co., Ltd. (TCI, Tokyo) were used for the chemiluminescence assays. 8-anilino-naphthalene-1-sulfonic acid (ANS) and micro-peroxidase were acquired from Wako. MGO manufactured by Sigma and dihydroxyacetone, H_2O_2, glucose, and fructose manufactured by Wako were used as standard bactericidal components. The enzymes catalase manufactured by Nacalai Teque and superoxide dismutase (SOD) manufactured by Wako, each prepared at concentrations ranging from 100 to 10,000 U/assay, were used to check for enzymatic generation of active oxygen. 2-bromo-4'-nitroacetophenone utilized as internal standards for HPLC was obtained from TCI. 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was obtained from Dojindo Laboratories. A pH paper (pH indicator strips, pH 0 - 14 universal indicator) manufactured by Merck was used for pH measurement. The water purified in a Milli-Q system (Merck Millipore) was used for all experiment.

2.3. Sample Preparation

The nine types of Japanese honey and the manuka honey within one year of collecting them were used. Some of the horse chestnut within one year of collection, while some has been stored for ten years were used. 0.1 - 0.5 g of each type of honey was dissolved in 1 mL of water (aqueous solutions from 10% to 50%) and these solutions passed through a hydrophilic 0.45 μm membrane filter (Kurabo Industries Ltd., Osaka) were used as samples. We have included values on the component concentrations in the various types of honey, converted into units of concentration (mol/L) in 100% aqueous solution of honey. In enzyme reactions, each solution of catalase and SOD was formulated by dissolving the respective enzyme (100 - 10,000 units/assay) in analytical grade water. A honey sample was combined with an enzyme solution and the mixture was incubated for 30 min at 37˚C.

2.4. Measurement Method for H_2O_2 using TCPO/ANS-CL

100 μL of 0.05 mmol/L TCPO dissolved in ethyl acetate and 100 μL of ANS solution (0.02% ANS, 0.1% BSA, 0.2 mol/L barbital) was mixed with 10 μL of sample. The mixture was allowed to react for 10 seconds, and then the luminescence (integrated value) was measured during 10 seconds with a BLR-301 luminescence reader (Aloka).

2.5. Identification of H_2O_2 (Hydroxy Radicals) Using ESR

270 μL of a spin trapping solution (containing 50 mmol/L phosphate buffer (PB) (pH 7.4), 0.5 mmol/L $FeSO_4$, 1 mmol/L diethylene triamine pentaacetic acid, and H_2O) and 5 μL of DMPO were mixed with a 30 μL sample aliquot, and the ESR spectrum was measured with a JES-FR30 ESR spectrometer (JEOL Resonance, Inc.). The ESR conditions included a microwave power of 4 mW, a modulation frequency of 9420.0 MHz, a field center of 335.250 mT (width 5.0 × 1 mT), a mod of 100 kHz (width 1.0 × 0.1 mT), an amplitude of 5.0 × 10, a time constant of 0.03 sec, and a sweep time of 1 min.

2.6. Simple Method for Measuring O_2^- and Reducing Agents Using Lucigenin-CL

100 μL of lucigenin luminescence reagent solution (2.4×10^{-5} mol/L lucigenin, 0.16 mol/L KOH, and 0.02% Triton X-100) was mixed with a 10 μL aliquot of the sample. This was allowed to react for 10 seconds, and the subsequent luminescence (integrated value) was measured during 10 seconds with a BLR-301 luminescence reader (Aloka).

2.7. Identification of O_2^- and Reducing Agents using ESR

200 μL of a spin trapping solution (2.4×10^{-5} mol/L lucigenin, 0.16 mol/L KOH, and 0.02% Triton X-100) and 5 μL of DMPO were mixed into 30 μL of the sample, and the ESR spectrum was immediately measured afterward. The xanthine-xanthine oxidase reaction was used as an O_2^- control, and 20 μL of 20 mmol/L xanthine, 20 μL of 1 U/mL xanthine oxidase, and 200 μL of 0.1 mol/L phosphate buffer (pH 7.4) were mixed with 5 μL of DMPO. Then an ESR spectrum of the mixture was measured. The ESR conditions were the same as those used to measure hydroxy radicals.

2.8. Method of Measuring Antioxidative Potential Using the Isoluminol Chemiluminescence Method

80 μL of 0.8 mol/L bicarbonate buffer (pH 9.5), 200 μL of the isoluminol luminescence solution (containing 2.5×10^{-4} mol/L isoluminol and 1.0×10^{-6} mol/L micro-peroxidase dissolved in 0.8 mol/L bicarbonate buffer), and 5 μL of 1.0×10^{-5} mol/L H_2O_2 was mixed with a 50 μL sample aliquot. This was incubated for 10 seconds to allow the reaction to attain completion, followed by measurement of the generated luminescence (integrated value) during 10 seconds with the

Luminescence-PSM AB-200. We calculated the antioxidative potential relative to H_2O_2 from the following formula:

$$\text{Antioxidative potential}(\%) = 100 - \left(\frac{X}{Y} \times 100\right)$$

X indicates "luminescence intensity when the sample was added". *Y* indicates "luminescence intensity when the sample was not added".

2.9. Method of Analyzing Bactericidal Components Using Lucigenin-CL-HPLC Assay

An intelligent HPLC pump (Jasco Co.) and a CLD-110 chemiluminescence detector (manufactured by Tohoku Electronic Industrial) were used for the HPLC system. A two linked Inertsil NH_2 columns (4.6 mm × 250 mm, 5 μm particle size, manufactured by GL Sciences) was used for the separation column. 90% Acetonitrile was used for the mobile phase. The lucigenin reagent (2.4×10^{-6} mol/L lucigenin, 0.016 mol/L KOH, and 0.002% Triton X-100) diluted by a factor of 10 was used for HPLC as the luminescence reagent solution. The flow rate was maintained at 1 mL/min. 5 μL of the sample was injected into the HPLC system, and a chromatogram was obtained using the generated luminescence intensity. 4.1×10^{-3} mol/L 2-bromo-4'-nitroacetophenone (2B) was used as an internal standard.

3. Results and Discussion

3.1. Detection of H_2O_2 Using TCPO/ANS-CL

In order to detect the presence of H_2O_2 in Japanese honey, a 10% aqueous solution of each honey was prepared and the TCPO/ANS-CL assay was performed. As shown in **Figure 1**, the amount of luminescence varied greatly depending on the type of honey; hence, we verified that the amount of H_2O_2 was different, depending on the honey plant from which the honey was derived. Chestnut honey showed the greatest value (13.8) for the signal-to-noise ratio (S/N ratio). Also, since the luminescence values of each honey on catalase treatment decreased, it was verified that H_2O_2 was included in all these types of honey. Next, the amount of H_2O_2 in each honey was calculated from an H_2O_2 calibration curve obtained with TCPO/ANS-CL. Chestnut honey showed the highest value of 3.2×10^{-4} mol/L, followed by the other types with concentrations of at least 2.0×10^{-5} mol/L. In order to verify whether the H_2O_2 in chestnut honey was derived from the honey plant, the source of H_2O_2 present in chestnut leaves and fruits were measured using TCPO/ANS-CL. 1 g each of chestnut leaves and chestnuts were grounded up in a mortar, were added 50 mL of water, were incubated for 30 minutes at 37˚C, and then were filtered the supernatant liquid with a 0.45 μm filter to prepare samples. Chestnut leaves and chestnuts were shown to have an S/N ratio of 10.7 and 11.8, respectively; which proves that H_2O_2 is found in honey plants.

3.2. Identification of H_2O_2 (Hydroxy Radicals) Using ESR

H_2O_2 generates hydroxy radicals in the presence of $FeSO_4$, thereby becoming a DMPO adduct. This leads to a characteristic four-line ESR spectrum with an intensity ratio of 1:2:2:1. In this work, The H_2O_2 present in chestnut honey characterized as the one with strongest luminescence among all types of Japanese honey was identified as hydroxy radicals using ESR. The samples at 50% concentration were prepared, the hydroxy radical content of chestnut honey was independently verified. The results of the ESR measurement were shown in Figure 2(a). Next, a complete absence of the hydroxy radical spectrum was observed after reaction with catalase, as shown in Figure 2(b).

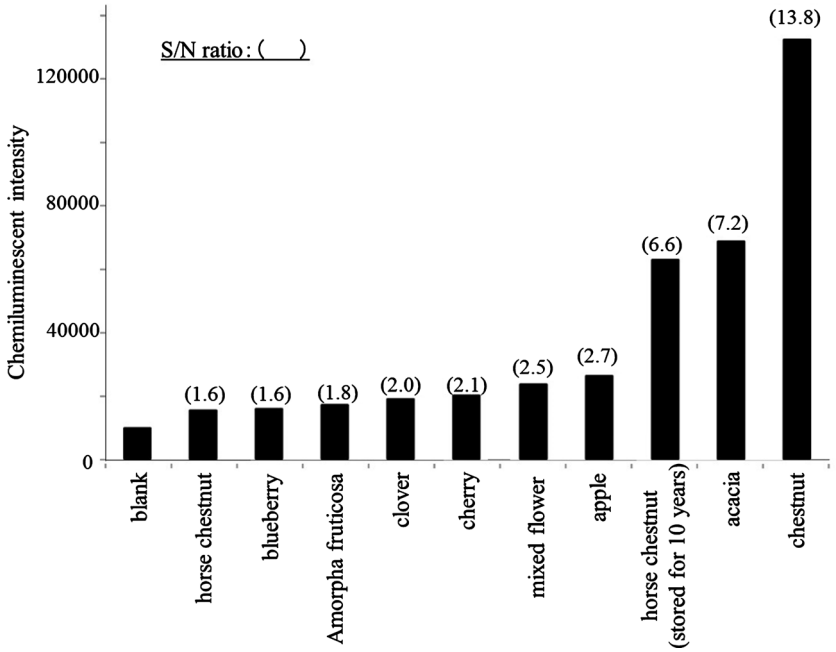

Figure 1. Chemiluminescence intensity and S/N ration of each honey by TCPO/ANS-CL (Comparison of relative concentrations for hydrogen peroxide). "Blank" indicates the result of the reaction with water.

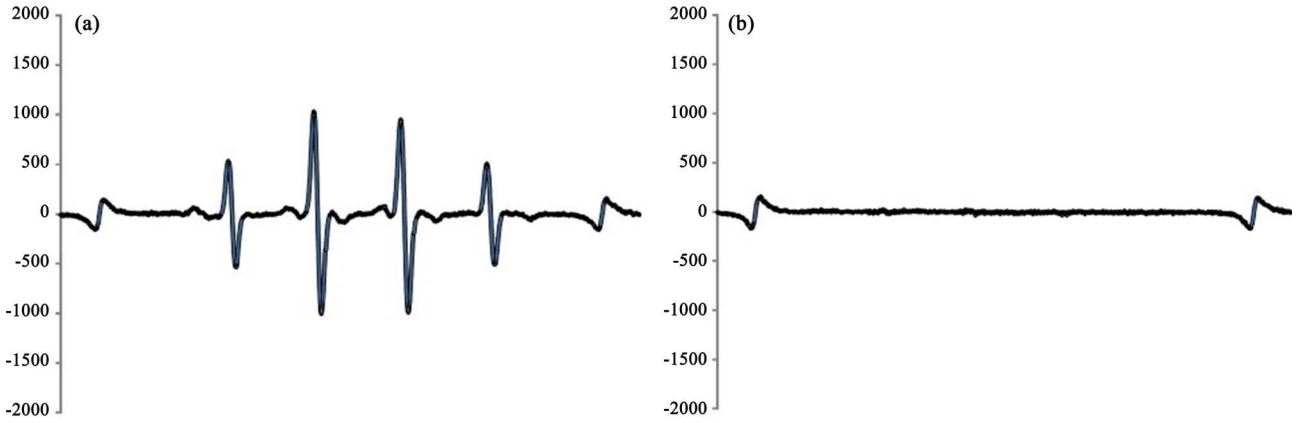

Figure 2. (a) ESR spectra of hydroxyl radical produced from chestnut honey; (b) ESR spectra of hydroxyl radical produced from chestnut honey with catalase.

3.3. Detection of O_2^- and Reducing Agents Using Lucigenin-CL

The authors have previously reported on the production of O_2^- from glucose, fructose, and MGO in manuka honey [17]. Japanese honey could include components other than H_2O_2 that are capable of generating active oxygen species. Therefore, in this study, a 10% aqueous solution of each type of honey was prepared and the lucigenin-CL assay was used to investigate the detection of O_2^- derived from bactericidal components. The results were shown in **Figure 3**, all honey types showed strong luminescence. Among them, acacia honey had the largest S/N ratio of 1350, due to its rather strong luminescence. On the other hand, the S/N ratio of chestnut honey was 208, which was the lowest among the types of honey measured at this time. The results of TCPO/ANS-CL (**Figure 1**) for chestnut honey showed the inclusion of a large amount of H_2O_2, but the lucigenin-CL results estimated the fraction of components that could generate species such as O_2^- tended to be small. From the above one could suppose that all types of honey generate O_2^- from some component.

Next, acacia honey, which showed the greatest luminescence with lucigenin-CL was carried out its reaction with catalase and SOD to investigate how the lucigenin chemiluminescence value would change. The amount of luminescence was weakened to almost a blank value. Therefore, it could be assumed to be due to the disappearance of O_2^- and H_2O_2 derived from some component, such that the chemiluminescence with lucigenin was weakened.

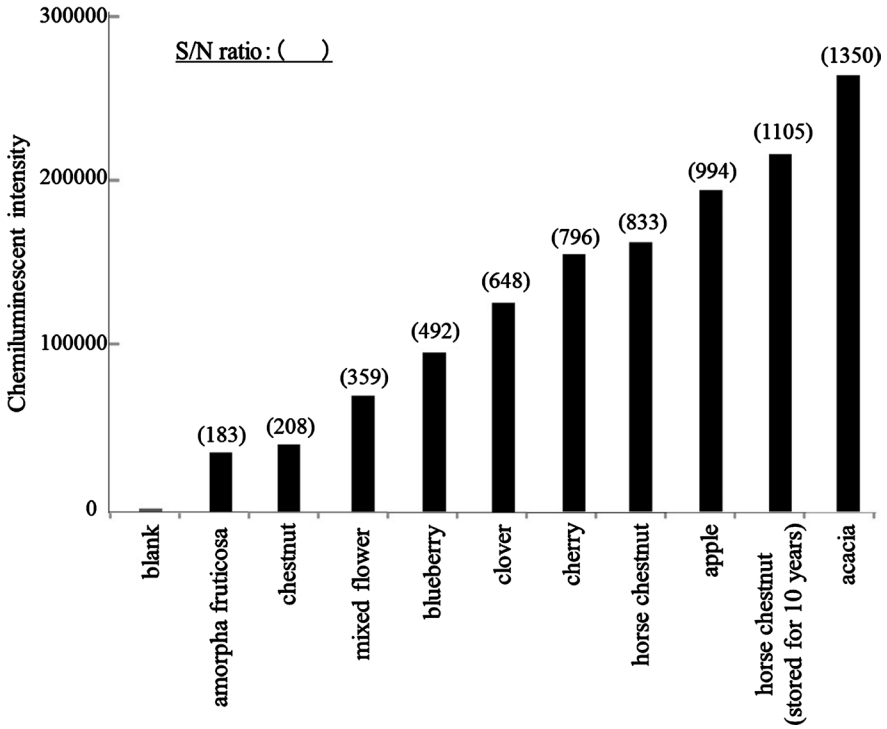

Figure 3. Chemiluminescence intensity and S/N ration of each honey by Lucigenin-CL (Comparison of relative concentrations for superoxide). "Blank" indicates the result of the reaction with water.

3.4. Identification of O_2^- Using ESR

The O_2^- generated from some components of Japanese honey was identified using ESR. A sample of 50% chestnut honey was used in this study. A xanthine-xanthine oxidase reaction was used as a control for the O_2^- spectrum. As a result, the ESR spectrum by O_2^- generated from a component in chestnut honey was detected. When the chestnut honey was incubated with SOD and the O_2^- spectrum in the ESR plot was found to disappear. This allowed an independent verification that O_2^- was generated from a component included in chestnut honey. **Figure 4(a)** shows the O_2^- spectrum at 50% chestnut honey, and **Figure 4(b)** shows O_2^- spectrum at 50% chestnut honey obtained by the SOD treatment.

3.5. Measurement of Antioxidative Potential Using the Isoluminol Chemiluminescence Method

In this study, we used the isoluminol chemiluminescence method to examine the antioxidative potential of each type of honey. The antioxidative potential based on the weakening of the amount of luminescence due to H_2O_2 was determined, when each type of honey was mixed with 1×10^{-5} mol/L H_2O_2. The strength of the antioxidative potential to that of 1×10^{-5} to 10^{-3} mol/L ascorbic acid was compared as control. As shown in **Figure 5**, chestnut honey had the greatest antioxidative potential, followed by acacia and then horse chestnut (stored for ten years) honey. From these results, it appears that the higher H_2O_2 concentration, as measured by TCPO/ANS-CL and shown in **Figure 1**, leads to a stronger antioxidative potential. Prior research has mainly studied the active oxygen species included in honey. It is held that plants generate active oxygen on their own for its antimicrobial (oxidative) action, in order to protect themselves from external enemies. On the other hand, data from the present study showed that the measured antioxidation potential is contrary to that of antimicrobial action. Plants are credited to produce antioxidants, such as polyphenol, in order to eliminate internally generated active oxygen, and thereby prevent oxidation of the plant. Since our data linked higher H_2O_2 content in a type of honey to greater antioxidative potential, certain components of honey must be capable of H_2O_2 production potential. This could be the same component as the factor that shows antioxidative potential. With regard to this relationship between H_2O_2 generation potential and antioxidative potential, similar results have been obtained for aroma oil [25]. Furthermore, in the results obtained with lucigenin-CL (**Figure 3**), large amounts of reducing agents and O_2^- were detected in acacia, horse chestnut (stored for ten years), cherry, and apple honey. These results may also influence the antioxidative potential. This could translate to increase in active oxygen on reduction of oxygen by an electron from some other component. It is considered that the remaining radical, that has lost an electron, could show antioxidative potential. Therefore, more detailed analysis of components that show antioxidative potential is needed.

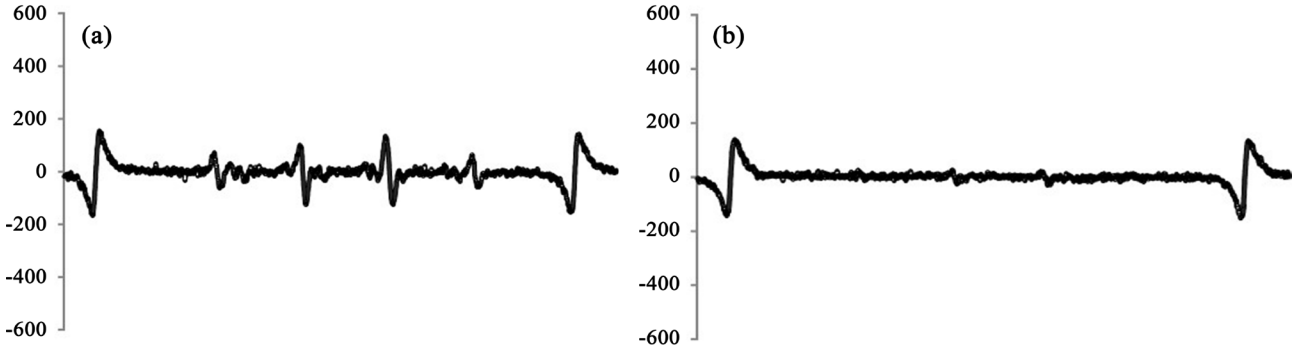

Figure 4. (a) ESR spectra of superoxide produced from components of chestnut honey; (b). ESR spectra of superoxide produced from components of chestnut honey with SOD.

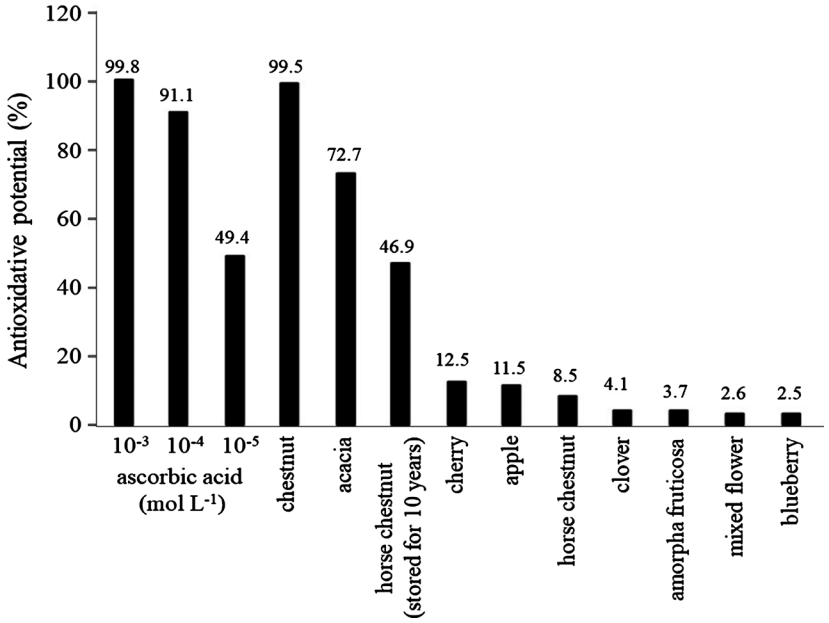

Figure 5. Antioxidative potential of Japanese honeys.

3.6. Analysis of Five Bactericidal Components Using Lucigenin-CL-HPLC

Simple analytical methods for the simultaneous detection of antibacterial components in honey like MGO, DHA, fructose, glucose, and H_2O_2, have not been reported. We have focused on reactive oxygen species (ROS) produced from these five components to permit easy detection of these compounds in honey. Lucigenin-CL is used primarily for the measurement of ROS such as O_2^-. The authors have previously developed lucigenin-CL-HPLC as a new method to simply and simultaneously detect five bactericidal components (H_2O_2, MGO, DHA, glucose, and fructose) in manuka honey [17]. We applied this lucigenin-CL-HPLC method to the analysis of Japanese honey used in this study. The samples of 40% honey were prepared and the nine types of honey were checked with the lucigenin-CL-HPLC method. The four species H_2O_2, glucose, fructose, and MGO have been detected in all types of honey. MGO is a bactericidal component believed to be specific to manuka honey, but the results showed that it is also included in

Japanese honey. On the other hand, DHA, which was included in manuka honey, was not detected in Japanese honey. Figure 6 shows two examples of chromatograms (corresponding to chestnut and acacia honey). In addition, the concentrations calculated from calibration curves for respective standard products of the included components were listed in Table 1. The H_2O_2 concentration for each type of honey measured with lucigenin-CL-HPLC was roughly the same as the value measured with TCPO/ANS-CL. Chestnut honey had the largest content at 1.8×10^{-4} mol/L. Glucose and fructose showed roughly the same values as in a previous report [22]. Even though the amount of MGO was smaller than that in manuka honey, its inclusion in all types of Japanese honey represented a novel finding. MGO increases on storage of manuka honey due to its gradual conversion from DHA [17]. On the other hand, since Japanese honey did not contain DHA, it was assumed that the MGO is generated from a different component.

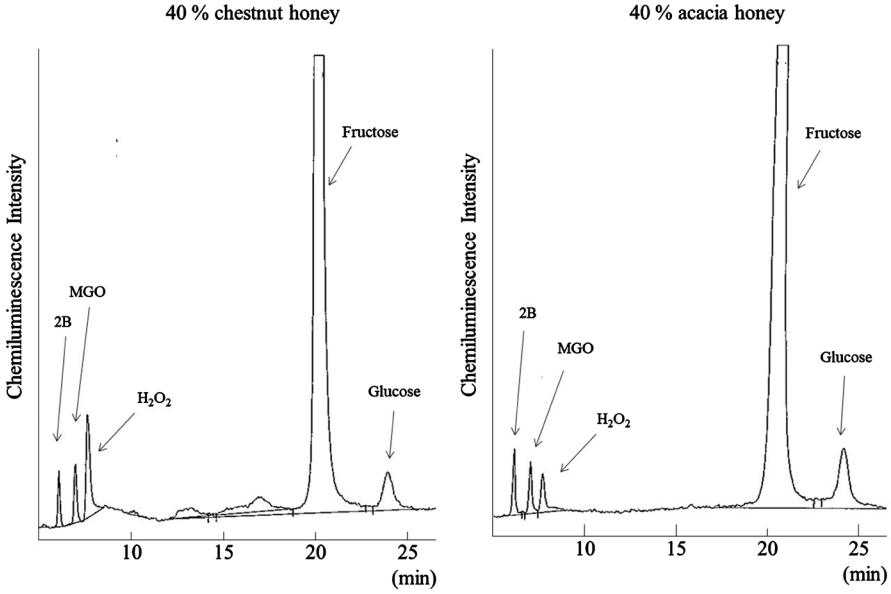

Figure 6. Chromatograms of chestnut and acacia honey by lucigenin-CL-HPLC.

Table 1. Concentrations of four components in Japanese honeys.

Honey	H_2O_2 (mol·L^{-1})	Fructose (mol·L^{-1})	Glucose (mol·L^{-1})	Methylglyoxal (mol·L^{-1})
horse chestnut	$5.8 * 10^{-5}$	2.4	2.2	$8.0 * 10^{-3}$
bluberry	$4.1 * 10^{-5}$	2.2	2.5	$4.0 * 10^{-3}$
amorpha fruticosa	$4.4 * 10^{-5}$	2.4	2.6	$1.6 * 10^{-3}$
clover	$4.7 * 10^{-5}$	2.4	2.1	$6.0 * 10^{-3}$
apple	$4.8 * 10^{-5}$	1.9	1.7	$4.0 * 10^{-3}$
mixed flower	$8.2 * 10^{-5}$	1.9	1.8	$8.0 * 10^{-3}$
cherry	$6.0 * 10^{-5}$	1.9	1.4	$5.0 * 10^{-3}$
acacia	$9.0 * 10^{-5}$	2.2	1.8	$1.4 * 10^{-3}$
chestnut	$1.8 * 10^{-5}$	2.1	1.4	$1.6 * 10^{-3}$

4. Conclusion

In this study, we analyzed bactericidal components present in nine types of Japanese honey. The component analysis of honey showed that the amount of H_2O_2 ranged from 4.1×10^{-5} to 1.8×10^{-4} mol/L, with the greatest amount found in chestnut honey. Glucose, fructose, and MGO were identified as the components in Japanese honey that generate active oxygen species such as O_2^-. Their respective concentrations were from 1.4 to 2.8, 1.9 to 2.4, and 4.0×10^{-3} to 1.6×10^{-2} mol/L. We also showed that higher H_2O_2 concentration generally translates to stronger antioxidative potential. The lucigenin-CL-HPLC assay allowed us to detect MGO in Japanese honey. Thus, we demonstrated the utility of lucigenin-CL-HPLC for simultaneous detection of the five honey components associated with antibacterial effect. Previously reported methods for the analysis of MGO required cumbersome and time-consuming processing. In contrast, the technique described in the present study permits the detection and identification of Glucose, Fructose, H_2O_2, DHA, and MGO within 30 min and without any special treatments; this technique is based on the detection of ROS production. Taken together, this study reports on the analysis of bactericidal components in Japanese honey. We hope that more detailed investigations in the future along similar lines would lead to safer use and quality control of honey, as well as its clinical applications.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Krasil'Nikov, N.A. (1952) [Antibiotic Properties of Honey-Dew]. *Mikrobiologiia*, **21**, 19-22.

[2] Ulker, N. (1967) [Antibacterial Action of Honey toward Different Types of Mycobacterium]. *Turk Tip Cemiyeti Mecmuasi*, **33**, 282-287.

[3] Haffejee, I.E. and Moosa, A. (1985) Honey in the Treatment of Infantile Gastroenteritis. *British Medical Journal* (*Clinical Research Ed*), **290**, 1866-1867. https://www.bmj.com/content/290/6485/1866

[4] Kwakman, P.H.S. and Zaat, S.A.J. (2012) Antibacterial Components of Honey. *IUBMB Life*, **64**, 48-55. https://doi.org/10.1002/iub.578

[5] Kwakman, P.H.S., te Velde, A.A., de Boer, L., Speijer, D., Vandenbroucke-Grauls, M.J.C and Zaat, S.A.J. (2010) How Honey Kills Bacteria. *Federation of American Societies for Experimental Biology journal*, **24**, 2576-2582. https://doi.org/10.1096/fj.09-150789

[6] Hawley, P., Hovan, A., McGahan, C.E. and Saunders, D. (2014) A Randomized Placebo-Controlled Trial of Manuka Honey for Radiation-Induced Oral Mucositis. *Supportive Care in Cancer*, **22**, 751-761. https://doi.org/10.1007/s00520-013-2031-0

[7] Adams, C.J., Boult, C.H., Deadman, B.J., Farr, J.M., Grainger, M.N.C., Manley-Harris, M. and Snow, M.J. (2008) Isolation by HPLC and Characterisation of the Bioactive Fraction of New Zealand Manuka (*Leptospermum scoparium*) honey. *Carbohydrate research*, **343**, 651–659. https://doi.org/10.1016/j.carres.2007.12.011

[8] McGovern, D.P.B., Abbas, S.Z., Vivian, G. and Dalton, H.R. (1999) Manuka Honey against *Helicobacter pylori*. *Journal of the Royal Society of Medicine*, **92**, 439. https://doi.org/10.1177/014107689909200832

[9] Jenkins, R., Burton, N. and Cooper, R. (2011) Manuka Honey Inhibits Cell Division in Methicillin-Resistant *Staphylococcus aureus*. *The Journal of antimicrobial chemotherapy*, **66**, 2536-2542. https://doi.org/10.1093/jac/dkr340

[10] Mavric, E., Wittmann, S., Barth, G. and Henle, T. (2008) Identification and Quantification of Methylglyoxal as the Dominant Antibacterial Constituent of Manuka (*Leptospermum scoparium*) Honeys from New Zealand. *Molecular Nutrition and Food Research*, **52**, 483-489. https://doi.org/10.1002/mnfr.200700282

[11] Kato, Y., Umeda, N., Maeda, A., Matsumoto, D., Kitamoto, N. and Kikuzaki, H. (2012) Identification of a Novel Glycoside, Leptosin, as a Chemical Marker of Manuka Honey. *Journal of Agricultural and Food Chemistry*, **60**, 3418-3423. https://doi.org/10.1021/jf300068w

[12] Adams, C.J., Manley-Harris, M. and Molan, P.C. (2009) The Origin of Methylglyoxal in New Zealand Manuka (*Leptospermum scoparium*) Honey. *Carbohydrate Research*, **344**, 1050-1053. https://doi.org/10.1016/j.carres.2009.03.020

[13] Atrott, J., Haberlau, S. and Henle, T. (2012) Studies on the Formation of Methylglyoxal from Dihydroxyacetone in Manuka (*Leptospermum scoparium*) Honey. *Carbohydrate Research*, **361**, 7-11. https://doi.org/10.1016/j.carres.2012.07.025

[14] Turk, Z., Nemet, I., Varga-Defteardarović, L. and Car, N. (2006) Elevated Level of Methylglyoxal during Diabetic Ketoacidosis and Its Recovery Phase. *Diabetes and Metabolism*, **32**, 176-180. https://doi.org/10.1016/S1262-3636(07)70266-5

[15] Oya, T., Hattori, N., Mizuno, Y., *et al.* (1999) Methylglyoxal Modification of Protein. Chemical and Immunochemical Characterization of Methylglyoxal-Arginine Adducts. *The Journal of Biological Chemistry*, **274**, 18492-18502. https://doi.org/10.1074/jbc.274.26.18492

[16] Nakayama, K., Nakayama, M., Iwabuchi, M., *et al.* (2008) Plasma α-Oxoaldehyde Levels in Diabetic and Nondiabetic Chronic Kidney Disease Patients. *American Journal of Nephrology*, **28**, 871-878. https://doi.org/10.1159/000139653

[17] Karasawa, K., Haraya, S., Okubo, S. and Arakawa, H. (2017) Novel Assay of Antibacterial Components in Manuka Honey Using Lucigenin-Chemiluminescence-HPLC. *Analytica Chimica Acta*, **954**, 151-158. https://doi.org/10.1016/j.aca.2016.12.004

[18] Ishii, R., Horie, M., Murayama, M. and Maitani, T. (2006) Analysis of Chloramphenicol in Honey and Royal Jelly by LC/MS/MS. *Journal of the Food Hygienic Society of Japan*, **47**, 58-65. https://doi.org/10.3358/shokueishi.47.58

[19] Kanematsu, H., Aoyama, M., Maruyama, T. and Niiya, I. (1982) Amino Acid Analysis of Honeys with Different Geographical and Floral Origin. *Journal of Japanese Society of Nutrition and Food Science*, **35**, 297-303. https://doi.org/10.4327/jsnfs1949.35.297

[20] Saito, S., Sakai, T., Nemoto, S. and Matsuda, R. (2011) Determination of Pindone in Animal Products, Fishery Products, and Honey by LC-MS/MS. *Food Hygiene and Safety Science*, **52**, 294-298. https://doi.org/10.3358/shokueishi.52.294

[21] Saito, T., Kishikawa, R., Sahashi, N., Takahara, H., Takahashi, H., Funaguma, T., Kondo, T. and Aida, Y. (2017) The Fiftieth Anniversary of the Palynological Society of Japan and Historical Review of the Field in Japan. *Japanese Journal of Palynology*, **62**, 41-74.

[22] Osatani, S., Aida, Y., Kawamura, U. and Sato, M. (1978) Quality and Analysis Method of Honey-5-Recent Honey Quality. *Report of National Food Research Institute*, **33**, 89-108.

[23] Nakajima, Y., Nakano, Y., Fuwano, S., Hayashi, N., *et al.* (2013) Effects of Three Types of Japanese Honey on Full-Thickness Wound in Mice. *Evidence-Based Complementary and Alternative Medicine*, **2013**, Article ID: 504537. https://doi.org/10.1155/2013/504537

[24] Arakawa, H., Kanemitsu, M., Tajima, N. and Maeda, M. (2002) Chemiluminescence Assay for Catechin Based on Generation of Hydrogen Peroxide in Basic Solution. *Analytica Chimica Acta*, **472**, 75-82. https://doi.org/10.1016/S0003-2670(02)00982-0

[25] Arakawa, H., Hosonuma, M., Nakano, E., Nagano, H., Tajima, N., Mitsuka, N. and Inamoto, T. (2015) Reactive Oxygen and Antioxidant Activity of Essential Oil and Aroma-Water Produced From Hydrodistillation of Tree. *Journal of Japanese Society of Aromatherapy*, **14**, 29-34.