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Antioxidant Properties of Royal Jelly Associated with Larval Age and Time of Harvest

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This study aimed to evaluate the antioxidant properties of royal jelly (RJ) collected from larvae of different ages that were transferred in artificial bee queen cells for 24, 48, and 72 h. RJ harvested from the 1 day old larvae 24 h after the graft displayed predominant antioxidant properties, including scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, inhibition of linoleic acid peroxidation, and reducing power. Regardless of the initial larval age, lower antioxidant activities were observed in the RJ harvested later than 24 h except for the activity of superoxide dismutase. In addition, higher contents of proteins and polyphenolic compounds were determined in the RJ harvested 24 h than that harvested 48 or 72 h after the graft. It implied that the polyphenolic compounds may be the major component for giving the antioxidant activities in RJ. In summary, the time of harvest and the initial larval age did affect the antioxidant potencies in RJ, and RJ collected 24 h after the larval transfer showed the most substantial antioxidant activities.

KEYWORDS: Royal jelly; antioxidant; DPPH radical; reducing power; superoxide dismutase

INTRODUCTION

Royal jelly (RJ), a milky-white secretion produced by the hypopharyngeal and mandibular glands of young worker bees (*Apis mellifera*), is an essential food for feeding all young larvae and the adult queen bee. It is also the only food for the larvae that is chosen to develop into the queen bee for her lifetime (*1*). The caste differentiation between worker bees and queen bees completely depends on the time and the amount of RJ intake during their larval development (*2*). From the fourth day on, the feeding of RJ to worker larvae immediately comes to an end, while queen larvae are continually supplied with excess RJ until cells are capped by nurse bees (*3*). The abundant RJ speeds up the development of queen larvae and sequentially provides a future queen bee with fully mature ovaries for reproduction and a longer life span (*4*).

RJ possesses high nutritional values due to the abundant amounts of proteins, free amino acids, lipids, vitamins, and sugars (*5*). Proteins are the major components amounting up to 50% of its dry weight (*6*). The bioactive components of RJ are the major royal jelly proteins (MRJPs), apisimin, and royalisin, which show immunoregulatory and antibacterial effects (*7*−*9*). The major fatty acid found in RJ is *trans*-10-hydroxy-2-decenoic acid (10-HDA), which has been demonstrated to exhibit antitumor, antibacterial, and immunomodulatory activities (*10*−*12*). Furthermore, several factors affect the production and composition of RJ, such as feeding (*13*), season (*14, 15*), time of harvest (*16*), and age of the larvae at grafting time (*13*).

Natural antioxidants have recently attracted attention from scientists and researchers because of its benefit for our health to prevent various chronic diseases caused by attack of free radicals (*17*). Antioxidant activities in RJ have been reported (*18*), and on the basis of its nutritional value, more and more commercial RJ products in various forms are available in global markets, while the consumers demand the good quality product. The effects of larval age and time of harvest on the antioxidant properties in RJ remain unknown. Accordingly, the aim of this study was to investigate in detail the antioxidant properties of RJ collected from larvae of different ages at different times of harvest.

MATERIALS AND METHODS

Preparation of RJ. RJ was prepared by Fu-Chang Beekeeping in Hualien, Taiwan. Larvae of different ages (1, 2, and 3 days) were transferred into queen cell cups on the frames, and each frame contained 30 queen cups. The frames were transferred into bee hives, and the RJ was collected 24, 48, or 72 h after transferring the larvae. Each RJ sample was collected from five hives, and each hive contained approximately 25000 honeybees. The collected RJ samples were kept at −20 °C until further analysis. The RJ samples were diluted in sterilized, distilled water (100 mg/mL, wt/vol) and ultrasonicated for at least 60 min with occasional shaking. Following sonication, the sample solution was centrifuged at 15000*g* for 10 min, and the supernatants were used as the sample solution for the following tests.

Determination the Contents of Proteins and Total Polyphenol of Water-Soluble Extract of RJ. The protein content of the water-soluble extract of RJ was determined by the method of Lowry et al.

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Table 1. Protein, Total Polyphenolic, and 10-HDA Contents of Water-Soluble Extract of RJ

	time of harvest (after the larval transfer)								
	protein contents (%)			total phenolic contents (μg/g)			10-HDA contents (%)		
initial larval age (days)	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
1	16.5 ± 0.2 a	10.1 ± 0.3 b	9.8 ± 0.2 b	219.2 ± 1.5 a	194.4 ± 3.3 b	131.7 ± 14.0 c	1.97 ± 0.07 b	2.05 ± 0.04 a	1.60 ± 0.04 c
2	14.5 ± 0.4 a	10.7 ± 0.3 b	7.7 ± 0.4 c	206.9 ± 3.1 a	163.6 ± 4.5 b	150.0 ± 15.6 b	2.80 ± 0.04 a	1.93 ± 0.03 c	2.04 ± 0.03 b
3	13.9 ± 0.6 a	9.6 ± 0.7 b	9.5 ± 0.5 b	215.4 ± 5.1 a	179.9 ± 7.6 b	168.0 ± 5.9 b	2.16 ± 0.03 a	1.75 ± 0.05 b	1.72 ± 0.03 b

(*19*) using bovine serium albumin as the standard. The total polyphenolic content of the water-soluble extract of RJ was measured by the Folin−Ciocalteu colorimetric method using gallic acid as a calibration standard (*20*). The procedure consisted of diluting an aliquot of the RJ sample solution (0.1 mL) in water (5 mL) followed by the addition of Folin−Ciocalteu reagent (0.5 mL). After mixing for 3 min, 1 mL of an aqueous Na_2CO_3 solution (35 g/L) was added and mixed thoroughly by vortex. After the mixture placed at room temperature for 1 h, the absorbance of the mixture was measured at 725 nm against a blank.

Determination of 10-HDA in RJ. The 10-HDA content of RJ was determined by high-performance liquid chromatography (HPLC) by a Hitachi L-7100 pumping system equipped with a diode array L-7455 detector, a ODS-II column (4.6 mm), and a Rheodyne injector. The RJ sample (2.5 g) was dissolved in 100 mL of methanol solution (50:50 v/v with ultrapure, deionized, and CO_2 free water) by sonication and adjusted at pH 2.5 with phosphoric acid. The sample solution was then diluted 10 times and filtered through a membrane (0.22 μm), and 20 μL was injected into the HPLC to quantify 10-HDA. The mobile phase was methanol solution (60:40 v/v with ultrapure and deionized water) adjusted with phosphoric acid to pH 2.5, filtered through a membrane (0.45 μm), and degassed for 5 min. The mobile phase flow rate was adjusted to 1.0 mL/min, and detection was performed at 225 nm.

Radical Scavenging Effect upon 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radicals. The DPPH assay was based on the method of Nagai et al. (*21*). The RJ sample solution (0.3 mL) was mixed with 0.3 mL of DPPH radical solution (1.0 mM) and 2.4 mL of ethanol (99%). The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was measured at 517 nm. The capability of the test material to scavenge DPPH radicals was calculated as (%) = [1 − (absorbance of the sample at 517 nm)/(absorbance of the control at 517 nm)] × 100.

Radical Scavenging Effect upon Hydroxyl Radicals. The scavenging of hydroxyl radicals by RJ was assayed according to the deoxyribose method (*21*). The RJ sample solution (0.075 mL) was mixed with 0.45 mL of sodium phosphate buffer (0.2 M, pH 7.0), 0.15 mL of 2-deoxyribose (10 mM), 0.15 mL of $FeSO_4$-EDTA (10 mM), 0.15 mL of H_2O_2 (10 mM), and 0.525 mL of H_2O. After incubation at 37 °C for 4 h, the reaction was terminated by adding 0.75 mL of thiobarbituric acid (TBA; 1%) in NaOH (50 mM). The solution was boiled for 10 min and then cooled in cold water. The absorbance of the solution was measured at 520 nm. Hydroxyl radical-scavenging ability was evaluated as the rate of inhibition of 2-deoxyribose oxidation by hydroxyl radicals. The relative inhibition of 2-deoxyribose oxidation was calculated as (%) = [1 − (absorbance of the sample at 520 nm)/(absorbance of the control at 520 nm)] × 100.

Radical Scavenging Effect upon Superoxide Radicals. The scavenging of superoxide anion radicals was estimated according to the method of Nagai and Inoue (*22*). The RJ sample solution (20 μL) was mixed with 0.48 mL of sodium carbonate buffer (0.05 M, pH 10.5), 0.2 mL of xanthine (3 mM), 0.02 mL of EDTA disodium salt (3 mM), 0.02 mL of bovine serium albumin (0.15%), and 0.02 mL of nitroblue tetrazolium (NBT, 0.75 mM). The mixture was preincubated at 25 °C for 10 min, and then, 6 mU of xanthine oxidase was added to initiate the reaction. After 20 min at room temperature, the reaction was stopped by adding 0.02 mL of CuCl (6 mM). The absorbance of the reaction mixture was measured at 560 nm, and the inhibition rate was calculated as (%) = [(absorbance of the control at 560 nm − absorbance of the sample at 560 nm)/absorbance of the control at 560 nm] × 100.

Inhibition Effect upon Linoleic Acid Oxidation. The antioxidant activity of RJ was determined by applying the linoleoic acid system (*23*). The RJ sample solution (0.0833 mL) was mixed with 0.2083 mL of sodium phosphate buffer (0.2 M, pH 7.0) and 0.2083 mL of linoleic acid in ethanol (2.5%, wt/vol). The peroxidation was initiated by adding 20.8 μL of 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH, 0.1 M) and carried out at 37 °C for 200 min in the dark. The degree of oxidation was measured according to the thiocyanate method (*23*), which involved the sequential addition of 4.7 mL of ethanol (75%), 0.1 mL of ammonium thiocyanate (30%), 0.1 mL of sample solution, and 0.1 mL of ferrous chloride (20 mM) in HCl (3.5%). After the mixture was stirred for 3 min, the peroxide was determined by reading the absorbance at 500 nm. The relative inhibition of linoleic acid peroxidation was calculated as (%) = [1 − (absorbance of the sample at 500 nm)/(absorbance of the control at 500 nm)] × 100.

Reducing Power. The reducing power of RJ was determined according to the method of Oyaizu (*24*). The RJ sample solution (2.5 mL) was mixed with an equal volume of sodium phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (1%). The mixture was incubated at 50 °C for 20 min. After this, an equal volume of thrichloroacetic acid (1%) was added to the mixture, which was then centrifuged at 1400*g* for 10 min. The upper layer was mixed with distilled water and ferric chloride (0.1%) at a ratio of 1:1:2, and the absorbance of the incident radiation by the solution was measured at a wavelength of 700 nm, as the absornace was proportional to the reducing power. Dibutyl hydroxytoluene (BHT) was used as a reference for comparison.

Superoxide Dismutase (SOD) Activity. The SOD activity of RJ was determined according to the method of Liu et al. (*25*). The RJ sample solution (0.1 mL) was mixed with 3 mL of reaction solution containing Tris-HCl (50 mM, pH 8.5), cytochrome *c* (24 μM), and xanthine oxidase (33 mU). After 20 min at room temperature, the absorbance of the mixture was determined at 550 nm. The SOD activity of RJ sample was calculated from the standard curve of purified bovine erythrocyte SOD. One unit of SOD activity was defined as the quantity of SOD required to cause a 20% inhibition of the cytochrome *c* reduction activity of the tested sample using the described conditions.

Statistical Analysis. All results were analyzed using the general linear model procedure available from Statistical Analysis System software package version 9.1 (Statistical Analysis System Institute, 2002). Duncan's multiple range test (*26*) was used to detect differences between means of the treatments. Each experiment was conducted in triplicate.

RESULTS AND DISCUSSION

Protein, Total Polyphenolic, and 10-HDA Contents of Water-Soluble Extract of RJ. Regardless of initial larval age, RJ collected 24 h after the larval transfer contained higher protein and total polyphenolic contents than the RJ collected 48 or 72 h after the transfer (**Table 1**). Moreover, RJ collected 24 h after the larval transfer of the 1 day old larvae showed the greatest protein and total polyphenolic contents. However, only the RJ samples collected 24 h after the larval transfer of 2 or 3 day old larvae had higher 10-HDA contents than the other RJ samples. Many kinds of polyphenolic compounds, including flavonoids and cinnamic acid derivatives, are presented in RJ (*27*). It is well-known that the antioxidant properties of phenolic acids or flavonoids could be predicted by investigating the availability of the phenolic hydrogens as the hydrogen donating radical scavengers according to their chemical structures (*28*).

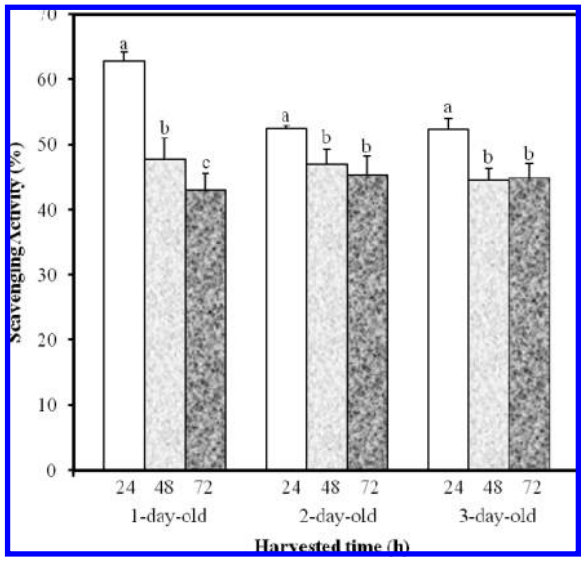

Figure 1. Effect of initial larval age and time of harvest upon the DPPH radical-scavenging activity of RJ. Vertical bars represent the standard deviation for each data point. Bars within the same initial larval age marked with the same letter are not significantly different ($n = 3$, $p < 0.05$).

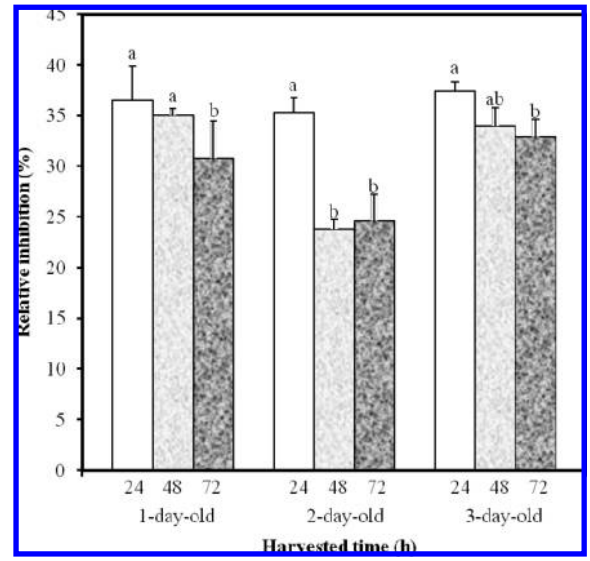

Figure 2. Effect of initial larval age and time of harvest upon the superoxide radical-scavenging activity of RJ. Vertical bars represent the standard deviation for each data point. Bars within the same initial larval age marked with the same letter are not significantly different ($n = 3$, $p < 0.05$).

Phenolic compounds of RJ could be originated from plants, where they are widely distributed, comprising at least 8000 different known structures (*29*). These compounds are reported to exhibit anticarcinogenic, anti-inflammatory, antiatherogenic, antithrombotic, immune modulating, and analgesic activities, among others, and exert these functions as antioxidants (*30*−*33*). In general, phenolic compounds can be divided into at least 10 types depending upon their basic structure: simple phenols, phenolic acids, coumarins and isocoumarins, naphthoquinones, xanthones, stilbenes, anthraquinones, flavonoids, and lignins. Flavonoids constitute the most important polyphenolic class, with more than 5000 compounds already described (*34*). Another bioactive compound of RJ is 10-HDA, a unique fatty acid found only in RJ but not in other natural products or other bee-related products. 10-HDA has been reported to exhibit antitumor, antibacteria, and immunomodulatory activities (*10*−*12*). Therefore, it is reasonable to suggest that RJ collected 24 h after the larval transfer, which contains more polyphenolic compounds and 10-HDA, may possess greater biological activity.

Scavenging Effect of RJ upon DPPH Radicals. Proton radical scavenging has been reported to be an important mechanism for antioxidation. The assay for assessment of proton radical-scavenging activity with DPPH is relatively simple and quite reproducible; this compound has been reported to be stoichiometrically decolorized by antioxidants. The reduction in the DPPH would be observed as its absorbance at a characteristic wavelength is decreased when it is treated with proton radical scavengers (*35*). **Figure 1** depicts the DPPH radical-scavenging activity of RJ collected from larvae of different ages and times of harvest. Each RJ sample displayed a DPPH radical-scavenging effect in the range from 43.0 to 62.8%. Regardless of initial larval age, RJ collected 24 h after the larval transfer demonstrated a significantly greater DPPH radical-scavenging activity than those collected 48 or 72 h after the transfer (**Figure 1**). Moreover, RJ collected 24 h after the larval transfer of the 1 day old larvae showed the greatest DPPH radical-scavenging activity among all of the RJ samples ($p < 0.05$).

Radical Scavenging Effect of RJ upon Superoxide Radicals. Superoxide radical, the one-electron reduced form of molecular oxygen, is a precursor to active free radicals that have the potential of reacting with biological macromolecules and thereby inducing tissue damage (*36*). According to **Figure 2**, each RJ sample inhibited the superoxide radical formation with an inhibition rate ranging from 37.4 to 23.9%. Furthermore, the inhibitory effect of the RJ collected 24 h after the larval transfer on superoxide radical formation was significantly higher than that of the RJ collected at 72 h ($p < 0.05$). These results reveal that RJ is a potent scavenger of superoxide radicals with a SOD-like ability.

Radical Scavenging Effect of RJ upon Hydroxyl Radicals. Hydroxyl radical is an extremely reactive species that can hydroxylate DNA, proteins, and lipids and cause lethal oxidative damage to the cells. Therefore, removal of hydroxyl radical is required to ensure cellular homeostasis. The Fenton reaction describes the oxidation of hydrogen peroxide by ferrous ion to hydroxyl radical and ferric ion. In the model employed in this experiment, the production of hydroxyl radical induced oxidation of the deoxyribose, which in turn reacted with TBA to produce a TBA reactive chromophore that was detectable at 520 nm, thus enabling the assessment of antioxidative activity of RJ. As shown in **Figure 3**, a reduction in the activity of hydroxyl radical removal was observed at each RJ sample from the larvae of different ages, as the time of harvest became longer. Consequently, the RJ samples collected at 24 h were the most effective for antioxidation in terms of scavenging hydroxyl radicals as compared to the samples collected 48 and 72 h after the larval transfer. On the other hand, the age of larvae did not have an effect on the hydroxyl radical-scavenging activity in the RJ.

Inhibition Effect of RJ on Linoleic Acid Oxidation. The process of lipid peroxidation is initiated by an attack on a fatty acid or fatty acyl side chain by any chemical species featuring sufficient reactivity to take a hydrogen atom away from a methylene carbon in the side chain. The resulting lipid radicals then undergo molecular rearrangement, followed by reacting with oxygen to produce peroxyl radicals, which are capable of abstracting hydrogen from adjacent fatty acid side chains and so propagating a chain reaction of lipid peroxidation (*37*). Lipid peroxides exert harmful effects on human health, and they are also associated with food deterioration. As shown in **Figure 4**,

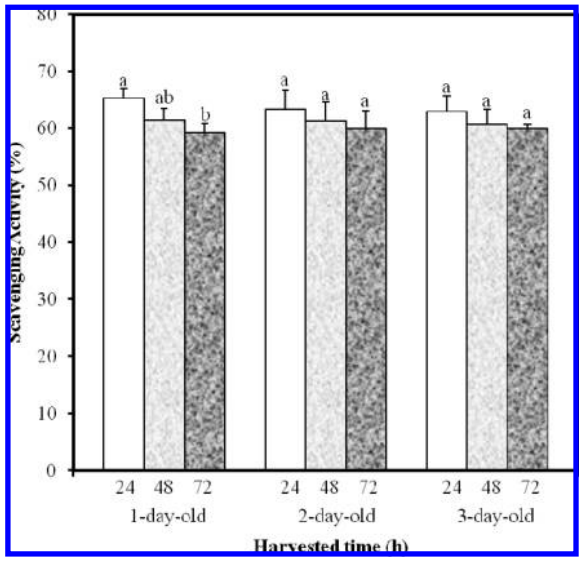

Figure 3. Effect of initial larval age and time of harvest upon the hydroxyl radical-scavenging activity of RJ. Vertical bars represent the standard deviation for each data point. Bars within the same initial larval age marked with the same letter are not significantly different ($n = 3$, $p < 0.05$).

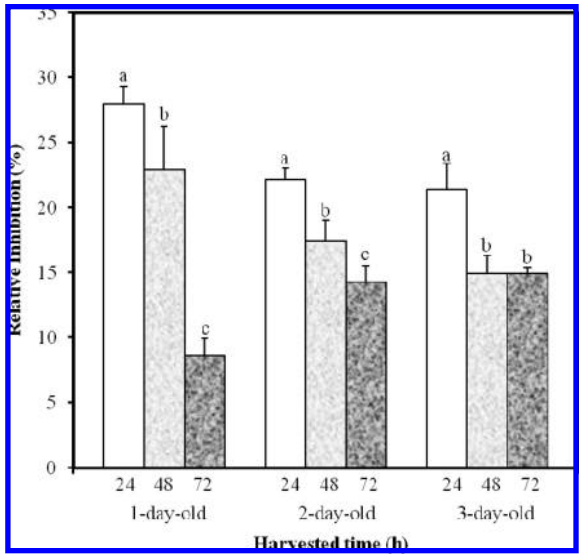

Figure 4. Effect of initial larval age and time of harvest on the inhibition of linoleic acid peroxidation by RJ. Vertical bars represent the standard deviation for each data point. Bars within the same initial larval age marked with the same letter are not significantly different ($n = 3$, $p < 0.05$).

each RJ sample demonstrated an inhibitory effect on linoleic acid peroxidation. Among the samples, RJ collected 24 h after the larval transfer of the 1 day old larvae demonstrated the highest inhibition rate at 27.9 ± 1.4%, whereas RJ collected 72 h after the larval transfer of the 1 day old larvae showed the lowest inhibition rate at 8.6 ± 1.3%. This result further confirms a greater antioxidative effect presented in the RJ collected 24 h than that collected 48 or 72 h after the larval transfer.

Reducing Power of RJ. The term "reducing power" indicates the ability of either enzymes (catalase, NADH oxidase, and NADH peroxidase) or nonenzymatic compounds (ascorbate, tocopherol, and glutathione) to reduce oxygen radicals or iron that then becomes unavailable for oxidative reations (*38*). Some studies have reported that the reducing power of a compound may serve as a significant indicator of its potential antioxidant activity (*39*). Thus, the reducing power of RJ collected from different larval age and time of harvest was investigated as well

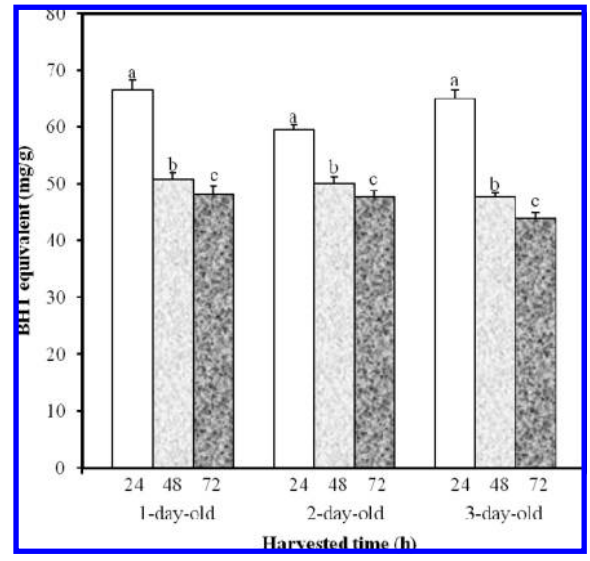

Figure 5. Effect of initial larval age and time of harvest on the reducing power of RJ. Vertical bars represent the standard deviation for each data point. Bars within the same initial larval age marked with the same letter are not significantly different ($n = 3$, $p < 0.05$).

Table 2. Specific Activities of SOD in RJ[a]

	time of harvest (after the larval transfer)		
initial larval age (days)	24 h	48 h	72 h
1	4.62 ± 0.58 b	5.60 ± 0.73 a	5.85 ± 0.48 a
2	3.95 ± 0.46 b	5.41 ± 0.23 a	5.62 ± 0.40 a
3	3.62 ± 0.55 c	6.10 ± 0.79 b	8.15 ± 0.22 a

[a] Means in a row with different superscripts are significantly different ($p < 0.05$).

to elucidate the relationship between their antioxidant effect and their reducing power. The reducing power of each RJ sample is shown in **Figure 5**. Among the samples, the RJ collected 24 h after the larval transfer of the 1 day old larvae demonstrated the highest reducing power equivalent to 66.5 ± 1.7 mg of BHT, and the RJ collected 72 h after the larval transfer of the 3 day old larvae had the lowest rate at reducing power equivalent to 43.9 ± 1.1 mg of BHT. Regardless of initial larval age, the RJ collected 24 h after the larval transfer always demonstrated a significantly greater reducing power than the RJ collected 48 or 72 h after the transfer.

SOD Activity of RJ. Highly reactive free radicals formed by exogenous chemicals or endogenous metabolic processes in the human body or in food processing are capable of oxidizing biomolecules, resulting in cell death and tissue damage. Almost all organisms are well-protected against free radical damage by antioxidative enzymes such as SOD, catalase, peroxidase, ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase, glutathione peroxidase, and glutathione reductase. Among a number of different antioxidative enzymes, catalase and SOD have been found in honey (*40*), although, to the best of our knowledge, no study has yet been published focusing on the area of antioxidative enzyme activity in RJ. The SOD activities of RJ are indicated in **Table 2**, in which it reveals that the RJ samples displayed a SOD activity ranging from 8.15 to 3.62 U/g. Among the samples, it was observed that the SOD activity of the RJ sample collected at 72 h after larval transferrring of the 3 day old larvae was significantly higher ($p < 0.05$) than the others. SOD is an important antioxidant enzyme that catalyzes the conversion of superoxides to hydrogen peroxide and oxygen. In this study, RJ collected 24 h after the larval transfer of the 1 day old larvae displayed

the highest inhibitory effect on superoxide radical formation but not the highest SOD activity. Therefore, it is suggested that the superoxide radical scavenging effect of RJ might be attributed to antioxidative compounds other than SOD.

In previous studies, high antioxidant activity has been found in RJ to protect against reactive oxygen species such as superoxide anion and hydroxyl radicals, and it has been suggested that its antioxidant activity may be attributed, in part, to the vitamins, polyphenolic compounds, or protein fractions (*18, 21, 22*). To the best of our knowledge, however, the effects of larval age and time of harvest on the antioxidant properties of RJ have not been reported before. In this study, we demonstrated that RJ collected 24 h after the larval transfer displayed a significantly greater scavenging activity upon DPPH radicals, an inhibition effect upon linoleic acid peroxidation, and more substantial reducing power, but a lower SOD activity than those from the RJ collected 48 or 72 h after the transfer. These findings have demonstrated that differences seem to exist in the antioxidant potencies of RJ harvested at different larval ages and times of harvest. Consequently, it is suggested that as the RJ harvested later than 24 h after the larval transfer, the total polyphenolic contents would decrease, leading to a reduction in the antioxidant activities.

ABBREVIATIONS USED

AAPH, 2,2′-azobis (2-amidinopropane) dihydrochloride; BHT, dibutyl hydroxytoluene; DPPH, 1,1-diphenyl-2-picrylhydrazyl; 10-HDA, *trans*-10-hydroxy-2-decenoic acid; HPLC, high-performance liquid chromatography; MRJP, major royal jelly protein; NBT, nitroblue tetrazolium; RJ, royal jelly; SOD, superoxide dismutase; TBA, thiobarbituric acid.

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