Glycative Stress Research

Online edition : ISSN 2188-3610
Print edition : ISSN 2188-3602
Received : February 6, 2015
Accepted : July 1, 2015
Published online : September 30, 2015

Original article

Anti-oxidative activity of royal jelly glycation products

Taku Kaneko

Department of Neurology, Oregon Health & Science University, Portland, Oregon, USA

Abstract

***Objective*:** To verify the anti-oxidative function of melanoidin-containing royal jelly (RJ) prepared by heating RJ which containing proteins and carbohydrates.

***Methods*:** A sample prepared by lyophilizing intact RJ was heated at a specified temperature (60-100°C) in a water bath for 1 hour to yield heated RJ (HRJ). To compare the anti-oxidative functions of RJ and HRJ, their 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical eliminating activity, superoxide anion radical eliminating activity [electron spin resonance (ESR)], activity to eliminate radicals from leukocytes stimulated with phorbol 12-myristate 13-acetate (PMA), and aldose reductase inhibiting activity were compared between the two groups.

***Results*:** The DPPH radical eliminating activity increased according to heat treatment time (from 0 to 1 hour) and HRJ concentration. The superoxide anion radical eliminating activity and leukocyte-derived radical eliminating activity increased according to HRJ concentration; it was markedly higher with HRJ than with RJ. The aldose reductase inhibiting activity did not differ between RJ and HRJ.

***Conclusion*:** HRJ, produced by heating RJ, and containing the glycation product melanoidin, may exhibit higher anti-oxidative action than conventional RJ.

***KEY WORDS*:** royal jelly, anti-oxidative action, melanoidin, glycation, aldose reductase

Introduction

Royal jelly (RJ) contains a wide variety of substances, including proteins, lipids, carbohydrates, minerals, and vitamins. Past studies have shown that RJ possesses a broad range of pharmacological effects, including antibacterial action, antitumor action, suppression of diabetes mellitus, sex hormone action, increase in blood flow, body weight gain, growth promotion, survival prolongation against radiation disorders, and suppression of arteriosclerosis [1-3]. RJ is widely utilized in nutritional supplements, cosmetics, quasi-drugs, pharmaceuticals, and other items. As a healthful food, RJ has long been utilized by many people; it is very safe for living organisms with a low incidence of side effects. Any side effect is likely to be due to additives [4].

When heated, RJ produces a glycation product of proteins and carbohydrates contained therein. In this study, we examined the anti-oxidative capacity of the glycation product of RJ.

Methods

Preparation of materials

In this study, intact RJ, and dry RJ prepared by lyophilizing intact RJ, were used. Since the components of RJ are likely to be denatured by light, heat, and oxygen, RJ was immediately frozen after collection and stored in a dry state under freezing conditions to maintain adequate storage stability.

Samples prepared by lyophilizing intact RJ were heated at a specified temperature (60-100°C) in a water bath for 1 hour to yield heated RJ (HRJ) [5].

HRJ, 1.0 g, was dissolved in 6 mL of 0.2 M NaOH, and 4 mL of 0.2 M phosphate buffer solution (pH 7.4) was added to yield a test solution, which was diluted with the same buffer solution to desired dilution rates. The resulting dilutions were assayed to evaluate the anti-oxidative capacity in the following experiments listed below.

Corresponding to: Taku Kaneko MD, PhD
Visiting Researcher, Department of Neurology,
Oregon Health & Science University, Portland, Oregon, USA.
TEL: +1-734-494-6695 FAX: +1-503-494-7499
eMail: kanekot@ohsu.edu

Parameters

DPPH radical eliminating activity

The absorbance of 1,1-diphenyl-2-picryl hydrazyl (DPPH), a radical species as potent as active oxygen in oxidizing capacity, is lost upon a reaction with a substance having anti-oxidative capacity [6]. In this experiment, the anti-oxidative power of each sample was determined by measuring the loss of the absorbance of DPPH using a spectrophotometer. RJ was heated at 100°C for 20, 40, 60, and 180 minutes, and the DPPH radical eliminating activity was determined using a spectrophotometer (wavelength: 517 nm).

An evaluation test was performed using a reaction system comprising 1.95 mL of 0.05 M Tris-HCI buffer solution (pH 7.4) supplemented with 1.0 mL of 0.1 mM DPPH and 0.05 mL of HRJ sample (5-fold dilution). The reaction mixture was centrifuged, and the resulting supernatant fraction was assayed 8 minutes later. The DPPH radical eliminating activity was calculated as a DPPH retention ratio (%) using the following equation:

DPPH retention ratio (%) = (A1/A2) x 100

A1: Absorbance after HRJ addition

A2: Absorbance before HRJ addition

Superoxide anion radical eliminating activity

This activity was determined by trapping superoxide anion radicals generated from the *in vivo* xanthine-xanthine oxidase system (a system for producing superoxide anion radicals) using the spin trapper 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), and detecting the resulting adduct using an electron spin resonance (ESR) system (JES-FR30, JEOL Ltd., Akishima-shi, Tokyo) [7,8]. The radical eliminating activity was evaluated on the basis of the ESR signal attenuation caused by the addition of HRJ.

An evaluation test was performed using a reaction system comprising 47 μL of 0.2 M phosphate buffer solution (pH 7.4) supplemented with 3.0 μL of 9.0 M DMPO, 5 mM hypoxanthine, HRJ (treated at 100°C for 1 hour), and 60 μL of 1.0 U/mL xanthine oxidase. The aforementioned buffer solution used to dissolve and dilute HRJ was used as the control.

Leukocyte-derived radical eliminating activity

Leukocytes produce radicals upon stimulation with phorbol 12-myristate 13-acetate (PMA). Such radicals were reacted with the sensitizer luminol, and the resulting chemiluminescence was measured over time using a luminescence detector (CLD-110, Tohoku Electronic Industrial Co., Ltd., Taihaku-ku, Sendai-shi, Miyagi, Japan) [9,10]. The radical eliminating activity was evaluated from the luminescence intensity attenuation with the addition of a sample having radical eliminating capacity to the system.

An evaluation test was performed using a reaction system comprising Hanks' buffer solution (pH 7.4) supplemented with leukocytes from rat blood ($1x10^8$ cells/mL), 0.56 mM luminol, an HRJ sample (treated at 100°C for 1 hour), and 100 mM PMA. The aforementioned buffer solution used to dissolve and dilute HRJ was used as a control in place of the sample.

Chemiluminescence suppression rate (%) = {1 – (C1/C2)} x 100

C1: Chemiluminescence level (photons) after HRJ addition

C2: Chemiluminescence level (photons) before HRJ addition

Aldose reductase inhibiting activity

Aldose reductase is an enzyme serving as an indicator of diabetes mellitus that increases in the disease. Abnormal increases in this enzyme are known to cause an abnormal accumulation of sorbitol, hence resulting in complications such as cataracts [11,12]. Therefore, inhibiting this enzyme may suppress diabetic complications.

The aldose reductase inhibiting activities of RJ and HRJ were determined as described below [13]. A reaction mixture containing 745 μL of 0.2 M phosphate buffer solution (pH 6.2), glyceraldehyde, 1.5 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), RJ or HRJ (treated at 100°C for 1 hour), and 5.0 μL of aldose reductase (final concentration: 5x10-3 units) was incubated at 25°C for 3 minutes, and the absorbance of NADPH (wavelength: 340 nm) was measured. The RJ and HRJ samples were used in the form of 5- and 10-fold dilutions, respectively. The aforementioned buffer solution used to dissolve and dilute HRJ was used as a control in place of each sample. The inhibiting activity rate was calculated using the following equation:

Chemiluminescence suppression rate (%) = {1 – (D1/D2)} x 100

D1: NADPH (340 nm) absorbance after sample addition

D2: NADPH (340 nm) absorbance before sample addition

Results

DPPH radical eliminating activity

Results for DPPH radical eliminating activity are shown in ***Table 1***. The DPPH radical suppression rate increased with increasing heat treatment time, but did not change with heating for 1 hour or longer. Therefore, a heating time of at least 60 minutes was required to prepare HRJ with higher anti-oxidative action by 100°C heat treatment.

Subsequently, samples were prepared at various dilution rates (1-160 fold) using HRJ that had been previously heated at 100°C for 1 hour, and DPPH radical suppression rates were determined in the same manner using non-heated RJ (1-, 2-, 4-, and 8-fold dilutions) as the control.

Results showed that the highest DPPH radical eliminating activity was observed at a dilution rate of 1, with the activity decreasing with increasing dilution rate (***Table 2***). Hence, the radical eliminating activity was found to depend on the concentration of HRJ. The radical eliminating activity of HRJ was significantly higher than that of RJ ($p < 0.05$).

Superoxide anion radical eliminating activity

When examining the ESR signal attenuation curve, HRJ was shown to eliminate superoxide anion radicals (***Fig. 1***). The elimination ratio increased according to the concentration of HRJ; it was remarkably higher with HRJ than with non-heated RJ (***Table 3***).

Table 1. Relationship between the heating time and DPPH radical suppression rate with HRJ

Heat treatment time (100°C)	(Minutes)	0	20	40	60	180
DPPH radical suppression rate	(%)	40.0	65.1	70.5	85.6	86.5

HRJ, heated royal jelly; DPPH, 1,1-diphenyl-2-picryl hydrazyl.

Table 2. Relationship between the sample dilution rate and DPPH radical suppression rate with HRJ or RJ

HRJ dilution rate		1	5	10	20	40	80	160
DPPH radical suppression rate	(%)	87.9	85.6	61.9	40.0	24.8	16.8	9.8
RJ dilution rate		1	2	4	8			
DPPH radical suppression rate	(%)	75	66	45	21			

HRJ, heated royal jelly; RJ, royal jelly; DPPH, 1,1-diphenyl-2-picryl hydrazyl. Results are expressed as mean values (n = 3). $p < 0.05$, significantly different in slopes between HRJ and RJ by Mann–Whitney U test. Standard error means are not presented due to a missing data problem of the computer.

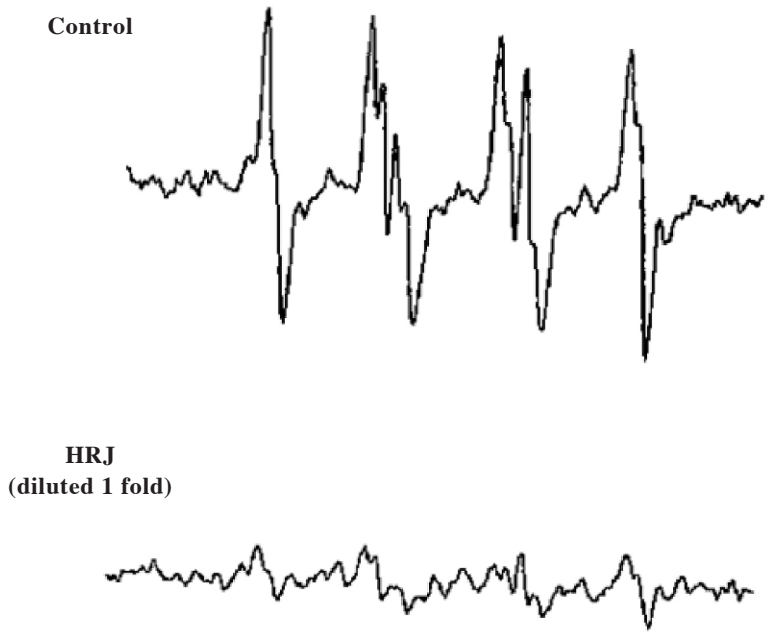

Fig 1. **ESR signal attenuation curves: superoxide anion radical eliminating action of HRJ**
Measured using the JES-FR30 analyzer (JEOL Ltd.). ESR, electron spin resonance; HRJ, heated royal jelly.

Table 3. Superoxide anion radical suppression by HRJ and RJ

Dilution rate			1	2	4	8	20
Superoxide anion radical suppression rate	HRJ	(%)	74	60	52	43	19
	RJ	(%)	44	22	17	–	–

HRJ, heated royal jelly; RJ, royal jelly; –, no inhibitory effect.

Leukocyte-derived radical eliminating activity

Measurements of chemiluminescence from leukocyte-derived radicals in 1-fold dilution of HRJ and control are shown in ***Fig. 2***. The radical-induced increase in the luminescence intensity in the leukocytes was suppressed by HRJ. Hence, HRJ was shown to also suppress radicals produced in cells.

Subsequently, the suppression rate with HRJ was compared with that with RJ at various dilution rates (1, 10, and 50 fold). The suppression rate for leukocyte-derived radicals was found to have concentration dependency like the radical-inhibitory activity described above, and the suppression rate was higher with HRJ than with RJ (***Table 4***).

Aldose reductase inhibiting activity

HRJ and RJ both exhibited high aldose reductase inhibiting activity (***Table 5***). The bioactivity of conventional RJ was not affected by heat treatment.

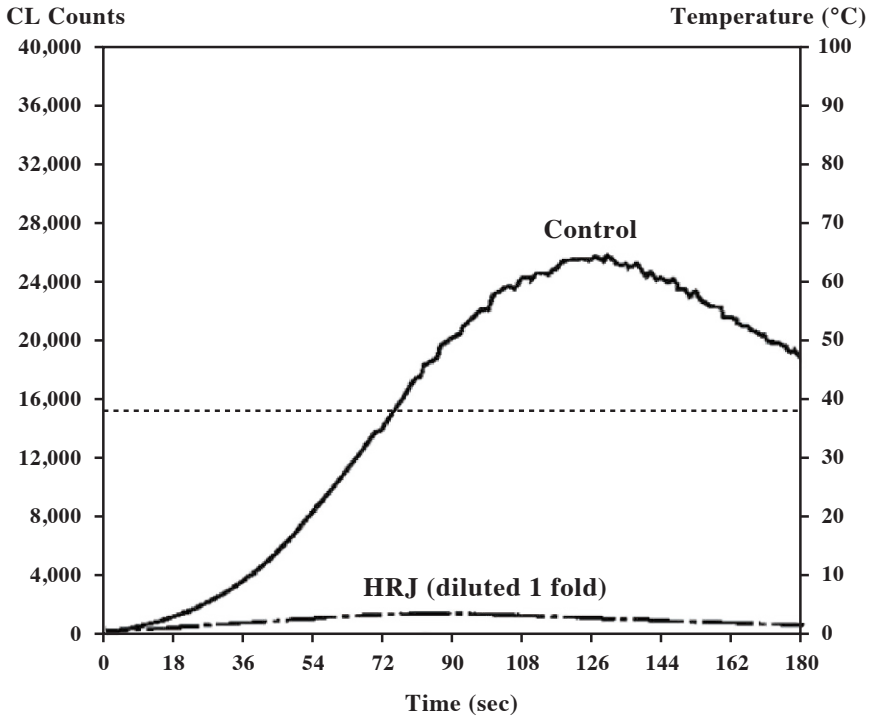

Fig 2. **Suppression of chemiluminescence from leukocyte-derived radicals by HRJ**

The amount of radicals produced upon stimulation of rat leukocytes with phorbol 12-myristate 13-acetate (PMA) was measured via a luminescence detector with the use of the sensitizer luminol. HRJ, heated royal jelly.

Table 4. Suppression of chemiluminescence from leukocyte-derived radicals by HRJ and RJ

Dilution rate		1	10	50
Chemiluminescence suppression rate	HRJ (%)	94.3	74.9	42.3
	RJ (%)	83.4	55.5	31.0

HRJ, heated royal jelly; RJ, royal jelly.

Table 5. Aldose reductase inhibiting activities of HRJ and RJ

Dilution rate		5	10
Aldose reductase suppression rate	HRJ (%)	84.7	74.9
	RJ (%)	81.5	77.5

HRJ, heated royal jelly; RJ, royal jelly.

Discussion

In this study, HRJ, prepared by heating RJ, was shown to exhibit higher anti-oxidative action than RJ in terms of DPPH radical eliminating activity, superoxide anion radical suppressing activity, and leukocyte-derived radical eliminating activity. Because the heat treatment did not alter the aldose reductase activity, HRJ was confirmed to possess increased anti-oxidative function while avoiding the loss of the bioactivity of conventional RJ.

Usually, heating alters food component proteins, lipids, and carbohydrates, and causes them to interact with each other, resulting in a wide variety of changes. Caramelization is a phenomenon in which saccharides change their colors to brown without reacting with amino compounds when heated to 100°C or higher [14]. Caramelization and glycation are strictly differentiated. Because food composition is diverse, in reality, coexisting components work catalytically during heating, resulting in the concurrent occurrence of caramelization and glycation. Although general foods rarely undergo caramelization only, the contribution of caramelization is high in cacao and coffee bean roasting, meat grilling, bakery, and other processes.

When heated alone to about 60-150°C, reducing sugars (glucose and fructose) undergo anomerization and isomerization, concurrently producing disaccharides with 1,6-glucoside bonds, such as isomaltose and gentiobiose, 1-6-anhydrosugars, oligosaccharides, and other substances [15-19]. Furthermore, a dehydration condensation reaction produces 3-deoxyglucosone (3DG), 5-hydroxymethylfurfural, and 2-hydroxyacetylfuran. Non-volatile acids such as succinic acid, tartaric acid, pyruvic acid, levulinic acid, and furanecarboxylic acid are also produced concurrently. Thus, the products produced due to heating are diverse.

A brown substance produced upon glycation of food is known as melanoidin. Although the chemical structure of melanoidin remains unidentified, it is thought to be an assembly of many coloring substances [20]. Melanoidin is a brown final product produced via pyrrole aldehyde and other substances following the conversion of D-glucosides from saccharides to amadori compounds via enaminol, and condensation-polymerization with amino compounds involving osones and furfurals as intermediates.

Food melanoidins have been suggested to have favorable effects on the body. Those produced from amino acids such as glycine, serine, isoleucine, and glutamic acid, in particular, are active in hydroxy radical elimination and exhibit anti-oxidative action [20-22]. With regard to coffee, advanced research has been conducted on the glycation product melanoidin, resulting in a number of reports on its functionality with favorable effects on human health, including anti-oxidative action [23,24]. When roasted, coffee beans have increased melanoidin contents, and exhibit enhanced anti-oxidative action [25,26].

RJ is composed of 60-70% water, 9-18% amino acids (including glycine, serine, isoleucine, and glutamic acid) and proteins (royalisin [27], royalactin [28], apisin [29]), 11-23% carbohydrates (glucose, fructose, sucrose, oligosacchrides), and 3-8% fatty acids (decenoic acid [30]), and it also contains small amounts of water-soluble vitamins and minerals [31-33]. RJ has a wide variety of effects, including anti-oxidative action [34-38].

The above-described facts suggest that the amino acids and reducing sugars contained in RJ underwent glycation due to heating to produce melanoidin, and that the anti-oxidative action of HRJ might be enhanced as an add-on effect of the anti-oxidative action of melanoidin on the anti-oxidative action of the starting material RJ.

Conclusion

HRJ, produced upon heat treatment of RJ, contains the glycation product melanoidin, and was shown to exhibit higher anti-oxidative action than conventional RJ, suggesting a potential for use as a new anti-oxidative food.

State of Conflict of Interest

Non contributory.

Acknowledgments

The author thanks Dr. Yoko Aniya, Professor-Emeritus of the University of the Ryukyu, for guidance in conducting this study.

References

1) Viuda-Martos M, Ruiz-Navajas Y, Fernández-López J, et al. Functional properties of honey, propolis, and royal jelly. J Food Sci. 2008; 73: R117-124.
2) Oršolić N. Royal jelly: Component efficiency, analysis, and standardisation. Arh Hig Rada Toksikol. 2013; 64: 445-461. (in Croatian)
3) Buttstedt A, Moritz RF, Erler S. Origin and function of the major royal jelly proteins of the honeybee (*Apis mellifera*) as members of the yellow gene family. Biol Rev Camb Philos Soc. 2014; 89: 255-269.
4) Yonei Y, Shibagaki K, Tsukada N, et al. Case report: Haemorrhagic colitis associated with royal jelly intake. J Gastroenterol Hepatol. 1997; 12: 495-499.
5) Kaneko T, Aniya Y. Heated royal jelly: Its manufacturing method and derivatives. Patent #: 2001-238615 (P2001-238615A). 2000 March 1. (in Japanese)
6) Kedare SB, Singh RP. Genesis and development of DPPH method of anti-oxidative assay. J Food Sci Technol. 2011; 48: 412-422.

7) Nishizawa C, Takeshita K, Ueda J, et al. Hydroxyl radical generation caused by the reaction of singlet oxygen with a spin trap, DMPO, increases significantly in the presence of biological reductants. Free Radic Res. 2004; 38: 385-392.
8) Takanami Y, Nakayama T. Evaluation of superoxide anion radicals generated from an aqueous extract of particulate phase cigarette smoke by electron spin resonance using 5, 5-dimethyl-1-pyrroline-N-oxide. Biosci Biotechnol Biochem. 2011; 75: 34-39.
9) Kitagawa RR, Raddi MS, Khalil NM, et al. Effect of the isocoumarin paepalantine on the luminol and lucigenin amplified chemiluminescence of rat neutrophils. Biol Pharm Bull. 2003; 26: 905-908.
10) Hatanaka E, Levada-Pires AC, Pithon-Curi TC, et al. Systematic study on ROS production induced by oleic, linoleic, and gamma-linolenic acids in human and rat neutrophils. Free Radic Biol Med. 2006; 41: 1124-1132.
11) Oka M, Kato N. Aldose reductase inhibitors. J Enzyme Inhib. 2001; 16: 465-473.
12) Kawanishi K, Ueda H, Moriyasu M. Aldose reductase inhibitors from the nature. Curr Med Chem. 2003; 10: 1353-1374.
13) Del Corso A, Costantino L, Rastelli G, et al. Aldose reductase does catalyse the reduction of glyceraldehyde through a stoichiometric oxidation of NADPH. Exp Eye Res. 2000; 71: 515-521.
14) Gato N, Oka K. Impact of 5-HMF as a fragrant micronutrient. Aroma Research. 2009; 10: 340-346. (in Japanese)
15) Pellegrino L, De Noni I, Cattaneo S. Formation of protein bound lysine-derived galactosyl and glucosyl pyrroles in heated model systems. Nahrung. 2000; 44: 193-200.
16) Goldberg T, Cai W, Peppa M, et al. Advanced glycoxidation end products in commonly consumed foods. J Am Diet Assoc. 2004; 104: 1287-1291.
17) Arribas-Lorenzo G, Morales FJ. Analysis, distribution, and dietary exposure of glyoxal and methylglyoxal in cookies and their relationship with other heat-induced contaminants. J Agric Food Chem. 2010; 58: 2966-2972.
18) Degen J, Hellwig M, Henle T. 1,2-dicarbonyl compounds in commonly consumed foods. J Agric Food Chem. 2012; 60: 7071-7079.
19) Frolov A, Schmidt R, Spiller S, et al. Arginine-derived advanced glycation end products generated in peptide-glucose mixtures during boiling. J Agric Food Chem. 2014; 62: 3626-3635.
20) Kato H, Ishikawa H, Kashio H, et al. Antioxidative activity of the oxidation and the reduction products prepared from melanoidins. Journal of Japan Society for Bioscience, Biotechnology, and Agrochemistry. 1975; 49: 179-183. (in Japanese)
21) Yamaguchi N, Fujimaki M. Studies on browning reaction products from reducing sugars and amino acids: Part XIV. Antioxidative activities of purified melanoidins and their comparison with those of legal anti-oxidatives. Nippon Shokuhin Kogyo Gakkaishi. 1974; 21: 6-12. (in Japanese)
22) Usui T, Shizukuuchi S, Watanabe H, et al. Protective effect of melanoidins against oxidative stress. Bulletin of Showagakuin Junior College. 2007; 43: 43-48.
23) Argirova MD, Stefanova ID, Krustev AD. New biological properties of coffee melanoidins. Food Funct. 2013; 4: 1204-1208.
24) Coelho C, Ribeiro M, Cruz AC, et al. Nature of phenolic compounds in coffee melanoidins. J Agric Food Chem. 2014; 62: 7843-7853.
25) Del Pino-García R, González-SanJosé ML, Rivero-Pérez MD, et al. Influence of the degree of roasting on the anti-oxidative capacity and genoprotective effect of instant coffee: contribution of the melanoidin fraction. J Agric Food Chem. 2012; 60: 10530-10539.
26) Smrke S, Opitz SE, Vovk I, et al. How does roasting affect the anti-oxidatives of a coffee brew? Exploring the anti-oxidative capacity of coffee via on-line anti-oxidative assays coupled with size exclusion chromatography. Food Funct. 2013; 4: 108210-108292.
27) Fujiwara S, Imai J, Fujiwara M, et al. A potent antibacterial protein in royal jelly. Purification and determination of the primary structure of royalisin. J Biol Chem. 1990; 265: 11333-11337.
28) Kamakura M. Royalactin induces queen differentiation in honeybees. Nature. 2011; 473: 478-483.
29) Yamaguchi K, He S, Li Z, et al. Quantification of major royal jelly protein 1 in fresh royal jelly by indirect enzyme-linked immunosorbent assay. Biosci Biotechnol Biochem. 2013; 77: 1310-1312.
30) Kodai T, Umebayashi K, Nakatani T, et al. Compositions of royal jelly II. Organic acid glycosides and sterols of the royal jelly of honeybees (*Apis mellifera*). Chem Pharm Bull (Tokyo). 2007; 55: 1528-1531.
31) Serra BJ. Sugars, acidity and pH of royal jelly. Anal. Bromatol.1992; 44: 65-69.
32) Sabatini AG, Marcazzan G, Caboni MF, et al. Quality and standardisation of royal jelly. JAAS 2009; 1: 1-6.
33) Bogdanov S. Royal jelly, bee brood: Composition, health, medicine: A review. 2012; http://www.bee-hexagon.net/files/file/fileE/Health/RJBookReview.pdf, accessed 08 August 2014.
34) Kuwabara Y, Hori Y, Yoneta T, et al. Anti-oxidatice effect of royal jelly. Japanese Pharmacology & Therapeutics. 1996; 24: 1463-1467. (in Japanese)
35) Nagai T, Inoue R, Suzuki N, et al. Antioxidant properties of enzymatic hydrolysates from royal jelly. J Med Food. 2006; 9: 363-367.
36) Jamnik P, Goranovic D, Raspor P. Antioxidative action of royal jelly in the yeast cell. Exp Gerontol. 2007; 42: 594-600.
37) Guo H, Ekusa A, Iwai K, et al. Royal jelly peptides inhibit lipid peroxidation *in vitro* and *in vivo*. J Nutr Sci Vitaminol (Tokyo). 2008; 54: 191-195.
38) Ferreira D, Rocha HC, Kreutz LC, et al. Bee products prevent agrichemical-induced oxidative damage in fish. PLoS One. 2013 Oct 3;8(10):e74499. doi: 10.1371/journal.pone.0074499. eCollection 2013.