Research Article

The Lyophilization Process Maintains the Chemical and Biological Characteristics of Royal Jelly

Andresa Piacezzi Nascimento,1,2 Larissa Ariana Roveroni Moraes,2 Nathália Ursoli Ferreira,1 Gabriela de Padua Moreno,1 Fernanda Grassi Mangolini Uahib,1 Edna Aparecida Barizon,3 and Andresa Aparecida Berretta2,3

1Laboratório de Microbiologia, Apis Flora Industrial e Comercial Ltda., 14020-670 Ribeirão Preto, SP, Brazil
2Laboratório de Pesquisa, Desenvolvimento e Inovação, Apis Flora Industrial e Comercial Ltda., 14020-670 Ribeirão Preto, SP, Brazil
3Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, 14040-903 Ribeirão Preto, SP, Brazil

Correspondence should be addressed to Andresa Piacezzi Nascimento; piacezzi@gmail.com

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The alternative use of natural products, like royal jelly (RJ), may be an important tool for the treatment of infections caused by antibiotic-resistant bacteria. RJ presents a large number of bioactive substances, including antimicrobial compounds. In this study, we carried out the chemical characterization of fresh and lyophilized RJ and investigated their antibacterial effects with the purpose of evaluating if the lyophilization process maintains the chemical and antibacterial properties of RJ. Furthermore, we evaluated the antibacterial efficacy of the main fatty acid found in RJ, the 10-hydroxy-2-decanoic acid (10H2DA). Chromatographic profile of the RJ samples showed similar fingerprints and the presence of 10H2DA in both samples. Furthermore, fresh and lyophilized RJ were effective against all bacteria evaluated; that is, the lyophilization process maintains the antibacterial activity of RJ and the chemical field of 10H2DA. The fatty acid 10H2DA exhibited a good antibacterial activity against Streptococcus pneumoniae. Therefore, it may be used as an alternative and complementary treatment for infections caused by antibiotic-resistant S. pneumoniae.

1. Introduction

Frequent occurrence of infections caused by bacteria resistant to antibacterial agents is a common problem in hospitals. Resistant strains of bacteria are not inhibited or killed by the antibacterial agents at concentrations of the drugs achievable in the body after normal dosage. The resistance may increase the severity of disease and drive up health care costs. Therefore, the alternative use of natural products, like bee products, may be an important tool for the treatment of these infections.

Royal jelly (RJ) is a bee product widely used in traditional Oriental medicine. It is secreted from the mandibular and hypopharyngeal glands of worker honeybees (Apis mellifera) [1] and used to feed young larvae (from one to three days old), the queen bee larva, and the adult queen bee. RJ is involved in the sexual determination of the queen, besides its longevity.

In its composition, RJ contains proteins (approximately 50% of its dry mass), free amino acids, vitamins, sugars, fatty acids, sterols, and minerals [2–4]. Therefore, it is used worldwide as a functional food.

RJ also presents several pharmacological properties, such as antibacterial [3, 5, 6], antifungal [3, 7], antihypertensive [8], and estrogenic [9, 10] activities. Furthermore, RJ can play a significant role against colitis [11], induces mineralization in osteoblasts [10], improves erythropoiesis, glucose tolerance, and mental health [12], and may have antiatherogenic activity [13] and be beneficial in controlling diabetes outcomes [14]. Due to its pharmacological properties, RJ is used to supplement the treatment of several diseases, in many countries.

A large number of bioactive substances are present in RJ, such as antimicrobial peptides (royalisin and jelleins) [15–19], peptides with antihypertensive activity [8], and sterols and
fatty acids with estrogenic effects [9], like the 10-hydroxy-2-decenoic acid (10H2DA) [10].

In this study, the abbreviation 10H2DA was used in order to differentiate 10H2DA from the other fatty acid present in RJ, the 10-hydroxydecanoic acid (10HDA), which is the saturated counterpart of 10H2DA.

Fresh RJ contains approximately 66% of water. Therefore, it is perishable and must be kept refrigerated to retain its nutritional value. Besides fresh RJ, the lyophilized one also is commercially available. Lyophilization process removes the water from RJ and is carried out by means of sublimation of the water (transition directly from the solid to the gaseous state). The advantage of the lyophilized RJ is that it can be stored at room temperature. Furthermore, it is usually sold in capsules in order to facilitate its use.

In the present study, we carried out the chemical characterization of fresh and lyophilized RJ and investigated their antibacterial effects, with the purpose of evaluating if the lyophilization process maintains the chemical and antibacterial properties of RJ. Furthermore, we evaluated the antibacterial efficacy of the 10H2DA.

2. Materials and Methods

2.1. Chemicals. Fresh RJ was purchased from Apis Nativá Produtos Naturais (Araranguá, SC, Brazil). Lyophilized RJ was obtained after lyophilization process of the fresh one, using a lyophilizer (Terroni, São Carlos, SP, Brazil). 10H2DA was purchased from Chromadex (Irvine, California, USA). Methanol HPLC grade was obtained from J.T. Baker. Water was treated in Milli-Q water purification system. The following culture media were used: Mueller Hinton agar and broth, which were purchased from Difco (Detroit, MI, USA); Mueller Hinton agar with 5% sheep blood (Plast Labor, Rio de Janeiro, RJ, Brazil); and Mueller Hinton broth supplemented with 5% lysed horse blood (Ebefarma Biológica e Agropecuária, Cachoeiras de Macacu, RJ, Brazil).

2.2. Chemical Characterization of RJ. Fresh and lyophilized RJ were analyzed by high-performance liquid chromatography (HPLC), using a Shimadzu apparatus equipped with a CBM-20A controller, a LC-20AT quaternary pump, a SPD-M 20A diode-array detector, and Shimadzu LC solution software, version 1.21 SP1. A Shimadzu Shim-Pack CLC-ODS (M) column (4.6 × 250 mm, particle diameter of 5 µm, pore diameter of 100 Å) was used. The mobile phase consisted of methanol in pump B and of a solution of water-phosphoric acid (0.02% v/v), pH 2.5, in pump D. The mixture was eluted using an isocratic elution with 50% B and 50% D over a period of 22 min at a flow-rate of 0.8 mL/min. Detection was set at 215 nm.

RJ was dissolved with 5 mL of methanol (HPLC grade) in 10 mL volumetric flasks, subjected to sonication for 10 min and diluted to volume with Milli-Q water. The samples were filtered through a 45 µm filter before analysis.

2.3. Antibacterial Activity. The following bacteria were used: Staphylococcus aureus ATCC 25923, Staphylococcus aureus ATCC 43300, Staphylococcus epidermidis ATCC 14990, Streptococcus pneumoniae ATCC 49619, Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 10031, Proteus mirabilis ATCC 12453, Salmonella enteritidis ATCC 13076, and Pseudomonas aeruginosa ATCC 27853.

The broth microdilution method [20] was used to test the antibacterial activity of the samples. Mueller Hinton broth was used in the test with most of the bacteria. For S. pneumoniae Mueller Hinton broth supplemented with 5% lysed horse blood was used. The final RJ concentrations in relation to the dry weight ranged from 0.02 to 6.19% w/v. The final 10H2DA concentrations ranged from 7.81 to 250 µg/mL.

The experiments were replicated three times for each bacterium.

2.4. Statistical Analysis. The data of the chemical characterization and antibacterial activity of the samples were submitted to two-way ANOVA. The data of the comparison of the bacteria were submitted to the one-way ANOVA and Bonferroni’s Multiple Comparison Test. The established significance level was 5%. Statistical analysis of data was performed using the software Graph Pad Prism 5.

3. Results

3.1. Chemical Characterization of RJ. The moisture contents of the lyophilized and fresh RJ were 0.96 and 69.21%, respectively. Chromatographic profile of the RJ samples showed similar fingerprints and the presence of 10H2DA in both samples (Figure 1). Furthermore, there was no significant difference between them (P > 0.05) (Figure 2).

3.2. Antibacterial Activity. Fresh and lyophilized RJ showed in vitro antibacterial activity against all bacteria evaluated (Table 1). There was no significant difference between them (P > 0.05). Results of MIC of the RJ samples were similar for most of the microorganisms, except P. aeruginosa and S. pneumoniae. The last one was the most susceptible microorganism to the samples (P < 0.05).
The fatty acid 10H2DA was not efficacious against most of the bacteria tested (Table 2). However, it exhibited antibacterial activity against S. pneumoniae.

4. Discussion

10H2DA is the major component of the lipid fraction of RJ; however, its content varies according to geographical origin of the sample [21]. Furthermore, 10H2DA is a unique RJ component [3] and is characterized like a biomarker of this bee product. Therefore, its detection and quantification may be considered as an identity and quality indicator of the RJ. In this study, chromatographic profile of the lyophilized and fresh RJ showed similar fingerprint and the presence of 10H2DA in both samples, demonstrating that the lyophilization process does not degrade the fatty acid.

The concentration of 10H2DA in fresh RJ is variable around the world and values of 0.33–2.54% were found by Genc and Aslan [22], 1.26–2.25% by Zhou et al. [23], and 1.58–3.99% by Garcia-Amoedo and Almeida-Muradian [24]. Sabatini et al. [25] suggest that 10H2DA content should be around the world and values of 0.33–2.54% were found by Genc and Aslan [22], 1.26–2.25% by Zhou et al. [23], and 1.58–3.99% by Garcia-Amoedo and Almeida-Muradian [24]. Sabatini et al. [25] suggest that 10H2DA content should be at least 1.4% for fresh royal jelly to attend quality control parameters. However, the data presented in this study showed that despite the low quantities of 10H2DA in the sample evaluated, the antibacterial activity was maintained.

Both samples of RJ were effective against all bacteria tested. It is important to mention that the samples were not submitted to any extraction process; that is, integral RJ samples were used (fresh or lyophilized raw material). Furthermore, our findings show that the lyophilization process maintains the antibacterial activity of RJ. In an in vivo study, Kayashima et al. [1] also demonstrated that lyophilized RJ maintains its developmental and physiological bioactivity in the fruit fly Drosophila melanogaster (model animal to examine the effects of RJ in multicellular organisms).

Gram-positive (staphylococci and S. pneumoniae) and Gram-negative bacteria (E. coli, K. pneumoniae, P. mirabilis, S. enteritidis, and P. aeruginosa) were killed by both samples. Two strains of S. aureus (ATCC 25923 and ATCC 43300) were evaluated in this study and both were killed by the RJ samples, including the S. aureus ATCC 43300, which is a methicillin-resistant S. aureus (MRSA), that is, a multidrug-resistant strain.

P. aeruginosa, the most frequent isolate from the burn wound [26], also was studied. The tested strain (ATCC 27853) also was evaluated by Boukraa [5], which demonstrated the efficacy of RJ from Algeria against this bacterium.

Besides P. aeruginosa, other bacteria usually isolated from the burn wound were evaluated in this study: K. pneumoniae, E. coli, and staphylococci [26]. Since RJ was effective against this bacterium, it may be considered as an identity and quality indicator of the RJ.
In conclusion, fresh and lyophilized RJ maintained their 10H2DA contents and were effective against all bacteria evaluated; that is, the lyophilization process maintains the chemical and antibacterial properties of RJ. The fatty acid 10H2DA exhibited a good antibacterial activity against S. pneumoniae. Therefore, it may be used as an alternative and complementary treatment for infections caused by antibiotic-resistant S. pneumoniae.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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**References**

[1] Y. Kayashima, K. Yamanashi, A. Sato, S. Kumazawa, and K. Yamakawa-Kobayashi, “Freeze-dried royal jelly maintains its developmental and physiological bioactivity in Drosophila melanogaster,” Bioscience, Biotechnology and Biochemistry, vol. 76, no. 11, pp. 2107–2111, 2012.

[2] A. Karaali, F. Meydanoglu, and D. Eke, “Studies on composition, freeze-drying and storage of Turkish royal jelly,” Journal of Apicultural Research, vol. 27, no. 2, pp. 182–185, 1998.

[3] E. Mellioiu and I. Chinou, “Chemistry and bioactivity of royal jelly from Greece,” Journal of Agricultural and Food Chemistry, vol. 53, no. 23, pp. 8987–8992, 2005.

[4] T. Kodai, K. Umebayashi, T. Nakatani, K. Ishiyama, and N. Noda, “Compositions of royal jelly II. Organic acid glycosides and sterols of the royal jelly of honeybees (Apis mellifera),” Chemical & Pharmaceutical Bulletin, vol. 55, no. 10, pp. 1528–1531, 2007.

[5] L. Boukraa, “Additive activity of royal jelly and honey against Pseudomonas aeruginosa,” Alternative Medicine Review, vol. 13, no. 4, pp. 330–333, 2008.

[6] L. Boukraa, A. Meslem, M. Benhanifia, and S. M. Hammoudi, “Synergistic effect of starch and royal jelly against Staphylococcus aureus and Escherichia coli,” The Journal of Alternative and Complementary Medicine, vol. 15, no. 7, pp. 755–757, 2009.

[7] A. N. Koç, S. Silici, F. Kasap, H. T. Hörmet-Öz, H. Mavus-Buldu, and B. D. Erçal, “Antifungal activity of the honeybee products against Candida spp. and Trichosporon spp,” Journal of Medicinal Food, vol. 14, no. 1-2, pp. 128–134, 2011.

[8] S. Takaki-Doi, K. Hashimoto, M. Yamamura, and C. Kamei, “Antihypertensive activities of royal jelly protein hydrolysate and its fractions in spontaneously hypertensive rats,” Acta Medica Okayama, vol. 63, no. 1, pp. 57–64, 2009.

[9] K.-M. Suzuki, Y. Isohama, H. Maruyama et al., “Estrogenic activities of fatty acids and a sterol isolated from royal jelly,” Evidence-Based Complementary and Alternative Medicine, vol. 5, no. 3, pp. 295–302, 2008.
[10] P. Moutsatsou, Z. Papoutsí, E. Kassi et al., “Fatty acids derived from royal jelly are modulators of estrogen receptor functions,” *PLoS ONE*, vol. 5, no. 12, Article ID e15594, 2010.

[11] T. Karaca, F. Bayiroglu, M. Yoruk et al., “Effect of royal jelly on experimental colitis Induced by acetic acid and alteration of mast cell distribution in the colon of rats.” *European Journal of Histochimistry*, vol. 54, no. 4, p. e35, 2010.

[12] H. Morita, T. Ikeda, K. Kajita et al., “Effect of royal jelly ingestion for six months on healthy volunteers,” *Nutrition Journal*, vol. 11, no. 1, article 77, 2012.

[13] E. P. Cherniack, “Eyes as drugs, part I: insects. The 'new' alternative medicine for the 21st century?” *Alternative Medicine Review*, vol. 15, no. 2, pp. 124–135, 2010.

[14] S. Pourmoradian, R. Mahdavi, M. Mobasseri, and E. Faramarzi, “Effect of royal jelly supplementation on glycemic control and oxidative stress factors in type 2 diabetic female: a randomized clinical trial,” *Chinese Journal of Integrative Medicine*, vol. 20, no. 5, pp. 347–352, 2014.

[15] S. Fujiwara, J. Imai, M. Fujiwara, T. Yaeshima, T. Kawashima, and K. Kobayashi, “A potent antibacterial protein in royal jelly. Purification and determination of the primary structure of royalisin,” *The Journal of Biological Chemistry*, vol. 265, no. 19, pp. 11333–11337, 1990.

[16] A. Romanelli, L. Moggio, R. C. Montella et al., “Peptides from Royal Jelly: studies on the antimicrobial activity of jelleins, jelleins analogs and synergy with temporins,” *Peptides*, vol. 25, no. 6, pp. 919–928, 2004.

[17] A. Romanelli, L. Moggio, R. C. Montella et al., “Peptides from Royal Jelly: studies on the antimicrobial activity of jelleins, jelleins analogs and synergy with temporins,” *Journal of Peptide Science*, vol. 17, no. 5, pp. 348–352, 2011.

[18] J.-M. Tseng, J.-R. Huang, H.-C. Huang, J. T. C. Tzen, W.-M. Chou, and C.-C. Peng, “Facilitative production of an antimicrobial peptide royalisin and its antibody via an artificial oil-body system,” *Biotechnology Progress*, vol. 27, no. 1, pp. 153–161, 2011.

[19] L. Shen, D. Liu, M. Li et al., “Mechanism of action of recombinant Acc-royalisin from royal jelly of Asian honeybee against Gram-positive bacteria,” *PLoS ONE*, vol. 7, no. 10, Article ID e47194, 2012.

[20] Clinical and Laboratory Standards Institute, *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved Standard*, CLSI Document M07-A9, 9th edition, 2012.

[21] W.-T. Wei, Y.-Q. Hu, H.-Q. Zheng, L.-F. Cao, F.-L. Hu, and H. R. Hepburn, “Geographical influences on content of 10-hydroxy-trans-2-decenoic acid in royal jelly in China,” *Journal of Economic Entomology*, vol. 106, no. 5, pp. 1958–1963, 2013.

[22] M. Genc and A. Aslan, “Determination of trans-10-hydroxy-2-decenoic acid content in pure royal jelly and royal jelly products by column liquid chromatography,” *Journal of Chromatography A*, vol. 839, no. 1-2, pp. 265–268, 1999.

[23] J. Zhou, J. Zhao, H. Yuan et al., “Comparison of UPLC and HPLC for determination of trans-10-hydroxy-2-decenoic acid content in royal jelly by ultrasound-assisted extraction with internal standard,” *Chromatographia*, vol. 66, no. 3–4, pp. 185–190, 2007.

[24] L. H. Garcia-Amoedo and L. B. De Almeida-Muradian, “Physicochemical composition of pure and adulterated royal jelly,” *Quimica Nova*, vol. 30, no. 2, pp. 257–259, 2007.

[25] A. G. Sabatini, G. L. Marczazzan, M. F. Caboni, S. Bogdanov, and L. B. Almeida-Muradian, “Quality and standardisation of Royal Jelly,” *Journal of ApiProduct & ApiMedical Science*, vol. 1, no. 1, pp. 16–21, 2009.

[26] S. Nasser, A. Mabrouk, and A. Maher, "Colonization of burn wounds in Ain Shams University Burn Unit," *Burns*, vol. 29, no. 3, pp. 229–233, 2003.

[27] M. Siavash, S. Shokri, S. Haghighi, M. Mohammad, M. A. Shahtalebi, and Z. Farajzadehgan, “The efficacy of topical Royal Jelly on diabetic foot ulcers healing: a case series,” *Journal of Research in Medical Sciences*, vol. 16, no. 7, pp. 904–909, 2011.

[28] M. Siavash, S. Shokri, S. Haghighi, M. A. Shahtalebi, and Z. Farajzadehgan, “The efficacy of topical royal jelly on healing of diabetic foot ulcers: a double-blind placebo-controlled clinical trial,” *International Wound Journal*, vol. 12, no. 2, pp. 137–142, 2013.

[29] F. B. Holetz, G. L. Pessini, N. R. Sanches, D. A. G. Cortez, C. V. Nakamura, and B. P. Dias Filho, “Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases,” *Memorias do Instituto Oswaldo Cruz*, vol. 97, no. 7, pp. 1027–1031, 2002.

[30] B. Yousefi, S. Ghaderi, A. Rezapour-Lactooyi, N. Amiri, J. Verdi, and A. Shoae-Hassani, “Hydroxy decenoic acid down regulates gtfB and gtfC expression and prevents Streptococcus mutans adherence to the cell surfaces,” *Annals of Clinical Microbiology and Antimicrobials*, vol. 11, article 21, 2012.