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ORIGINAL RESEARCH ARTICLE

Antibacterial activity of Royal Jelly against bacteria capable of infecting cutaneous wounds

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Summary

The antibacterial activity of two royal jelly (RJ) samples against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Streptococcus uberis*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae* was tested using the well diffusion method. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined by broth dilution tests. Raw RJ sample A did not inhibit the growth of *K. pneumoniae* and *S. uberis*, while raw RJ sample B did not inhibit the growth of *K. pneumoniae*. MIC values of RJ sample A were in the concentration range between 3.3 and 10.3 mg/mL, while that of RJ sample B were in the concentration range between 7.1 and 14.5 mg/mL. MBC concentration ranges were between 125 and 250 mg/mL, and between 63 and 250 mg/mL, for RJ samples A and B, respectively. The differences observed in MIC and MBC values may be related to components of RJ associated with their geographical provenance or with genetic variability between bee colonies.

Keywords: royal jelly, antibacterial activity, skin wounds

Introduction

Antibiotic-resistant bacteria continue to be a major health concern world-wide. Since the use of antibiotics became widespread over 50 years ago, bacteria have progressively developed resistance (Hsueh *et al.*, 2005). Consequently, scientific efforts have been made to study and develop new compounds to be used beyond conventional antibiotic therapy. These comprise probiotic strains (Reid, 2001; Petti *et al.,* 2008), new organic compounds (Hoellman *et al.*, 2002), peptide isolates (Yau *et al.*, 2001), plant extracts (Tereschuk *et al.,* 2004; Chomnawang *et al.*, 2009), natural foodstuffs such as kefir (Leite Rodrigues *et al.,* 2005), honey (Molan, 2002; Basualdo *et al.,* 2007), propolis (Sawaya *et al.,* 2004) and royal jelly (Ratanavalachai and Wongchai, 2002; Eshraghi, 2005). Moreover, the use of alternative medicines has increased substantially over the past fifteen years (Romero *et al.*, 2005). It is, therefore of great interest to study the biological properties of natural products likely to be used as new health remedies.

Royal jelly (RJ) is a secretion produced by the hypopharyngeal and the mandibular glands in the head of the nurse bees. It has a thick, milky appearance, with a slightly acid, pungent odour and a somewhat bitter taste. The water content is fairly uniform at greater than 60%, and with an activity (a_w) above 0.92. The dry matter of RJ is composed of protein (27-41%, including free amino acids), carbohydrates (approximately 30%), lipids (8-19%), trace elements and some vitamins (Sabatini *et al.,* 2009). RJ serves as nourishment for honeybee larvae during their first three days of life and as the sole food for the queen bee during its entire life span.

Beyond its excellent nutritious properties, RJ has widely recognized biological actions, including vasodilative and hypotensive activities, antiseptic action, antitumour, antihyper-cholesterolemic, and anti-inflammatory activities (Nagai and Inoue, 2005). It was also

reported that RJ has antibacterial activity against both Gram positive and Gram negative bacteria due mainly to fatty acids present in RJ, such as trans-10-hydroxydec-2-enoic acid, 3-hydroxydodecanoic acid, 11-oxododecanoic acid, and 11-*S*-hydroxydodecanoic acid (Melliou and Chinou, 2005). However, some peptides (Fontana *et al.,* 2004) and proteins (Fujiwara *et al.,* 1990) present in RJ have also been shown to possess strong antibacterial properties. It has been proposed that the short extension of the peptides, their poor cytolytic and mast cell degranulating activities, together with their broad spectrum antibiosis, make them attractive models for the development of new antibacterial agents, (Fontana *et al.,* 2004).

A diverse range of different bacteria are responsible for wound contamination, wound colonization and clinical infection. Microorganisms such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Streptococcus uberis*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae* are frequently isolated from skin wounds in humans and animals. Methicillin-resistant and -sensitive *Staphylococcus aureus* (MRSA and MSSA) are the main strains involved in difficult-to -treat skin and underlying tissue infections associated with Gram positive bacteria (Halcón and Milkus, 2004). *Staphylococcus epidermidis* infections are commonly acquired in hospitals as a result of contamination of surgical incision sites with microorganisms from the patients themselves or from hospital personnel (Vuong and Otto, 2002). Infection with *Pseudomonas aeruginosa* is the most serious complication in burn patients (Nasser *et al.,* 2003; Altoparlak *et al.,* 2005) followed by infections with *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus* and other pathogen microorganisms (Nasser *et al.,* 2003). Hence there is a need to undertake studies to determine the antibacterial activity of natural products against these important pathogens.

Argentina is one of the world's leaders in the production and commercialization of honey. It also has a recognized reputation in the production and quality of royal jelly. The present study was carried out to determine the antibacterial activity of royal jelly against bacteria implicated in infections of skin wounds such as *S. aureus*, *S. epidermidis*, *M. luteus*, *S. uberis*, *E. faecalis*, *P. aeruginosa*, *E. coli*, and *K. pneumoniae*.

Materials and methods

Royal jelly samples

Royal jelly samples were obtained from two sources: one royal jelly sample produced in southern Córdoba (Argentina) was acquired in the local market (sample A) and the other sample was provided by Estación Experimental Agropecuaria INTA Famaillá, Tucumán (Argentina) (sample B). Both royal jelly samples were stored in sterile plastic flasks in the cold (4–8 °C) and in the dark. Both samples fulfilled the requirements of the Argentinean Código Alimentario (Código Alimentario Argentino, 1995). Royal jelly samples were tested without centrifugation.

Bacterial strains

Reference strains of *S. aureus* MS 1 (ATCC 25923), *M. luteus* (ATCC 9341) and *E. faecalis* 1 (ATCC 29212) were used. Strains of *S. epidermidis* isolated from cow's milk, *S. uberis* isolated from cow mastitis, *E. coli* isolated from well water and *K. pneumoniae* isolated from poultry manure were also used in this work. These strains were kindly donated by the Microbiology Laboratory of Universidad Nacional de Río Cuarto. Strains of *S. aureus* MS 2, *S. aureus* MR 1 and *S. aureus* MR 2, isolated from milk obtained from cows with mastitis, were kindly donated by the Bacteriology Laboratory of Universidad Federal Rural de Rio de Janeiro. The strain of *E. faecalis* 2 was isolated from skin infections in humans, and the strain of *P. aeruginosa* was isolated from a patient with catheter infection. Bacterial cultures were kept in brain–heart infusion with 15% glycerol and maintained in 3 mL plastic bottles at -20°C. Inocula for experiments were prepared immediately before use by diluting an overnight culture in trypticase soy broth (TSB) until an optical density at 620 nm in the range of 0.02–0.04 was obtained.

Assessment of antibacterial activity

Agar-well diffusion assays

Agar-well diffusion assays were carried out with each of the above-mentioned bacterial strains. One milliliter of the bacterial culture was added to 30 mL molten, sterile Mueller Hinton Agar (MHA; Britania, Argentina). After mixing, the inoculated culture medium was poured into 90 mm-diameter Petri dishes, allowed to set and stored at 4°C for 1 h. Wells of 4 mm diameter were cut in each plate and 50 µL of royal jelly sample were added to each well. Undiluted royal jelly and a range of concentrations between 10 and 90% (w/v) were tested. Sterilized water was used as negative control. Plates were incubated at 37°C for 24 h. The diameters of the zones of inhibition obtained were measured with a caliper. The experiments were repeated in triplicate on one occasion. The average diameters of the inhibition zones were calculated.

Broth dilution assays: Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of the royal jelly samples were determined for each of the above-mentioned bacterial strains. A set of eight tubes was prepared, each containing 1.0 mL of Mueller Hinton Broth (MHB), then, 1.0 g of RJ was added to the first tube, and thoroughly mixed. From this tube a serial doubling dilution was prepared. Tubes were inoculated with 1 mL inoculum and incubated at 37°C during 24 h on a 300 rpm agitated platform. A series of blank

Table 1. Inhibition of bacteria by Royal Jelly in an agar well diffusion assay determined on three separate occasions.

Mean diameter of zones of inhibition (in millimetres ± S.D.) of Royal Jelly samples.

RJ sample	RJ % (w/v)	*S. aureus* MS 1	*S. aureus* MS 2	*S. aureus* MR 1	*S. aureus* MR 2	*S. epidermidis*	*M. luteus*	*E. faecalis* 1	*E. faecalis* 2	*S. uberis*	*P. aeruginosa*	*E. coli*	*K. Pneumoniae*
	100	14.7 ± 0.6	17.3 ± 0.6	14.7 ± 0.6	15 ± 0	12.7 ± 0.6	12 ± 0	7.7 ± 0.6	8.7 ± 0.6	-	5.3 ± 1.2	7.7 ± 0.6	-
	90	12.3 ± 0.6	15.3 ± 0.6	12.3 ± 0.6	8.7 ± 0.6	8.7 ± 0.6	10.7 ± 0.6	7.7 ± 0.6	7.3 ± 0.6	-	-	-	-
	80	11.3 ± 0.6	14.7 ± 0.6	11.3 ± 0.6	8.7 ± 0.6	6.7 ± 0.6	10 ± 1	7.3 ± 0.6	7.3 ± 0.6	-	-	-	-
	70	8.7 ± 0.6	9.3 ± 0.6	8.7 ± 0.6	7.3 ± 0.6	-	12 ± 1	-	-	-	-	-	-
A	60	5.7 ± 0.6	7.7 ± 0.6	5.7 ± 0.6	-	-	9.3 ± 0.6	-	-	-	-	-	-
	50	-	5.7 ± 0.6	-	-	-	8.7 ± 0.6	-	-	-	-	-	-
	40	-	-	-	-	-	7.7 ± 0.6	-	-	-	-	-	-
	30	-	-	-	-	-	-	-	-	-	-	-	-
	20	-	-	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-	-	-
	100	15.7 ± 0.6	18.7 ± 0.6	12.3 ± 0,6	20.7 ± 0.6	13.7 ± 0.6	15.3 ± 0.6	12 ± 0	10.7 ± 0.6	11 ± 0	5.7 ± 0.6	9.7 ± 0.6	-
	90	15.3 ± 0.6	15 ± 0	10.7 ± 0.6	19 ± 0	11.7 ± 0.6	11 ± 0	9.3 ± 0.6	8 ± 0	7.7 ± 0.6	-	8 ± 0	-
	80	11 ± 0	12.7 ± 0.6	9,3 ± 0,6	18.7 ± 0.6	9.3 ± 0.6	9 ± 0	9.3 ± 0.6	-	7.7 ± 0.6	-	7.7 ± 0.6	-
	70	10.7 ± 0.6	11.7 ± 0.6	8.3 ± 0.6	12.7 ± 0.6	8.7 ± 0.6	8.7 ± 0.6	-	-	7 ± 0	-	6.7 ± 0.6	-
B	60	8.7 ± 0.6	11.3 ± 0.6	7.3 ± 0.6	6.7 ± 0.6	8 ± 0	8.3 ± 0.6	-	-	-	-	6 ± 0	-
	50	8 ± 0	9.7 ± 0.6	7 ± 0	-	7.7 ± 0.6	-	-	-	-	-	-	-
	40	7.7 ± 0.6	-	6.3 ± 0.6	-		-	-	-	-	-	-	-
	30	7 ± 0	-	-	-	-	-	-	-	-	-	-	-
	20	6.3 ± 0.6	-	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-	-	-

- = No zone of inhibition observed.

tubes were prepared in the same way, by replacing the bacterial inoculum with BHI. A positive control was prepared with 1 mL MHB and 1 mL of bacterial inoculum in TSB. A negative control was prepared with 1.0 mL MHB and 1.0 mL of TSB. After incubation optical density (O.D) was measured at 620 nm against the blank tubes; the MIC was determined graphically by plotting O.D vs RJ concentration (mg RJ/mL). Concentrations of RJ between 250 and 1.95 mg/mL were tested.

The MBC was obtained by streaking 100 µL from each tube onto Petri dishes containing MHA. Plates were incubated at 37°C for 24 h, bactericidal action and the lowest concentration to prevent bacterial growth was recorded as MBC.

Results

Agar-well diffusion assays

Overall RJ sample B had higher antibacterial activity than Sample A (Table 1). The size of the inhibition zones produced by Sample B were larger than those produced by Sample A for all test organisms, except for *S. aureus* MR1. Also, Sample B mostly gave zones of inhibition at lower concentration than Sample A. The mean diameter of zones for undiluted RJ samples against *S. aureus* were calculated as 15.4 ± 1.3 mm for Sample A and 16.8 ± 3.6 mm for Sample B.

On the whole Gram positive bacteria were more susceptible to RJ than Gram negative bacteria, although sample A did not inhibit *S. uberis*. *K. pneumoniae* was not inhibited and only undiluted Sample A inhibited *E. coli and P. aeruginosa*. Of the Gram positive bacteria, the largest zones of inhibition were observed with staphylococci, particularly *S. aureus.*

Broth dilution assays:

Contrary to the agar well diffusion assay, royal jelly sample A was more effective at inhibiting test bacteria than sample B, with the exception of *S. epidermidis.* Also there was no marked differences in susceptibilities of Gram positive and Gram negative bacteria and even *K. pneumoniae* was inhibited by 8 mg/mL RJ (Table 2).

The difference between MIC and MBC values for both RJ samples and all test organisms was greater than 4 indicating that the mode of inhibition was bacteriostatic rather than bactericidal.

Discussion

The results obtained in our study showed that both RJ samples, obtained from a different ecological region of Argentina, inhibited the Gram negative and Gram positive bacteria strains used here.

It can be observed from Table 1 that both MR and MS *S. aureus*, bacteria usually isolated from skin wounds (Basualdo *et al.,* 1996), were the most sensitive strains of either RJ sample tested here. Similar results have been reported by other authors (Ratanavalachai and Wongchai, 2002; Eshraghi, 2005). Among the *S. aureus* strains tested here, MS 1 was the most sensitive strain taking into account that its growth was inhibited by RJ sample B diluted up to a 20% w/v. The results of the inhibition tests obtained with *S. aureus* MR may be important considering that this species remains a serious problem in the treatment of infected wounds in humans and animals (Scott Weese and van Duijkeren, 2009). Thus, the discovery of natural products, such as royal jelly, honey, propolis and plant extracts, that could be used alone or with regular antibiotics, is interesting because the effectiveness of antibiotics might be increased while minimizing the possibility of the development of resistant strains (Ratanavalachai and Wongchai, 2002; Lowy, 2003; Sato *et al.*, 2004; Sawaya *et al.*, 2004; Basualdo *et al.*, 2007). Considering the low production yields and high costs of RJ, this possibility requires further investigation. Efficacy of RJ may be enhanced by a synergistic effect when RJ is mixed with starch (Boukraâ *et al.*, 2009).

Lower concentrations of RJ sample B were necessary to inhibit the growth of *S. epidermidis*, as compared to RJ sample A. The growth of *M. luteus* and *E. faecalis*, strains 1 and 2, showed a remarkable sensitivity to the presence of either RJ sample. In a previous study we demonstrated that honey samples inhibited the growth of *S. epidermidis* but not *M. luteus* or *E. faecalis* (Basualdo *et al.*, 2007). Other authors have shown moderate inhibition of the growth of these bacteria by different plant extracts (Cao *et al.,* 2009; Lim *et al.,* 2009). Our results suggest that royal jelly may contain active components which are not present in honey. For example, the protein royalisin was identified as a bactericidal compound active against Gram positive bacteria, while 10-hydroxy decenoic has antibacterial activity against both Gram positive and Gram negative bacteria. These two compounds are generally present in royal jelly (Fujiwara *et al.,* 1990; Hornitzky, 1998; Melliou and Chinou, 2005), but only royalisin has been found in honey (Kwakman *et al.,* 2010).

Our results show that *S. uberis* was resistant to RJ sample A and moderately sensitive to RJ sample B. *S. uberis*, an environmental organism, is the most common pathogen agent for mastitis and the main cause of re-infection during the dry period. It has been noted that *S. uberis* is more resistant to antibiotics than other *Streptococcus* species like *S. dysgalactiae* or *S. agalactiae* (Guérin-Flaubeé *et al.,* 2002).

A diversity of results were obtained in the agar-well diffusion assays performed with both RJ samples against the growth of the Gram negative bacteria used in our study. Thus, the growth of *P. aeruginosa* was inhibited only by undiluted RJ samples of A or B. This bacteria is usually found in skin wounds, particularly those

Table 2. Inhibition of bacteria by Royal Jelly in broth dilutions assays. MIC and MBC values are expressed as mg / mL Royal Jelly.

Bacterial strains	Sample A		Sample B	
	MIC	**MBC**	**MIC**	**MBC**
Staphylococcus aureus MS 1	7.8	125	9.0	<250
Staphylococcus aureus MS 2	3.4	<250	8.8	125
Staphylococcus aureus MR 1	8.0	125	14.5	125
Staphylococcus aureus MR 2	8.0	250	12.5	125
Staphylococcus epidermidis	10.3	125	8.7	125
Micrococcus luteus	7.5	125	11.8	125
Enterococcus faecalis 1	5.0	<250	13.7	<250
Enterococcus faecalis 2	3.7	<250	7.6	125
Streptococcus uberis	5.8	<250	14.5	250
Pseudomonas aeruginosa	3.3	250	14.4	63
Escherichia coli	7.0	<250	7.1	<250
Klebsiella pneumoniae	8.1	125	8	250

related to burns. It causes a variety of systemic infections, particularly in victims of severe burns. Therefore, the antibacterial activity showed by the royal jelly samples studied against *P. aeruginosa* may be of importance in the development of ointments for the treatment of wounds (Abdelatif *et al., 2008*). *E. coli* showed higher sensitivity to RJ sample B as compared to RJ sample A and the growth of *K. pneumoniae* was not affected by the presence of neither RJ sample. *Klebsiella pneumoniae* can cause pneumonia, but more often it is linked with urinary tract diseases and wound infections acquired from the environment, especially in individuals with weakened immune systems. Its virulence is increased by the growing emergence of antibiotic-resistant strains, especially to the beta-lactam antibiotics because *K. pneumoniae* is a producer of beta lactamases (Martínez Ramos, *et al*., 2005; Gaitán, *et al*., 2009). More research is necessary to understand the mechanism of antimicrobial action of the active compounds in royal jelly.

The RJ samples used in this study showed bacteriostatic effects (Table 2). Considerable differences between the MIC and MBC values are observed in Table 2 for RJ sample A and RJ sample B. MIC values were in the range between 3.3 mg/mL and 14.5 mg/ mL, while MBC values were in the range between 63 mg/mL and 250 mg/mL. A somewhat unexpected result was observed for *P. aeruginosa*, as RJ sample A showed a MIC value almost four times lower than that of RJ sample B. However, the MBC value was approximately four times lower for RJ sample B than that observed for RJ sample A.

The agar-well diffusion technique is widely employed to screen for antibacterial activity. It has been recognized to be precise and reliable. However, some debate about whether it is a semi-quantitative technique or a qualitative technique exists, since the results are not always quite reproducible. The last point seems meritorious given the difficulty of hydrophobic compounds to diffuse in the agar. Nevertheless, the agar-well diffusion technique is easy to implement and provides an estimation of bacterial growth inhibition (Wilkinson, 2006; Kalemba and Kunica, 2003). On the other hand, the broth dilution technique is considered precise in assessing the susceptibility of a microorganism to the antibacterial compound being tested. It is widely used to obtain quantitative results (Finegold and Baron, 1992). We recognize that the results obtained with agar-well diffusion techniques are not directly comparable with those obtained with broth dilution techniques. In our study these techniques were used for different purposes: agar-well diffusion techniques were used to screen for antibacterial activity while the broth dilution technique was used to quantify that activity.

Our findings suggest that both RJ samples were active against the growth of the bacterial strains tested. The differences observed in MIC values, as well as in MBC values, may be related to components of RJ associated with their geographical provenance or be linked with genetic variability between colonies (Bachanová *et al*., 2002). Sensitivity of bacteria to bee products varies considerably within the product and the varieties of the same product (Boukraâ and Sulaiman, 2009). These facts lead us to continue in the near future with studies on the chemical composition of RJ and its relationship with the biological activity. Also, dosage and safety of royal jelly must be tested before its possible *in vivo* application.

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