

AN EVALUATION OF THE POTENT INHIBITORY EFFECTS OF ROYAL JELLY FRACTIONS AGAINST *STREPTOMYCES* BACTERIA

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ABSTRACT

Objective: To investigate the anti-bacterial properties of royal jelly against six different bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Streptomyces griseus*, and three unclassified strains of *Streptomyces*).

Design: To find out the antibacterial properties of royal jelly by means of agar distribution method (drop-plate).

Setting: The diameter of the clean zone formed in each concentration was measured and correlated to the ability of the extracts to inhibit the growth of microorganisms. Interestingly the results show inhibitory effects of royal jelly on different strains of the bacteria *in-vitro*. Four concentrations of water soluble extracts of pure royal jelly were prepared and added drop wise to the bacterial strains seed layer cultured individually.

Main outcome measures: Ether-soluble and non-soluble fractions of the royal jelly were also examined using the above method.

Results: It was found that the antibacterial effect of ether-soluble fraction is substantially higher than the effect of ether non-soluble fraction. The zones formed by ether-soluble fractions of royal jelly in the route of distribution in agar belong to the potency of anti-bacterial properties of fatty acids present in this fraction of royal jelly (10-HDA).

Conclusions: The non ether-soluble fraction of royal jelly contains a bactericidal substance called royalisin that was found to have potent antibacterial activity. This fraction of royal jelly revealed weaker antibacterial effect than the ether-soluble fraction and even of pure royal jelly. Hence apart from its nutritional and medicinal effects, royal jelly's bactericidal ability should be considered and employed whenever appropriate in clinical medicine.

KEYWORDS: Royal jelly, *Streptomyces*, Royalisin, *Apis mellifera*, *trans*-10-hydroxy decenoic acid

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INTRODUCTION

Honey and royal jelly are complex heterogeneous mixtures of flower's nectar sugars proteins and bee's glandular secretions. Royal jelly is the hypo-pharyngeal gland excretion of the young worker honeybees (*Apis mellifera*). The wonderful effects of royal jelly on the sexual ability and maturity of the queen has been an interesting topic and controversial issue for researchers for many years.

The present descriptive study was designed to investigate inhibitory effects of royal jelly against variety of bacteria. Royal jelly is the

milky-white gelatinous substance produced in the worker bees' stomach by the incomplete digestion of honeydew. The substance is secreted from the cephalic glands of nurse worker bees (*Apis mellifera*) for apparently the sole purpose of stimulating the growth and development of the queen bee. Every cell of the honeycomb which is named shakhoon is a frame made of bee wax and is the place of the queen's larva which is always filled by excess amounts of royal jelly for nutritional requirements of larvae. The queen's larva is floating in a dense jelly-like, milky white-colored substance¹⁻⁴.

A new potent antibacterial protein, designated royalisin, was found in royal jelly of the honeybee *A. mellifera* by Fujiwara *et al.*, 1990. The substance was purified to homogeneity for the first time by acid extraction, gel filtration, and reverse-phase high pressure liquid chromatography. The primary structure of royalisin was determined to consist of 51 residues, with three intramolecular disulfide linkages, having a calculated molecular mass of 5523 Da⁵.

Royal Jelly Compounds

It is a milky white highly viscous secretion from the salivary gland of the worker hive bees *Apis mellifera* (Apidae) which is essential for the development of queen bees⁶⁻⁷. All useful effects of royal jelly cannot be attributed to an individual component. It is noticeable that the combination of the elements together produces the useful effects⁶⁻⁸. It is supposed that royal jelly makes a balance metabolism in the human body. It might be because of a similar balance between its components which are mixed in a suitable proportion⁶⁻¹¹.

Medicinal Properties

Studies conducted previously show a wide range of medical activities in royal jelly. Some of these effects are as follows: anti-microbial effects¹²⁻¹³, Suppression of allergic reactions^{2,7,14}, lowering the amount of blood cholesterol¹⁵⁻¹⁷, preventing cell damage in cancer and HIV patients¹⁸⁻¹⁹, as well as wound healing and growth acceleration²⁰.

The potency of anti-bacterial properties of

royal jelly could be related to a particular fatty acid present in ether-soluble fraction of royal jelly called *trans-10-hydroxy decenoic acid (10-HDA)*,²¹⁻²². The contents of 10-HDA, a bioactive component of royal jelly, and several vitamins did not change during storage at 40 degrees °C for 7 days. The substance has the chemical formula of (C₁₀ H₈ O₃) and amount to 10% of the total weight of royal jelly^{6,21-22}.

Microbiology

Streptomyces species are aerobic gram-positive bacteria. They produce filaments that are long highly branched and non-fragmenting²³⁻²⁴. The bacteria are generally harmless saprophytes of the soil though some strain e.g. *Streptomyces griseus* and *Streptomyces somaliensis* have been found in cases of mycetoma. More than 3000 species of *Streptomyces* have been described which are important as producers of antibiotics²⁵⁻²⁹.

As regards the results obtained, we were interested to investigate antibacterial effects of royal jelly against above mentioned bacteria.

MATERIALS AND METHODS

Streptomyces griseus (ATCC 11746), *Staphylococcus aureus* (ATCC 14776), *Escherichia coli* (ATCC 29532), freeze-dried ampoules were obtained from American Type Culture Collection 10801 University Boulevard Manassas USA. Three unclassified strains of *Streptomyces* indicated as (*S*₄₆) (*S*_{F8}) and (*S*₆₆) were provided from School of Public Health, Tehran University of Medical Sciences. The royal jelly was supplied by the faculty of Agriculture, University of Tehran located in Karaj City. Royal jelly was transferred to Tehran in ice. The antimicrobial susceptibility test discs {ceftazidime (31633), neomycin (31313), gentamicin (31299), tobramycin (31569), vancomycin (31353) and streptomycin (31328)} were supplied from Becton Dickinson Microbiology Systems Cockeysville Maryland (BBL products).

Preparation of royal jelly extract:

The appropriate amounts of royal jelly had been collected during spring time (May-June)

from hive bee's located in Karaj city in Iran. The water solutions extract were prepared with the concentrations of 1000, 333, 200 and 143 mg/ml. As the filtration procedure may reduce the bactericidal activity of royal jelly³⁰, to avoid any contamination, the extraction procedures were performed aseptically under flow cabinet.

Different concentrations of water soluble extracts of royal jelly were applied to the surface of Starch Casein Agar plates inoculated with a pure culture of *Streptomyces* strain. The same extracts were applied to the surface of Muller Hinton Agar plates inoculated with a pure culture of *Escherichia coli* and *Staphylococcus aureus* strain. The plates were prepared using seed layer culture procedure. The susceptibility test discs containing appropriate amounts of anti-microbial agents were applied to the surface of Starch Casein Agar plates inoculated with a pure culture of *Streptomyces* strain. Twenty micro liters of each concentration (1000, 333, 200 and 143 mg/ml) of royal jelly which corresponded with 20, 6.66, 4 and 2.86 mg per 20 µl of royal jelly respectively, were added in the middle of every marked area using Gilson samplers. Following incubation, the plates were examined and the clear zones of inhibition surrounding the drop points were measured and compared with established zone size ranges for antimicrobial agent discs. In the present experimental work the appropriate control strains were used along with the test strains mentioned in material section.

The amount of existing effective matter (X) in each concentration was obtained by the

following equation:

$$X = V \times M$$

V = volume of extract, M = dilution of extract.

Ether-soluble (30 mg/ml) and non-soluble (30 and 300 mg/ml) fractions of the royal jelly were also examined using the above method.

At the first stage all fatty acids from the royal jelly were extracted using the solvent diethyl ether. When the solvent was evaporated, 70% ethanol was added to the mixture as an inert solvent. The remaining is ether non-soluble fraction in the royal jelly, which had not been dissolved in di-ethyl ether. Water was also used as an ineffective solvent to the latter fraction.

RESULTS

The royal jelly showed clearly inhibitory effects against a variety of bacteria and in similar concentrations demonstrated different inhibitory effects on different strains of *Streptomyces* (Table-I). The antibacterial activity of pure royal jelly is lesser than the ether-soluble fraction of royal jelly (Tables I & II).

The inhibitory effect of ether-soluble fraction of royal jelly against the bacteria is clearly stronger than the ether-non-soluble fraction (Tables II & III).

The results indicate that non-soluble fraction of royal jelly at a concentration similar to soluble fraction of royal jelly (30 mg/ml) does not have any inhibitory activity and even at a higher concentration (300 mg/ml) it has a low activity (Tables II & III).

The antimicrobial susceptibility test against *Streptomyces strain* showed that all bacteria are

Table-I: The inhibitory activities of pure royal jelly against Bacteria using drop plate method

Test strain	Royal jelly Concentration (mg/ml)			
	143	200	330	1000
	Inhibitory zone diameter (mm)			
<i>Streptomyces strain</i> (46)	-	7	13	20
<i>Streptomyces strain</i> (F _g)	3	9	13	17
<i>Streptomyces strain</i> (66)	1	8	10	16
<i>Streptomyces griseus</i> (ATCC 11746)	6	13	14	20
<i>Staphylococcus aureus</i> (ATCC 14776)	3	13	15	20
<i>Escherichia coli</i> (ATCC 29532)	-	10	12	17

susceptible to most of standard antimicrobial discs (Table-IV).

DISCUSSION

The results of the experiments on the aqueous solution of the pure royal jelly are indicative of the antibacterial effect on various types of bacteria (Table-I).

The studies of royal jelly against a wide variety of microorganisms all over the world, show

Table-II: The inhibitory activity of ether-soluble fraction of royal jelly against bacteria using drop plate method

Test strain	Royal jelly Concentration	
	30 (mg/ml)	
	Inhibitory zone diameter (mm)	
<i>Streptomyces strain</i> (46)	23	
<i>Streptomyces strain</i> (F ₈)	22	
<i>Streptomyces strain</i> (66)	24	
<i>Streptomyces griseus</i> (ATCC 11746)	29	
<i>Staphylococcus aureus</i> (ATCC 14776)	40	
<i>Escherichia coli</i> (ATCC 29532)	22	

Table-III: The inhibitory activity of ether-non-soluble fraction of royal jelly against Bacteria using drop plate method

Test strain	Royal jelly Concentration (mg/ml)	
	30 (mg/ml)	300 (mg/ml)
	Inhibitory zone diameter (mm)	
<i>Streptomyces strain</i> (46)	-	9
<i>Streptomyces strain</i> (F ₈)	-	10
<i>Streptomyces strain</i> (66)	-	7
<i>Streptomyces griseus</i> (ATCC 11746)	-	10
<i>Staphylococcus aureus</i> (ATCC 14776)	-	12
<i>Escherichia coli</i> (ATCC 29532)	-	4

Table-IV: The antimicrobial susceptibility test against *Streptomyces* strain using disc plate method

Bacteria strain	Antimicrobial discs					
	Ceftazidime	Gentamicin	Neomycin	Streptomycin	Tobramycin	Vancomycin
<i>Streptomyces strain</i> (46)	-	24	25	19	29	28
<i>Streptomyces strain</i> (F ₈)	-	23	29	23	24	28
<i>Streptomyces strain</i> (66)	-	18	20	17	26	23
<i>Streptomyces griseus</i> (ATCC 11746)	-	27	23	25	25	27

strong antimicrobial activity^{12,18}. However none of the investigation was related to filamentous bacteria (e.g. *Streptomyces* strain) and thus the present investigation is unique.

The characterization of major proteins of honeybee and royal jelly has been described by researchers^{1,31}. The results of royal jelly fresh specimen analysis that has been presented in the Merck's Index and Martindale books^{6,11} are as follows (% wt at pH 5): moisture 65-70, protein 15-20, carbohydrate 10-15, lipid 1.7-6, ash 0.7-2.0; trace elements: Na, K, Fe, Cu, Mg, Mn, Ca. Vitamins (µg/g): thiamine 2, riboflavine 10, pyridoxine 2, nicotinic acid 75, biotine 2, folic acid 0.3, inositol 100, pantothenic 250, ascorbic acid 3-5, vitamin D & E trace. When stored at room temperature it changes to a light yellow gum and after some weeks to brittle amber solid^{6-7,10-11}.

The interesting result in this study was the maximum clear zones of inhibition surrounding the soluble fraction by applying the minimum concentration (30 mg/ml) of royal jelly (Table II and Figure 1). It seems that the activity is attributed to the 10 HDA content of soluble fraction of royal jelly.

Generally the major antibacterial effect of royal jelly is attributed to a particular 10-carbon molecule of fatty acid (10-hydroxy-decenoic acid)²¹⁻²². Since this acid and the other existing fatty acids are portions of the ether-soluble fraction of royal jelly they create a strong antibacterial activity in this fraction¹²⁻¹³ while the non-soluble fraction in ether and the other peptide fractions of royal jelly have weaker antibacterial effect than ether-soluble fraction and even of pure royal jelly¹². The bactericidal activity of decenoic acid which is a

long chain fatty acid is stronger than other 10 carbon fatty acids and is not neutralized by addition of alkaline while the short chain fatty acids may be inactivated during the extraction procedures²¹⁻²².

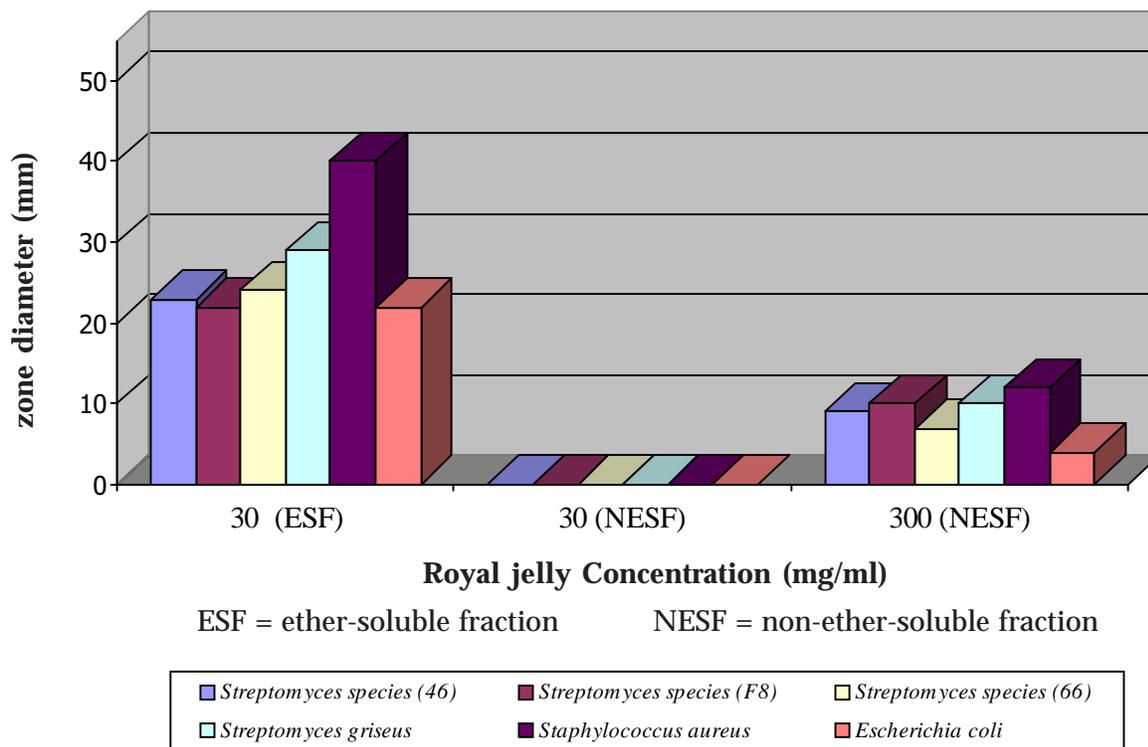
The concentration of 30 mg/ml of ether-soluble fraction of royal jelly (containing 10-HDA) inhibited all bacteria (Table-II) while ether non-soluble fraction with the same concentration of royal jelly (containing royalisin) was not effective against the same microorganisms (Table-III). Royalisin is an amphipathic protein, with the C-terminal half of the molecule being rich in charged amino acids; and it showed extensive sequence homology to two other antibacterial proteins, sapecin from embryonic *Sarcophaga peregrina* cells and phormicins from *Phormia terranova* larvae. Royalisin was found to have potent antibacterial activity against Gram-positive bacteria at low concentrations, but not against Gram-negative bacteria. Royalisin may be involved in a defense system active against bacterial

invasion of the honeybee⁵.

Although the inhibitory effect of ether non-soluble fraction of royal jelly increased marginally by application of ten fold higher concentration of the fraction (*i.e.* 300 mg/ml) however the extent of inhibition was substantially lower than that achieved with 30 mg/ml of the ether soluble fraction (Tables II & III Figure 1). These results suggest that the inhibitory effect is most likely associated to lipid-containing molecules present in ether soluble fraction of royal jelly. The weaker antibacterial activity in ether-non-soluble fraction of royal jelly may be attributed to royalisin which is a portion of the ether-non-soluble fraction of royal jelly¹⁰ thus the potent antibacterial activity against gram-positive bacteria at high concentrations (300 mg/ml) is probably due to the presence of royalisin.

Comparison of the results presented in Tables-III & IV revealed approximately similar antibacterial activity in royal jelly and standard antibiotics against the strain of *Strepto-*

Figure 1: The comparison of the inhibitory activities of different fractions of royal jelly against Bacteria using drop plate method



myces. Therefore apart from the wonderful effects of royal jelly in nutritional and medicinal field its bactericidal ability should also be considered and employed whenever appropriate in clinical medicine.

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