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Propolis, royal jelly and pollen from beehive have antibacterial effect on aquatic pathogenic bacterial isolates

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ABSTRACT

New approaches for treatment of infectious diseases in aquatic animals have important roles in aquaculture technology progress. In the present study, In vitro effects of different extracts of propolis, royal jelly and pollen obtained from beehives have been investigated on aquatic pathogenic bacterial isolates. The isolated bacteria identified on the basis of their biochemical properties and sequence alignment of the amplified genome fragments. Antimicrobial activities of ethanol extracts of royal jelly, propolis and pollen, and acetone extract of propolis were determined through well diffusion and microdilution methods. The isolated bacteria identified as *Aeromonas* and *Vibrio* spp., based on biochemical characterization. Alignments of the amplified sequences showed most similarites to *Vibrio cholerae* and *Aeromonas hydrophila*. The results obtained from antibacterial effects of extracts showed that the acetone extract of propolis as well as the ethanol extract of royal jelly, had the greatest effect on *Aeromonas hydrophila* (MIC=25 mg ml^{-1}); and the ethanol extracts of pollen and royal jelly as well as the acetone extract of propolis, showed the greatest effect on *Vibrio cholerae* (MIC=50 mg ml^{-1}). The results of present in vitro study propose the beehive compounds (royal jelly, propolis and pollen) as powerful natural products to control pathogenic bacteria in aquaculture systems.

1. Introduction

The most common infectious diseases of aquatic organisms are caused by bacteria, particularly Gram-negative bacteria. Environmental factors (such as stress) and deficiency in immune system can lead to increasing susceptibility to infection (Toranzo et al., 2005).

Hony bees carry resin substances to their hive, add other substances such as wax to it and modify it to another substance that is called propolis. Propolis layers in the hive are used to block the cracks and pores in the hive in order to keep out light, air, and potential predators such as spiders, ants, and other insects that might attack warm environments and food supplies. Propolis is composed of resin, wax and oily acids, volatile oils and other organic and mineral substances. The chemical compounds of propolis differ on the basis of the source area. Falvonoids are the major antibacterial compounds in propolis (Mlagan and Sulimanovic, 1982; Greenaway et al., 1990).

Royal jelly is the main food used to feed larvae. Unlike other hive products, royal jelly is

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not an herbal product collected and transformed by honeybees; rather, it is a substance that is actually produced by them. Nurse bees digest flower pollen and nectar and then secrete royal jelly (Eshraghi and Seifollahi, 2003). Royal jelly is creamish white in colour, somewhat sticky, with a homogeneous mass, which physically looks like glue and has a special pleasant smell, like that of concentrated milk or yogurt. The main components of royal jelly include water, protein, lipids, hydrocarbons and mineral salts (Kodai et al., 2007). Proteins with antimicrobial properties isolated from royal jelly include lysine, jelin 1, 2, 3 and aspirin. The royal jelly collected from the three-days larvae has the highest quality and antimicrobial activity (Fujiwara et al., 2008).

The pollen collected by honeybees, which stick together with bee secretions and flower nectars, are called pollen grains as they shape like small seeds with bright colours. The 15 main sources of proteins for bees are nectars that are collected in pollen baskets on the bee’s rear legs and are carried as small tablets. Pollen grains are the reproductive cells of flowers. It could also be stated that before leaving the hive to collect pollen, bees fill their crop or honey stomach with some nectar from the hive. During pollen collection, they return some of the pollen and use it for dampening their rear legs. This causes pollen to stick to the hair on the pollen basket of the bees. Worker bees collect these seeds and keep them in their pollen baskets after mixing them with their own saliva. The bacterial environment that has been created includes 5 to 8 genera of lactic bacteria and 3 genera of yeast, which prevent the growth of any other type of bacteria capable of corrupting pollen (Bell et al., 1983). The antibacterial effect of pollen also is due to the existence of phenolic compounds and carotenoids, which induces the immune system (Solberg and Remedios, 1980; Socha et al., 2009).

2. Material and Methods

2.1. Samples

The studied bacteria were isolated from aquarium fish such as goldfish (*Carassius auratus auratus*), and guppy (*Poecilia reticulate*) on blood agar, nutrient agar and thiosulfate citrate bile salt sucrose agar media. The isolated bacteria were initially identified via microscopic examination and biochemical tests (Pier et al., 1978; Brooks et al., 2010).

2.2. Molecular identification

The number of 2×10^7 bacterial cells grown on brain heart infusion broth was used for DNA extraction by high yield DNA purification kit (DN 8115C, Cinnagen, Iran). Considering the results of the biochemical tests, the PCR reaction was done using specific primers for DNA replication of 16SrDNA of each identified bacterium. The sequences of primers were 5'-AAC CTG GTT CCG CTC AAG CCG TTG-3' and 5'-TTG CTC GCC TCG GCC CAG CAG CT-3' for *Aeromonas* spp.; and 5'-CGG TGA AAT GCG TAG AGA T-3' and 5'-TTA CTA GCG ATT CCG AGT TC-3' for *Vibrio* spp. (Cascón et al., 1996; Tarr et al., 2007).

2.3. Sequencing of amplified fragments

The amplified final products were sequenced by ABI3730XL system (Bioneer Corporation, Korea) and aligned with the current sequences in the BLAST database.

2.4. Extract preparation

Propolis, royal jelly and pollen were obtained from the Honey Bee group of Isfahan Center for Research in Agricultural Science, Iran. For preparation of ethanol extract of propolis, royal jelly and pollen, 250 gr of each substance were mixed with 1 litter of 96% ethanol in separate lided containers and shaked with 120 rpm in room temperature for 3 days. The mixture of royal jelly and ethanol (for 2 days), and the mixture of propolis and ethanol (for 14 days) were kept in a stable place in room temperature. Then, each extract was filtered through sterile filter papers and gauzes. To evaporate alcohol, the extracts were poured into big glass plates and dried for 24 to 28 hours in 45°C. The extracts were stored in 4°C (Dagostin et al., 2010). For preparation of acetone extract, 25 gr of propolis was mixed with 100 ml acetone in tightly fitted container. The mixture was placed on 120 rpm for 72 hours in room temperature. Afterwards, the extract was filtered and evaporated as the previous methods (Manish et al., 2006; Dagostin et al., 2010).

2.5. Antibacterial activity assays

In order to investigate the antibacterial activity of ethanol extracts of propolis, royal jelly and the acetone extract of propolis, concentrations of 3.125, 6.25, 12.5, 25, 50, 100, 200 and 400 mg ml^{-1} of the ethanol and acetone extracts were prepared in 10% dimethyl sulfoxide (DMSO). The antibacterial activity of the extracts was investigated by well diffusion and microdilution methods.

In the well diffusion method, a suspension of 2×10^8 bacterial cells per ml was prepared for each bacterium in Muller Hinton Broth (MHB). The suspension was cultured on Muller Hinton Agar (MHA) in 4 directions. The amount of 100µl of each concentration obtained from the ethanol and acetone extracts were separately added to bottom sealed wells with 6 mm in diameter and 25 cm distance with each other. DMSO 10% was used as negative control and 30µg of each doxycycline and cephalothin antibiotics were used as positive controls. The diameter of each growth inhibition zone was measured after 24 hours incubation at 32°C, (Kognou et al., 2011). The experiments were repeated three times.

In the microdilution method, 100 µl of each concentration was separately added to a well of sterile lidded microplates. Then, an amount of 10^5 bacterial cells was separately added to each well. An amount of 10^5 bacterial cells was used as positive control, while 200 µl of sterile MHB medium was used as negative control. The optical absorptions of microplate wells prior to incubation and 24 hours after incubation were measured via ELISA reader in the wavelength of 630 nm at 32^0C. The minimum concentration of the tested extract, which revealed no opaqueness, was considered as minimum inhibitory concentration (MIC). In order to determine the minimum bactericidal concentration (MBC) of each extract, 20µl from the wells related to MIC and three wells related to the greater concentrations that revealed no detectable opaqueness were streaked on MHA medium, and were kept for 24 hours in 32^0C. The inoculums from each extract concentration that had no growth on solid medium were taken as MBC (Dagostin et al., 2010; Kognou et al., 2011). The experiments were repeated three times.

3. Results

3.1. Biochemical identification

The results of biochemical tests for identification of bacterial isolates are illustrated in table 1. Both bacteria were Gram-negative. According to these results the isolates were initially detected as *Aeromonas hydrophilla* and *Vibrio cholera*.

Table 1. Biochemical tests results obtained for entification of bacterial isolates.

Bacterial isolate	*Aeromonas* spp.	*Vibrio* spp.
Indol	+	-
MR	-	+
VP	+	-
Catalase	+	+
Oxidase	+	-
Urease	-	+
Hemolysis	α	β
H_2S production	+	+
Citrate tilization	-	+
OF (Glucose)	Fermentative	Oxidative
Motility	+	+
Fermentation of:		
Lactose	-	-
Glucose	+	+
Manitol	+	+
Xylose	-	+
Sorbitol	+	+
Sucrose	+	+

3.2. Molecular identification

The 760 bp band resulting from amplification of *Aeromonas* spp. genome and the 750 bp resulting from amplification of *Vibrio* spp. genome are illustrated in Figure 1.

3.3. Sequence analysis

The results of sequence alignment of amplified fragments in BLAST program are illustrated in Table 2. The bacterial isolates showed the highest levels of similarites to *Vibrio cholerae* and *Aeromonas hydrophilia,* while they showed lower levels of similarities to *Aeromonas jandaei* and *Aeromonas veronii,* too.

3.4. Antibacterial activity of the extracts

The average diameter of inhibition zone of different extracts on *Aeromonas* and *Vibrio* spp. are illustrated in table 3. The MIC and MBC values of various extracts against the tested bacteria are illustrated in figures 2 and 3. The highest bactericidal activity of ethanol extracts of royal jelly, pollen and propolis and aceton extract of propolis were observed against *Aeromonas hydrophila*, while they all showed a lower activity against *Vibrio cholera*, too.

4. Discussion

In the present study, we investigated the antibacterial effect of three beehave natural materials (propolis, royal jelly and pollen) on pathogen bacteria isolated from ornamental guppy and goldfish. The isolated bacteria showed the most similarities to *Aeromonas hydrophila* and *Vibrio cholerae* in genome alignment analysis. The results also showed that the acetone extract of propolis had the strongest effect and the ethanol extract of pollen had the weakest effect on the *Aeromonas hydrophila* and *Vibrio cholerae* isolated from fish samples.

A previous study investigated the effects of ethanol extract of propolis in 2.74 and 5.48 mg ml^{-1} concentrations on *Staphylococcus aureus* and *Escherichia coli* based on inhibition zone diameter and reported that the ethanol extract of propolis has stronger antibacterial activity against *Staphylococcus aureus* (gram-positive) in contrast to *Escherichia coli,* which is Gram-negative (Rahman et al., 2010). The isolated Gram-negative bacteria in our research also showed highly sensitivity to propolis extracts, especially the aceton extract (MIC=25 mg ml^{-1}).

There is some inconsistency in the findings over the effect of propolis, which results from its different sources. Antimicrobial activity of propolis is because of high concentrations of flavonoids in this natrul substance, but this activity differs on the basis of geographical areas and environmental factors such as pH (Meresta and Meresta, 1980). Therefore, the results obtained from different studies may show some degrees of variety.

In another study, the royalisin protein were extracted from royal jelly and reported that it was strongly effective against *Lactobacillus, Clostridium, Corynebacterium, Leuconoctoc, Staphylococcus* and *Streptococcus* (Pavel et al., 2011). Also, the effect of antimicrobial activity of royalisin extracted from royal jelly was studied on inhibition of the growth of Gram-positive bacteria in a previous study. The MIC values of royalisin on *Bacillus subtilis*, *Micrococcus flavus* and *Staphylococcus aureus* were 62.5,125 and 250 mg ml^{-1} respectively (Shen et al., 2012). It has been shown that the antibacterial activity of royal jelly differs on the basis of collection days, and this leads to the inhibition of the growth of *Aeromonas hydrophila*, *Bacillus subtilis*, *Listeria monocytogenes*, *Salmonella enteritidis*, and *Escherichia coli* in cocentrations 32, 64, and 128 mg ml^{-1} (Attalla et al., 2007). The findings of the present study showed that the minimum concentration of 25 mg ml^{-1} of royal jelly ethanol extract can inhibit the growth of *Aeromonas hydrophila* and the minimum concentration of 50 mg ml^{-1} from the same extract can inhibit the growth of *Vibrio cholera*.

It has been shown that ethanol extract of poppy flower had the highest antibacterial activity against *Staphylococcus aureus* (Sramkova et al., 2013). Also, in a previous study the antimicrobial effect of aqueous, methanol and dichloromethane extracts of Greece pollen were compared on Gram-negative and Gram-positive bacteria as well as pathogenic human fungi. All the three extracts showed strong antibacterial activities against Gram-positive bacteria. The dichloromethane extract of pollen had no effect on human pathogen candidas. The methanol extract of pollen has a weak effect on Gram-negative bacteria (Graikou et al., 2011). The present research demonstrated that the minimum concentration of 50 mg ml^{-1} of ethanol extract of royal jelly is able to inhibit the growth of *Aeromonas hydrophila* and *Vibrio cholerae*. Because of the ease of the ethanol extraction and its potential antibacterial effect, we propose it can be used as an alternative to methanol or dichloromethane extracts at least for Gram-negative bacteria.

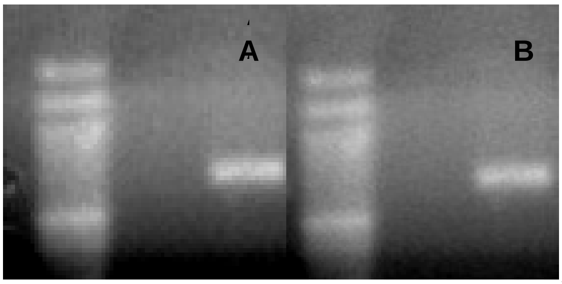

Figure1. A: The 760 bp band resulting from DNA replication of *Aeromonas* spp., B: The 750 bp band resulting from DNA replication of *Vibrio* spp.

Table 2. The results obtained from sequencing alignment of amplified fragments.

Aeromonas spp.	*Vibrio* spp.
Organism report (*Aeromonas* spp.)	**Organism report (*Vibrio* spp.)**
gb|JQ040113.1| *Aeromonas hydrophila* strain B51 16S ribosom... 1040 0.0	gb|DQ068935.1| *Vibrio cholerae* 16S ribosomal RNA gene, par... 880 0.0
gb|JQ040109.1| *Aeromonas veronii* strain B41 16S ribosomal ... 1040 0.0	gb|JN555611.1| *Vibrio cholerae* strain SX-1 16S ribosomal R... 878 0.0
gb|JQ040106.1| *Aeromonas hydrophila* strain B30 16S ribosom... 1040 0.0	gb|GU296110.1| *Vibrio cholerae* strain F2 16S ribosomal RNA... 878 0.0
gb|JQ040105.1| *Aeromonas jandaei* strain B29 16S ribosomal ... 1040 0.0	gb|GU272068.1| *Vibrio cholerae* strain V28 16S ribosomal RN... 878 0.0
gb|JQ040101.1| *Aeromonas hydrophila* strain B10 16S ribosom... 1040 0.0	gb|GU272064.1| *Vibrio cholerae* strain V20 16S ribosomal RN... 878 0.0
gb|KC150866.1| *Aeromonas hydrophila* strain Ah-13 16S ribos... 1112 0.0	gb|GU272062.1| *Vibrio cholerae* strain V6 16S ribosomal RNA... 878 0.0
gb|JQ040113.1| *Aeromonas hydrophila* strain B51 16S ribosom... 1112 0.0	gb|GQ871451.1| *Vibrio cholerae* strain KK3 16S ribosomal RN... 878 0.0
gb|JQ040109.1| *Aeromonas veronii* strain B41 16S ribosomal ... 1112 0.0	gb|GQ205447.1| *Vibrio cholerae* strain LD081008B-1 16S ribo... 878 0.0
gb|JQ040106.1| *Aeromonas hydrophila* strain B30 16S ribosom... 1112 0.0	gb|EF684899.1| *Vibrio cholerae* isolate RC483 16S ribosomal... 878 0.0
gb|JQ040105.1| *Aeromonas jandaei* strain B29 16S ribosomal ... 1112 0.0	gb|AY494843.1| *Vibrio cholerae* strain TP 16S ribosomal RNA... 878 0.0
gb|JQ040101.1| *Aeromonas hydrophila* strain B10 16S ribosom... 1112 0.0	gb|AY513500.1| *Vibrio cholerae* strain VC12-Ogawa 16S ribos... 874 0.0
emb|HE979858.1| *Aeromonas salmonicida* partial 16S rRNA gen... 1105 0.0	gb|JN836452.1| *Vibrio cholerae* strain VC30 16S ribosomal R... 872 0.0

Table 3. Average inhibition zone diameters of different extracts on *Aeromonas* and *Vibrio* spp.

	Concentration (mg ml^{-1})	Ethanole extract inhibition zone diameter (mm)			Inhibition zone diameter of propolis acetone extract (mm)
		Pollen	royal jelly	propolis	
Aeromonas hydrophila	3.125	00.00±0.000	00.00±0.000	00.00±0.000	00.00±0.000
	6.25	00.00±0.000	00.00±0.000	00.00±0.000	00.00±0.000
	2.5	00.00±0.000	11.67±1.000	00.00±0.000	00.00±0.000
	25	9.67±0.578	15.00±1.527	13.67±0.577	14.00±2.000
	50	13.67±3.005	20.33±1.527	17.00±1.000	21.67±2.081
	100	19.00±1.000	25.00±1.527	21.00±1.000	25.33±2.081
	200	26.33±1.527	27.00±1.055	27.33±3.055	29.00±1.000
	400	30.33±1.527	32.67±1.154	30.33±1.154	29.00±1.000
Vibrio cholerae	3.125	00.00±0.000	00.00±0.000	00.00±0.000	00.00±0.000
	6.25	00.00±0.000	00.00±0.000	00.00±0.000	00.00±0.000
	12.5	00.00±0.000	00.00±0.000	00.00±0.000	00.00±0.000
	25	11.33±1.527	12.00±2.000	00.00±0.000	11.33±1.527
	50	13±20.6457	14.67±1.527	00.00±0.000	13.33±1.527
	100	17.67±1.527	19.00±1.000	14.67±1.527	20.00±1.000
	200	26.00±2.645	25.67±1.527	19.67±1.527	22.67±1.527
	400	29.67±2.517	29.33±1.527	25.00±1.000	26.00±1.000

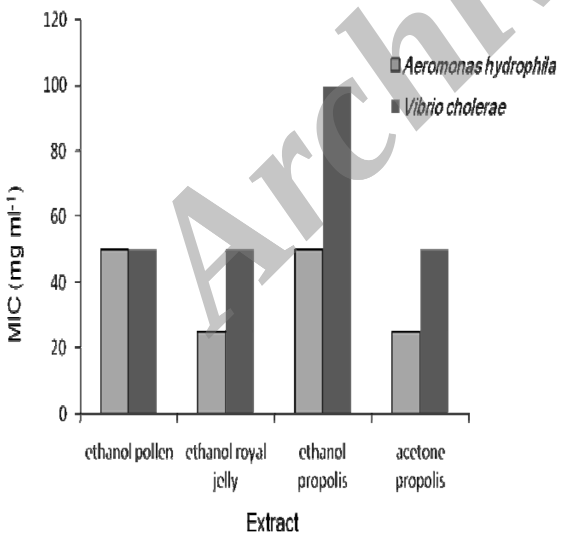

Figure 2. Minimum inhibitory concentration (MIC) of various extracts on the studied bacteria.

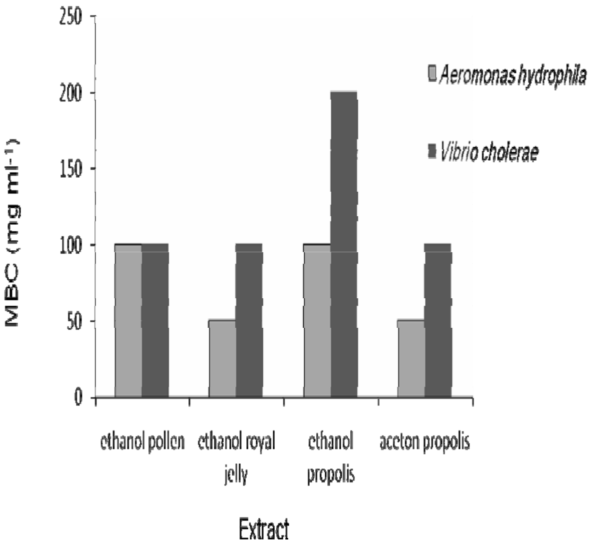

Figure 3. Minimum bactericidal concentration (MBC) of various extracts on the studied bacteria.

Conclusion

The findings of this study revealed that the ethanol extracts of pollen, royal jelly and propolis and the acetone extract of propolis had strong effect on *Aeromonas hydrophila* and *Vibrio cholerae*, which are potential aquatic pathogens. In general, the findings showed that *Aeromonas hydrophila* was more sensitive than *Vibrio cholerae*; and, compared to other extracts, acetone extract of propolis had the strongest antibacterial effect on the studied bacteria.

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