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Original Article

Effect of propolis on gene expiration *IL-1β* in *Cyprinus carpio* challenged with *Aeromonas hydrophilia*

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Abstract: Aquaculture has become a significant source of income in areas facing declining natural fishery productivity. This work aimed to investigate the effect of Water Ethanol Extract propolis (WEEP) on peritoneal macrophages in vitro production of cytokines, including interleukin-1beta (IL-1β) in common carp, *Cyprinus carpio* challenged with *Aeromonas hydrophilia*. The results of hematological parameters revealed substantial alterations, particularly significant in the 2g/kg propolis group (T2), where red blood cells, packed cell volume, and hemoglobin exhibited changes, indicating potential advantages for fish health by intriguing immunomodulatory effects. The 2 g/kg propolis group (T2) increased WBC count, suggesting propolis's potential as an immunostimulant. Interleukin-1β (IL-1β) gene expression results showed a significant decrease in T2 compared to T3. This finding revealed the intricate relationship between propolis concentrations and the modulation of the immune response. The results showed the positive effect of propolis on as potential application as an immunostimulant in aquaculture.

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Introduction

Aquaculture has become a significant source of income in areas where natural fishery productivity has declined (Alwash et al., 2022). Currently, molecular techniques are used in fisheries research, such as identifying and differentiating the stock and the pathogen's origin. One of them is identifying *Aeromonas hydrophila*, a heterotrophic, rod-shaped, gram-negative, free-living bacteria that range in size from 1.0 to 3.5 μm in length and 0.13 μm in diameter. Typically, this bacterium is found in freshwater, although it was also occasionally detected in seawater (Suhail et al., 2022).

Gene expression analysis is a common and successful technique for assessing the transcriptional activity of biological systems, determining disease-related cell states, and serving various other purposes (Lovén et al., 2012). An increasingly used method for analyzing gene expression is real-time polymerase chain reaction (PCR). The manufacturer (Promega Corporation) states that there are two main ways to do real-time PCR. One-step reverse transcription is used in the first method, which includes the reverse transcription step in the same tube as the PCR reaction. Two-step reverse transcription is used in the second method to create complementary deoxyribonucleic acid (cDNA), which is first generated through a separate reverse transcription reaction and then added to the PCR reaction.

A vast range of ribonucleic acid (RNA) types may be discovered inside cells because it acts as the template for protein synthesis. Messenger RNA (mRNA) is the most crucial component of life (Guo et al., 2023). Immunological balance during infection is essential for sustaining the body's immunological defense and limiting an overly aggressive immune response.

Honey bees make propolis, a viscous, waxy, resinous material (Farag et al., 2021). Propolis involves over 100 different substances (Anjum, 2019).

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Most ethanolic extracts from dark brown propolis consist of flavonoids, derivatives of cinnamic acid, and caffeoylquinic acid (Tani et al., 2019). This investigation aimed to investigate propolis's immunomodulatory effect on peritoneal macrophages in vitro production of cytokines, including interleukin-1beta (IL-1β) in common carp, *Cyprinus carpio* challenged with *Aeromonas hydrophilia*.

Materials and Methods

Experimental feeding: The experiment was conducted in the laboratory of Qasim Green University. A total of 40 common carp (average weight: 100±10 g/fish and total length: 15 cm) were randomly selected from fish farm ponds near AL-Musib, Babylon. The extract of crude propolis and the Water Ethanol Extract propolis (WEEP) propolis was prepared according to Eraslan et al. (2007). After 14 days of adaptation, fish were distributed into six treated groups in duplicate as follows: (1) two control groups (positive and negative), where fish were given a basic feed without propolis added, (2) T1: fish were fed a basal diet supplemented with 10 g/kg crude propolis, and (3) T2, (4) T3, and (5) T4: fish were fed WEEP at concentrations of 2, 4, and 8 g/kg diet for 56 days. Fish were given manual feed twice daily as desired, and the dissolved oxygen level was maintained at 6.82±0.2 mg/L.

***Aeromonas hydrophila* isolation**: Bacteria were isolated from the skin and kidneys of infected fish. The bacterial colonies were densely grown and incubated in a Rimler-shot medium on Brian heart infusion agar, 5% sheep blood agar, and MacConkey agar. The cultures were then incubated at 25°C for 24 hours under aerobic conditions. Biochemical characteristics and Avitek2 were used to identify the bacteria. The LD50 of *A. hydrophila* was cultured in Brain Heart Infusion Agar at 28°C for 24 hours, with the LD50 at a concentration of 0.1 ml (1.57×10^6 colony forming units CFU/ml).

Blood collection: Fish were anesthetized using clove powder at a concentration of 25 mg/L (Witeska et al., 2022). Blood samples were collected from fish randomly from caudal vein punctures using plastic syringes, and the samples were transferred into tubes containing K3EDTA.

Hematological parameters: According to Blaxhall and Daisley (1973), hemoglobin concentration was measured using the cyano-methemoglobin method. The packed cell volume was determined using hematocrit capillary tubes (Acharya and Mohanty, 2018). Total red blood and white blood cell counts were counted in mm^3 according to Khalifa (2023), and differential leukocyte counts were done according to Bajimaya et al. (2021).

Total RNA extraction: Total RNA extraction was performed according to the Promega company's instructions using the Easy-spin™ (DNA-free) total RNA extraction kit. The procedure involved preparing 50-100 mg of fresh tissue. The steps were: (1) Add 1 mL of lysis buffer (Easy-BLUE reagent) to the tissue sample, and use a homogenizer or similar device to homogenize the tissue. Stir vigorously at room temperature for 10 seconds, (2) add 200 µL of chloroform and vortex, (3) after centrifugation at 13,000 rpm (4°C) for 10 minutes, transfer 400 µL of the top fluid to an empty 1.5 ml tube, (4) mix 400 µL of binding buffer thoroughly with a pipette or gently invert 2-3 times. Do not centrifuge and let it sit for 1 minute at room temperature, (5) load the top solution to the column, but not all of it, as the column reservoirs can only hold 800 µL. Once the column has been loaded with the optimal amount of the upper solution, centrifuge for 30 seconds at 13,000 rpm, (6) after centrifuging, discard the flow-through and replace the spin column in the same 2 mL gathering tube, (7) perform the same action one more time, (8) fill the column with 700 µL of washing buffer A. To wash the column, carefully close the tubes and centrifuge for 30 seconds at 13,000 rpm, (9) reinstall the spin column in the same 2 ml collection tube after discarding the flow-through, (10) after adding 700 µL of washing buffer B to the column, centrifuge at 13,000 rpm for 30 seconds to wash, (11) reinstall the spin column in the same 2 ml collection tube after discarding the filtrates, (12) to dry the column membrane, centrifuge at 13,000 rpm for one to two minutes, (13) transfer the column to a sanitized 1.5 ml microcentrifuge tube (not

Table 1. Primers were used for gene expression experiments in this study.

Target gene	Primer name	5'−3'	PCR product	Reference	Accession number
IL-1B	F	AAGGAGGCCAGTGGCTCTGT	69 bp	Przybylska (2012)	AJ245635.1
	R	CCTGAAGAAGAGGAGGCTGTCA			
40S	F	GTTGAAGGAAGTGGCAAGGA	146 bp	Gonzalez et al. (2006)	AB012087.1
	R	AGAATACGGCCTCTGATGGA			

Table 2. Preparation steps of real-time PCR solutions.

Components	Concentration	Volume (20 µl)
Go−TaqTM qPCR master mix, 2X	1X	10 µl
Forward primer	10 µM/µl	2µl
Reverse primer	10 µM/µl	2 µl
GoScript^{TM} RT mix for one-step RT−qPCR	1X	0.4 µl
ddH_2O	-	3.6 µl
RNA template	250 ng	2µl

Table 3. Real-time PCR conditions (According to the instructions of the GoTaq® one-step RT-qPCR System).

Stage	Ta (°C)	Time	Cycles
Reverse transcription	42	15 min	1
RT inactivation/Hot-start activation	95	10 min	1X
Denaturation	95	10 sec.	40X
Annealing/data collection	60	30 sec.	
Extension	72	30 sec.	
Dissociation	72	2 min	1X

included) and immediately apply 50 µL of Elution Buffer to the membrane, and (14) after one minute of RT incubation, elute by centrifuging for one minute at 13,000 rpm.

Preparation of primers: To prepare the primers for storage at -20°C, they were initially dissolved in free ddH_2O to a final concentration of 100 pM/µL based on the company's recommendations and then created a 10 pM/µL concentration. The used primers are listed in Table 1.

Protocol of Go-Taq® One-Step RT-qPCR System for Real-Time qPCR (gene expression assay): The protocol used was (1) configuring a real-time device for one-step RT-qPCR in standard or fast mode (Table 3), (2) defrosting the RNA templates and primer pair, as well as the GoTaq One-Step RT-qPCR System components, either at ambient temperature or at 37°C; combining all thawed ingredients immediately; reducing the speed of the vortex mixer during mixing to minimize aeration; storing thawed chemicals refrigerated, (3) preparing RNA samples (mRNA, 500 fg to 100 ng) using water or any qPCR suitable diluent, (4) combining reaction components (Table 2) in a non-stick, sterile tube on ice; mixing gently after each addition and pipetting reaction volumes carefully onto an ice-covered plate, (5) transferring the plate from the ice into the pre-programmed instrument. Begin running immediately, and (6) gather the data and evaluate the results after the run.

Statistical analysis: The data was analyzed using the Complete Randomized Design (CRD) method and the pre-made statistical application in SAS. The LSD and Tukey tests were used to determine whether there were any significant differences between treatments.

Results and Discussion

Hematological parameters: Table 4 displays the impact of propolis on the blood parameters of common carp subjected to various propolis concentrations. The results showed a significant decrease in RBCs ($P \leq 0.05$) in T1, T2, T3, and T4 compared to the control group. The PCV% and Hb

Table 4. Hematological parameters of the common carp challenged with *Aeromonas hydrophilia* in different studied treatments.

Blood test	LYMPH%	MID%	WBCs×10^3 /mm^3	RBCs×10^6/mm^3	PCV%	HB (g/dL)
C-	94.67±0.88AB	3.20±1.50A	45.83±3.35B	0.79±0.01A	20.93±0.74A	6.93±0.24A
C+	96.53±0.47A	2.17±0.22A	59.23±7.21AB	0.69±0.06AB	17.23±1.55ABC	5.70±0.50ABC
T1CR	95.43±1.23AB	2.60±0.51A	46.93±8.12B	0.57±0.07BC	12.93±1.27CD	4.27±0.43CD
T2	94.77±0.84AB	3.20±0.50A	64.20±4.79A	0.73±0.04AB	20.10±1.05AB	6.67±0.37AB
T3	93.63±1.62AB	3.60±0.81A	56.07±2.51AB	0.64±0.05ABC	16.03±1.67BCD	5.33±0.55BCD
T4	91.83±2.84B	4.60±1.49A	48.10±2.93AB	0.47±0.09C	11.63±2.17D	3.83±0.72D
LSD	4.686	3.0062	0.1434	0.1833	4.5626	1.5211

recorded a significant increase in T2 compared to T1 and T4, while there was no significant difference in T3. All treatment groups showed a significant decrease compared to the C-group.

There was no significant difference ($P \geq 0.5$) in total leukocyte counts, but there was a significant increase in T2 compared to the C-group. However, there were no significant alterations ($P > 0.05$) in the granulocyte levels (LYMPH and MID). In WBCs, there was a significant increase in T2 compared to T1, while there were non-significant differences between the control (C-) and C+ groups and T3 and T4. In MID%, there was no significant difference between all treatment groups. LYMPH% showed a significant decrease in T4 compared to the C+ group.

The changes in blood parameters, including hemoglobin, packed cell volume, and RBC in T4 and T3 at 4 and 8 g/kg in common carp challenged to *Aeromonas* species produced a range of virulence factors, such as adhesins, cytotoxins, hemolysis, and enterotoxins (Martins et al., 2002). This infection led to anemia and nutritional deficiency (Choobkar et al., 2017), because *A. hydrophilia* can lyse RBCs (Subramani et al., 2016). The head of the kidney and spleen is the site of hematopoietic organs in fish (Maymounah, 2023).

The increased RBC in T2 (2 g/kg) for 56 days positively affected fish health and increased their Hb and PCV (6.67 g/dl and 20.10%). According to Orun and Erdogan (2014), propolis contains bioflavonoids and vitamins B1, B2, B6, C, E, and F. The level of mineral elements such as manganese, iron, calcium, aluminum, and vanadium varies based on the region and type of plant.

Using crude propolis in T1 decreased RBC, Hb, and PVC due to its high amount of wax and low concentration of propolis. This also resulted in decreases in PCV and Hb due to erythrocytes being damaged with increased leukocytic activity in common carp infected with *A. hydrophila*, which agrees with the findings of Choobkar et al. (2017).

To assess the effectiveness of an immunostimulant, it is crucial to evaluate the bacterial resistance of treated fish. Propolis has bactericidal and immunostimulant components in ethanol extract, which can increase the immunological response against *A. hydrophila*, leukocyte activity, and antibody titer in treated fish. It may also be used as an adjuvant or immunostimulant in fish. Based on the results, fish fed varying amounts of WEEP (experimental groups), the leukocyte differential count (MID%; basophil and eosinophil) and LYMPH% (lymphocytes, neutrophils, and monocytes) did not significantly differ from the control group ($P < 0.05$), similar to the findings of Alishahi et al. (2018).

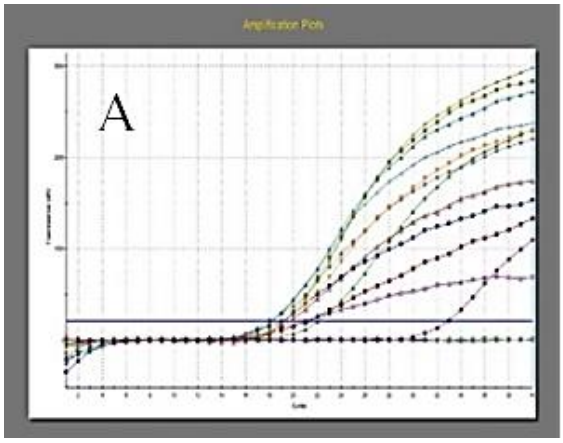
Immunological parameters

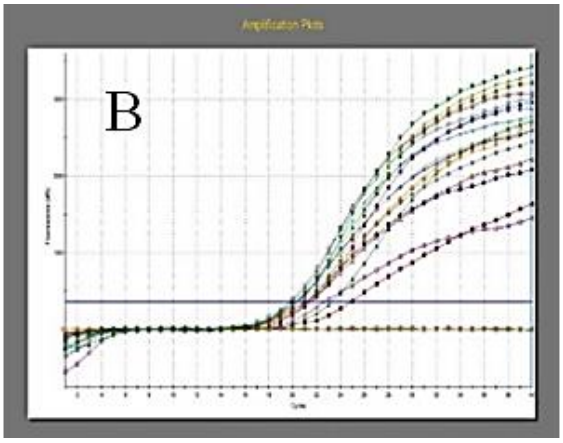
Expression of *IL-1β* and *40S* genes: Figure 1 presents amplification plots of the target *IL-1β* and *40S* genes in *C. carpio* challenged with *A. hydrophilia*. The studied pro-inflammatory cytokine is interleukin-1 beta (IL-1β), which is essential for generating local and systemic responses to immune activation, injury, and infection (Reis et al., 2012).

Inflammation (both acute and chronic) plays a role in the cause of autoimmune diseases. Excessive production of mediators, such as inflammatory cytokines, by macrophages, occurs with inflammatory diseases (Jung et al., 2007). A pleiotropic inflammatory cytokine, IL-1β, induces systemic and

Table 5. Tukey's multiple comparisons test between treats in the expression of IL-1β gene.

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	P
Control Negative vs. Control Positive	0.3946	-0.3731 to 1.162	No	Ns	0.3729
Control Negative vs. Propolis 10g/kg	0.04287	-0.7248 to 0.8106	No	Ns	0.9998
Control Negative vs. Extracted Propolis 2g/kg	-0.3379	-1.278 to 0.6023	No	Ns	0.6635
Control Negative vs. Extracted Propolis 4g/kg	0.6338	-0.1339 to 1.401	No	Ns	0.0999
Control Negative vs. Extracted Propolis 8g/kg	0.4454	-0.3223 to 1.213	No	Ns	0.2826
Control Positive vs. Propolis 10g/kg	-0.3517	-1.119 to 0.4160	No	Ns	0.4665
Control Positive vs. Extracted Propolis 2g/kg	-0.7325	-1.673 to 0.2077	No	Ns	0.1212
Control Positive vs. Extracted Propolis 4g/kg	0.2392	-0.5285 to 1.007	No	Ns	0.7629
Control Positive vs. Extracted Propolis 8g/kg	0.05082	-0.7169 to 0.8185	No	Ns	0.9996
Propolis 10g/kg diet vs. Extracted Propolis 2g/kg	-0.3808	-1.321 to 0.5594	No	Ns	0.5689
Propolis 10g/kg diet vs. Extracted Propolis 4g/kg	0.5909	-0.1768 to 1.359	No	Ns	0.126
Propolis 10g/kg diet vs. Extracted Propolis 8g/kg	0.4025	-0.3652 to 1.170	No	Ns	0.3573
Extracted Propolis 2g/kg diet vs. Extracted Propolis 4g/kg	0.9717	0.03146 to 1.912	Yes	*	0.0441
Extracted Propolis 2g/kg diet vs. Extracted Propolis 8g/kg	0.7833	-0.1569 to 1.724	No	Ns	0.0968
Extracted Propolis 4g/kg diet vs. Extracted Propolis 8g/kg	-0.1884	-0.9561 to 0.5793	No	Ns	0.8836





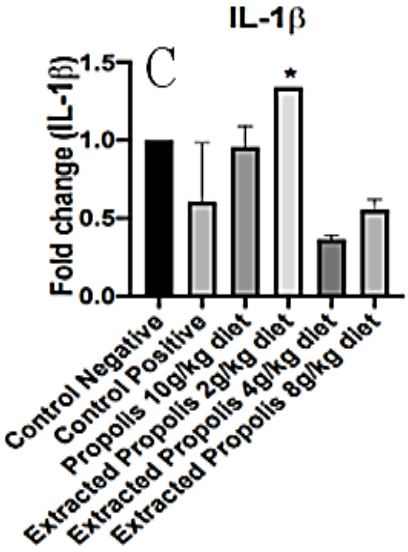


Figure 1. (A) Amplification plot of the target *IL-1β* gene, (B) amplification plot of the reference *40S* gene, and (C) gene expression analysis in *Cyprinus carpio*.

local responses to infection. It causes chemotactic and endothelial cells to produce adhesion molecules, which filter inflammatory and immune cells (Dinarello, 2009). When lipopolysaccharides (LPS) activate macrophages, a number of inflammatory mediators, including IL-6, IL-10, and IL-1β, are released (Parker and Schimmer, 2001).

There is no significant difference between

treatments in Table 5. The results showed a significant difference in the effects of propolis at treatment 2g/kg diet compared to a 4g/kg diet. Enhanced macrophages cultivated with propolis were shown to produce IL-1β in response to modest amounts of cinnamic and coumaric acids (2 g/kg) reported *in vitro*. A previous study from our laboratory has examined the immune system's reaction to propolis to comprehend how it works on the spleen cells' first phases of the immune response. This agrees with recent studies (Szliszka et al., 2013; Touzani et al., 2019; Rebouças-Silva et al., 2017; Xool-Tamayo et al., 2020) that demonstrated propolis's capacity to either increase or decrease pro-inflammatory cytokines. Additionally, the pro-inflammatory cytokines' action was dosage dependent because concentrations of 10 cruds, 4 WEEP, and 8 WEEP g/kg of propolis were needed to inhibit cytokine production. Zhang et al. (2014) reported that apigenin, a compound found in propolis, can decrease the mRNA levels of IL-1β and IL-6. Brazilian propolis extract, dissolved in DMSO, contains Artepillin C, which exhibits potent antioxidant activity by inhibiting the production of reactive nitrogen species (RNS), reactive oxygen species (ROS), nitric oxide (NO), and various cytokines, including IL-1β. This leads to Brazilian green propolis scavenging ROS and inhibiting chemiluminescence, exhibiting antioxidant properties (Szliszka et al., 2013).

The propolis can have pro-inflammatory and anti-inflammatory effects, depending on the dose, and modulate the immune/inflammatory response (Pagliarone et al., 2009a; Orsatti et al., 2010b). Our results suggest propolis's effect on cytokine production involves cinnamic and coumaric acids. The synergistic action of propolis's constituent parts may contribute to its medicinal benefits. It is essential to note that propolis's effectiveness in immunological tests can vary depending on the type of study, the amount used, and the incubation period (*in vitro* or *in vivo*). In conclusion, the findings showed that propolis does not affect macrophage viability, indicating that it is a naturally occurring chemical that is non-toxic to cells.

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