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ORIGINAL ARTICLE

Cell wall disruption, membrane damage, and decrease in the expression of Yrp1 virulence factor in *Yersinia ruckeri* by propolis ethanol extract

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ABSTRACT

Background and Objectives: Instead of antibiotics, propolis is a promising alternative for treating bacterial diseases. The aim of this study was to evaluate the effect of propolis ethanol extract (PEE) on *Yersinia ruckeri (Y. ruckeri)*, a fish pathogen, by examining its impact on the cell wall, cytoplasmic membrane, and gene expression.

Materials and Methods: The effect of propolis on the bacterial cell wall, membrane, and DNA using scanning electron microscopy (SEM) was investigated. Its effect on the NAD+/NADH ratio, reactive oxygen species (ROS) production, as well as the expression of a virulence factor (yrp1) was also determined.

Results: It was demonstrated that PEE has multiple antibacterial mechanisms against *Y. ruckeri* involving cell wall damage, membrane lysis, and a decrease in gene expression.

Conclusion: The obtained results indicated that the mode of propolis action against *Y. ruckeri* is both structural and functional, while others showed propolis only could inactivate bacteria in a structural way.

Keywords: Animals; *Yersinia ruckeri*; Propolis; Virulence factor; Anti-bacterial agents; Gene expression

INTRODUCTION

Propolis, bee glue, is a resinous mixture produced by honeybees from various parts of the plant such as flowers, leaves, buds, and exudates (1). The propolis is produced by masticating the resin and the addition of the salivary enzymes to the partially digested material (2). It contains natural mixtures of various secondary metabolites that are responsible for various bioactivities like antioxidant, antibacterial, anti-inflammatory, and anti-viral actions (3). The major ingredients of propolis collected from Iran have been characterized by many researchers (4-7). Propolis has a long history of medicinal use dating back to 350 BC during the time of Aristotle. The Greeks used propolis for wound healing, while the Egyptians used it for embalming the dead. Arabs and Persians also used propolis for treating various diseases. Today, propolis is widely used in traditional medicine for disinfecting wounds, treating burns, bedsores, and as a mouthwash (7).

So far, a number of scientists demonstrated that propolis has anti-bacterial characteristics. Veiga et al. (8) reported that propolis has an anti-bacterial effect on both Gram-negative and Gram-positive bacteria. Furthermore, Tukmechi et al. (5) investigated the anti-bacterial activity of Iranian propolis (IP) against some important fish pathogenic bacteria such as *Aeromonas hydrophila*, *Streptococcus iniae*, and *Yersinia ruckeri.*

Yersinia ruckeri is a Gram-negative bacterium that caused enteric red mouth disease (ERM) or yersini-

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osis in Salmonids. Serological methods are limited in the diagnosis of *Y. ruckeri* due to cross-reactivity with other fish-origin bacteria, while the polymerase chain reaction (PCR) is a fast and specific method for identifying this bacterium. Yersiniosis is an infectious disease in the rainbow trout farming industry that imposes economic losses in several countries as well as in Iran. Recently, multidrug-resistant (MDR) strains of *Yersinia ruckeri* emerged with growing frequency in rainbow trout farming in Iran (9-11). One of the most important virulence factors of the bacterium is serralysin metalloprotease (Yrp1) which previously has been described by Secades and Guijarror (12).

A significant step in the advancement of any new evidence-based antibacterial treatment is the clarification of its mechanism of action, specifically diagnosing the cellular function that is inhibited by the agent and the cellular structure that agent binds to. Clarification of these details lets anticipation of problems relating to clinical safety, and bacterial resistance, as well as a comprehension of antagonistic and synergistic drug interactions (13). According to Huang et al. (14) many scientists demonstrated the antimicrobial activity of propolis as only a match to their research, without ever determining the real mode of action. While Bryan et al. (2) indicated that the action mechanism of Russian PEE is structural rather than functional. It resulted that propolis is acting on bacterial DNA.

In the present study, Iranian PEE was used as an antibacterial substance against *Yersinia ruckeri* for investigating the mechanism of action. Specifically, the study aimed to investigate the effect of Iranian PEE on the integrity of the cell wall using scanning electron microscopy (SEM), as well as on important membrane functions and gene expression (Yrp1, a virulence gene) using quantitative polymerase chain reaction (qPCR).

MATERIALS AND METHODS

Preparation of Iranian PPE. Crude propolis samples were collected from the *Apis mellifera* carnica colonies placed in the apiaries of Iran. Hand-collected propolis was stored in a dry place at 4°C until its usage. The sample was cut into small pieces. Twenty five g of grounded propolis was extracted by 250 mL of 80% ethanol using orbital shaking by 150 rpm at 25°C for 48 h. The ethanol extract was filtered using a Whatman #42 filter paper. Propolis samples were dried, weighed, and then diluted in ethanol to obtain a 10% (w/v) solution. Samples were kept in the dark place at 4°C for use (5).

Bacterial strain and bactericidal activity testing. The type strain of *Y. ruckeri* (LMG 3279) was purchased from Belgian Co-ordinated Collections of Micro-organism. *Y. ruckeri* was cultured in tryptic soy broth medium (Merck, Germany) at 28ºC with a shaking speed of 200 rpm and it was used as inoculums. Minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) were determined using procedures of the Clinical and Laboratory Standards Institute (15). The culture at or larger than the MIC was plated onto the agar plate. The MBC was defined as the lowest concentration of Iranian PEE that resulted in a ≥99.9% reduction in growth compared to the starting test inoculums.

Time-kill studies. Time-kill assays were conducted with Iranian PEE and *Y. ruckeri* to find proper treatment times and concentrations for SEM studies. For assays, Mueller-Hinton broth (Merck, Germany) was selected as the growth medium. Sterile Erlenmeyer comprising 20 mL of growth medium only, 20 mL of growth medium supplemented with 2% (v/v) dimethyl sulfoxide (DMSO), and 20 mL of growth medium supplemented with Iranian PEE were inoculated with *Y. ruckeri* (final cell density: 5×10^5 CFU/mL bacteria). The extract was tested at 1×MIC, 1×MBC, and 2×MBC levels. This equated to 200 µg/mL (1×MIC), 400 µg/mL (1×MBC) and 800 µg/mL (2×MBC) for *Y. ruckeri*. Inoculated Erlenmeyer was incubated at 28ºC under aerobic conditions and with shaking (100 rpm). Viable counts were performed after several time intervals (0, 1, 2, 4, 8, 12, and 24 h) by removing 1 mL samples, preparing a dilution series in 0.1% (w/v) sterile normal saline (10^{-1}-10^{-8}) and plating out this dilution series (100 µL per dilution) on agar. Time-kill assays were conducted duplicate to confirm the reproducibility of the results (13).

Preparation of specimens for scanning electron microscope (SEM) analysis. Mueller-Hinton broth was applied as the growth medium and sterile Erlenmeyer containing 40 mL of the growth medium, 40 mL of growth medium added with 2% (v/v) DMSO, and 40 mL of growth medium complement-

ed with Iranian PEE were inoculated so as to contain 5×10^5 CFU/mL bacteria. *Y. ruckeri* was treated with 2×MBC (800 µg/mL) levels of the Iranian PEE and incubated at 28ºC under aerobic conditions for a time period of 8 h. Bacterial cells were collected at 6000 rpm centrifugation rate at 4ºC for 10 min. The pellets were fixed with 2.5% (w/v) glutaraldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.2) at 4ºC overnight. After the fixation process, the cells were washed three times with 1 mL of PBS (each cell for 10 min), and post-fixed with 1% (w/v) osmium tetroxide in PBS for 1 h. They were then washed three times with 1 mL of distilled water (for 10 min) and en-bloc stained in the dark with 5% (w/v) uranyl acetate for 1 h. This was continued by three further items of washing with 1 mL of distilled water (for 10 min). The cells were dehydrated by suspension in rising concentrations of aqueous acetone solution [20%, 40%, 60%, 80%, and 100% (v/v); twice at 100% (v/v)]. They were centrifuged at 2500 rpm for 5 min at 4ºC between each dehydration processes (13).

After the dehydration process in acetone, three 25 mL samples of bacterial suspension were withdrawn and used on the surface of three coverslips. These specimens first were exposed to air-dry, then coated with gold via a sputter coater. Morphology of the bacterial cells was analyzed using SEM images (XL 30 FEG, Philips, Netherlands). Bacterial cell length was calculated using Image J software (version 1.46).

NAD^+/NADH extraction, NAD^+ cycling assay, and ROS generation. The dinucleotide extraction and cycling assay were conducted using the protocols proposed by Kohanski et al. (16). To extract NAD^+ and NADH, 1 mL samples were collected from *Y. ruckeri* cultures every half hour between 0 and 3 hours after adding the Iranian PEE at a concentration of 200 µg/mL (1×MIC). The samples were collected by centrifugation at 13,000 rpm for 1 minute. The supernatant was removed and the pellets frizzed instantly in a dry ice-ethanol bath. The pellets were stored at -80ºC. 75 mL of 0.2 M HCl (for NAD^+ extraction) or 25 mL of 0.2 M NaOH (for NADH extraction) were added to the ice-cold pellets. The samples were first heated at 100ºC for 10 min and then centrifuged at 10,000 rpm for 5 min. The NAD^+/NADH containing supernatant was transferred to fresh tubes and kept in a dark place on ice until its usage time.

The NAD^+ cycling assay was performed in a 96-well plate. The reaction mixture contained 40 mL sample extract, 16 mL of bicine (1.0 M, pH 8.0; Sigma-Aldrich, USA), 40 mL neutralizing buffer, 16 mL phenazine methosulfate (Sigma-Aldrich, USA), 16 mL 3-(4,5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT; Merck, Germany), 16 mL 100% ethanol and, 30 mL of 40 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0). Finally, phenazine methosulfate (PES) and MTT were added to the 96-well plate and were incubated for 3 min at 30ºC. 3.2 mL of alcohol dehydrogenase (500 U/mL; Sigma-Aldrich, USA) was added to the bicine buffer (pH 8.0). This prepared solution was added to the reaction mixture to start the assay. The increased absorbance was recorded at 570 nm within 10 min. Notably, the rate of MTT reduction relates to the concentration of NAD^+ or NADH in the sample. NAD^+ and NADH standards (Sigma-Aldrich, USA) can be used between 0.0375 and 0.75 nanomole to calibrate the assay.

A Reactive Oxygen Species Assay Kit (Sigma-Aldrich, USA) was used based on 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) to determine the generation of ROS. Bacterial samples were collected in the logarithmic phase after treatment with 200 µg/mL of Iranian PEE for 4 h by centrifugation and stained with 10 mM DCFH-DA. The fluorescence intensity was estimated using a microplate reader (BMG Labtech, Germany) with excitation at 488 nm and emission at 525 nm.

Virulence gene expression. The expression of a virulence gene (*yrp1*) was evaluated in *Y. ruckeri* treated and untreated with Iranian PEE by qPCR. Briefly, 18 h culture of *Y. ruckeri* was treated with a 100 µg/mL sub-inhibitory concentration of Iranian PEE at 28°C in aerobic conditions. After incubation, bacterial cells were centrifuged at 8000 rpm for 5 min and washed with PBS. Total cellular RNA was extracted by Trizol® (Yektatazhiz, Iran). DNase I treatment as well as reverse transcription of the RNA samples was carried out using instructions of the cDNA Synthesis Kit (RevertAid First Strand, Thermofhsher, USA). The expression of *yrp1* gene was quantified by a real-time PCR assay using SYBR green master mix (Pishgam, Iran) and the primer sets (Table 2). Housekeeping genes involving 16S rRNA and *recA* were used as references for data normalization. The difference in gene expression between Iranian PEE treated and untreated cells was determined using the $2^{-\Delta\Delta Ct}$ formula. It means $\Delta\Delta Ct = \Delta Ct_{(\text{treated sample})} - \Delta Ct_{(\text{untreated sample})}$, where, $\Delta Ct = Ct$,

–Ct, and Ct are target genes, 16S rRNA or *recA*, and the threshold cycle value of the amplified target or reference genes, respectively (17-19).

Statistical analysis. The data collected were analyzed using ANOVA and Tukey's HSD test in SPSS software Version 24, with statistical significance determined at $p<0.05$.

RESULTS

Antimicrobial activity of Iranian PEE against *Y. ruckeri*. It was shown that Iranian PEE inhibited the growth of *Y. ruckeri* at concentration from 200 µg/mL. Since ethanol extract of propolis was evaluated in this study, ethanol 96% (v/v), was used as solvent control. Ethanol did not show any inhibition against the *Y. ruckeri*, suggesting the antimicrobial effect was due to the Iranian propolis. Correspondingly MBC of Iranian PEE was obtained from 400 µg/mL. Agar well diffusion method was applied to determine the zone of inhibition. The minimum concentrations of propolis that allowed measurement of the diameter of the inhibition zone was 200 µg/mL (9 mm; Fig. 1). Enrofloxacin was used as a control antimicrobial drug for each assay (Table 1).

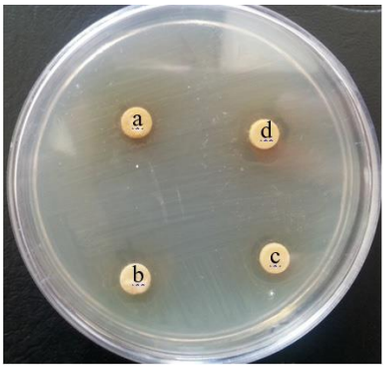

Fig. 1. Visible inhibition zone (mm) produced by Iranian PEE against *Y. ruckeri*: a) 200 µg/mL; b) 400 µg/mL; c) 600 µg/mL and d) 800 µg/mL.

Table 1. The results of the evaluation of the antimicrobial activity of Iranian PEE against *Y. ruckeri.*

Substance	MIC (µg/ml)	MBC (µg/ml)	Zone of inhibition (mm)
Iranian PEE	400	800	9
Enrofloxacin	62.5	125	33

Time-kill studies. Iranian PEE showed a concentration-dependent influence against *Y. ruckeri* (Fig. 2). Bactericidal activity was first recorded at a treatment time of 8 h and a concentration of 2×MBC. These treatment time and concentration were selected for use in the following SEM study to know how Iranian PEE kills *Y. ruckeri.*

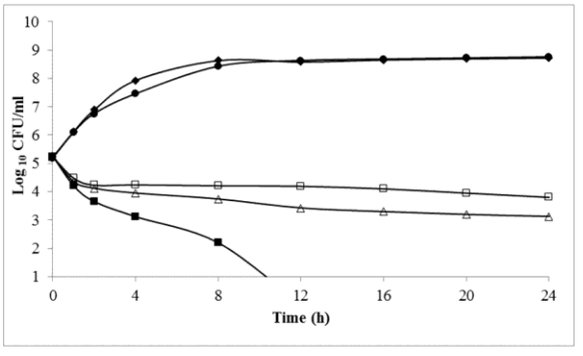

Fig. 2. The effect of Iranian PEE on *Y. ruckeri* (5×10^5 CFU/ml).◆: Growth medium; ●: 2% (v/v) ethanol; □: 1×MIC propolis; ◘: 1×MBC propolis; ■: 2×MBC propolis.

SEM analysis of treated and untreated *Y. ruckeri.* SEM examination of control *Y. ruckeri* (treated with DMSO) showed these cells had not been deformed by the DMSO. The bacteria were rod-shaped in their morphology. They had typical dimensions, a smooth cell surface, and were present in large numbers (Fig 3b). Specimens of *Y. ruckeri* treated with 2×MBC Iranian PEE had very different shapes and appearances. These bacteria were meaningfully enlarged (up to 1.2-fold than control cells; Fig. 3c) and many had deformity and swelling.

Catabolic NADH and ROS generation. Our results showed that treatment with a bactericidal concentration of Iranian PEE significantly induced an apparent change in NAD^+/NADH ratio in *Y. ruckeri* (Fig. 4a). Also, the cells' ROS content increased after 2 h of treatment with the same concentration of the propolis (Fig. 4b). Additionally, within 30 min an obvious increase of ROS was observed in the cells treated with enrofloxacin (62.5 µg/mL; Fig. 4b).

Gene expression. To study the effect of Iranian PEE on *Y. ruckeri*, the expression of the gene involved in virulence (*yrp1*) was examined by qPCR. For expression analysis, *Y. ruckeri* cells in the exponential phase were exposed to a sub-inhibitory concentration of Iranian PEE (100 µg/mL) for 18 hours. In comparison,

Table 2. The sequences of primers set that were used for amplification of *yrp1*, 16s rRNA, and *recA* genes of *Y. rucker* in qPCR.

Primer	Sequence	Product size (bp)	Reference
Yrp1-F	5′-TGCGCAAACCAATATCAGCG-3′	477	17
Yrp1-R	5′-TGCGCAAACCAATATCAGCG-3′		
16s rRNA-F	5′-TTTGTTGCCAGCACGTAATGGT-3′	148	18
16s rRNA-R	5′-GCGAGTTCGCTTCACTTTGTATCT-3′		
recA-F	5′-TCTGGACATCGCTCTGG-3′	188	19
recA-R	5′-AGTTTTTTTGCGTAGATAGGA-3′		

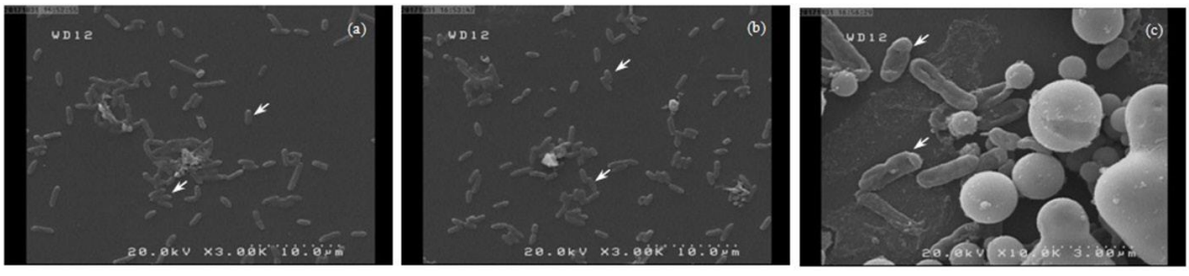

Fig. 3. SEM Images of *Y. ruckeri* showing treatment with 2% (v/v) DMSO (control: a and b) and 2×MBC propolis. Arrowheads indicate the location of normal cells in control (a and b), and the location of treated cells with enlarged and deformity appearance (image c).

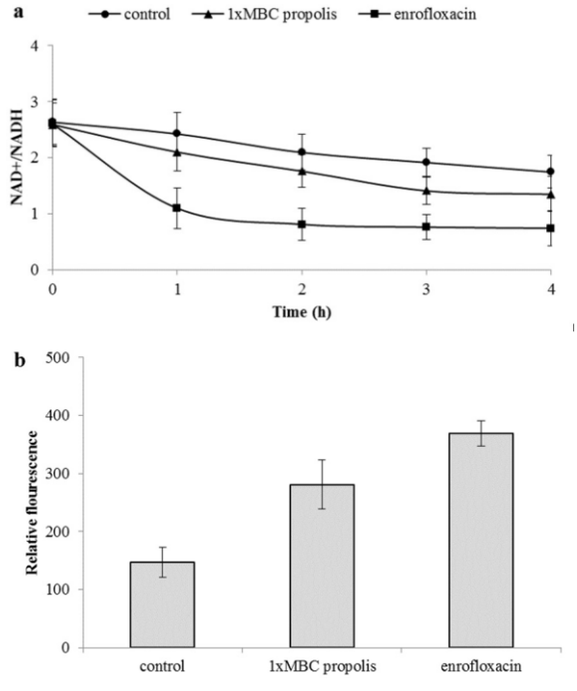

Fig. 4. (a): The NAD^+/NADH ratio for treated (Iranian PEE and enrofloxacin) and untreated (control) *Y. ruckeri* at different treatment times, (b): Determination of ROS in untreated and treated *Y. ruckeri*. All data were shown as the mean ± standard deviation of triplicate examinations.

treatment with Iranian PEE down-regulate 22-fold the expression of *yrp1* in the untreated cells (Fig. 5a). Our results showed transcription of housekeeping genes *recA* and 16s rRNA were not significantly affected (Fig. 5b).

DISCUSSION

Even though *Y. ruckeri* is sensitive to some antibiotics, there is an ever-increasing lot of reports about acquired resistance of *Y. ruckeri* strains to different antibacterial agents. Currently, the most used antibiotics against yersiniosis (red mouth disease) in rain-

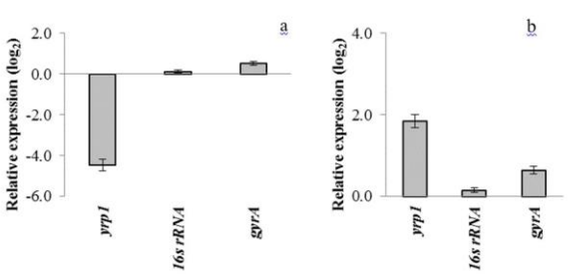

Fig. 5. Different virulence gene expressions between Iranian PEE treated (a) and untreated (b) *Y. ruckeri*. ΔΔCT of each gene is presented in $\log_2$ value.

bow trout farms in Iran are florfenicol and quinolones (especially enrofloxacin). It was reported by some researchers that some isolates of *Y. ruckeri* are fully resistant to therapeutic doses of sulfonamides, tetracycline, florfenicol, ampicillin, gentamicin, amoxicillin, doxycycline, and ciprofloxacin (20, 21). Due to the bacterium's capacity to acquire antibiotics resistance, use of quinolones must be limited and they should be used in emergency cases only. Furthermore, *Y. ruckeri* belongs to the *Enterobacteriaceae* family which includes a number of important human pathogens. In this way, it is very presumed that transport of antibiotic resistance could occur. Moreover, because of environmental and health viewpoints, the application of antibiotics in the aquaculture industry must be reduced.

The antimicrobial activity of propolis has been confirmed against fungi, viruses, bacteria, and protozoa (22). To date, the antibacterial efficacy of propolis has been examined against more than 600 different bacterial strains. Although numerous research data documented that propolis was more potent against Gram-positive bacteria compared to Gram-negative bacteria (23). Notably, Middle Eastern propolis was exceptionally effective against both Gram-negative and Gram-positive bacteria (24). Also, in the previous work, we indicated that Iranian propolis was effective against *Y. ruckeri* and its MIC and MBC were 390.62 and 1562.5 µg/mL respectively (5). In this study, the same standard strain of the bacterium was tested. However, different outputs were obtained in relation to Iranian PEE antibacterial efficacy (Table 1). In explaining this, the antibacterial activity of propolis is dependent on plant species, seasonal, geographic location factors and etc.

The results of the time-kill assay of Iranian PEE confirmed the results obtained from antimicrobial analysis against *Y. ruckeri.* A time-kill assay is a kinetic technique to determine the ability to kill in relation to time and with various concentrations of antimicrobial substances. As can be seen in Fig. 2 bacterial control cultures (growth medium and ethanol) grew during 24 hours of evaluation. But bacterial cultures in contact with 1×MIC, 1×MBC, and 2×MBC began to decrease after a few hours of contact. The most effective Iranian PEE concentration was 2×MBC (800 µg/ml), which significantly inhibited the growth of *Y. ruckeri* after 8 h from the moment of contact ($P<0.05$). Although, it is the first investigation that the time-kill assay was performed for propolis against *Y. ruckeri*, some researchers have obtained similar results for other bacteria (25-28). All studies confirmed the results obtained in this research. The optimal concentrations of propolis against a number of bacteria were greater than or equal to 2×MIC (29) and the effective time of exposure was between 4 and 8 h from the first hour of contact (30). These results suggested that the time-kill assay was dependent on the test bacterial strain and chemical composition of the propolis.

In order to estimate the bactericidal characteristics of Iranian PEE, SEM images were used to determine the cell wall integrity of the treated *Y. ruckeri.* Fig. 3 shows dramatic damage to the cell wall after 8 h of exposure to 800 µg/mL of propolis. SEM images provide reasonable evidence for the opportunity of cell lysis in *Y. ruckeri* as a result of the propolis. Bryan et al. (2) documented that one of the bactericidal mechanisms of action of Russian propolis against *Staphylococcus aureus* and *Escherichia coli* was owing to cell lysis. They explained how the interaction between propolis and bacterial cell wall can occur. These researchers by conducting the large unilamellar vesicles (LUV) experiments, showed propolis interacts with the lipid molecules in the bacterial cell walls and induces leakage and disruption. Therefore, on the base of our SEM imaging and the above scientists' results, it is suggested that one of the mechanisms of action of propolis is structural.

Interestingly, we observed propolis decreased the NAD+/NADH ratio in *Y. ruckeri* within 1 h from the contact (Fig. 4a). In comparison to the control, a significant decrease in the NAD^+/NADH ratio was seen after 3 h from the moment of contact (>2-fold). Enrofloxacin significantly decreased the NAD+/NADH ratio after 1 h from the exposure (>4-fold than the control). These findings agree with other observations that some antibiotics lead to an increase in the NADH levels in bacteria (31, 32). The NAD^+/NADH ratio has a key role in the metabolic state of bacteria and is thus considered an important antibacterial agent target. Besides, the reduction of the NAD^+/NADH ratio endorses the formation of ROS that contribute to bacterial death (33). Our results indicated propolis increased ROS production in *Y. ruckeri* (Fig 4b). Based on these results, another antibacterial mechanism of Iranian PEE in *Y. ruckeri* is membrane damage.

From the transcriptional analysis of the virulence gene, we found the expression of *yrp1* was significantly decreased in response to a sub-inhibitory con-

centration of propolis. *yrp1* encodes an extracellular protease that is a clear virulence factor in *Y. ruckeri* (34). In support of our findings, previous studies proved that the sub-inhibitory concentration of propolis could down-regulate the expression of bacterial important genes. For example, Veloz et al. (35) documented that polyphenol-riched extract of propolis reduces the expression of glucosyltransferases in *Streptococcus mutans.*

There are a lot of reports that demonstrate some antimicrobial agents decrease the bacterial virulence gene expression at sub-inhibitory concentration (36-38). These findings are important evidence that another antibacterial mechanism of propolis is modulation of the gene expression.

CONCLUSION

Based on the obtained results, the authors propose multiple mechanisms for the action of Iranian PEE on *Y. ruckeri* due to (A) propolis interacts with constructional lipids, which induces cell wall damage, disrupts bacterial membrane integrity, and leads to membrane leakage; (B) bactericidal concentration of propolis changes the cellular NAD+/NADH ratio and causes the production of ROS resulting bacterial death and (C): this natural substance down-regulate virulence gene expression at sub-MIC concentration.

Results of this study suggests that Iranian propolis has strong antibacterial activity against an important fish pathogen mainly by disrupting cell wall formation, changing membrane integrity and causing membrane leakage, ROS production and DNA damage.

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