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Comparison of the adjuvant effect of propolis and Freund on the efficacy of *Aeromonas hydrophila* vaccine in common carp (*Cyprinus carpio*)

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Abstract

The success of many vaccines relies on their association with selected adjuvants in order to increase their immunogenicity and ensure long-term protection. Propolis is a natural compound, mostly known for its immunostimulatory properties. In this study the adjuvant effects of propolis in combination with formalin-killed *Aeromonas hydrophila* vaccine in Common carp (*Cyprinus carpio*) were evaluated and compared with Freund's adjuvant. Three hundred juvenile carp (weighing 50.4±3.2 g) were divided randomly into four groups in triplicates. The fish were intraperitonealy injected with *A. hydrophila* bacterin (A.h) in combination with normal saline (Formalin Killed Bacterin, FKB group), Freund (FKB +F group), and Propolis (FKB +P group) respectively. The control group was injected with normal saline. Serum samples were taken from fish in each group every other week (days 0, 14, 28 and 42) of the experiment and immunological parameters including anti *A. hydrophila* antibody titer, serum lysozyme and antibacterial activity, complement activity, Nitro blue tetrazolium activity and serum total protein and globulin were compared among the groups. At the end of study the remaining fish in each group were challenged with virulent *A. hydrophila* and mortality was recorded for 10 days and Relative Percentage Survival (RPS) was calculated and compared among the groups. Results showed that although antibody titer and most of none specific immune responses increased in groups 2 and 3 compare to control group ($p<0.05$), no significant difference were seen in group 1 and 3 ($p>0.05$). Besides no significant change was observed in mortality rate following the challenge in the propolis adjuvanted group compared with the FKB group ($p>0.05$). According to results, it can be concluded that although propolis as an adjuvant can promote some immune responses of common carp, it can't affect efficacy of vaccine and Ab titer of injected antigens, so it seems more work is needed to present propolis as a proper candidate for the development of a natural adjuvant in fish vaccines.

Keywords: Propolis, Common carp, Adjuvant, *Aeromonas hydrophila*, Freund adjuvant, Killed vaccine

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Introduction

Active immunization or vaccination is the process of preventing or reducing the manifestations of a pathogenic infectious agent in humans or animals (Aguilar and Rodriguez, 2007). The design of potent vaccines aims to induce a strong, long lasting, and highly specific immune response against the targeted pathogen (Aguilar and Rodriguez, 2007). Disease prevention by vaccination is, on economic, environmental and ethical grounds the most appropriate method for pathogen control currently available to the aquaculture sector. Most commercial vaccines are comprised of inactivated pathogens. Inactivated vaccines (particularly *Aeromonas hydrophila*) based on either killed pathogens are in many cases, weakly immunogenic. Thus, adjuvants or immunopotentiators, are highly required for the elicitation of immune responses that may be 100% protective against certain pathogens (Harikrishnan *et al*., 2009). In general, adjuvants are chemical substances that boost the immune response against the associated antigens Moreover, the use of adjuvants may reduce the required amount of antigen or the number of immunization doses necessary to induce a protective immune response and improve the efficacy of vaccines in immuno-compromised candidates.

Freund's adjuvant is a well-known classical oil based adjuvant with strong adjuvanticity effects that has been used for years in veterinary vaccines (Gjessing *et al*., 2012). Also different adverse effects including granuloma formation, necrosis and tissue impairment have been observed with Freund's adjuvant (Jiao *et al*., 2010).

Propolis is a dark resinous material that honey bees produce by mixing saliva and beewax with exudate gathered from tree buds. Its color varies between green, red and brown. Although its composition slightly varies between regions and among seasons as a result of the variation in flora, in general it is composed of 50% resin and balsams, 30% wax, 10% essential and aromatic oils, 5% pollen grains, and other substances. Bees use it to seal holes in their comb and to embalm carcasses of invading insects to mummify them and prevent their putrefaction. Moreover, it protects the bee colony from diseases through its antiseptic and antimicrobial properties (Sforcin and Bankova, 2011).

A. hydrophila has been recognized as an opportunistic pathogen of a wild variety of hosts (Yin *et al*., 2009). *A.hydrophila* caused critical damage in pond culture, mainly common carp). In the last decade, the disease has caused extensive losses in cultured freshwater fish (Xia *et al*., 2004), so pathogenic *A. hydrophila* have become the most important pathogenic bacteria for cyprinoids fish. Due to resistance to antibiotics the use of vaccines in the aquaculture industry has been important in reducing economic losses which occur as a result of disease and reduction in the use of antibiotics (Hatha *et al*., 2005). A number of different types of vaccines have been developed against *A. hydrophila*. One of the best strategies against

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aeromonads infection is vaccination. There are lots of local vaccines against *A. hydrophila* in different parts of the world. Although these different preparations have provided varying degrees of protection in fish, there is no commercial vaccine available for *A. hydrophila* (Fang *et al*., 2004).

Then in this study formalin killed *A. hydrophila* were used along with a natural adjuvant (Propolis) and a positive control (Freund), to evaluate the specific and non-specific immune responses of common carp, as well as the efficacy of vaccines with different adjuvants after 6 weeks.

Materials and methods

Fish

Common carp (50.4±3.2 g) were obtained from a fish farm in Ahvaz, Iran. Fish were adapted for 2 weeks in 100 liter glass aquaria filled with chlorine free water and supplied continuous aeration using electronic air pumping compressors and fed with commercial pelleted diet twice a day. The basal practical diet was formulated to contain approximately 37 % crude protein and 9 % lipids, which has been shown to be sufficient to support the optimal growth of juvenile *T. grypus*. Water quality factors were recorded during the experiment as: temperature, 25±1°C; dissolved oxygen, 8-10 ppm; pH, 7.9±0.3; NO_2, <0.01ppm and NH_3, <0.1 ppm. Water exchange rate was 20% of water volume daily.

Preparation of killed Aeromonas hydrophila bacterin

A. hydrophila AH04 was kindly donated by Professor Soltani, Tehran University, Iran. Preparation of the vaccine was performed according to Soltani *et al*. (2007). One colony of *A. hydrophila* isolate was inoculated in trypticase soya broth and inoculated at 30ºC for 24h. The broth culture flask was checked for purity and the total colony count was adjusted to 10^{10}. Bacteria were inactivated by 0.5% formalin. Formalin was removed by washing the bacterial solution with normal saline.

Propolis adjuvant preparation

Alcoholic extracted of propolis was obtained as described by Xiao (Xiao *et al*., 2007). Briefly, 15g of propolis collected from Khozestan Provine was suspended in 60 ml of ethanol 95% by shaking at 25ºC for 1 day in a water bath. Subsequently, the extract of propolis was filtered through a sterile 0.4µm membranes and used as stock solution. Before use, the propolis was resuspended in PBS at a concentration of 30 mg ml^{-1} and it was used with killed *A. hydrophila* at a ratio of 1:1.

Experimental design

For the evaluation of the adjuvant effects of propolis, four groups in triplicates were designed as follows: Fish in group 1, immunized with formalin killed Bacterin (FKB), without adjuvant. Fish in groups 2 and 3 immunized with FKB adjuvanted with Freund and propolis respectively (FKB + F and FKB+ propolis). Control group injected with PBS in the same way of

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other groups without bacterin and adjuvant.

Injection of vaccine was done interapritoneally on day 0 and 14. Before injection all fish were anesthesized with phenoxy ethanol (300 mg L^{-1}). Fish were injected intraperitoneally with 0.2 ml of vaccine and after injection, each group of fish was kept in a separate aquarium and fed daily for 42 days. Nine fish were randomly collected from each group on days 0, 14, 28 and 42 of the experiment and anesthetized with 100 ppm MS-222 in de-chlorinated water. Blood samples were taken from the caudal vein with a 2cc sterile syringe. Heparinized blood was used for hematological assays. Sera were separated from blood samples via centrifugation, for immunological assays. The sera were stored at -80 ºC until used.

Immune responses parameters

Total protein and globulin concentrations were determined in each group (Zist Shimi kit Iran) according to Nayak *et al*.(2008) and Sahoo *et al*. (2008).

Bacterial microagglutination titer (MAT)

The agglutination test was conducted in 'U' shaped microtiter plates. Two-fold serial dilutions of the 25 ml serum of fish was made with an equal volumes of PBS in each well, to which, 25 ml of formalin-killed *A. hydrophila* (10^7 cells ml^{-1}) suspension was added. The plates were incubated overnight at room temperature. The titer was calculated as the reciprocal of the highest dilution (based on log_2) of serum showing complete agglutination of the bacterial cells (Swain *et al*., 2006).

Lysozyme activity

Serum lysozyme activity was measured as described by Ellis (1990). Briefly, 10 µl of serum was mixed with 200 µl of a *Micrococcus lisodeichticus* (Sigma) suspension at 0.2 mg ml in 0.05 M sodium phosphate buffer (pH 6.2). The mixture was incubated at 27 °C, and its OD was detected after 1 and 6 min at 530 nm using an ELISA (enzyme-linked immunosorbent assay) plate reader. One unit of lysozyme activity was defined as the amount of enzyme that produced a decrease in absorbance of 0.001 min ml^{-1} serum.

Nitro blue tetrazolium assay (NBT)

The respiratory burst activity was measured by the reduction of nitro blue tetrazolium (NBT) by intracellular superoxide radicals (Anderson and Siwicki, 1994). Briefly, 100 ml of heparinised blood from fish of each group was mixed with 100 ml of 0.2% NBT (Sigma, USA) solution for 30 min at 25 ˚C. After incubation, 50 ml from the above mixture was added with 1 ml of N,N diethylmethyl formamide (Qualigens, India) and then centrifuged at 3000 g for 5 min. The optical density of the supernatant was measured at 540 nm.

Alternative complement activity

The complement activity was assayed using Rabbit Red blood cells (RaBRC) as target. RaBRC was provided in 1.5% agaros (pH 7.2 containing 0.5 mM

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$MgCl_2$, 1.5 mM $CaCl_2$). RaBRC in agaros were washed with PBS by centrifugation at 750g for 5min and the cell concentration is adjusted to 1×108 cell ml^{-1}. Agaros containing RaBRC was dispensed into plate and were incubated at 4ºC overnight before agaros was punched (diameter 3mm). Subsequently each hole was filled with 20 microliter of serum of *Barbus grypus* and they were incubated at room temperature for 48h. After 48h diameter of lysis was measured (Navinchandran *et al*., 2014).

Serum bactericidal activity (SBA)

Bactericidal activity was studied following procedure by (Kajita *et al*., 1990) with slight modifications. Sera samples were diluted three times with 0.1% gelatin-veronal buffer (GVBC2) (v/v), (pH 7.5, containing 0.5 mM ml^{-1} Mg^{2+} and 0.15 mM ml^{-1} Ca^{2+}). *A. hydrophila* (live, washed cells) suspended in the same buffer at a concentration of 105 CFU ml^{-1}. The diluted sera and bacteria were mixed at 1:1 v/v, incubated for 90 min at 25 ºC on a shaker. The control group containing bacterial suspension was also included. The number of viable bacteria was then determined by counting the colonies after culturing on Tripticase Soy Agar (TSA) plates for 24 h at room temperature 25 ºC.

Total serum protein and globulin

Samples were analyzed for total protein using the method outlined by Lowry *et al*. (1951). Albumin content was measured using a standard albumin estimation kit (Zistchem Diagnostics, Iran) and the globulin content was estimated by subtracting albumin from total protein.

White blood cell count

White blood cell count (WBC) was calculated in a Neubauer counting chamber as described by Schaperclaus *et al*.(1991).

Challenge experiment

Challenge with virulent *A. hydrophila* were done after 42 days post–vaccination. Fish from each group were injected intraperitoneally with 100µl of live and virolent *A. hydrophila* at a LC50 dose concentration of 2×108 CFU ml-1. The corresponding control fish received 0.1 ml of PBS (n =24). All the groups were maintained separately in triplicate tanks for 10 days,

Mortality of challenged fish was recorded daily for 14 days. The cause of death was ascertained by re-isolating the infecting organism from kidney of dead fish according to (Deng *et al*., 2011).

Statistical analysis

Statistical analyses were performed using SPSS 19 software. Data were tested for normal distribution with Shapiroe Wilk's test and for homogeneous variance with Levene's test. Differences among the extract supplemented fed and control groups were tested with One-Way ANOVA followed by Duncan's multiple range test using a probability level of 0.05.

Ressult

Bacterial agglutination titer

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Effect of propolis and freund adjuvants on anti *A. hydrophila* agglutination titer is showed in Fig. 1. All Immunized fish showed significant increase in anti *A. hydrophila* antibody titer on days 14, 28 and 42 compare with control group (p<0.05). No significant difference in anti *A. hydrophila* antibody titer observed among three vaccinated groups regardless to their adjuvants (p>0.05).

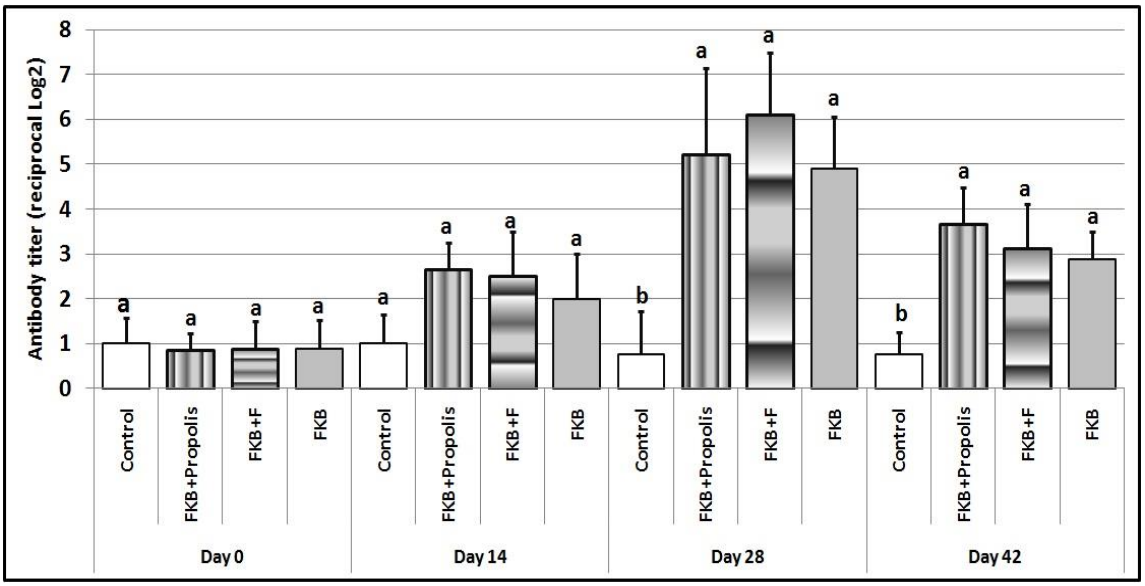

Figure 1: The effect of different vaccine adjuvants on anti *Aeromonas hydrophila* antibody titer in vaccinated common carp. Data showed as Mean±SD. FKB+Propolis: Carp vaccinated with FKB in combination with propolis as adjuvant, FKB+F: Carp vaccinated with FKB in combination with freund adjuvant, FKB: carp vaccinated with Formalin Killed Bacterin without adjuvant. Significant differences (p<0.05) are marked by different letters.

Lysozyme activity

Although relative increases in serum lysozyme activity were seen in vaccinated groups compared with the control, these increases were not significant. Increases in lysozyme activity in the FKB+propolis group were recorded on day 28 of the experiment (Table 1).

Respiratory burst (NBT)

On day 28 of the trial a significant increase in NBT was observed in FKB+Propolis and FKB+Freund groups (p<0.05) compared with the control group (Table 1), but on day 42 of the experiment the NBT activity significantly increased only in the FKB+propolis group (p<0.05).

Serum bactericidal activity

The bactericidal activity of *Cyprinus carpio* serum against *A. hydrophyla* was increased in all vaccinated groups on day 14 of the experiment and in the FKB+ Propolis and FKB+Freund groups on day 28 compared with the control. An significant increase in the serum bactericidal activity was seen only in the FKB+Freund group on day 42 of the experiment (p<0.05).

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Table 1: The effect of different vaccine adjuvants on some immunological indices in vaccinated common carp in four sampling points. Data showed as Mean±SD, n=15. FKB+Propolis: Carp vaccinated with FKB in combination with propolis as adjuvant, FKB+F: Carp vaccinated with FKB in combination with freund adjuvant, FKB: carp vaccinated with Formalin Killed Bacterin without adjuvant. Significant differences (p<0.05) are marked by different letters.

day	groups	Lysozyme	bactericidal activity	WBC	Complement	NBT	Total protein	Total globulin	Albomin
Day 0	Control	172.37±25.3^{a}	61.57±14.95^{a}	8.36±0.91^{a}	19.65±6.37^{a}	0.13±0.01^{a}	3.84±0.68^{a}	1.74±0.34^{a}	2.84±0.51^{a}
	FKB+Propolis	176.05±26.8 ^{a}	63.75±14.38^{a}	8.03±2.14^{a}	19.95±5.83^{a}	0.13±0.02^{a}	3.64±0.74^{a}	1.61±0.26^{a}	2.64±0.50^{a}
	FKB+F	183.95±32.6 ^{a}	65.57±15.68^{a}	8.75±1.29^{a}	18.16±3.64^{a}	0.13±0.03^{a}	3.86±0.67^{a}	1.67±0.32^{a}	2.86±0.50^{a}
	FKB	179.37±27.8 ^{a}	64.50±9.89^{a}	8.78±0.77^{a}	18.61±2.6 ^{a}	0.12±0.02^{a}	3.51±0.61^{a}	1.70±0.35^{a}	2.51±0.48^{a}
Day 14	Control	190.18±19.4^{a}	65.14±12.7^{b}	8.24±2.73^{b}	18.41±6.11^{a}	0.12±0.04^{a}	3.96±0.94^{b}	1.60±0.20^{b}	2.96±0.57^{a}
	FKB+Propolis	236.80±49.2^{a}	55.71±12.9^{a}	14.01±2.17^{a}	23.44±7.71^{a}	0.14±0.06^{a}	4.55±0.83^{a}	2.17±0.72^{a}	3.05±0.77^{a}
	FKB+F	209.40±16.0^{a}	59.38±12.4^{a}	11.42±2.52^{ab}	24.42±4.07^{a}	0.13±0.04^{a}	4.40±0.95^{ab}	1.49±0.29^{b}	3.10±0.62^{a}
	FKB	207.30±24.7^{a}	59.00±18.1^{ab}	10.09±1.61^{b}	21.63±4.5^{a}	0.13±0.01^{a}	4.01±0.66^{b}	1.71±0.35^{b}	3.01±0.50^{a}
Day 28	Control	184.78±34.1^{b}	62.25±18.2^{b}	8.76±1.26^{b}	18.68±7.15^{b}	0.13±0.03^{b}	3.82±0.56^{b}	1.68±0.36^{b}	2.92±0.46^{a}
	FKB+Propolis	219.79±32.4^{ab}	52.43±10.2^{a}	13.46±1.66^{a}	38.11±10.2^{a}	0.16±0.01^{a}	4.42±1.07^{a}	2.04±0.44^{a}	2.82±0.75^{a}
	FKB+F	206.47±35.8^{b}	55.50±15.1^{ab}	12.35±1.83^{a}	34.61±6.9^{a}	0.15±0.02^{ab}	4.31±0.87^{a}	1.90±0.59^{b}	2.81±0.73^{a}
	FKB	203.13±31.4^{b}	59.00±14.2^{b}	11.51±2.39^{ab}	25.28±7.7^{a}	0.14±0.02^{b}	4.15±0.79^{ab}	1.81±0.35^{b}	2.95±0.57^{a}
Day 42	Control	171.19±41.3^{a}	65.14±13.6^{a}	8.86±1.16^{b}	23.45±6.4^{b}	0.13±0.02^{a}	3.92±0.73^{a}	1.64±0.31^{a}	2.92±0.52^{a}
	FKB+Propolis	204.54±28.8^{a}	61.00±9.7^{a}	11.76±1.74^{a}	33.75±3.6^{a}	0.14±0.02^{a}	4.23±0.97^{a}	2.02±0.18^{a}	3.23±0.58^{a}
	FKB+F	206.09±19.5^{a}	61.14±14.3^{a}	10.20±2.11^{ab}	34.86±7.7^{a}	0.14±0.02^{a}	4.05±0.63^{a}	1.97±0.43^{a}	3.05±0.53^{a}
	FKB	195.18±48.0^{a}	62.57±16.9^{a}	9.06±1.77^{b}	26.51±6.28^{b}	0.13±0.01^{a}	4.15±0.63^{a}	1.76±0.39^{a}	3.15±0.5^{a}

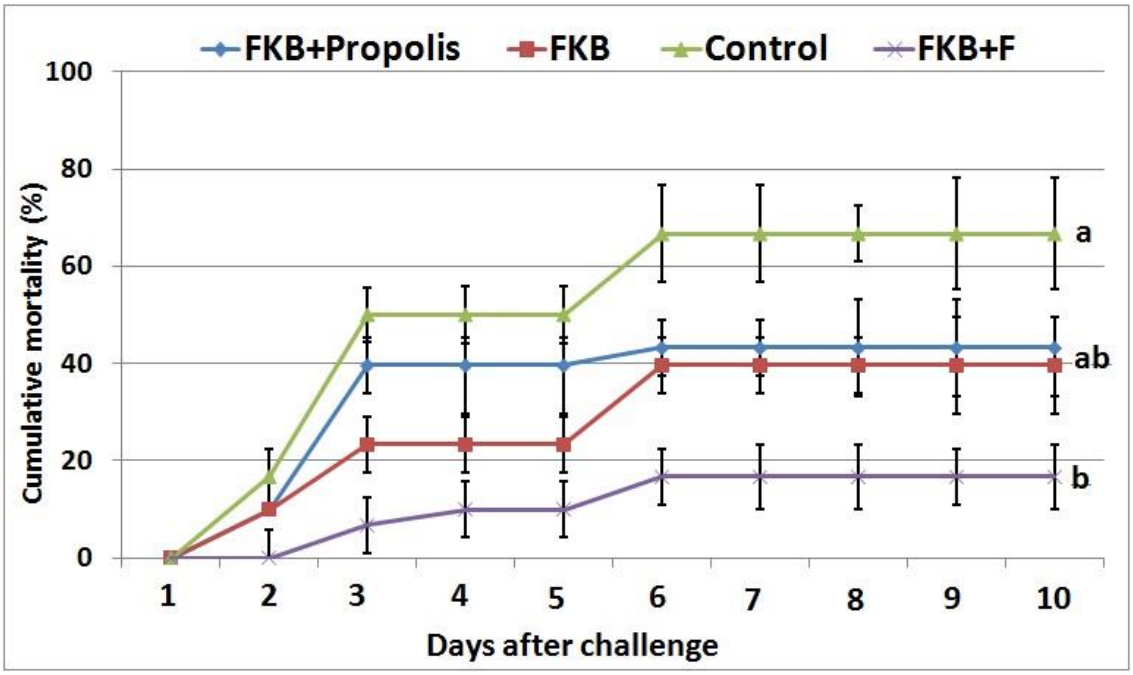

Figure 2: RPS of vaccinated carp with different adjuvants after challenge with *Aeromonas hydrophila*. Data showed as Mean±SD. FKB+Propolis.

Carp vaccinated with FKB in combination with propolis as adjuvant, FKB+F: Carp vaccinated with FKB in combination with freund adjuvant, FKB: carp vaccinated with Formalin Killed Bacterin without adjuvant. Significant differences (p<0.05) are marked by different letters.

Complement activity

Complement activity increased in all vaccinated groups on day 28 of the experiment, and in propolis and ferund adjuvanted vaccine groups on day 42 of the experiment (p<0.05) (Table 1).

Serum proteins, globulin and albumin

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The levels of total protein and IgM showed significant increase in the FKB+ propolis group on days 14 and 28 compared with the control group (p<0.05). Other groups showed no significant change in total protein and IgM values (Table 1). Besides, albumin levels weren't affected by various adjuvanted groups in four sampling points (p>0.05).

WBC count

A significant increase in WBC value was observed in the FKB+ Propolis group on days 14, 28 and 42 of the experiment compared with the control (p<0.05), whereas the FKB+ Freund group showed increase in WBC value only on day 28 compared with the control group (p<0.05).

Post challenge protection

Cumulative mortality of all groups after challenge with *A.hydrophila* has been shown in Fig 2. All immunized groups showed notable decrease in mortality rate compared with the unimmunized control (p<0.05). The lowest mortality rates, were recorded in FKB+ Freund groups which were significantly lower than mortality rate of FKB and FKB+propolis groups (p<0.05). No differences were recorded between the FKB and FKB+propolis groups (p>0.05).

Discussion

The success of many vaccines relies on their association with selected adjuvants in order to increase their immunogenicity and ensure long-term protection. All available adjuvants, particularly Freund, have adverse effects mostly due to their toxicity and reactogenicity (Fuat Gulhan *et al*., 2017). Several studies have confirmed the different medicinal benefits of propolis in fish (Alishahi and Jangeran, 2012; Alishahi *et al*., 2018). However a few studies addressed their use as a potent, safe, vaccine adjuvant in fish (Chu, 2006; Zheng *et al*., 2012). In this study the effect of ethanolic extract of propolis as an adjuvant on some immunological parameters of common carp were investigated. Results showed that although anti *A.hydrophila* vaccine formulated with propolis and Freund as an adjuvant improved the specific and non-specific immune response of common carp, efficacy of *A. hydrophila* vaccine was only improved in the Freund adjuvanted vaccine group.

The results of experimental challenge via injection of live *A. hydrophila* showed that mortality rate in the FKB+Freund, FKB+Propolis and FKB groups were 16.7%, 43.3% and 39.7% respectively. The mortality rate in the vaccinated groups with FKC+Freund was significantly higher than other groups (p<0.05), but no difference were seen in the FKB+Propolis and FKB groups (p>0.05). The results indicated that unlike Freund adjuvant, propolis didnt improve efficacy of *A. hydrophila* vaccine in common carp. Contrary to our findings, Chu (2006) indicated that the use of the water extract of propolis as an adjuvant to *A.hydrophila* bacterin in *Carasious auratus* resulted in 67.8% RPS versus 49.9% for the adjuvant free vaccine. In another study Hao *et al*.

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(2010) compared the adjuvant effects of propolis, Freund and white oil in *Aeromonas sobria* vaccine administrated intraperitonealy in soft shelled turtle. They reported stronger adjuvant effect of Freund compared with propolis and white oil, but they indicate that the vaccine with propolis was less toxic than white oil and cheaper than Freund's adjuvant.

Zheng *et al*. (2012) studied efficacy of penta-valent vaccine consisting of four species of virulent vibrio and *Edwardsiella tarda*, which had been formulated with astragalus polysaccharide (APS), alcoholic extract of propolis, and Freund adjuvant. Although the highest immune response and efficacy was obtained with Freund, its high price and toxicity (Chilling and Donaldson, 2003) limit its potential usage. Both the APS and the propolis extracts elicited a similar immune response with a high RPS. In a similar work in chicken, a comparison between Freund adjuvant, Quil A, Cochinchina extract and propolis, proved that propolis may be used as a possible adjuvant for a protein subunit vaccine for the *E. coli* infection in chicken to boost the immune system of the egg by inducing increased antibody content in the eggs without decreasing egg reduction as occurs with Freund's adjuvant (Sun *et al*., 2008). The different results of works on the adjuvant effect of propolis in vaccine efficacy can be related to several reasons; the geographical origin, quality and even the season of sampling of propolis may affect its effective materials. Besides the procedure of extraction can deeply affect the quality of propolis. Different effective components were extracted in water and ethanolic extraction procedure. Chu (2006) evaluated the water extract of propolis with the origin of China, and Zheng *et al*. (2012) worked with ethanolic extract of the same propolis, whereas our propolis was originally from the southern region of Iran. Silici and Kutluca (2005) reported that honeybee race has a great impact on the chemical composition and bioactivity of propolis yield. The difference between the origins of propolises, and extraction procedure may be the main reasons for the incoherence among the different works. The animal species showed difference in immune mechanisms. Another major reason of incompatibility of results of this work on propolis effect on vaccine efficacy with similar works may be related to fish species.

The results of the present study show that bacterin of *A. hydrophila* formulated with propolis or Freund as adjuvant, when given intraperitoneally to common carp, induce higher Ab titer compared with the control group ($p<0.05$). Although higher Ab production was seen in the FKB+Propolis and FKB+Freund compared with FKB free adjuvant, this increase was not significant ($p>0.05$). Numerous researches about the contribution of adjuvant to immune response of fish have been reported. Most of them proved that adjuvant could enhance immune response through increasing activity of leukocyte and plasmocyte as well as speeding up

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production of specific antibody (Choobkar, 2014; Gunathilaka *et al*., 2015). Some studies have looked at the action of propolis on humoral response. Scheller *et al*. (1998) suggested that propolis immunostimulant activity may be associated with macrophage activation and phagocytosis capacity. In a similar work, *Carassius auratus* were injected with bacterin of *A. hydrophila* or the propolis formulated vaccine intraperitonealy. The propolis formulated vaccine induced higher Ab titer against *A. hydrophila*, and increased leucocytes activity. In some reports propolis has been shown to stimulate antibody formation. Spleen cells producing antibodies in mice administered with propolis as an adjuvant were three times greater than that of controls. Scheller *et al*. (1998) also reported that propolis was able to increase the number of plaque-forming cells in the spleen of immunized mice, demonstrating their ability to produce antibodies. In a similar work in mammals, propolis has been shown to stimulate an immune response in increasing the Ab titer in rabbits (Nassar *et al.*, 2012). Japanese researchers have shown an extract of propolis to produce a macrophage activation phenomenon related to the immune function in humans (Moriyasu *et al*., 1994). Probably propolis activates immune cells which produce cytokines which are necessary for formation of Abs. Increased Ab titer in propolis treated group probably is due to the presence of some effective compounds such as flavonoids, phenolic acids and their esters in propolis.

Serum lysozyme is mostly used to measure the innate immune response in fish (Ellis, 1990). It presents bactericidal activity and opsonin effects by activating some immune mechanisms in fish (Magnadottir, 2006). Lysozyme activity is a measurable humoral component of the non-specific defense mechanism. In the present study, although relative increase in serum lysozyme activity was recorded in all vaccinated groups compared with the control, this increase was significant only in the FKB+propolis group on day 28 of the experiment. The reports on effects of propolis in lysozyme activity in fish are often contradictory. Similar to the present results Alishahi and Jangeran (2012) found that food supplemented with 0.5% and 1% propolis increased the serum lysozyme activity in *Barbus barbulus*. Increased lysozyme activity has been reported after supplementing the fish diet with propolis as well as propolis and Chinese herbs (Zhang *et al*., 2009). In spite of later reports Dotta *et al*. (2014) indicated that lysozyme activity of Nile tilapia didn't change following administration of propolis via injection and oral routes. Sahu *et al*. (2007), did not find any differences in the lysozyme activity of *Labeo rohita* supplemented with 1 g and 5 g *Magnifera indica* in the diet for 20 days. The contradictory reports about the effect of propolis on fish immune response can be related to origin and quality of propolis as well as the experimental fish species.

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According to the results of this work, phagocytosis activity presented as NBT reduction showed significant increase in the FKB+Propolis and FKB+Freund groups compared with the control group in most of sampling times (p<0.05). Similar reports in *C. auratus* showed that formulation of *A. hydrophila* vaccine with propolis caused higher phagocytosis activity (69.2% versus 54.6% for the adjuvant free vaccine) and increased leucocytes activity. The fact that propolis compounds can enhance macrophage mobility and efficacy has previously been confirmed. Cuesta *et al*. (2005) reported that leucocyte phagocytosis, cytotoxicity and peroxidase content were enhanced in sea bream vaccinated with propolis adjuvanted vaccine. They described faster and higher adjuvant effects when propolis was administered intraperitoneally than when administered orally. Propolis enhanced the immune response of the vaccine, activated phagocytosis, increased lymphocyte count, and protected the experimental chickens. To overcome the instability of propolis flavonoids, the extract was formulated with lecithin and cholesterol to form a safe synthetic bilayer liposome. The propolis' flavonoid encapsulated in the liposome significantly enhanced the vaccine's humoral immunity, prolonged its effect, produced better peripheral lymphocyte proliferation, and a higher level of interleukin-2 (IL-2) and INF- γ levels especially in the later period of vaccination (Yuan *et al*., 2012). Ivanovska *et al*. (1995) showed changes in the phagocyte activity of mice injected with water-soluble derivatives of propolis, in which the number of leucocytes and the percentage of CD8+ and CD4+ cells were significantly increased (Ivanovska *et al*., 1995). Although several studies have demonstrated the phagocytic activity of leukocytes in fish, phagocytic activity data varies among the fish species and experimental conditions.

In this study the levels of total protein and IgM showed significant increase in the FKB+ propolis group on days 14 and 28 compared with the control group (p<0.05). Other groups showed no significant change in total protein and IgM values (p>0.05). Total plasma protein and IgM concentration is used as a broad clinical indicator of health, stress, and welfare in both aquatic organisms (Riche, 2007). These results agree with others, which describe increased total protein and IgM in propolis treated fish. Dotta *et al*. (2014) reported increased protein and IgM level in sea bream in vaccinated fish with propolis adjuvanted vaccine. Besides Yonar *et al*. (2012) report similar results in common carp fed with propolis supplemented food. The increase in serum protein content might be in part due to an increase in the WBC, which is a major source of serum protein production such as lysozyme, complement factors and bactericidal peptides (Misra *et al*., 2006). This is supported by an enhancement in WBC level in the immunized group that received propolis as an adjuvant.

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The serum bactericidal activity of *C. carpio* against *A. hydrophyla* was increased in all vaccinated groups on day 14 of the experiment and in the FKB+ Propolis and FKB+Freund groups in all sampling points (p<0.05). Similar to the present results Dotta *et al*. (2014) reported that supplementation of food with 0.5 and 1% propolis for 20 days induced no significant change in antimicrobial potency (evaluated *against A. hydrophila, Enterococcus durans* and *E. coli*) in tilapia. Also Alishahi and Jangeran Nejad (2012) did not found differences in the serum bactericidal activity of *Barbus barbulus* supplemented with 0.5 g and 1 g propolis in the diet for 60 days. Tukmechi *et al*. (2014) found notable increase in serum antibacterial activity of propolis treated rainbow trout. Abd-El-Rhman, (2009) reported significant increase in serum bactericidal activity following the administration of propolis in tilapia. They suggest that antibacterial activity can be attributed to the effect of propolis on liver and leukocyte production, the important sites for the synthesis of antibacterial proteins. Cuesta *et al*. (2005) reported that intraperitoneal administration of propolis and dietary EEP inclusion (0.1 or 10 g kg^{-1} EEP) had no effect on serum antibacterial activity in gilthead seabream (*Sparus aurata*). The apparent discrepancy among these studies may be attributed to the propolis source, dose, and fish species.

WBC count and complement activity, and other components of the fish immune system increased in all vaccinated groups on day 28 of the experiment, and in propolis and ferund adjuvanted vaccine groups on day 42 of the experiment (p<0.05). The present results concur with the reports of previous workers which describe increments in the complement activity, intensity, mobility and activities of leukocytes, and activating factors of leucocytes after in vitro or in vivo treatment with propolis (Scheller *et al*., 1998; Alishahi *et al*., 2010) suggesting that propolis immunostimulant activity may be associated with macrophage activation and phagocytosis capacity. Probably, the increase in the leucocyte count might have resulted in the enhancement of the nonspecific defence mechanisms, because leucocytes are the key elements in the immune system and are the major affecter cells on which propolis affects.

Regarding the safety of propolis, Nassar *et al*. (2012) found that the ethanolic extract of propolis as an adjuvant with the inactivated *Pasteurella multocida* vaccine in rabbits were safe, without adverse reactions on the rabbit's health, enhanced specific and non-specific immune response of the vaccine, and reduced the severity of adverse clinical symptoms and mortality rate among the tested rabbits (Nassar *et al*., 2012).

To conclude, based on our findings, `Although ethanol extract of propolis as an adjuvant can promote immunogenicity of *A. hydrophial* vaccine in common carp, which this stimulation is comparable with even better effects than freund adjuvant, its effect on vaccine efficacy were

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significantly lower than freund adjuvant. Then in spite of the strong immunogenicity of propolis as an adjuvant more research should be conducted on increasing the experimental efficacy of propolis.

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