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Can dietary jujube (*Ziziphus jujuba* Mill.) fruit extract alter cutaneous mucosal immunity, immune related genes expression in skin and growth performance of common carp (*Cyprinus carpio*)?

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ABSTRACT

The present study investigates the effect of dietary *Ziziphus jujube* fruit extract [ZJFE] on skin mucus non-specific immune parameters as well as mRNA levels of immune related gene in the skin of common carp fingerlings. An eight-week feeding trial was performed using different levels of dietary ZJFE (0, 0.25, 0.5 and 1%). At the end of the trial skin mucus immune parameters (total Ig, lysozyme and protease activity), cytokines genes (*il1b*, *il8*, *il10* and *tnf-alpha*) expression in skin and growth performance were studied. The result showed highest and lowest skin mucus total Ig were observed in fish fed diet containing 0.5% ZJFE and control group, respectively ($P < 0.05$). There were no significant difference among treatments regarding skin mucus lysozyme activity ($P > 0.05$). It should be noted that, feeding on 0.5 and 1% ZJFE significantly increased skin mucus protease activity ($P < 0.05$). Likewise, gene expression studies in skin showed significant increase of *il1b* expression in fish fed 0.5% ZJFE compared other treatments ($P < 0.05$). Also, *il8* gene was noticeably up-regulated in 0.5 and 1% treatments compared to the control group ($P < 0.05$). While there were no significant difference between 0.25% JFE treatment and control in case of relative *il10* gene expression ($P > 0.05$), feeding on diets containing 0.5% or 1% ZJFE significantly down-regulated *il10* gene ($P < 0.05$). Our study indicated that relative expression of *tnf-alpha* gene significantly increased in treated groups ($P < 0.05$). Also, feeding on ZJFE supplemented diet improved growth performance parameters. Overall, this experiment demonstrated the potentially useful effects of ZJFE on skin mucosal immunity and performance of common carp fingerlings.

1. Introduction

Over the years, the prophylactic use of chemicals and antibiotics has become commonplace. This excessive use resulted in consequences such as the emergence of resistant bacteria and antibiotic residues in fish meat which pose a risk in human nutrition and environment [1,2]. Therefore, herbal compounds have been considered as one of the most promising alternatives due to the lower side effects on living creatures and environment, lack of drug resistance, cheapness and sustainability [3]. Therefore, this method can be used as an alternative for antibiotics, vaccines, and synthetic compounds [4]. The use of medicinal plants and their derivatives has an ancient basis [5]. Replacement of herbal medicine instead of antibiotics could be a new strategy in fish health management [6–9]. During the previous decade, diseases outbreak caused extensive losses in aquaculture [10]. The use of immunostimulants in aquaculture was successful in reducing antibiotics administration and economic losses as a result of disease [11–13]. Cyprinids are among the most important fish species which estimated to comprise more than 72% of world aquaculture production in freshwater [14,15]. One of the most important species in this family is Common carp (*Cyprinus carpio*) [16].

Ziziphus jujube is one of the medicinal plants belonging to the Romnaceae family [17]. This plant is grown in vast regions of the world, including the Mediterranean, Australia and southern European countries such as Spain, Greece and Cyprus [18], and also is cultivated on the Iranian plateau, mainly in the provinces of South Khorasan,

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Isfahan, Golestan, Mazandaran, Fars, Yazd, Hamedan and Qazvin [17]. Many nutrients and phytochemical elements are available in Jujubes which can contribute to a healthy diet. Chemically, jujube contains vitamins (especially ascorbic acid), minerals, fatty acid, amino acid, polysaccharide, fibre, flavonoids (flavones and flavone- 3- ols), triterpenic acids and nucleosides [19–24]. Interestingly, ZJFE contain a wide range of phenolic compound such as flavonols22, flavan-3-ols, procyanidin B2, epicatechin, catechin, rutin, quercetin-3-O-rutinoside, quercetin-3-robinobioside, quercetin-3-O-galactoside, kaempferol-glucosyl-rhamnoside, and kaempferol-glucosyl-3″-rhamnosid [18]. Besides, it contains polysaccharides, Nucleosides and Nucleobases, α-Tocopherol and Carotene. These bioactive compunds result in several beneficial effects on host such as Anticancer, Anti-inflammatory, Immunostimulating, Antioxidant, Hepatoprotective as well as Gastrointestinal Protective activites (see the review of Gao et al. [18]. The previous pharmacological studies revealed that *Ziziphus jujube* has sedative, anti-inflammatory and hepatoprotective effects, immunological and antioxidant activities [23,25,26]. These studies confirmed the potential of Jujube to be used as effective immunostimulant in aquaculture. However, to the best of our knowledge, the possible effects of *Ziziphus jujube* as feed additive have not been studied in aquaculture. Given the importance of introduction of new immunostimulant for so called green aquaculture (antibiotic free) from one side and the potential of jujube clearly indicate the necessity of performing this study.

One the other hand, when we talk about fish immunity, skin mucus is one of the important component. This is due to the function of mucus for preventing the formation of parasites, bacteria and pathogenic fungi on the outer surfaces of the body (because of constant secretion) and replacement [27]. Besides, the cutaneous mucus contains several compounds, including glycoproteins, agglutinins, lectins, antimicrobial peptides, proteolytic enzymes, flavoenzymes, immunoglobulins, lysine, lysosomes, natural antibodies, which play an important role against pathogens [28–30]. During past years there were increasing interests about dietary modulation of skin mucosal immunity; mainly focused on prebiotics, probiotics and medicinal plants [4,13,28]. The findings of those researches revealed beneficial effects on skin mucosal immunity.

Based on the above mentioned facts, the present study was designed to investigate the impacts of dietary *Ziziphus jujube* fruit extract on skin mucus immune parameters and cytokines genes expression (*il1b*, *il8*, *il10* and *tnf-alpha*) in the skin of common carp (*Cyprinus carpio*).

2. Materials and methods

2.1. Fish husbandry and feeding trial

Common carp (*Cyprinus carpio*) juvenile with average weight of (10.78 ± 0.05 g) were obtained from Sijaval, Bandar–e Turkeman Fish farm, Golestan Province, Iran. Upon delivery of fish to laboratory, carps were acclimatized in 500-L fiberglass tanks for two weeks. Then, they were randomly stocked into twelve 100-L tanks (15 fish/replicate; n = 45 fish/group). The culture system was static water with constant aeration provided by air stones. The tanks were cleaned daily and the feces siphoned every day. Also to maintain optimum water quality 50% of tanks water was exchanged with fresh water. The physiochemical parameters of water was regularly checked using portable instrument (WThW, Munich, Germany) and the Water temperature, pH and dissolved oxygen were checked daily by and maintained at 24 ± 1.1 °C, 7.5 ± 0.3 and 7.1 ± 0.1. The cultural period was lasted for 8 weeks and during this period fish were fed three time a day at 8:00, 12:00 and 16:00. The feeding ratio was 3% of body weight which was corrected every 10 days based on the biometry of fish. Utmost care was considered to avoid feed lose and uneaten feed.

2.2. Preparation of Ziziphus jujube fruit extract

Juicy fruits (half red pear-jujube stage) were purchased to prepare the extract. After separating the nucleus from the fruit gust to eradicate excess moisture, the flesh was placed in the oven for 24 h at 40 °C. Then the jujube fruits were completely powdered and preserved in glass containers. To prepare the hydro-alcoholic extract, 200 g powder was mixed in 1 L ethanol (75%) and placed on shaker for 18 h to dissolve the soluble compounds in ethanol. Thereafter, the suspension was passed through a 200 nm filter paper. The solution passed the filter paper to glass containers and placed inside a rotary machine (Rv10 digital, Germany) at 45 °C to entirely dry [31].

Table 1
Dietary formulations (%) and proximate composition.

Ingredient	Control	0.25%	0.5%	1%
Fish meal	40.0	40.0	40.0	40.0
Wheat flour	21.0	21.0	21.0	21.0
Soybean meal	13.5	13.5	13.5	13.5
Gluten	5.5	5.5	5.5	5.5
Soybean oil	6.0	6.0	6.0	6.0
Fish oil	6.0	6.0	6.0	6.0
Mineral premix[a]	3.0	3.0	3.0	3.0
Vitamin premix[a]	2.0	2.0	2.0	2.0
Binder[b]	2.0	2.0	2.0	2.0
Anti fungi[c]	0.5	0.5	0.5	0.5
Antioxidant[d]	0.5	0.5	0.5	0.5
JFE	0	0.25	0.5	1
Proximate analysis				
Dry matter	91.5	91.8	91.4	91.9
Crude protein[a]	36.1	35.9	36.1	36.0
Crude lipid[a]	11.2	11.0	11.4	11.3
Ash[a]	3.5	3.8	3.7	3.4

[a] Premix detailed by [Hoseinifar et al., 2012].
[b] Amet binder ™, Mehr Taban-e− Yazd, Iran.
[c] ToxiBan antifungal (Vet-A-Mix, Shenan-doah, IA).
[d] Butylated hydroxytoluene (BHT) (Merck, Germany).

2.3. Preparing experimental diets

The ingredients used for preparation of the basal diet as well as proximate composition are mentioned presented in Table 1. The experimental diets were prepared by addition of different levels of ZJFE (0.25, 0.5 and 1%) to basal diet as described elsewhere [32]. Briefly, the ingredients were passed through a 1.0-mm sieve to be homogenized and then thoroughly mixed. Then, distilled water (25% v/w) was added to ingredient to make it a dough and then passed through grinder to be make pellet. The pellets were oven dried (at 60 °C) for 24 h and stored in sealed plastic bags at 4 °C until use.

2.4. Immunological assays

2.4.1. Skin mucus collection

Three fish (9 fish per experimental group) were randomly sampled at the end of the feeding trial. Fish were rapidly anesthetized using clove powder (500 mg L^{-1}) dissolved in water and transferred into a polyethylene bag containing 5 mL of 50 mM NaCl (Sigma, Steinheim, Germany). Skin mucus were taken using indirect method as suggested previously [33] by gently rubbing the fish inside the plastic for 1–2 min. The obtained samples were immediately transferred to 15 mL sterile centrifuge tubes, centrifuged (5810 Eppendrof, Engelsdorf, Germany) (1500 × *g* for 10 min at 4 °C) and supernatant was collected and kept at −80 °C until analysis.

2.4.2. Evaluation of non-specific immune parameters in the skin mucus

The skin mucus total Ig level was determined as suggested previously [34]. This technique was based on determination of total protein level before and after precipitating down the immunoglobulin

molecules (using 12% solution of polyethylene glycol (Sigma)). The lysozyme activity in samples was measured by the turbidimetric method assay using a lysozyme-sensitive Gram-positive bacterium *Micrococcus luteus* (Sigma) as described in previous work [35]. Briefly, the same amount of mucus sample (50 μL) and lyophilized *Micrococcus luteus* (Sigma) suspension was added to 96 well plate, incubated for 15 min (at 30 °C) and the absorbance was observed for 50 min. A unit of activity classified as the amount of enzyme diminishes the absorbance 0.001 min^{-1} at 450 nm. Protease activity in skin mucus was determined by the method explained by Ref. [36], 0.7% azocasein solution (Sigma) was combined with 100 μl of mucus sample and incubated for 19 h at 30 °C with constant agitation. Then, 4.5% trichloroacetic acid was added to stop the reaction and the supernatant was obtained by centrifuging the reaction mixture at 15000 g for 5 min. The supernatant was pipette to a 96-well flat bottom plate containing 100 mL 1 N sodium hydroxide (NaOH). The optical density (OD) was measured at 450 nm.

2.5. Evaluation of immune related genes expression in skin

2.5.1. RNA extraction, cDNA synthesis

Three fish from each replication (nine fish) were taken at random and anesthetized using clove powder (500 mg L^{-1}). The samples were taken from skin and subsequently deep-frozen without delay in liquid nitrogen and retained at −80 °C for later isolation of RNA [37]. The RNA was extracted from the skin samples according to the manufacturer's instructions (RNAx Plus Cinna Gen, Iran). Subsequently, the quantity and concentration of RNA were controlled by spectrophotometer at 230 and 260 nm. To avoid pollution with genomic DNA, total RNA was treated with DNase I (Fermentas, Lithuania) following the manufacturer's guidelines. The quantity and quality of RNA samples were evaluated with a Nanophotometer (NanoDrop (Nanodrop technology, Wilmington, DE, USA) and agarose gel (1.5%), respectively, as previously described [35,38]. Next, the cDNA synthesis was prepared using cDNA synthesis master mix according to the manufacturer's instructions (Sinagen Company).

2.5.2. Primer design and real time PCR

The qPCR primers for immune related genes were prepared based on the conserved region available in Gene Bank sequences using Oligo 7 (Table 2). Quantitative real-time PCR assays were implemented to study the effects of JFE on the expression of cytokines genes (interleukin1 beta (*il1b*), interleukin 8 (*il8*), interleukin 10 (*il10*) and necrosis factor alpha (*tnf-alpha*)) in common carp skin as described by Khodadadian Zou et al. [35]. The expression of the selected genes was modified by the β-actin RNA content of each sample. The IQ5 optical system software (Bio-Rad) and ΔΔCt method was used for data analysis.

2.6. Growth performance

At the end of feeding trial, fish were not fed for 24 h and then all fish in tanks were weighed and growth performance parameters were measured according to the below formula:

Weight gain = $[(W_2\,(g) - W_1\,(g))/\,W_1] \times 100$; W_1 is the initial weight, W_2 is the final weight

Specific growth rate (SGR) = $100 \times [(\ln W_2 - \ln W_1)/T]$; W_1 is the initial weight, W_2 is the final weight and T is the duration of the feeding trial; Feed conversion ratio (FCR) = feed intake (g)/weight gain (g); Survival = (final number of fish/initial number of fish) × 100.

2.7. Statistical analysis

Prior to statistical analysis, all data were tested for normality by the Kolmogornov-Smirnov test. Then, with regard to the normal distribution of data, one way ANOVA was used to compare the experimental treatments. Data were tested for significant difference using Duncan test at $P < 0.05$. SPSS13.0 statistical package was used to perform statistical analyses.

3. Results

3.1. Mucus immune parameters

The effects of feeding ZJFE supplemented diets on skin mucus non-specific immune parameters in Fig. (1–3). Evaluation of mucus immune parameters revealed significant difference in total Ig levels of common carp fed ZJFE and basal diet ($P < 0.05$). However, there were no significant difference between different inclusion levels ($P > 0.05$). In our study, maximum and minimum total Ig were observed at diet containing 0.5% ZJFE and control group respectively ($P < 0.05$) (Fig. 1). Meanwhile, no significant difference was noticed in case of skin mucus lysozyme activity of treated fish and control ($P > 0.05$) (Fig. 2). Regarding skin mucus protease activity, lower inclusion level (0.25%) of ZJFE had no significant effects when compared with control treatment ($P > 0.05$). However, feeding on 0.5 and 1% ZJFE significantly increased protease activity in mucus of common carp ($P < 0.05$) (Fig. 3).

3.2. Immune related gene expression

Figs. 4–7 represents the effects of different levels of ZJFE on expression of *il1b*, *il8*, *il10* and *tnf-alpha* in common carp skin. The results showed the relative expression of *il1b* was significantly different among treatments ($P < 0.05$) that the relative expression of *il1b* was higher in fish fed with diets containing 0.5% of ZJFE (Fig. 4). The results showed that the relative expression of *il8* had significant difference among treatments ($P < 0.05$), however the higher was in fish that fed with diet containing 0.25% ZJFE (Fig. 5). According to the results, the relative expression of *il10* was significantly higher in fish fed with 0.25% ZJFE ($P < 0.05$) and the lowest relative expression of *il10* was observed in fish fed with 0.5% ZJFE (Fig. 6). Our study indicated that relative expression of *tnf-alpha* gene in experimental treatment was significantly

Table 2
The sequence and accession number of primers of selected immune related genes.

Gene name	Gene abbreviation	Sequences of primers	Accession no
Beta-actin	*b-actin*	Forward: AGACATCAGGGTGTCATGGTTGGT Reverse: CTCAAACATGATCTGTGTCAT	M24113.1
Interleukin 1 beta	*il1b*	Forward: ACCAGCTGGATTTGTCAGAAG Reverse: ACATACTGAATTGAACTTTG	AB010701.1
Interleukin 8	*il18*	Forward: GTCTTAGAGGACTGGGTGTA Reverse: ACAGTGTGAGCTTGGAGGGA	AB470924.1
Interleukin 10	*il10*	Forward: CGCCAGCATAAAGAACTCGT Reverse: TGCCAAATACTGCTCGATGT	AB110780
Tumor necrosis factor alpha	*tnf-alpha*	Forward: GGTGATGGTGTCGAGGAGGAA Reverse: TGGAAAGACACCTGGCTGTA	AJ311800

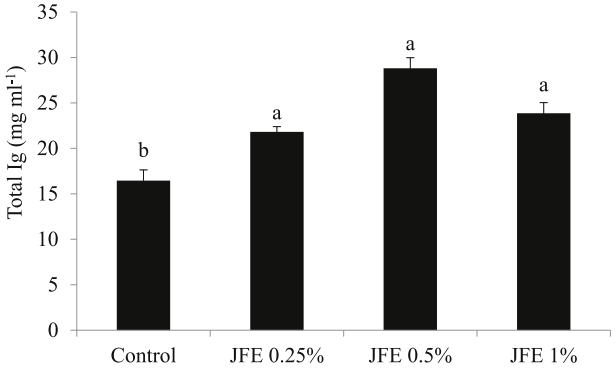

Fig. 1. Total immunoglobulin levels in the skin mucus of common carp fingerling fed different levels of dietary *Ziziphus jujube*, for 8 weeks. The different letters present significant difference ($P < 0.05$).

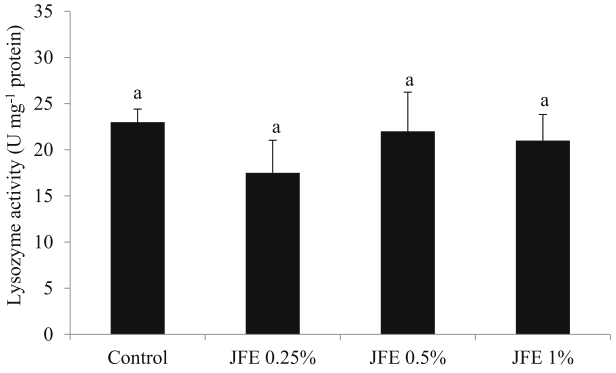

Fig. 2. Lysozyme activity in the skin mucus of common carp fingerling fed different levels of dietary *Ziziphus jujube*, for 8 weeks. The same letters present not significant difference (P > 0.05).

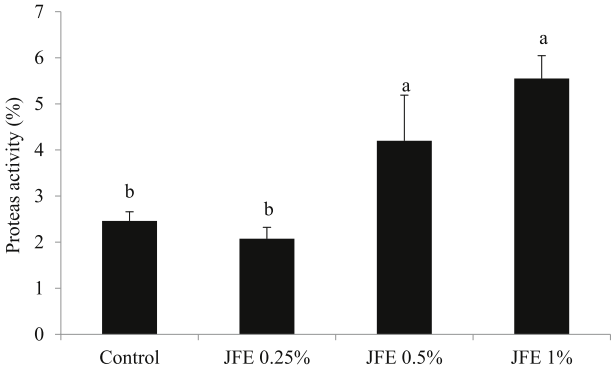

Fig. 3. Protease activity in the skin mucus of common carp fingerling fed different levels of dietary *Ziziphus jujube*, for 8 weeks. The different letters present significant difference ($P < 0.05$).

higher than control group ($P < 0.05$) (Fig. 7).

3.3. Growth performance and survival rate

The results of the JFE effects on the growth indices of common carp are presented in Table 3. At the beginning of the period, there was no significant difference between treatments in terms of weight ($P < 0.05$). At the end of the study period, growth indices were indicative of an increase in growth indices in juvenile fish fed JFE, which was statistically significant compared to control ($P < 0.05$). There was a significant difference ($P < 0.05$) between control treatment and jujube extracts in case of the WG, FCR and SGR. In the treatment fed 0.5% JFE, the best results were obtained from when compared with other

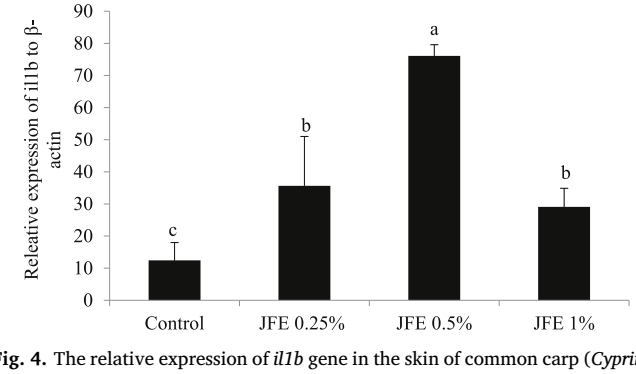

Fig. 4. The relative expression of *il1b* gene in the skin of common carp (*Cyprinus carpio*) fed different levels of dietary *Ziziphus jujube* fruit extract for 8 weeks. Values are shown as mean ± SE. The different letters on the bars indicate significant difference between treatments ($P < 0.05$).

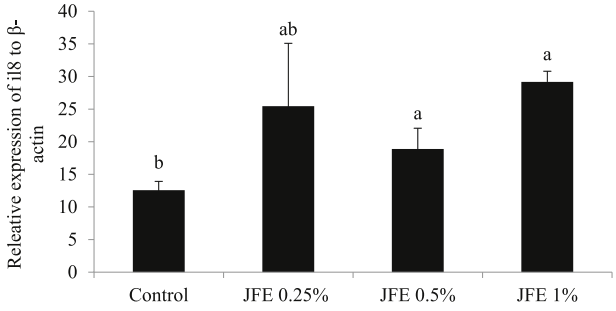

Fig. 5. The relative expression of *il18* gene in the skin of common carp (*Cyprinus carpio*) fed different levels of dietary *Ziziphus jujube* fruit extract for 8 weeks. Values are shown as mean ± SE. The different letters on the bars indicate significant difference between treatments ($P < 0.05$).

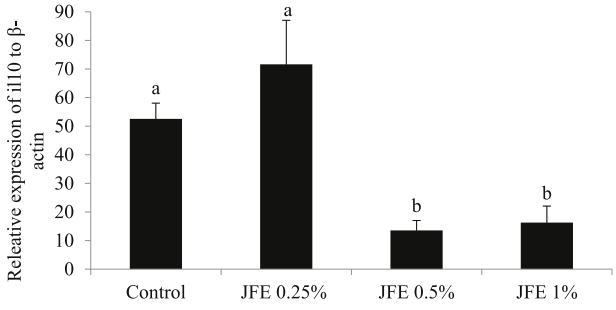

Fig. 6. The relative expression of *il110* gene in the skin of common carp (*Cyprinus carpio*) fed different levels of dietary *Ziziphus jujube* fruit extract for 8 weeks. Values are shown as mean ± SE. The different letters on the bars indicate significant difference between treatments ($P < 0.05$).

treatments and the control group. The survival rate at the end of the period was 100% in both control and treatment groups and there was no mortality during the period of the trial.

4. Discussion

The outcome of the present study illustrated that the dietary administration of ZJFE noticeably affected some crucial defense molecules in the skin mucus of common carp. As far as it is known, there is no document about alteration of skin mucus immune response or mucosal immune-related genes expression using ZJFE in aquaculture. Positive effects of ZJFE addition on total Ig and protease activity were noticed by the assessment of mucus immune factors. In the immune system of fish, the Ig is a vital constituent and contributes to eliminating viruses, fungi, bacteria and the escalation of these activities revealed heightened immunological performance. In line with our findings, Hoshyar

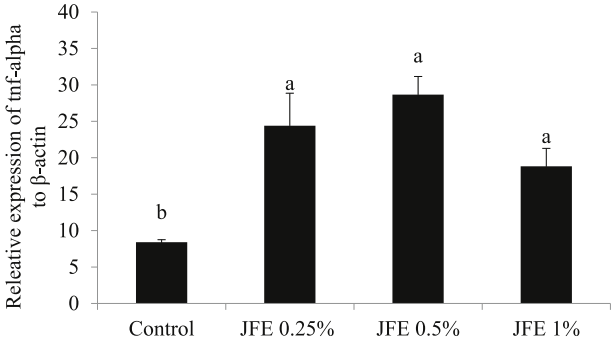

Fig. 7. The relative expression of *tnf-alpha* gene in the skin of common carp (*Cyprinus carpio*) fed different levels of dietary *Ziziphus jujube* fruit extract for 8 weeks. Values are shown as mean ± SE. The different letters on the bars indicate significant difference between treatments ($P < 0.05$).

et al. [39] illustrated positive effect extract of *Ziziphus jujube* as an immunostimulant in rat that caused increase lymphocyte in rat. Similarly, Harikrishnan et al. [4] showed increase in the serum immune parameters can be due to presence of polysaccharides (in ANAB) which has shown to stimulate humoral parameters and stimulate T lymphocyte proliferation and humoral immune parameters. A number of researchers have also discovered that innate immunity is enhanced by plant or herbal extracts [5,40]. In accordance with present findings, Hoseinifar et al. [41] observed a rise of non-specific immune parameters in the skin mucus of common carp (*Cyprinus carpio*) fed dietary medlar (*Mespilus germanica*) extracts. In the same way, non-specific immune parameters in the skin Caspian brown trout (*Salmo trutta caspicus*) were enhanced by dietary administration of peppermint (*Mentha piperita*) [42]. Moreover, the total immunoglobulin level in *Oncorhynchus mykiss* elevated after 3-week feeding on black cumin seed extract [43]. Comparably, *Angelica membranaceus* and *Angelica sinensis*, two herbal medicines, were regularly administered to boost the immune systems of common carp and large yellow croaker, respectively [44]. Similar to our results, studies on rats, broiler chickens and human macrophages proved the immunostimulant and anti-tumor characteristics of ZJFE [39,45]. The existence of bioactive compounds such as polysaccharides, vitamin C and flavonoids in *Ziziphus jujube* is probably the reason for its beneficial characteristics on non-specific immune parameters [22,46]. The immunopotentiating quality of polysaccharides taken from various traditional medicinal herbs both in vivo as well as in vitro has been proved [11,40]. Also, it was found that β-glucan is one of the most important compounds of *Ziziphus jujube* [20]. In animals, β – glucan is considered as a stimulator of the immunity system [18].

In the present study we have studied expression of some of the cytokines genes (include *il1b*, *il8*, *il10* and *tnf-alpha*) in the skin of common carp ZJFE. *Tnf-alpha* as a cytokine plays a role in regulation of immune cells and systemic inflammation [47]. What is more, various pathogens can be destroyed by this cytokine. *Tnf-alpha* accomplishes this through stimulating diverse cellular responses, and for this reason, has been deemed an exceptional health indicator and biomarker for both fish and mammals [48]. Owing to its capacity to amplify lysozyme synthesis, phagocyte activity, leukocyte migration, macrophage production, *il1b* is another main mediator of inflammation that is intensely involved in the host response to tissue injury and microbial invasion [49]. Macrophages generate *il8* which is a valuable chemokine that makes chemotaxis and phagocytosis happen [50]. According to Hoseinifar et al. [41] common carp fed with medlar (*Mespilus germanica*) leaf extract caused an up-regulation of skin mucosal immunity. Also, in accordance with our findings, Chen et al. [46] reported up-regulation of pro-inflammatory cytokines (*il1b* and *tnf-alpha*) murine macrophages treated with jujube. Likewise, Przybylska-Diaz et al. [51] revealed that mucosal immune response, healing wounds and up-regulating the expression of cytokine in carp skin can be augmented by enrichment of common carp diet with beta-glucan. The up-regulation of cytokine by jujube was in good arrangement with previous studies in which jujube activated the immune response by the spleen cell proliferation [52]. As mentioned in case of skin mucus immune parameters, up-regulation of cytokines gene expression following feeding with ZJFE, can be due to presence of bioactive compounds such as polysaccharides, vitamin C and flavonoids.

The results of this study showed that by adding jujube fruit extract in the 8 week period, the growth performance was significantly increased compared to the control; the highest incerement occurred in fish fed 0.5% JFE. Considering that the cost of food in aquaculture, the use of such cost effective additives will be very beneficial for farmers. Based on the review of literatures, there have been no studies on the use of JFE in aquatic diets and their effects on growth indices. However, the improvement in growth indices in juvenile fish fed with jujube fruit extracts seems to be due to a better nutritional status created by the nutritional supplement of JFE. It has been reported that JFE contains beneficial compounds such as triterpenic acids, nucleosides, flavonoids (flavones and flavone- 3- ols), polysaccharide and fibre. Besides, it has been repoted to have a high amounts of citric acid, sugars and proteins, organic salts and vitamin C [19–24]. In line with present finding, previous studies on other medicinal herbs also revealed beneficial effects on growth performance parameters [1,4,5,40,53].

In conclusion, the present study revealed beneficial effects of ZJFE as immunostimulant in common carp. The findings highlighted elevation of mucosal immunity following dietary administration of ZJFE: both immune parameters and cytokines gene expression. These results encourages furthers researches on determination of different aspects (inclusion level, optimum route of administration, mode of actions and etc.) of ZJFE as feed additive in aquaculture.

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Table 3
The effects of different levels of dietary *Ziziphus jujube* on growth performance, feed utilisation and survival rate of common carp fingerling.

	JFE (%)			
	0.00 (control)	0.25	0.50	1.00
Initial weight (g)	10.81 ± 0.08^{a}	10.71 ± 0.14^{a}	10.75 ± 0.11^{a}	10.84 ± 0.06^{a}
Final weight (g)	22.40 ± 0.10^{c}	23.03 ± 0.14^{b}	24.27 ± 0.19^{a}	23.67 ± 0.33^{b}
WG (g)	11.34 ± 0.16^{c}	12.32 ± 0.16^{b}	13.52 ± 0.26^{a}	12.82 ± 0.39^{b}
WG (%)	109.94 ± 2.22^{c}	115.04 ± 2.61^{b}	125.80 ± 3.53^{a}	118.27 ± 4.31^{b}
SGR (% day^{-1})	0.82 ± 0.02^{c}	0.93 ± 0.02^{b}	1.04 ± 0.03^{a}	0.96 ± 0.03^{b}
FCR	1.65 ± 0.04^{c}	1.51 ± 0.38^{b}	1.38 ± 0.04^{a}	1.47 ± 0.05^{b}
Survival (%)	100	100	100	100

Values are mean ± S.D and in each row with different letters denote significant differences ($P < 0.05$).

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