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Research Articles:

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Instruction for Authors

Types of paper: Research papers on fisheries and fishery-related subjects will be considered for publication. All material submitted must be original and unpublished works. Three types of manuscripts will be accepted for peer review: "Original Research Papers", "Short Communications" and "Review Papers".

- **Original Research Papers** should consist of original research. The body text should have the following contents: Title, Author(s), name(s) and affiliation(s), Abstract, Keywords, Introduction, Materials and methods, Results, Discussion, Acknowledgements and References.

- **Short Communications** are concise but complete description of limited investigations, which should consist of the following contents: Title, Author(s), name(s) and affiliation(s), Keywords, Introduction, Materials and methods, Results and discussion (combined), Acknowledgements and References. The number of figures and/or tables combined is limited to 4. They should not exceed 3000 words, including figures and tables, with up to 30 references. Because of their nature, these communications will go through a more accelerated review and publication process.

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7-Materials and methods: This section should be brief but sufficient information about the materials studied, instruments used, specialized chemical source and related experimental details must be provided so that other investigators can replicate the research.

Note: Sub-title either for this section or for "Results" should be given as italic with no bolded style. For instance:

Materials and methods (bold) *Sampling methods*...(Italic)

8-Results: In the Results section, only findings should be described without discussion of their significance. This section provides information by means of text, tables, and figures. The results should be concisely presented.

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b-Figures: All figures should be numbered sequentially in the text (i.e., Fig 1, Figs 3, 4–6), and must be cited in the text. Titles of figures go underneath the figure. If a figure is outsized it may be reduced photographically. Be sure to include clear, sharp pictures. The resolution must be set to at least 300 DPI (dots per inch). Figures, graphs and drawings normally should be all in black and white, not color. If figures are in parts, please label with upper case letters (A, B, C etc.) in the top

left corner of each part. Writing any text on the figures should be avoided as much as possible.

9-Discussion: The Discussion should not repeat the results; instead, the Discussion deal with the interpretation of results, citing relevant published research, and should also be as concise as possible. General information irrelevant to the subject matter should not be dealt with in prolonged discussions. The final paragraph should highlight the main conclusions of the study; do not include "Conclusions" as a separate heading.

10-Acknowledgement: This section includes acknowledgment of people, grant details, funds, etc.

11-References: The new version of Harvard system of referencing has been adopted by the journal as follows. The full list of references should be in alphabetical order. Journals names should be given in full without abbreviation.

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- Sakai, M., 1999. Current research status of fish immunostimulants. Aquaculture, 172, 63-92.
- Coad, B. W. and Najafpour, N., 1997. Barbus sublimus, a new species of cyprinid fish from Khuzestan Province, Iran. Ichthyological Exploration of Freshwaters, 7(**3**), 273-278.
- Rosenthal, H., McGlade, J. and Gollasch, S., 2001.The role of aquaculture in integrated coastal zone management. Bulletin of Aquaculture Association of Canada, 101(**1**), 5-10.
- AOAC, 2000. Official methods of analysis. 17th ed. Association of Official Analytical Chemists. Gaithersburg, MD, USA, 2200P.
- Noga, E. J., 2010. Fish disease: Diagnosis and treatment. 2nd ed.USA: Wiley-Blackwell.
- Bezirtzoglou, E., Alexopoulos, A., Voidarou, c. and Noussias, H., 2009. Occurrence of Mycobacterium sp.in euryhaline fishes in Greek aquaculture farms. 1st International Congress on Aquatic Animal Health Management and Diseases. Iranian Veterinary Council. Iran. Tehran. February. 2009.

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Research Article

Effect of solvent type on phytochemical properties of burdock (*Arctium lappa*) extract and their effect on some pathogenic bacteria strains in rainbow trout, *Oncorhynchus mykiss*

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Abstract

The extraction method and particularly the type of solvents used in the procedure are one of the most crucial steps for isolating antimicrobial compounds and extracts that potentially affect various microorganisms. This study aimed to evaluate the effects of water, ethanol (100%), methanol (100%), ethanol-water (50:50), and methanol-water (50:50) burdock (*Arctiumlappa*) root extracts (BE) on total phenol content and polyphenols as well as the antioxidant activity. In the next step, the solvent with the highest efficiency to have phenolic compounds were selected and employed to investigate the antibacterial effects of BE on the pathogenic bacterial strains (*i.e. Yersinia ruckeri*, *Lactococcus garvieae*, *Pseudomonas putida* and *Aeromonas hydrophila*) in rainbow trout (*Oncorhynchus mykiss*) by using disc diffusion test and tube dilution techniques. Maximum mean total phenolic content and antioxidant capacity were detected in the water-methanol mixture. Additionally, High-performance liquid chromatography(HPLC) analysis revealed that both hesperidin and trans-ferulic acid compounds existed in all solvents. The antimicrobial activity of water-methanol BE was varied based on the type of microorganism. In this context, *Y. ruckeri* as a gram negative bacterium was found to be the most susceptible one compared to other pathogens. According to our findings, water-methanol solvent was more efficient to isolate phenolic compounds and exert antioxidant capacity, as the level of phenolic compounds in the extract was increased. Moreover, *Y. ruckeri* was recognized as the most sensitive pathogen in all tests.

Keywords: Polar and nonpolar solvents, *Arctium lappa* root extract, Antibacterial, Rainbow trout, Phenol

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Introduction

Burdock plant (*Arctium lappa*) is an herbaceous biennial plant belonging to the Asteraceae family. Having a thick branched stem, this plant grows wild in the humid and moderate plains and regions in Europe and Asia (Predes *et al.*, 2009). Burdock has antifungal, antiviral, anti-inflammatory, and detoxification properties (Ferracane *et al*., 2010). Burdock root contains chemical compounds including inulin, volatile oils, tannins, resin, sugar, iron, calcium, quercetin, arctigenin, and vitamin C. Furthermore, this flavonoid-containing plant has also remarkable antioxidant activities (Ferracane *et al*., 2010). Burdock contains different kinds of lignan compounds, notably lapazole C, F, A,matairesinol, arctigenin, arctinin, arctignan E. Arctin as a lignin has been found in the root and it can significantly reduce the elevated level of Malondialdehyde (MDA) as a marker of oxidative stress (Chan *et al*., 2011).

Due to their hydroxyl groups, phenols have the ability for scavenging free radicals, they are among the very important ingredients in plants and may directly contribute to the oxidative effect of the plants (Gulcin *et al.*, 2002). Effects of phenolic compounds may be included to their capacity for scavenging free radicals, metal-chelating properties, gene expression regulation and also their co-antioxidant activity (Neergheen *et al*., 2006). Apparently, there is a close relationship between antioxidant activity and total phenolic contents (Davarynejad *et al.*, 2012). A large number of studies have been conducted on antioxidant compounds and several synthetic antioxidants are also available which using them has been limited due to their toxicity. Therefore, finding natural antioxidants remarkably derived from plants and using them in food and drug industries are a pressing need. Employing these plant-derived natural compounds has some advantages including extensive biological effects, especially in controlled concentrations (Milos *et al*., 2000). It should be noted that a single method isn't efficiently enough to survey antioxidant activity, but a combination of several procedures is an acceptable approach to assess antioxidant activity of various extracts (Kulisic *et al.*, 2004). In general, there are water and organic solvents including ethanol, acetone and diethyl-ether to extract polyphenols from the plants (Sun and Ho, 2005; Turkmen *et al.*, 2006; Hayouni *et al.*, 2007). There are obvious differences between phenolic contents in different extracts that may have been caused by variations in sample preparation approaches, the method used in the procedure, the duration of extraction process, and the physicochemical properties of the used solvents. By Rezaei *et al*. (2015) the effects of different groups of solvents and total phenolic content of Saqez (*Pistacia atlantica*) shell and the efficiency of their extraction were investigated and there was a significant difference that was attributed to the polarity, viscosity and unique vapor pressure of each

solvent . Thus, introducing a solvent with a defined concentration and maximum efficacy to isolate phenolic and antioxidant compounds from a certain plant is not an easy task.

Rainbow trout is the most important species among cold-water fish species in most parts of the world. Due to the increasing desire to breed this species, there are growing reports of arising different diseases such as bacterial diseases. *Lactococcus garvieae*, *Yersinia ruckeri*, *Aeromonas hydrophila* and *Pseudomonas putida* are the important underlying causes of septicemia and high mortality in fish farms especially in rainbow trout farms (Zorriehzahra *et al*., 2017). Since different extracts display varied levels of antimicrobial activity against various strains of bacteria, it seems that this antibacterial activity is derived from different chemical properties of particular ingredients in the plants. Isolation efficacy of these substances depends upon the type of solvent used for dissolving the solute. Hence, taking different approaches in extraction and employing various kinds of solvents may lead to more efficient extraction of biological substances with antibacterial properties, giving rise to developing new products with enhanced antibacterial activities (Harborne, 1998).

The aim of the current study is to investigate the effects of solvent type on the total phenolic compounds content, polyphenols, and antioxidant activity of Burdock root extract and finally evaluate the impacts of the extract with the most efficient solvent (regarding total phenolic content and antioxidant activity) against several pathogenic bacteria (gram-positive and gram-negative) in rainbow trout aquaculture industry.

Materials and methods

Preparing burdock plant and extraction procedure

Burdock plant was collected in May 2016 from Sepidan region (Fars Province). The plant then was taxonomically identified and confirmed by Herbarium and Plant Systematics Laboratory in the Faculty of Basic Sciences of Shiraz University. Protecting from exposure to light and humidity, roots were air-dried at room temperature until a constant weight was obtained and then were grinded by an electric grain mill grinder.

To prepare aqueous, methanol, ethanol-water and, ethanol-water extracts, 100g of powdered roots was added to an erlenmeyer flask containing 500 mL of methanol (100%) or ethanol(100%) or distilled water or methanol-water mixture (1:1) or ethanol-water mixture(1:1). The mixture was mixed gently on a magnetic stirrer at room temperature for 72 hours till extraction was completely accomplished.

Then to obtain primary extracts, the mixture of solvent and root powder was filtered by whatman filter paper. Supernatant was centrifuged at 3000 rpm for 10 minutes and was passed through a 0.45-µm pore size filter. To remove microbial contaminations

single-use syringe filters were employed and ultimately primary extracts were concentrated in a vacuum distillation apparatus. Concentrated extracts were collected in aluminum sterile containers and stored at 4°C until the later use (Ahmad and Beg, 2001).

Quantitiation of total phenolic content

Total phenolic content was measured in terms of the data obtained from folin-ciocalteu colorimetric method. Gallic acid was employed as the reference standard (Marinova *etal*., 2005). Then 1.8 mL of distilled water and 0.2 mLof diluted folin-ciocalteu reagent (1:15 v/v) were added to 0.2 mL of each extract or reference standard (0-100 mg/mL gallic acid in distilled water). After 5 minutes, 3 mL of sodium bicarbonate solution (7%) was added and the volume was brought to 5 mL with distilled water. The samples were incubated at room temperature for 90 minutes and then the absorbance was measured at 765 nm and total phenolic content was calculated as mg GA g^{-1} DW with regard to the calibration curve.

Investigation of antioxidant activity by DPPH (1, 1diphenyl-2-picryl hydrazyl)

In this method DPPH, a lipophilic stable radical compound with an absorption maximum at 517 nm was used. Ability to donate hydrogen atom or electron was evaluated by various chemical compounds and extracts according to their ability to change purple DPPH into a colorless form. To assess the level of antioxidant properties of this plant, 35-50 µL Burdock root extract dissolved in different solvents were added to 1 mL absolute ethanol. Then 1 mL DPPH solution (0.004%) was added in dark and after 30 minutes absorbance was read by CECILL9900 spectrophotometer (made in Britain). Finally, by using the following formula, the ability of DPPH to capture free radicals was calculated (Akowuah *et al*., 2005):

$$I\ \% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

In this formula A_{blank}, A_{sample} and I% represent the optical density of the negative control (without extract), the optical density of the extracts and the percentage of DPPH free radical scavenging activity, respectively. Using the linear chart, IC50 value was calculated and percentage of inhibition was determined in mg/mL.

Quantification of polyphenolic compounds using HPLC (High-performance liquid chromatography)

An Agilent HPLC system was used for this assay. Flow rate was 0.5 mL/min and the solvents used in the mobile phase include acetonitrile, water and acetic acid (2%). C18 reversed phase was employed as the filling material. Column height and particle size were 25 cm and 5µm, respectively. Chromatograms were acquired at 320 nm by the detector and the injection volume was 20 µL. All the steps were performed at room temperature (25°C) and Chrom Gate software was used to analyze data. 0.1 mg of each standard

was dissolved in HPLC grade methanol and 0.005, 0.02, 0.04, 0.06 and 0.1 mg/mL concentrations were prepared and were loaded along with other samples in triplicates. In order to perform quantitative analysis, the resultant chromatograms were compared with standards and the concentrations of these compounds were calculated on a dry-weight basis (µg/g) (Materska and Perucka, 2005).

Preparation of bacteria

In the present study, four bacterial strains notably *Lactococcus garvieae* (ATCC 49156), *Aeromonas hydrophila* (ATCC7966), *Yersinia ruckeri* (entrobacteria) and *Pseudomonas putida* (KT2440) were isolated from rainbow trout (*Oncorhynchusmykiss*) in farms of Fars Province and their genus and species were validated by biochemical and molecular tests (Austin and Austin, 2007). Bacteria were initially cultivated in liquid medium (Tryptic Soy Broth or briefly TSB) and after 48 hours of exponential growth, were centrifuged at 3000 rmp for 10 minutes. The pellet was washed thrice with physiological serum and then was resuspended and a bacterial suspension was prepared. Turbidity was measured at 530 nm by a spectrophotometer and the suspensions were diluted until their turbidity was adjusted to the turbidity of a 0.5 McFarland standard. The suspension must contain 1.5×0^{8} CFU/mL(Valero and Salmerón, 2003).

Determining antibacterial activity of burdock extract using disc diffusion method

After determining the most efficient solvent to prepare the extract, antibacterial activity of this plant against gram-positive and gram-negative bacteria was investigated. We used the sterile blank disks manufactured by Padtan-Teb Company (Tehran, Iran). Blank disks were put into the tubes containing 50, 100, 400, 200 and 600 mg/mL extract and after 50-30 minutes (after complete absorption of extracts to the disk), the disks were completely dried at 45°C. Then 100µL of bacterial suspension was prepared with reference to the 0.5 McFarland standards and uniformLy distributed over the entire surface of the Mueller Hinton agar plates. The disks were placed on the surface of agar at a certain distance from each other and also from the edge of the plate and were fixed with gentle pressure. Plates were incubated for 24 hours at 37°C and then the diameter of the bacterial growth inhibition halo was measured precisely by a ruler (in mm). Each measurement was repeated three times. In all steps standard tetracycline disks (30µg/disk) manufactured by Padtan-Teb Ltd. (Tehran, Iran) were employed (Bauer *et al*., 1966).

Measuring minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Taking tube dilution method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration

(MBC) of burdock extract were determined. To determine MIC we used a series of 11 sterile test tubes. 10 out of 11 test tubes were used to test various dilutions and one as control. After culturing, all test tubes were incubated at 37°C for 24 hours and the level of turbidity in each tube was evaluated. In comparison with the control, the concentration of the first clear tube was regarded as MIC value. This test was performed in triplicate for each microorganism (Benger *etal*., 2004).

To determine minimum bactericidal concentration (MBC) of the extract, 1 mL of the concentration with no turbidity was cultured over the entire surface of the Mueller Hinton agar plates by using the streaking method. Plates were incubated at 37°C for 24 hours and then the lowest concentration in which no bacteria growth was detected was regarded as minimum bactericidal concentration (MBC) value (Espinel-Ingroff *et al*., 2002).

Statistical analysis

All statistical analysis was performed using SPSS v.19.0. One-way analysis of variance (ANOVA) was used to compare the means and Duncan's test to investigate the difference between the means at significance level of 0.05.

Results

The means of total phenolic content in extracts dissolved in different solvents have been compared and illustrated in Figure 1. According to our results, the greatest total phenolic content was detected in the aqueous-methanolic extract at 417±13.9 mg GA g^{-1} DW and the lowest was found in aqueous extract at 205.1±10.18 mg GA g^{-1} DW. Total phenolic content in methanolic extract, ethanolic extract and aqueous-ethanollic extract was higher than phenolic content of aqueous extract. However the difference was not significant (p>0.05).

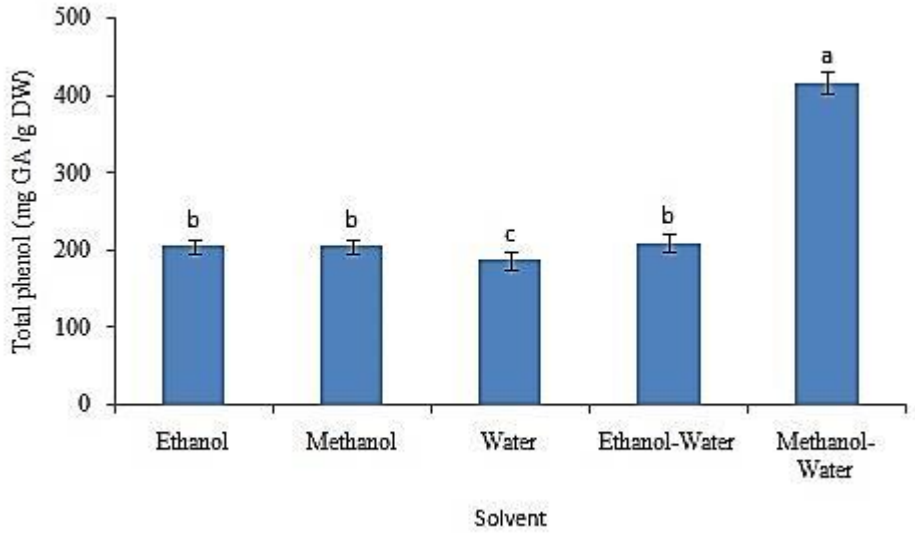

Figure 1: comparing total phenolic content of burdock root extracts dissolved in various solvents.

Unlike letters represent a significant difference between different solvents (p<0.05). The data obtained from DPPH test to measure antioxidant activity have been displayed in Figure 2. Our findings demonstrated that aqueous-methanolic extract showed the minimum IC50 of 540 and then ethanolic extract and methanolic extract were the most effective ones, respectively. Nonetheless the latter extracts showed no significant difference (p>0.05). Among them, aqueous extract represented the least antioxidant activity (IC50=590) and compared with other extracts, the difference was significant (p<0.05).Unlike letters indicate a significant difference between various solvents (p<0.05).

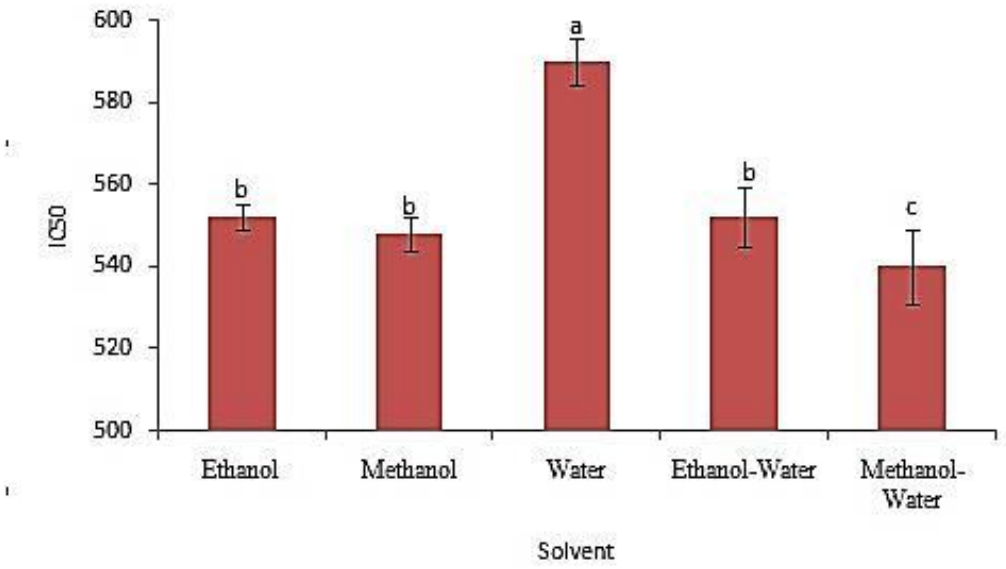

Figure 2: comparing antioxidant capacity of burdock root extracts dissolved in various solvents.

Correlation between phenolic compounds and antioxidant activity

Relationship between phenolic content and antioxidant capacity has been shown in Table1. An inverse correlation between phenolic content and IC50 of the extracts in antioxidant activity evaluations was detected (97%), indicating an increase in antioxidant activity by increasing phenolic compounds content.

Table 1: Results of correlation analysis between phenolic compounds and antioxidant activity.

Test type	Total phenol	IC50
Total phenol	1	0.975*
IC50	-	1

*Correlation is significant at the 0.05 level.

Comparing polyphenolic compounds in burdock root extracts dissolved in various solvents

HPLC analysis revealed that both hesperidin and trans-ferulic acid were found in all solvents, vanilin in all solvents except aqueous-ethanol solvent, rosmarinic acid in all solvents except ethanol and methanol, quercetin and hesperetin restrictively in aqueous solvent and chloregenic acid in ethanol and aqueous-ethanol. Other compounds were detected in none of the solvents (Table 2).

Table 2: polyphenol compounds (mg/mL) in burdock root extracts dissolved in various solvents.

Polyphenol compound (mg/L)	Water	Ethanol-Water	Methanol-Water	Methanol	Ethanol
Chloregenic acid	-	-	1.35	-	1.73
Trans- ferulic acid	15.56	21.36	16.13	32.02	31.72
Hesperidin	104.66	98.99	85.56	90.71	42.60
Vanilin	1.08	1.51	-	4.53	2.64
Quercetin	3.57	-	-	-	-
Hesperetin	6.79	-	-	-	-
Rosmarinic acid	27.90	30.10	28.80	-	-

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

With reference to the results of antioxidant activity tests and total phenolic content, burdock root extracts dissolved in aqueous-methanol solvent was the most efficient one to survey antibacterial activity. Minimum inhibitory concentration (MIC) values of this extract have been summarized in Table 3. According to our findings, MIC value of methanol-ethanol extract to inhibit *Y. ruckeri* growth was 1.25 mg/mL. MIC values of methanol-aqueous against *Y. ruckeri* , *A. hydrophila* and *P. putida* werefound to be1.25, 2.5 and 2.5(mg/mL), respectively. The results of minimum bactericidal concentration tests on burdock methanol extract have been represented in Table 3. According to our findings, MBC values of aqueous-methanol extract on *Y. ruckeri* , *A. hydrophila* and *P.putida* were 5, 10 and 10 (mg/mL), respectively (Table 4).

Table 3: Quantifying minimum inhibitory concentrations (MIC) of aqueous-methanol burdock extract different concentrations. Plus (+) sign represents bacteria growth and minus (-) sign represents no growth.

Bacteria	aqueous-methanol burdock extract different concentrations (mg/L)										
	control	0.02	0.04	0.08	0.16	0.63	1.25	0.31	2.50	5	10
L.garvieae	+	+	+	+	+	+	+	+	+	+	-
A.hydrophila	+	+	+	+	+	+	+	+	-	-	-
Y. ruckeri	+	+	+	+	+	+	-	-	-	-	-
P. putida	+	+	+	+	+	+	+	+	-	-	-

Table 4: Determining minimum bactericidal concentration (MBC) of aqueous-methanol burdock extracts in different concentrations.

Bacteria	aqueous-methanol burdock extract different concentrations (mg/L)										
	control	0.02	0.04	0.08	0.16	0.63	1.25	0.31	2.50	5	10
L.garvieae	+	+	+	+	+	+	+	+	+	+	+
A.hydrophila	+	+	+	+	+	+	+	+	+	+	-
Y. ruckeri	+	+	+	+	+	+	+	+	+	-	-
P. putida	+	+	+	+	+	+	+	+	+	+	-

Growth has been indicated as plus symbol (+) and no growth has been represented as minus symbol (-).

Determining antibacterial activity of burdock aqueous-methanol extract by agar diffusion assay

The results of investigating antibacterial activity of burdock aqueous-methanol extract and standard antibiotic (tetracycline) by disk-diffusion agar method have been shown in Table 5. According to the results, aqueous-methanol extract had inhibitory effects on *Y. ruckeri* in all concentrations (5, 10, 15, 20, 25 mg/mL). As aqueous-methanol extract concentration increased, the diameter of the bacterial growth inhibition halo also increased significantly and there was a significant difference among all the pathogens under survey except *P. putida* and *A. hydrophila* in 400 and 600 mg/mL concentrations (p>0.05).

Table 5: The diameter of the bacterial growth inhibition (in mm) in the presence of different concentrations of aqueous-methanol burdock extracts.

Bacteria	Tetracycline antibiotic (30µg / Disk)	aqueous-methanol burdock extract different concentrations (mg/mL)				
		50	100	200	400	600
Y. ruckeri	10.45±3.34	8.10±0.57	9.8±0.28	11.6±0.52	13.5±0.50^{a}	16.80±0.50^{a}
L.garvieae	14.38± 0.94	-	-	-	-	7.3±0.57^{c}
P. putida	12.75±0.87	-	-	-	8.3±0.37^{b}	9.9±0.87^{b}
A.hydrophila	18.15±0.45	-	-	-	8±0.67^{b}	9.8±0.49^{b}

Similar letters in each row indicating no significant difference in different concentrations (p>0.05).

Discussion

The increased incidence of bacteria resistance to many antibacterial drugs is of great concern and medicinal plants have proven as an alternative source of antibacterial agents.A recent study evaluated the antibacterial activity of a phytotherapeutic agent prepared from an ethyl acetate fraction (AcOEt) extracted from *Arctium lappa*. This agent inhibited the growth of all tested microorganisms (*Pseudomonasaeruginosa*, *Escherichia coli*, *Lactobacillus acidophilus*, *Streptococcus mutans* and *Candida albicans*) (Popescu *et al*., 2010). Solvent type significantly influences total phenolic content (p<0.05). The highest phenolic content was reported in aqueous-methanol extract. Overall, the best results were obtained from water-methanol mixture (417±0.9 mg GA g^{-1} DW), while the lowest content was found in water extract (Fig. 1). Phenol compounds contain one or more hydroxyl groups (polar part) attached to an aromatic ring. Due to its polarity, this conformation distinct phenols from

other molecules and phenols solubility in solvents can be explained by this conformation (the coexistence of polar and non-polar parts in a molecule) and intermolecular forces between the molecules and solvent as well (Galanakis *et al*., 2013). Water as a solvent creates a hyper polar environment, appropriate for isolation of bioactive compounds with higher polarity, whilst water-methanol, water-ethanol, ethanol and methanol solvents are convenient to extract the molecules with a broad spectrum of polarities due to the presence of the environment created by adding water to these organic solvents (Sun *et al*., 2015). On the other hand, water is necessary for cell swelling and hence in the presence of adequate volume of water in organic solvent, cell wall permeability enhances and can easily break down. Nonetheless, in absolute ethanol or methanol (100%) there is no water available for swelling, so the mixture of two solvents (the mixture of 50% (v/v) water with methanol or methanol) is more optimal and effective (Charpe and Rathod, 2014). According to the reports, due to the higher polarity, the mixture of water and ethanol or methanol are more effective to extract phenolic compounds compared with their corresponding absolute alcohols (Sultana *et al*., 2009). In the current study, in spite of water high polarity compared with water-ethanol, water-methanol, methanol and ethanol solvents, this inorganic solvent displayed a low efficiency to extract phenolic compounds. The mechanism underlying this lesser efficiency may be explained by the water potential to dissolve proteins, polysaccharides and other polar compounds. As another reason, due to the semi-polar nature of phenolic compounds present in burdock root, these compounds have dissolved in water with less efficiency. In a research performed by Ghasemzadeh *et al*.(2015) on rice bran extract, the highest amount of total phenolic content was reported 288.40 mg gallic acid in 100 g of dry matter. The extraction was performed by ultrasound using water-ethanol mixture as solvent (50:50) and was compared with the extract dissolved in ethanol. Furthermore, Nazir *et al*. (2013) conducted a study on phenolic compounds content dissolved in different solvents in persimmon and in consistent with our results, demonstrated that phenolic content was higher in water-ethanol mixture compared with ethanol or water solvents, implying that polarity and viscosity are two properties involved in releasing phenolic compounds from cells (Turkmen *et al*., 2006; Hayouni *et al*., 2007).

DPPH radical scavenging activity testing is an important technique to evaluate extract potential to donate hydrogen or inhibit free radicals. DPPH is a stable free radical which shows a maximum absorption at 517 nm. Due to the reaction between DPPH with antioxidants or hydrogen donor compounds, absorption is reduced which consequently causes a color change from purple to yellow (Ansari *et al*., 2013). Our results evinced that the

maximum calculated IC50 for different extracts differ significantly (p<0.05). The lowest IC50 value was 0.955 mg/mL for aqueous-methanolic extract (50:50 v/v), implying on the higher antioxidant capacity of this extract and aqueous extract showed the lowest free radical scavenging activity. Ling *etal*. (2009) accomplished a research and demonstrated that mango leaves extract dissolved in ethanol solvent and water displayed the highest and lowest free radical scavenging and antioxidant activity, respectively. Rezaeizadeh *et al*. (2011) investigated antioxidant activity of methanol and chloroform extracts of *Momoridica charanita* plant and claimed that the higher free radical scavenging activity of methanol extract (compared with chloroform extract) is due to the presence of higher phenolic content in this solvent and also there are a linear relationship between phenolic content and DPPH radical scavenging activity which is consistent with our findings. Radical scavenging activity of phenolic compounds can be attributed to their hydroxyl groups and enhances by increasing the concentration of phenolic compounds and increased number of hydroxyl groups and thus the probability of donating hydrogen to DPPH free radical, giving rise to the elevation of extract antioxidant and radical scavenging activity.

According to the correlation results in Table 1, phenolic compounds account for 97% of DPPH antioxidant and radical scavenging activity. This correlation has been proven by Ramli and colleagues in 2014, as well. Anokwuru *et al*. (2015) conducted a study on the association between phenolic compounds and antioxidant activity of Terminalia sericea burch plant and reported that there was a negative correlation between phenolic content and IC50 of DPPH radical scavenging activity (r=0.209) which the findings are consistent with our results. Therefore, it can be concluded that due to the presence of a large quantity of phenolic compounds, burdock root extract is associated with increased antioxidant potential.

We examined the effects of different solvents on phenolic compounds content of burdock root extract by HPLC and identified the compounds namely trans-ferulic acid, hesperidin, ferulic acid, vanilin, rosmarinic acid, quercetin, hesperetin and chloregenic acid in the root extract. In our results, methanol was recognized as the best solvent to extract trans-ferulic acid and vanilin. The highest rosmarinic acid content was found in water-methanol solvent and 100% ethanol contained the highest quantity of chloregenic acid. Water had the maximum extraction yield of quercetin, hesperidin and hesperetin. Nevertheless, collectively water-methanol mixture has exhibited a higher yield for isolation of phenolic compounds and this may be due to the semi-polar nature or the less polarity of phenolic compounds in burdock root extract which make them less soluble in water, causing to decreased yield. Kallithraka *et al*.(1995) investigated the effects of different solvents on the phenolic compound extraction yield

from grape seeds and identified gallic acid compounds, catechin, epicatechin, epicatechin gallate and procyanidin dimers and trimmers using HPLC approach. According to their findings, methanol yielded the greatest quantity of catechin (+), epicatechin(-) and epicatechin gallate. Additionally, acetone and 75% ethanol extracted the highest amount of proanthocyanidins and gallic acid, respectively. Overall, 75% acetone has yielded higher phenolic compounds quantity **Pekić** *et al.*(1998) conducted a study on grape seed extracts dissolved in water-acetone and water-ethyl acetate solvents and revealed that proanthocyanidins is not able to be efficiently extracted in the absence of water and increasing water content will remarkably enhance yield. However, the excessive increase in water content causes a less selective extraction. Overall results indicated that proanthocyanidins can be selectively extracted by ethyl acetate containing 10% water.

Due to higher efficiency of water-methanol mixture to isolate active ingredients, this solvent was employed to investigate antimicrobial activity of burdock root extract. Alizadeh Behbahani *et al*. (2013) examined antimicrobial activity of *Avicennamarina* leaves extracts dissolved in water, ethanol, methanol or glycerine against *Penicilliumdigitatum* and reported that due to the higher efficiency of ethanol and methanol solvents to isolate active ingredients and subsequent increased dry mass, methanol or ethanol leaves extracts exert greater inhibitory and fungicidal effects against *Penicillium digitatum*. Our study demonstrated that the higher the concentration of water-methanol burdock root extract, the larger the diameter of the bacterial growth inhibition halo of *Y. ruckeri* and in all concentrations the diameter of halos were significantly different with tetracycline halo. There was a significant difference among all bacterial strains treated with 400 and 600 (mg/mL) concentrations of burdock root extract except *P. putida* and *A. hydrophila* (Table 2). Sagdic *et al*. (2002) conducted a study to investigate antibacterial capacity of sage, thyme, oregano and cumin essences against *E. coli* and in consistent with our results they found that, as the concentration of the extracts increase, the diameter of inhibition also increases. Depending upon the type of organism, antimicrobial activity of burdock water-methanol extract varied, so that *Y. ruckeri* as a gram negative bacterium was found to be the most susceptible one, compared with other pathogens (Table 2) while *L. garvieae* as a gram positive bacterium was recognized as the most resistant strain. Moreover, comparing the results of disk diffusion testing by using tetracycline as control evinced that water-methanol extract of burdock root extract displayed a relatively higher antibacterial activity against *Y. ruckeri*, compared with control group. Hitherto, few studies have been performed focusing on antibacterial activity of medicinal herbs against *Y. ruckeri.* Alishahi *et al*.(2012)

showed that pomegranate peel, *Nigella Sativa* and *Zataria multiflora* extract had appropriate antibacterial effects against this strain, so that the diameter of growth inhibition halo was 22, 20 and 16 mm, respectively. Habibipour and Rajabi (2015) accomplished an in vitro survey to evaluate antimicrobial activity of burdock extract and *Artemesia absinthium* extract and found that burdock extract had antibacterial effects on *Haemophilus influenza* and *P. aeraginosa* and the diameter of bacterial growth inhibition halo was 18.4 and 11.9 (in mm), respectively. This activity can be clarified by the presence of lignan compounds including arctigenin and arctin which are among the major components in burdock root extract. These secondary metabolites function as anti-cancer, anti-inflammtion and antibacterial agents exert their effects by targeting cell membrane (Kamkaen *et al*., 2006).

The results of Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) testing of burdock root methanol-water extract indicated that *L. garvieae* was the most resistant pathogen (MIC, 10 mg/mL), while MIC values of burdock root methanol-water extract for *Y. ruckeri*, *P. putida* and *A. hydrophila* were 1.25, 2.5 and 2.5(mg/mL), respectively. MBC of burdock root methanol-water extract against *Y. ruckeri*, *P. putida* and *A. hydrophila* was obtained 5, 10 and 10 mg/mL, respectively. Habibipour and Rajabi (2015) reported that MIC and MBC of burdock root methanol-water extract against *H. influenza* was 230 and 540 mg/mL, respectively. However, these values were 45 and 90 mg/mL for *A.absinthium*. MIC and MBC values of burdock root methanol-water extract against *P. aeruginosa* were 500 and 750 mg/mL and of *A. absinthium* were 285 and 430 mg/mL. In both of them, MIC value against *Bacillus cereus* was 166 mg/mL. Antimicrobial properties of burdock plant can be explained by the presence of lignan and phenolic compounds, giving rise to enhancing permeability and, bacterial cell wall breakdown and subsequently extracellular discharge of cellular content (Kamkaen *et al.*, 2006).

The results of this study indicated that burdock root extract contains a substantial quantity of phenolic compounds with antioxidant capacity which can be used as natural antioxidant source. According to our findings, compared with other solvents, water-methanol solvent is more efficient to isolate phenolic compounds and antioxidants and antioxidant capacity enhances, as the quantity of phenolic compound in extract increases. Therefore, due to the higher efficiency to isolate active ingredients from burdock root extract, methanol-water solvent was employed to examine antimicrobial activity of burdock root extract. Among all the pathogens tested, *Y. ruckeri* was recognized as the most sensitive strain. However conducting extensive studies in clinical settings in fish farms seems necessary. We believe that after determining effective concentrations, understanding the

underlying mechanisms and proving its safety, this extract can be introduced as a promising efficient natural antimicrobial agent to aquaculture industry as an alternative to chemical drugs and antibiotics to treat yersiniosis.

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Research Article

Isolation, identification and evaluation of the anti-diabetic activity of secondary metabolites extracted from bacteria associated with the Persian Gulf sponges (*Haliclona* sp. and *Niphates* sp.)

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Abstract

Sponge-associated bacteria have a special position in marine biotechnology due to their unique biological activities. The main objective of this study was to isolate and identify the bacteria associated with sponges around Qeshm Island, Iran, which inhibit the activity of alpha-glucosidase and alpha-amylase enzymes by the produced metabolites. Samples were collected from sponge species living in the study area, including the genera *Haliclona* and *Niphates*. Isolation was performed using culture-dependent techniques. A total of 155 bacterial isolates were collected. The diversity pattern of bacteria in the sponge samples showed that the *Vibrio* and *Bacillus* constituted the predominant bacterial population. The assessment of alpha-glucosidase inhibitory activity of metabolites extracted from the isolated bacteria showed that 6 isolates could inhibit the enzyme activity with IC_{50} values ranging from 153.5 to 495.4 µg/ml, while 9 bacterial isolates inhibited the activity of alpha-amylase enzyme in IC_{50} values at the range of 112.9 to 670.9 µg/ml. The cytotoxic activity of the metabolites extracted on human umbilical cord endothelial cells showed the toxicity of the three extracts at effective concentrations, while seven isolates showed no toxicity. Genetic identification indicated 97% to 100% similarity of the potent isolates with the NCBI gene bank including *Bacillus pumilus, Bacillus safensis, Vibrio alginolyticus, Pseudomonas stutzeri, Vibrio parahaemolyticus, Pseudomonas lurida, Bacillus tequilensi*, and *Streptomyces enissocaesilis.* The results of this study provided a new understanding of the diversity pattern of cultivable sponge-associated bacteria and their inhibitory activity on alpha glucosidase and alpha amylase enzymes.

Keywords: Alpha-amylase, Alpha-glucosidase, Anti-diabetic, Persian Gulf, Secondary metabolites, Sponge-associated bacteria

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Introduction

In total, 34 of the 36 living animals live in the oceans, while only 18 groups live on land (Fenical, 2020). Due to the extraordinary difference between marine and terrestrial environments, it is estimated that marine bacteria have different characteristics from their soil origins. Therefore, they can produce a variety of bioactive compounds (Amelia *et al.*, 2022). Despite the wide biodiversity in marine environments, research on natural marine products is still in its infancy and remains unexplored compared to terrestrial habitats (Brinkmann *et al.*, 2017). Most studies have focused on the neurotoxic, antiviral, anti-cancer, anti-diabetic, antimicrobial, and cytotoxic properties of marine metabolites. There are some documents investigated regarding to natural feed additives as potential substitute for antibiotics in aquaculture nutrition (Pourmozaffar *et al.*, 2019). In general, natural marine resources have provided a great opportunity for the development of the pharmaceutical industry due to their diverse and unique bioactive compounds (Barzkar *et al.*, 2021).

Secondary metabolites produced by marine bacteria represent various biological activities (Manivasagan *et al.*, 2015; García-Jiménez *et al.*, 2018). These metabolites provide various activities, including antibacterial, antifungal, anti-cancer, anti-tumor, anti-diabetic, cytotoxic, cytostatic, anti-inflammatory, anti-parasitic, anti-malarial, anti-viral, antioxidant and anti-angiogenic, etc. (Gozari *et al.*, 2016; Gozari *et al.*, 2018; Gozari *et al.*, 2019a). Marine sponges belong to the foraminifera branch and feed by filtering large volumes of water through an advanced system. Thereby absorbing many microorganisms, algae, and organic particles and digesting them through phagocytosis (Nazemi *et al.*, 2017; Tamadoni Jahromi *et al.*, 2021). Sponges are able to efficiently retain different marine microorganisms in a coexistence relationship. Therefore, the presence of a large number of microorganisms in the mesophyll of sponges has been proven in various studies (Hofer, 2021). Bacteria can contain up to 60% of sponge biomass, equivalent to 108 to 109 bacteria per gram of sponge tissue (Posadas *et al.*, 2022). The high biosynthetic ability of sponges is due to symbiotic microorganisms (Zhang *et al.*, 2022). One of the major roles of sponge-associated bacteria is to participate in chemical defense mechanisms against invasive organisms and pathogens and to prevent bio adhesion by producing bioactive compounds (Gozari *et al*, 2020). By binding to the active site of the enzyme, these molecules inhibit the entry of the substrate and prevent the catalysis of the reaction by the enzyme. Relatively few enzyme inhibitors have been reported from marine bacteria (Lloyd, 2020). They are isolated from *Streptomyces* sp. SA-2289. Pyrrostatin A and B are inhibitors of the enzyme enacetyl beta-glucosamidase produced by *Streptomyces* sp. SA-3501 (Trang *et al.*, 2021). Diabetes is a metabolic disorder in the body, in which the body

loses the ability to produce insulin or becomes resistant to insulin production. Therefore, the insulin produced cannot function normally (Azam *et al.*, 2022). These biologically active compounds are members of different families including terpenoids, isoprenes, alkaloids, and flavonoids. Flavonoids are compounds that act as alpha-glucosidase inhibitors (Agrawal *et al.*, 2022). Despite advances in drug design, there is still an urgent need for new drugs with natural origins to combat various diseases such as diabetes. Therefore, this study aimed to evaluate the anti-diabetic activity of metabolites extracted from bacteria associated with some sponges in the Persian Gulf.

Material and methods

Sampling

Marine organisms were sampled in May 2020 from 10-15 m depth in Qeshm Island. About 25 samples of sponges were collected by diving and placed in sterile containers. The samples were kept to the ice until reaching the Persian Gulf and Oman Sea Ecology Research Institute laboratory in Bandar Abbas. The collected samples were isolated based on morphological features, including morphometric and morphometric features such as spicule identification by the Persian Gulf and Oman Sea Ecology Research Institute (Hooper, 2003). Sponge samples were identified from 2 species: *Niphates* sp. and *Haliclona* sp.

Preparation of the Samples

The samples were washed with sterile seawater just received at the laboratory to separate the attached particles. Subsequently, sponge samples were cut into 1 cubic cm pieces with sterile scissors under aseptic conditions and placed in a sterile mortar for homogenization. Then, serial dilutions up to 5-10 were prepared by sterile seawater dilution from homogenized samples (Gozari *et al.*, 2019b).

Cultivation and purification of bacteria

The sponges were cultured in culture media with formulations compatible with their natural conditions. Marine Zobell agar, Glycerol asparagine agar, and Marine sponge agar were used to isolate bacteria (Gozari *et al*, 2020).

Production and extraction of secondary metabolites from bacteria

The bacteria were cultured at 10^5 CFU/ml densities in a modified Nutrient Broth fermentation medium. After incubation at 30°C, the fermented liquid was removed and filtered using a vacuum pump (Gozari *et al.*, 2020).

Assaying the inhibition of alpha-glucosidase activity.

Different concentrations of the extracted metabolite were dissolved in sodium phosphate buffer (pH 6.8). 10 µL of each concentration was mixed with 20 µL of sodium phosphate buffer and 20 µl of para-nitrophenyl alpha-di-glucoside (2 mM) in a 96-well microplate and incubated at 37°C for 5 min. 10 µl of diluted alpha-glucosidase

enzyme at a 0.2 U/ml rate was added to each well using 0.01 M sodium phosphate buffer. After incubation at 37°C for 15 min, the adsorption of the samples at 405 nm was recorded by a microplate reader. In the blank form, sodium phosphate buffer was used instead of the enzyme. Acarbose was assayed as a positive control (Chen *et al.*, 2016).

Assaying the inhibition of alpha-amylase activity

Alpha-amylase inhibition by metabolites was measured based on DNSA (Dinitrosalicylic acid) colorimetric method (Hansawasdi *et al.*, 2000).

Evaluation of the cytotoxic activity of inhibitory metabolites

The cytotoxic activity of metabolites was investigated at different concentrations and had enzymatic inhibitory activity against normal human umbilical cord endothelial cell lines (HUVEC). MTT tetrazolium reagent was used for this purpose. IC_{50} of each extract was determined (Peng and Zhao, 2009).

Identification of isolates producing secondary inhibitory metabolites

The microscopic morphology of the selected productive isolates was investigated using the gram stain method. Macroscopic morphology, including strain growth rate, colony color, spore formation, and water-soluble pigment production isolates with inhibitory activity, were identified based on the polyphasic identification strategy of bacteria, which includes morphological, physiological, biochemical, and genetic characteristics (Goto, 2005).

Investigation of the production of some enzymes

The production of hydrogen sulfide, nitrate reductase, and amylase enzymes was performed using a modified broth neutrinos medium. The production of oxidase enzyme was also measured using 1% tetramethyl - phenylenediamine reagent and 3% oxygenated catalase (Pollack *et al.*, 2018).

Genetic identification DNA extraction of productive isolates

Genomic DNA extraction of productive isolates was performed using the CTAB extraction method. For this purpose, different stages of cell lysis, de-proteinization, and purification of genomic DNA were performed after preparing the appropriate biomass. The amount of DNA extracted at 230, 260, and 280 nm was examined by spectrophotometry, and the concentration of genomic DNA was calculated. The quality of DNA extracted by electrophoresis on 1% agarose gel was evaluated (Kieser *et al.*, 2000).

16S rRNA gene amplification of selected isolates

Genetic identification of productive isolates was based on the similarity of the 16S rRNA protected gene of

productive isolates. Primers 27F (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1492R (5′ ACGGCTACCTTGTTACGA 3′) were used to amplify the gene (Heuer *et al.*, 1997). The optimal temperature chain of the PCR reaction is shown in Table 1. After performing the reaction, the quality of the PCR product was evaluated using agarose gel electrophoresis (Fig. 4). 16S rRNA gene sequencing was performed using the Sanger method and a Dye Deoxy Terminator Cycle Sequencing kit technique (Tamadoni Jahromi *et al.*, 2019).

Phylogenetic analysis of productive isolates

Genetic and evolutionary distance of the selected isolates was calculated by comparing them with the closest strains in the NCBI gene bank using phylogenetic analyses. The phylogenetic tree was mapped by MEGA X software based on the neighbor-joining model (Kumar *et al.*, 2018).

Statistical analysis

The IC_{50} of the samples was determined by plotting the dose-response curve using GraphPad Prism 6 software. Results were expressed as mean, standard error (SE). Mean means were compared using one-way ANOVA and the Bartlett test. Data differences at the level of $p<0.05$ were considered statistically significant. The amount of inhibitory activity was calculated by the following equation (Le Berre *et al.*, 2022).

Results

Biodiversity of bacteria isolated from sponge samples

We obtained 106 bacterial isolates from the sponge *Haliclona* sp. and 49 isolates from sponge *Niphates* sp. Collected from Qeshm Island. The results of bacterial identification showed that the diversity pattern was different in two sponge species (Fig. 1).

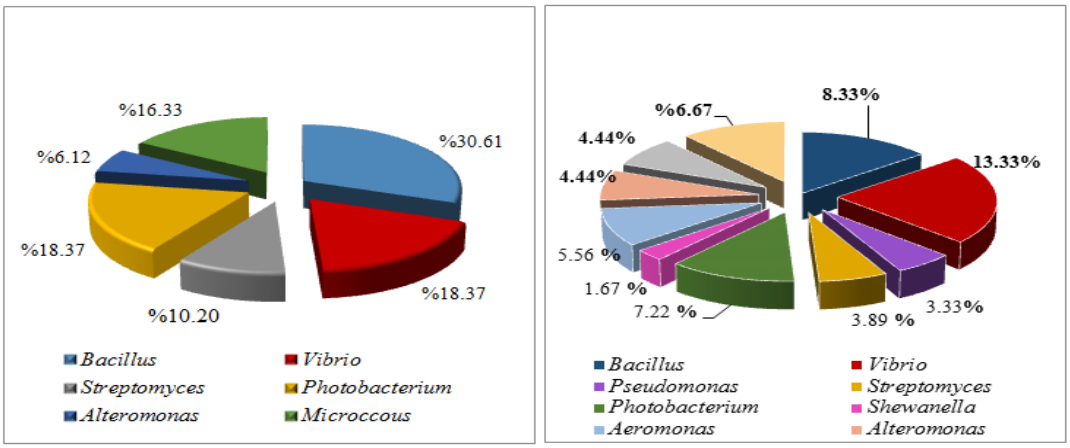

Figure 1: Biodiversity of bacteria isolated from *Haliclona* sp. (right) and *Niphates* sp. (left) collected from Qeshm Island

Measurement of inhibition of alpha-glucosidase activity

Evaluation of the inhibitory activity of metabolites extracted from 155 isolates obtained from sponge samples showed that 9 isolates producing alpha-glucosidase inhibitory compounds with IC_{50} values were less than 1250/g/ml. The IC_{50} inhibitory properties of the metabolites extracted from the generator isolates are listed in Table 1-3. Among the extracted metabolites, the highest inhibitory activity belonged to QH 26 isolate with IC_{50} value of 177.6 µg/ml. While the lowest level of IC_{50} belonged to the metabolites extracted from QN 63 isolate (495.4) (Table 1).

Table 1: Assessment of alpha-glucosidase inhibitory activity of the extracted metabolites.

Isolate	IC_{50} ±SE (µg/ml)
QH 26	177.6±11.38
QH 30	453.8± 19.73
QH 36	153.5± 15.02
QH 37	441.0± 12./20
QN 45	341.8± 14.54
QN 63	495.4±20.13

Measurement of inhibition of alpha-amylase activity

Evaluation of the inhibitory activity of metabolites extracted from 155 isolates obtained from sponge samples showed that 6 isolates producing amylase inhibitory compounds with IC_{50} values were less than 1250/g/ml. The IC_{50} inhibitory properties of the metabolites extracted from the generator isolates are listed in Table 3. Among the extracted metabolites, the highest inhibitory activity belonged to QH 26 isolate with an IC_{50} value of 112.9 µg/ml. While the lowest IC_{50} level belonged to the metabolites extracted from QN 45 isolate at the rate of 411.1 µg/ml (Table 2).

Measurement of inhibition of alpha-amylase activity

Evaluation of the inhibitory activity of metabolites extracted from 155 isolates obtained from sponge samples showed that 6 isolates producing amylase inhibitory compounds with IC_{50} values were less than 1250 g/ml. The IC_{50} inhibitory properties of the metabolites extracted from the generator isolates are listed in Table 3. Among the extracted metabolites, the highest inhibitory activity belonged to QH 26 isolate with an IC_{50} value of 112.9 µg/ml. While the lowest IC_{50} level belonged to the metabolites extracted from QN 45, isolate at the rate of 411.1 µg/ml (Table 2).

Table 2: Assessment of inhibitory activity of amylase enzyme by extracted metabolites.

Isolate	IC_{50} ±SE (µg/ml)
QH 26	9.03±112.9
QH 30	25.01±210.8
QH 36	15.56±314.8
QH 37	19.32±347.8
QN 45	21.53 ±411.1
QN 63	201.9±17.17
Acarbose	78.32± 15.14

Table 3: Determination of IC50 cytotoxic activity of extracted metabolites against HUVEC cell line.

Isolate	IC_{50}± SE (µg/ml)
QH 36	196.7± 25.93
QH 45	510.6± 11.27

Evaluation of the cytotoxic activity of extracted metabolites

The results of the cytotoxic activity assay of enzymes inhibiting enzyme activity showed that isolates of QH 36 and QH 45 against the cervical endothelial cell line of HUVEC showed cytotoxic activity with lethal values. The results showed cytotoxic activity dependent on the concentration of these isolates (Table 3). Determination of IC_{50} of the extracted metabolites showed that QH 36 isolate represented high cytotoxic activity against the HUVEC cell line with an IC_{50} value equal to 196.7 g/ml (Table 2). Examination of microscopic images of treated cells and treatment without treatment confirmed a significant reduction in the number of living cells and their morphological changes (Fig. 2).

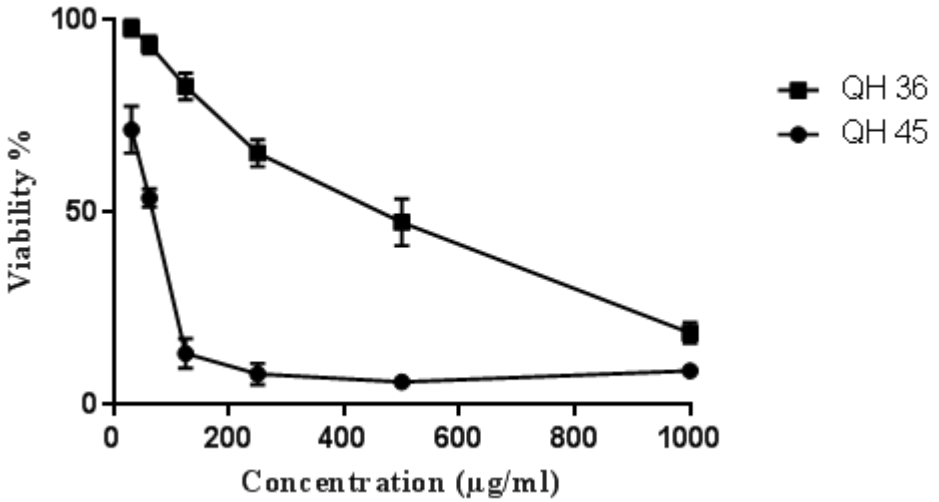

Figure 2: Dose-response diagram of cytotoxic activity of inhibitory metabolites against Human Umbilical Vein Endothelial Cells (HUVEC cells).

Genetic identification of inhibitory isolates

The results of genomic DNA electrophoresis extracted from the isolates confirmed the success of the extraction process. A comparison of the length of genomic DNA extracted from selected isolates with a gene ruler (Ladder) showed that the size of the extracted sequences was more than 10 kbp. As shown in Figure 3, the integrity and structure of the genomic DNA are protected during extraction. Also, the results of PCR gene electrophoresis of 16S rRNA of selected isolates indicated the production of sequences with a length of about 1500 bp. These results showed that the 16S rRNA gene amplification process was performed specifically by the polymerase chain reaction (Fig. 4).

16S rRNA sequence matching assay of productive isolates

The 16S rRNA sequence Blastn analysis results showed about 97 to

100% homology between 6 selected isolates with the type strains registered in the gene bank. The results showed that QH 26 strain had the highest homology with *Bacillus pumilus* strain ATCC 7061 strain at 100%. QH 30 isolate showed the highest homology at 99.88% with *Bacillus safensis* strain FO-36b. The 16S rRNA sequence of the QH 36 isolate showed 99.12% similarity with Vibrio alginolyticus strain ATCC 17749. Also, 99.88% similarity of QH 37 isolates with *Pseudomonas stutzeri* strain CCUG 11256 was confirmed. The results of this study also showed that QN 63 isolate had the highest similarity with *Vibrio alginolyticus* strain NBRC 15630 with 99.87%. While the 16S rRNA gene belonging to QN 45 strain showed the highest similarity with *Vibrio parahaemolyticus* strain ATCC 17802 strain at 100%.

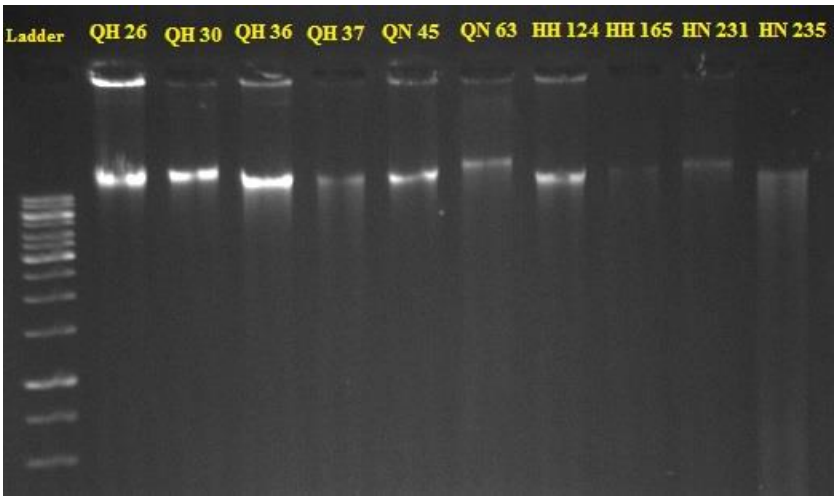

Figure 3: Genomic DNA electrophoresis extracted from selected isolates on agarose gel.

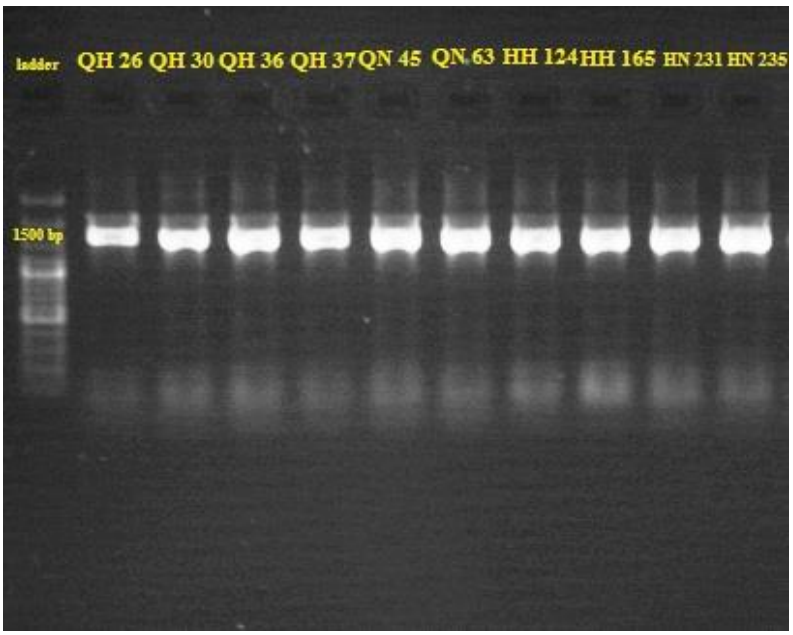

Figure 4: PCR product electrophoresis of 16S rRNA amplification of selected isolates.

Phylogenetic analysis of productive isolates

The results of phylogenetic analysis between inhibitory metabolite-producing strains and the nearest index strains based on 16s rRNA gene sequence or using Neighbor-joining pattern showed that the strains in the phylogenetic tree drawn were located in 4 separate clusters. The QN 63, QN 45, and QN 36 strains were found in a common cluster, although the QN 45 strain formed a separate evolutionary pathway. In the second cluster, strains belonging to the genus Pseudomonas were placed, forming a separate evolutionary path from the other strains (Fig. 5).

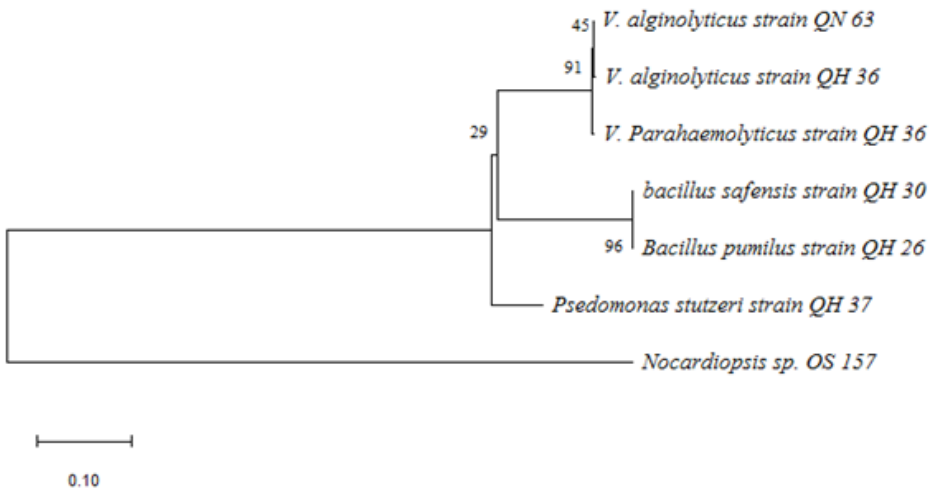

Figure 5: Phylogenetic tree of the potent strains based on Neighbor joining method. The numbers shown next to the nodes indicate the Bootstrap value. The scale bar indicates the replacement of 5 nucleotides per 100 nucleotides.

Discussion

In recent years, extensive programs have been designed and implemented to explore natural compounds, especially enzyme-inhibiting compounds from marine resources worldwide (Barzkar *et al.*, 2022). The present project was planned and implemented to isolate the bacteria that produce inhibitory compounds of enzymes related to diabetes. In this project, an attempt was made to isolate sponge samples from Qeshm Island. Bacteria associated with sea sponges have been prominent in microbial biotechnology and were screened for this purpose (Manivasagan *et al.*, 2015).

Examination of the biodiversity pattern of bacteria from two genera (*Haliclona* and *Niphates*) in both sampled areas showed that the samples belonging to the genus *Haliclona* hosted a more diverse range of bacteria. In *Haliclona* sponge samples, about 10 different genera of different bacteria were present, while in *Niphates* sponge samples, this diversity was limited to 6 bacterial genera. It is remarkable that the reported pattern is limited to culturable bacteria only with the

isolation methods used in this study and cannot indicate bacterial diversity in the sponge microbiome, especially non-culturable species. However, to compare the diversity of culturable bacteria in the studied samples, the isolation techniques are significant.

In this study, *Vibrio* and *Bacillus* genera predominance in the studied samples was significant. In other studies aimed at isolating actinobacteria, the predominance of Streptomyces in sponge samples has been reported. For example, a study reported that 73.6% of the isolates obtained from the Hymeniacidon perceive sponge belonged to *Streptomyces* (Zhang *et al.*, 2022). Results of another study on the biodiversity of actinobacteria associated with the marine sponge Iotrochota sp. showed that members of the genus Streptomyces formed the predominant population of actinobacteria associated with this sponge (Jiang *et al.*, 2008). The results of a biodiversity study of actinobacteria associated with *Dendrilla nigra* sponge showed that Streptomycetes accounted for 23.91% of actinobacteria isolates (Selvin *et al.*, 2009). Achieving enzymatic activity-inhibiting bacteria is difficult, complex, and important in at least two respects. In the first aspect, the bacterial profile of sponges in different sampling locations varies according to various parameters, and in the second aspect, most bacteria are in vitro uncultivable (Schirmer *et al.*, 2005). Therefore, studying the discovery of natural products produced by bacteria that coexist with marine invertebrates is a promising risk. The biggest challenge for the systematic development of marine organisms' bioactive secondary metabolites industry is access to large-scale source organisms. Given the ease and possibility of sustainable drug production by microorganisms on an industrial scale, the achievement of symbiotic microorganisms as the main source of production of target metabolites presents a clear perspective in this area. The results of inhibitory activity of the extracted metabolites showed that 9 and 10, equivalent to 3.46 and 3.84% of bacterial isolates, respectively, inhibiting the activity of alpha-glucosidase and alpha-amylase inhibitors, were effective. Compounds that inhibit the activity of these enzymes can play an important role in reducing blood glucose uptake by inhibiting the activity of alpha-amylase or alpha-glucosidase enzymes as carbohydrate hydrolyzing enzymes. The IC50 activity of these metabolites ranged from 112.9 to 670.9 μg/ml for alpha-amylase. However, this rate has been reported for algal extracts sampled from the Persian Gulf, about 420 μg/ml to 7.5 mg/ml (Pirian *et al.*, 2017). Another study reported the IC_{50} inhibitory activity of metabolites extracted from *Streptomyces longisporoflavus* at 162.3 μg/ml (Akshatha *et al.*, 2014). In the present study, the toxicity test was used on human endothelial human umbilical cord cell lines to evaluate the toxicity of the compounds. The cytotoxic activity of the secondary metabolites extracted

was assessed by MTT cell proliferation assay. This method has played an important role in studying new marine anticancer drugs in the last 30 years (Nga *et al.*, 2020). The results of cytotoxic activity showed that the secondary metabolites extracted from isolates QH 36 and QN 45 against the HUVEC cell line showed cytotoxic activity equivalent to 196.7 and 510.6 µg/ml, respectively. Therefore, these three bacteria will be excluded from future studies due to the toxicity of metabolites. The production of cytotoxic compounds by bacteria that coexist with sponges has been reported in various studies. Marine organisms, such as sponges, produce cytotoxic compounds to exert their defense strategies against predators and pathogens in their habitats. According to the theory of the microbial origin of some of the secondary metabolites of marine organisms, certain species of symbiotic bacteria must be permanently and specifically associated with the host organism and function (Sabrina Pankey *et al.*, 2022). According to the results of measuring the inhibitory activity of the extracted metabolites against alpha-glucosidase and alpha-amylase enzymes, the multiphase identification of the isolates was performed up to the strain level. Genetic identification of capable isolates was performed following the study of morphological, physiological, and biochemical characteristics of the obtained isolates. The results of genetic identification based on a comparison of 16S rRNA gene sequence showed that the potential isolates from the production of inhibitory compounds of the target enzymes had a 97 to 100% similarity with the index strains registered in the NCBI database. According to the results of multiphase identification tests, the results showed that most of the selected isolates are different strains from the index strains and can be further studied as new sources of bioactive metabolites. Phylogenetic analysis of selected strains with the closest strains was performed based on the 16s rRNA gene. The sequence of this gene is highly conserved and provides accurate information for species and genus differentiation (Abellan-Schneyder *et al.*, 2021). The phylogenetic analysis results based on the Neighbor-joining distance method indicated the existence of 4 separate clusters. It seems that evolved strains distinct from their counterparts in adapting to the conditions of their microenvironments within the host have undergone mutations. Of course, these mutations do not only target the 16s rRNA evolutionary genes and may be involved in the other biosynthetic genes. Therefore, it is possible to alter the biosynthesis pathways of secondary metabolites. The present study led to a new understanding of the biodiversity of cultivable bacteria associated with sponge communities in the Persian Gulf around Qeshm Island. It also showed the potential inhibitory activity of metabolites produced by these bacteria against alpha-amylase and alpha-glucosidase enzymes. These results can further prove the ecologically active

role of significant sponge-related bacterial populations. The present study presented an efficient strategy for isolating bacteria from marine sponges with different culture media. The result of this study was the acquisition of 7 strains of bacteria that produce inhibitory compounds against enzymes involved in diabetes. The results of this study confirmed the non-toxicity of the extracted metabolites against normal human cells. Therefore, if the pharmacological tests are approved, the mentioned strains can be used as a candidate to study the native sources of anti-diabetic drugs.

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Research Article

Effects of nickel on liver and bone metabolic functions, biochemical and histopathological responses in common carp (*Cyprinus carpio*)

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Abstract

This study is performed to investigate the effects of water-borne Ni^{2+} on common carp (*Cyprinus carpio*) liver and bone metabolisms. Fish (*N*=60; 184.40±18.56g) were exposed to background concentrations of Ni^{2+} (based on measured LC_{50}-96h: 5.820 mg/L), including 0.058, 0.291, 0.580, 1.750, 2.910 mg/L for 30 continuous days. Ni^{2+}-exposed fish showed a rising trend in the case of serum aspartate transaminase (AST). Serum alkaline phosphatase (ALP) elevated (p<0.05) in all Ni^{2+} treatments. Serum total protein, globulin, and albumin showed a transient reduction in 0.058, 0.291 and 0.580 mg/L Ni^{2+} exposures (p<0.05). Although serum calcium level did not change significantly, serum inorganic phosphorus was elevated (p<0.05) in 0.580 mg/L Ni^{2+}. Bone isoenzyme of ALP observed in higher levels in all Ni^{2+} treatments than the control group (p<0.05). Pathological damages, such as focal necrosis, pycnosis and cytoplasm degeneration were observed in liver tissues of Ni^{2+}-exposed fish. A higher number of osteocytes as well as osteoclasts in bone of Ni^{2+}-exposed fish revealed dual effect of this metal in the case of bone metabolism. Generally, low level of nickel had no significant effect on metabolic parameters of liver and bone while highest nickel treatment had adverse effects, reflecting dual effects of this metal on carp.

Keywords: Nickel, Common carp, Liver histopathology, Bone histology, Blood chemistry

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Introduction

Aquatic environmental pollution is increasingly recognized due to the continuous introduction of various xenobiotics into aquatic ecosystems such as industrial effluents, mining activities, urban sewage, and agricultural fertilizers (da Silva and Martinez, 2014; Shamsaie Mehrgan *et al.,* 2019). Because of the improper idea of "self-cleaning capacity of waters", wastewaters were discharged untreated to the surrounding water ecosystems, even until the latter part of the twentieth century (Nikinmaa, 2014). Although pollutants enter aquatic ecosystems as a result of human lifestyles, their natural sources are currently responsible for most of them and together they are considered as one of the major threats to aquatic life (Ng *et al.,* 2003; Justino *et al.,* 2016).

Contamination of water ecosystems by heavy metals is increased, which in turn, results in greater pollution uptake by aquatic animals (da Silva and Martinez, 2014; Janbakhsh *et al.,* 2018). Low concentration of Ni^{2+} is detected nearly throughout the water ecosystem, which is reported to be 1.43 to 159.48 µg L^{-1} in different sampling areas, and possibly differ from time to time as well as different ranges of physicochemical parameters (Zhou *et al.,* 2020). In addition, some anthropogenic activities rise this metal in most surface waters (Yu, 2000). Therefore, fish possibly are exposed to different levels of Ni^{2+} during their life, consequently, different levels of adverse effects are expected in aquatic organisms (De Boeck *et al.,* 1995; Dreyfuss *et al.,* 2014).

Ni^{2+} can enter into the fish body through different routes, such as food, gills, drinking water, and skin; adsorbed through its uptake through gills accounted a prominent role in ion uptake and homeostasis (McGeer *et al.,* 2000). Ni^{2+} absorption from food or water regulates lipid metabolism and cell membrane, hormone secretion, and bone strength within the animals' body (Kumar *et al*., 2012). Furthermore, this metal can affect serum chemistry as well as whole-body metabolism and growth performance (Javed, 2013; Moeinnejad *et al.,* 2019). Adverse pathological degeneration in the liver of *Hypophthalmichthys molitrix* is reported at high concentrations of water-borne Ni^{2+} (Athikesavan *et al*., 2006). Research shows that Ni^{2+} accumulation in the plasma and tissues (gill, stomach, and intestine) that have direct contact with water, is roughly commensurate to Ni^{2+} concentration in ambient water (Pane *et al.,* 2006), and likewise leads to changes in metabolism, disturbances in the content of other trace metal in tissues (Misra *et al.,* 1990; Funakoshi *et al.,* 1996).

The effect of trace elements on fish bone metabolism are also reported (Lall and Lewis, 2007; Malekpouri *et al.,* 2011). Macro elements like Ca and Pi have an important role in the hard tissue of animals and their blood levels could reflect bone function, *i.e.,* mineral deposition and absorption (Burtis *et al.,* 2012). Alkaline phosphatase enzymes are accounted as multifunctional membrane enzymes, distributed in different tissues, such as the liver, bone, kidney, and

intestine. Moreover, liver enzymes, such as serum aspartate transaminase (AST) and alanine transaminase (ALT) are important parameters in assessing the state of liver tissue (Coz-Rakovac *et al*., 2005; Khodaei *et al.*, 2019), due to liver being rich in these enzymes and any changes in this organ tissue lead to release of them into the bloodstream (Taghavizadeh *et al*., 2020).

The aim of this study was to evaluate the effects of low levels of Ni^{2+} on serum biochemical parameters related to liver enzyme, protein, and carbohydrate metabolisms. In addition, possible changes in liver and bone histology in common carp, *Cyprinus carpio*, following 30 days of water-borne exposure were examined. In detail, we aimed to pursue the role of different concentrations of nickel (dose-dependent manner) in some physiological and biochemical functions of *C. carpio* to explore whether higher concentrations of nickel can weaken the organism (leading to adverse effects), or low levels induce beneficial effects.

Materials and methods

Chemicals and fish maintenance

All materials were purchased from Merck Chemical Company (Germany) unless otherwise stated. Nickel sulfate hexahydrate ($NiSO_4.6H_2O$) was obtained from ACROS Organics (USA). Common carp, *C. carpio* were purchased from a local fish farm and then transferred to the laboratory. All fish were treated using 5% saline bath upon arrival and were acclimated to laboratory conditions for at least 2 weeks prior to commencement of the experiment.

Experimental design

Acute toxicity test

Forty-eight fish (125.6±11.06 g) were subjected to the acute toxicity test according to OECD protocol, no 203 (OECD, 1994). Briefly, acclimated fish (30 days) were divided into 5 geometric serial concentrations of Ni^{2+} (0.058, 0.291, 0.580, 1.750, and 2.910 mg/L) for 96h and a control group (0 mg/L). Mortality rates were recorded daily. The tested fish did not feed throughout the experiment. Probit regression analysis was applied to estimate the concentration of Ni^{2+} that caused the death of 50% of the animals, i.e., LC_{50}. This was applied to the main experiment.

Nickel treatment

To investigate the effect of Ni^{2+} on the liver and bone functions of carp, a total of 60 fish weighing 184.4±18.56 g were randomly moved into 6 glass aquaria containing well-aerated tap water under natural photoperiod. Half of the water was replenished every other day. Fish were fed *ad libitum* with a diet, containing 31.3% crude protein, 11.67% crude fat, and 11.7% ash. The fish were exposed to background concentrations of Ni^{2+} for 30 continuous days, including 0.058 (1%), 0.291 (5%), 0.580 (10%), 1.750 (30%), and 2.910 mg/L (50%) of LC_{50}-96h.

Water quality parameters were determined daily according to American Public Health Association (APHA, 1998) method (Table 1).

Table 1: Physical and chemical parameters of water during the experiment.

Parameters	Range
pH	7.69-7.93
EC	448-488 µs/cm
DO	5.7-6.5 mg/L
Temperature	22.2-23.4°C
NO_2^-	0.057-0.080 mg/L
NO_3^-	7.52-9.34 mg/L
NH_4^+	<0.1 mg/L
Po_4^{3-}	78.92-79.62 µg/L
Hardness	232-284 mg $CaCO_3$/L
Ca^{2+}	188-246 mg/L
Mg^{2+}	16-32 mg/L
TS	349.2-382.6 mg/L
TDS	320.8-421.6 mg/L
TSS	5.80-6.18 mg/L

Water samples were collected randomly for measuring dissolved Ni^{2+} concentrations in each treatment using atomic absorption spectrophotometry (Perkin Elmer A Analyst 700). Background and measured concentrations of Ni^{2+} were summarized in Table 2.

Serum chemistry

At the end of the experiment, blood samples were withdrawn from the caudal vein of 5 starved fish (at least 24 h) from each treatment. The blood was then centrifuged at 3,500 ×g (10 min). Serum AST and ALT activities were determined using 2,4-dinitrophenyl-hydrazones in an alkaline solution at 505 nm (Reitman and Frankel, 1957).

Table 2: Background and measured concentrations of nickel in different treatments.

Background concentration (mg/L)	0	0.058	0.291	0.580	1.75	2.91
Measured concentration (mg/L)	<0.001	0.055± 0.027	0.275± 0.008	0.572± 0.012	1.687± 0.009	2.909± 0.037

Data are presented as mean ± standard deviation for 3 measurements randomly during the experiment. Nickel as $NiSO_4$. $6H_2O$ concentrations were measured by atomic absorption spectrophotometry.

Serum alkaline phosphatase (ALP) activity was measured at 405 nm using P-nitrophenyl phosphate as substrate (Malekpouri *et al.,* 2011). Triglycerides and glucose were determined by measuring formed H_2O_2 by adding phenol and 4-aminoantipyrine in the presence of peroxidase at 490 and 546 nm, respectively (Fossati and Prencipe, 1982). Total protein (TP) content of serum was determined according to the Biuret method as reported elsewhere (Gornall *et al.,* 1949), as a formation of a Cu^{2+}-protein complex in alkaline reagent at 540 nm and serum albumin (Alb) was measured at 540 nm using bromocresol green complex. Globulin (Glb) was calculated by subtracting Alb from TP. Serum pH was also measured by an electrical pH meter (Metrohm, UK).

The o-cresolphthalein complexone method was used to determine Ca level of serum at 570 nm (Moorehead and Biggs, 1974) and P_i was determined using the ammonium molybdate method (Fiske and Subbarow, 1925). For determining ALP isoenzyme, a heat stability test was performed. Briefly, different ALP isoenzymes have resistance to temperature (at 56 and 65°C). In this regard, fresh serum was heated in Bain Marie at 56°C for 10 min and transferred to an ice bath immediately to stop the reactions

(Romslo *et al.*, 1975). Finally, serum ALP activity was determined as described above. The bone-specific ALP (B-ALP) was determined due to higher stability to this temperature, *i.e*., other isoenzymes were deactivated.

Histopathology

At the end of blood sampling, three liver and bone tissues were sampled immediately from each treatment and were fixed in 10% neutral buffered formalin (pH=7.2). Serial sections with 5µm thickness were then processed and stained using hematoxylin and eosin method. Bone tissue was pretreated with 10% EDTA before the tissue was processed for staining.

Statistical analyses

If normality and homogeneity were achieved, analysis of variance (one-way ANOVA) was used for this study with a complimentary Duncan multiple test. Statistical analyses were carried out using SigmaPlot 12 program and data are presented as mean±standard deviation for all cases. Each treatment's mean value was compared with their specified control at *P*-value lower than 0.05.

Results

No difference in body weight was observed following 30 days' exposure among different treatments. The results of acute toxicity test are provided in Table 3. 96-h LC_{50} value of Ni^{2+} for *C. carpio* was found to be 5.82 mg/L. Among all treated groups, there was no mortality during the 30 days' experimental period.

Table 3: Determination of the LC_{50} for nickel in common carp (*Cyprinus carpio*).

Exposure time (h)	24	48	72	96
LC_{50} mg/L	31.97	20.13	12.38	5.82
(95% confidence limits)	-	(19.08-21.52)	(11.47-13.67)	(4.73-6.24)

Data (*n=8*) are presented as median and confidence intervals. Nickel was applied as $NiSO_4$. $6H_2O$.

The obtained results indicated that serum TP was reduced significantly ($p<0.05$) following low concentrations of Ni^{2+} (0.058, 0.291, and 0.580 mg/L), while there were no significant changes between the highest Ni^{2+} and the control treatment. Serum Alb did not show any significant change among the first three treatments as compared with the control. Fish exposed to 1.750 mg/L waterborne Ni^{2+} showed an increase in Alb level ($p<0.05$). Similar to TP, Glb content of carp serum indicated a significant decrease in the first three treatments, while 1.750 and 2.910 mg/L of Ni^{2+} treatments showed no significant difference as compared with the control. Serum TG reduced significantly ($p<0.05$) in 0.580 and 2.910 mg/L of Ni^{2+} treatments, whereas other treatments did not lead to any significant change as compared with the control. Although glucose level in carp serum did not change in 0.058 and 0.291 mg/L of Ni^{2+}treatments, other highest treatments led to a significant ($p<0.05$) increase compared to the control group. The serum pH level was detected to be high

following 0.291, 1.750, and 2.910 mg/L of Ni^{2+} as compared with other treatments. AST level of serum did not change significantly in 0.058 mg/L Ni^{2+} as compared with the control. Other treatments resulted in a higher level of AST in carp, with the highest level observed in 2.910 mg/L Ni^{2+} treatment (p<0.05). Serum ALT reduced (p<0.05) following 0.058 and 0.291 mg/L Ni^{2+}, while the middle treatment, *i.e*., 0.580 mg/L Ni^{2+} did not show any significant change. Higher levels of Ni^{2+} also showed a significant decrease in ALT level of serum in comparison with the control (p<0.05). Serum ALP was elevated following all Ni^{2+} treatments as compared with the control. A higher level of ALP was observed in 0.580 and 1.750 mg/L Ni^{2+} treatments (Fig. 1).

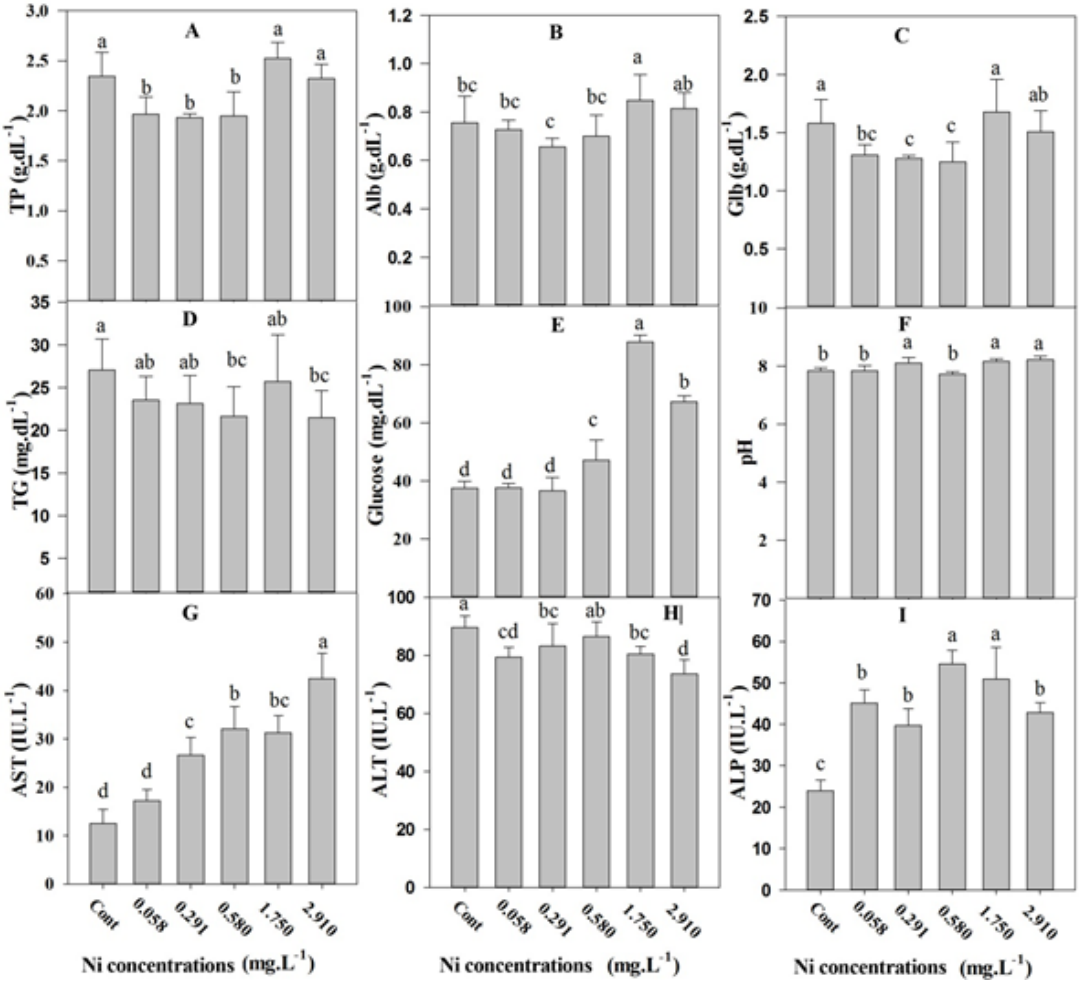


Figure 1: Serum biochemical parameters of *C. carpio* after 30 days of nickel ($NiSO_4$. $6H_2O$) exposures. Each value represents mean ± standard deviation of five separated samples, *i.e*, a total of 60 fish with 184.4±18.56 g initial weight were applied in this experiment. Different letters indicate significant differences among treatments at p<0.05. TP: total protein, Alb: albumin, Glb: globulin, TG: triglyceride, AST: aspartate transaminase, ALT: alanine transaminase, ALP: alkaline phosphatase.

Serum parameters related to bone metabolism were monitored and the results showed that there was no significant change in the case of serum Ca level following all Ni^{2+} treatments. Serum P_i elevated (p<0.05) only in 0.580 mg/L Ni^{2+}, while other changes in this parameter were not significant as compared with the control. Bone ALP isoenzyme elevated (p<0.05) following all treatments in comparison with the untreated fish (control). The highest level observed in 0.580 and 1.750 mg/L Ni^{2+}treatments, showing a maximum level in those treatments (Fig. 2).

Liver histopathological investigations indicated focal necrosis, lateral nuclei, pycnosis, and cytoplasm degeneration in all Ni^{2+} treatments but with different degrees (Fig. 3). Number of osteocytes elevated as fish exposed to 0.058 mg/L Ni^{2+}. The higher number of osteocytes as well as osteoclasts was detected, when the fish were exposed to a higher level of Ni^{2+}, *e.g.*, 1.750 and 2.910 mg/L (Fig. 4).

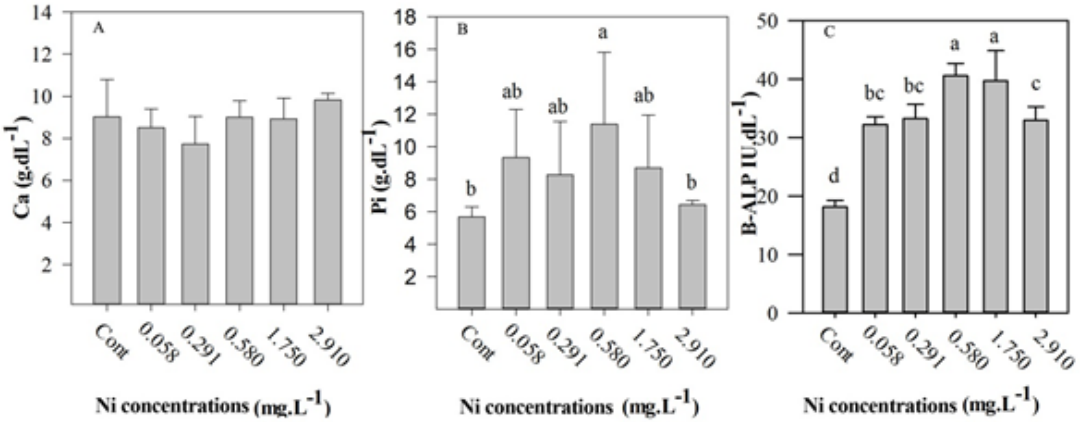
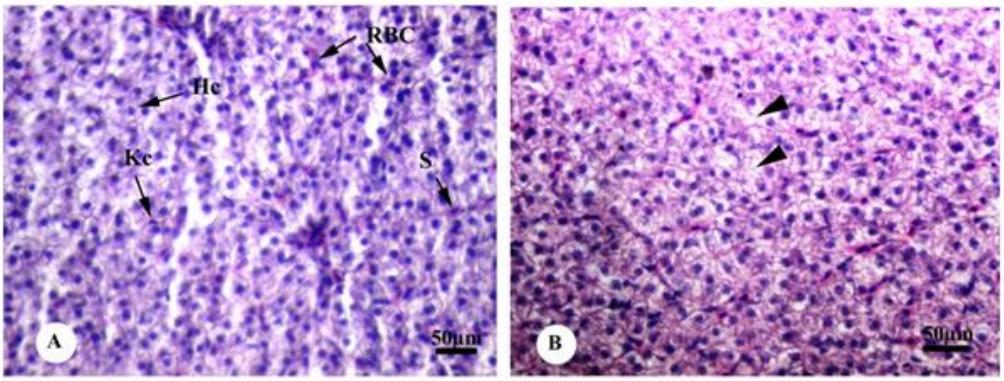


Figure 2: Serum biochemical parameters of bone metabolism in *C. carpio* after 30 days of nickel ($NiSO_4$. $6H_2O$) exposures. Each value represents mean ± standard deviation of five separated samples, *i.e*, a total of 60 fish with 184.4±18.56 g initial weight were applied in this experiment. Different alphabetical letters indicate significant differences among treatments at *p<0.05*. Ca: calcium, P_i: inorganic phosphorus, B-ALP: bone-specific alkaline phosphatase.



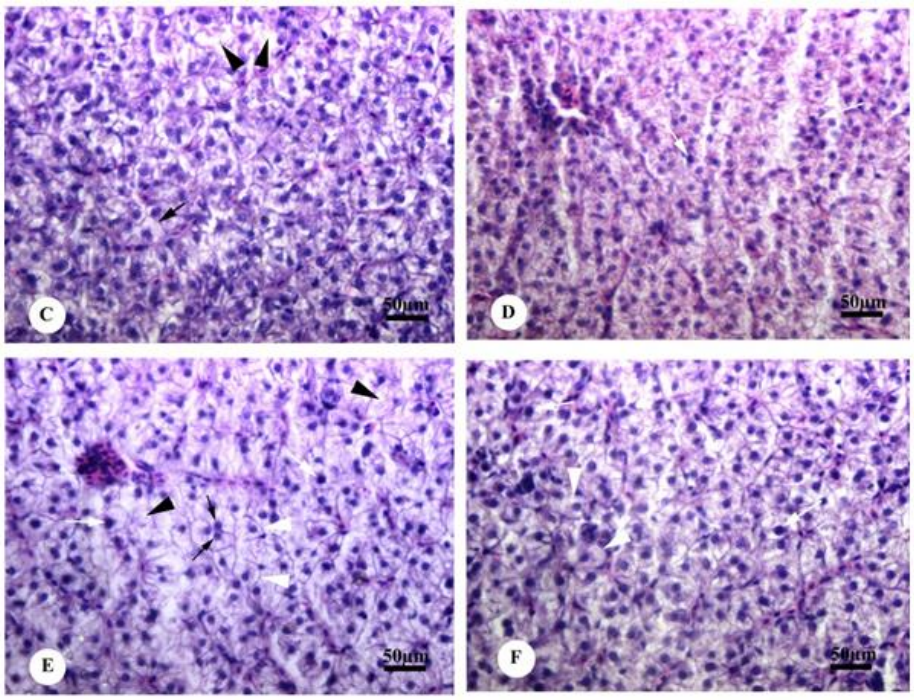


Figure 3: Photomicrograph of common carp histological sections of liver (hematoxylin and eosin), under control condition (A) showing normal structure of liver tissue, (B) exposed to 0.058 mg/L Ni^{2+} (as NiSO4. 6H2O) showing focal necrosis (black arrow head), (C) exposed to 0.291 mg/L Ni^{2+} showing focal necrosis (black arrow head) and lateral nuclei (black arrow), (D) exposed to 0.580 mg/L Ni^{2+} pycnosis (white arrow), (E) exposed to 1.750 mg/L Ni^{2+} showing focal necrosis (black arrowhead), later nuclei (black arrow), cytoplasm degeneration (white arrowhead) and pycnosis (white arrow) and (F) exposed to 2.910 mg/L Ni^{2+} showing cytoplasm degeneration (white arrowhead) and pycnosis (white arrow) following 30 continuous days. RBC: red blood cells, Hc: hepatocytes, Kc: Kupffer cells, S; sinus. Nickel was applied for 30 days as $NiSO_4$. $6H_2O$.

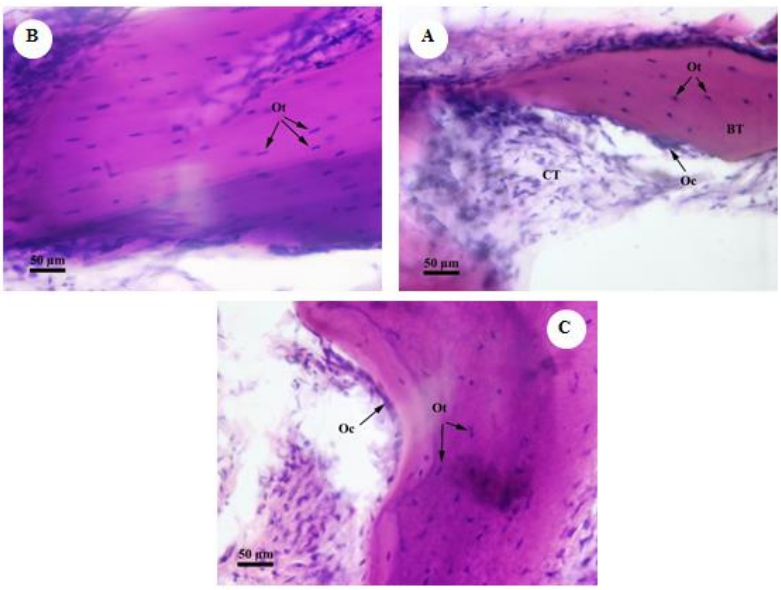


Figure 4: Selected photomicrographs of histological sections of bone (hematoxylin and eosin) in common carp under control condition (A), showing a normal structure of bone tissue, (B) exposed to 0.058 mg/L Ni^{2+} showing higher number of osteocytes, (C) exposed to 1.750 mg/L Ni^{2+} showing a higher number of osteocytes as well as osteoclasts. BT: bone tissue, CT: connective tissue, Ot: osteocytes, Oc: osteoclasts. X40. Nickel was applied for 30 days as $NiSO_4$. $6H_2O$.

Discussion

Trace elements play different biological roles in the body of living organisms. Extensive and detailed studies are needed, since interactions between the elements and physiological activities are complex (Sauliutė and Svecevičius, 2015). Therefore, attention to interactions between toxic elements and essential elements in biochemical pathways and the physiological function of aquatic animals is increased (Ali *et al.*, 2019). Nickel is an essential metal that is not shown to play a biological role in high doses, while in small amounts it can be used as an essential element in many bodily functions, including ossification, synthesis, and activation of metalloenzymes (Chowdhury *et al.*, 2008).

Serum protein content of common carp decreased only in nickel treatment at low concentrations. It may be related to increase protease activity and free amino acids in gills of the exposed fish to the lethal concentration of nickel. This possibly suggests the predominance of proteolytic sensitivity following metal exposure (Sreedevi *et al.*, 1992). Because fish gill tissues are in direct contact with the ambient water, high concentrations of nickel can destroy its resistance by disrupting cellular components. In addition, a high level of soluble protein in the kidneys indicates the dissolution of enzymes necessary to detoxify and eliminate the metal. Sharma and Davis (1980) reported that methylmercury disrupts carp protein synthesis. Most serum proteins are synthesized in liver, and therefore total serum protein is used as an indicator of liver dysfunction. Rivarola and Balegno (1991) reported that pesticides can decrease plasma protein owing to changes in protein and free amino acid metabolism and synthesis. Generally, a decrease in blood protein may be due to loss of protein through decreased protein synthesis or increased proteolytic activity or degradation as mentioned above. Decreasing in total protein can be partly attributed to the effects of the metal on liver cells, which is confirmed by increasing the serum AST and ALT activities observed in this study.

Blood glucose levels are shown to increase in fish exposed to a variety of environmental changes that are considered stressful. Higher level of carbohydrate in fish blood is well evidenced to be a general secondary response to acute intoxication and is considered a reliable indicator of environmental stress (Mazeaud *et al.*, 1977). Al-Attar (2007) suggested that high blood glucose levels could be a reliable indicator of nickel toxicity in fish. In the present study, low levels of nickel tested did not induce any significant change in the serum glucose concentration. Ptashynski *et al*. (2002) also found no difference in glucose concentration between nickel dietary treatment and the control group. In contrast, a high level of nickel exposure led to a significant increase in the blood glucose of this study. Eisler and Jacknow (1985) observed that blood glucose level can be elevated in nine species of freshwater fish following exposure to plating effluent containing cyanide and

Ni, chrome, copper, and zinc salts. Generally, exposure to Ni caused a significant amount of stress in fish, which may have led to a decrease in energy storage, followed by an increase in blood glucose. During the stress period, fish increased levels of glucocorticoids and catecholamines, which raise blood glucose (Reid *et al.,* 1998).

The glycemic response shown in the present study is a sign of impaired carbohydrate metabolism, possibly due to increased hepatic glucose 6-phosphatase activity, increased hepatic glycogen breakdown, or glucose synthesis from extra-hepatic tissue proteins and amino acids (Kubrak *et al.,* 2012). Combined exposure to metals (Nickel, Cadmium, and Lead) increases blood glucose content due to intense glycogenolysis and glucose synthesis from extra-hepatic tissue proteins and amino acids (Vinodhini and Narayanan, 2009). Fırat and Kargın (2010) suggested that elevated blood glucose during pesticide treatment may indicate impaired carbohydrate metabolism due to increased hepatic glycogen breakdown, possibly resulting in increased adrenocorticotrophic and glucagon hormones or decreased insulin activity.

Changes in the activity of hepatic enzymes indicate liver cell damages or a disruption in the metabolic process. Therefore, the study of enzyme activity as an important biochemical indicator is considered an important strategy to assess environmental conditions and the presence of toxic compounds (Baghshani and Shahsavani, 2013). ALT, AST, and ALP play very important roles in the metabolic processes of the body and fish health and are introduced as appropriate biomarkers in toxicological studies (Benincá *et al*., 2012; Kaviani *et al*., 2018; 2020). These enzymes are present in cells of various tissues, such as liver, heart, kidneys, muscles, and brain. Some physiological conditions, such as liver damage and skeletal disorders change (as observed here) the activity of these enzymes (Bogé *et al.*, 1992). Similarly, Öner *et al*. (2008) observed that levels of ALT and AST in the blood increased due to cell damage in liver and concluded that high levels of these enzymes in serum usually indicate disease and necrosis in animals' liver.

Fish showed different enzymatic responses (including decreasing or increasing enzyme activity) to heavy metal contamination, depending on species, metals, concentrations, and physicochemical conditions of water as contributing factors (Jiraungkoorskul *et al*., 2003; Sanchez *et al*., 2005). Fırat and Kargın (2010) showed that heavy metal poisoning can increase the activity of AST in tilapia liver, but the activity of ALT might be lessened due to poisoning in this fish. Some studies reported no significant change in these two enzymes in tissues and blood serum of some fish under the influence of heavy metals (De Smet and Blust, 2001). ALP is made up of several isozymes that are found in almost all tissues of the body, especially in cell membranes. This enzyme accelerates the hydrolysis of monophosphate esters and plays an important role in transporting substances through cell membranes and is also

effective in bone formation (Molina *et al.,* 2005). ALP enzyme is considered a suitable indicator due to sensitivity to cell toxicity due to xenobiotic substances (Lohner *et al.,* 2001).

The results of this study showed that water-borne nickel (at levels, applied here) does not change serum Ca concentration in common carp. However, a decreasing trend in serum Ca is somewhat evident in different treatments of nickel. Serum Ca concentration at the highest concentration of nickel (2.910 mg/L) showed a change compared to the control group but this change was not statistically significant. The route of uptake for essential or even non-essential elements (such as cadmium, zinc, nickel, copper, etc.) from water is the same as that of Ca. Therefore, it is expected that there is a possible interaction between nickel and Ca in uptake through the gills, although the present study did not show any significant change. In the studies of Knox *et al.* (1982) who examined different levels of copper and zinc in the diet of rainbow trout (*Oncorhynchus mykiss*) and Grosell *et al.* (2004) who examined changes in copper concentration in water, there was no significant difference in serum calcium concentration. Berntssen *et al.* (2003) also reported no change in serum Ca levels of Atlantic salmon (*Salmo salar*) fed with cadmium supplementation compared with the control group. Overall, antagonistic effects between the elements on Ca metabolism in common carp may have inhibited some of Ca uptake from the water, although no significant negative effect of nickel was observed here.

However, another study showed that feeding red drum, *Sciaenops ocellatus*, with a minimum level of zinc can increase Ca levels (Gatlin *et al.,* 1991). There are also several reports of decreased plasma Ca concentrations due to the presence of lead, cadmium, and copper in water (Dhanapakiam and Ramasamy, 2001; Pizent *et al.,* 2003; Alves and Wood, 2006). In the present study, the amount of Ca was reduced to some extent, therefore, the development of hypocalcemia under the influence of water-soluble nickel in common carp is somewhat predictable. Hypocalcemia may not be far from expectation as the duration of nickel exposure increases or the concentration changes (using higher concentrations). In fish, hypocalcemia can occur as a result of competition between metal ions and Ca for absorption through gills or competition for replacement in bone structure. In this regard, we can refer to the study conducted by Muramoto (1981), showing that cadmium ions can affect the metabolism of bone tissue and damage its structure and even lead to hypocalcemia. In addition, Zohouri *et al.* (2001) reported hypocalcemia in rainbow trout due to cadmium exposure. Similarly, Malekpouri *et al.* (2011) declared that hypocalcemia is the most prominent effect of toxic elements in common carp.

In the present study, increases (significant or insignificant) in serum concentrations of P_i were observed in common carp following Ni^{2+} treatments. Bone tissue contains an organic bone

matrix with minerals. The organic matrix of bone tissue often contains collagen, hydroxyapatite, a hydroxylated polymer of calcium phosphate $Ca_{10}(PO_4)_6(OH)_2$ (Mondal *et al.,* 2016). Therefore, phosphate is one of the main components of bone tissue. Studies showed that toxic elements such as aluminum and cadmium can damage bone tissue (Rodríguez and Mandalunis, 2018). Nickel can also disrupt kidney function, upsetting the balance of calcium and phosphorus, as the reabsorption and excretion of these ions from the renal tubules may also be impaired (Guo *et al.,* 2016).

In the present study, the level of serum ALP in fish was elevated. This enzyme, acting as a transphosphorylase in alkaline environments, plays a key role in bone mineralization. Thus, the highest concentration of that can be found in bone osteoblast cells (Leung *et al.,* 1993). As previously stated, toxic metals destroy bone tissue and it can be concluded that bone destruction causes separation of this enzyme from bone cells and its release into the blood (He *et al.,* 2020). As a result, the concentration of ALP in the serum of fish increases owing to the effect of nickel. On the other hand, synthesis of this enzyme takes place in hepatocytes; hence any damage to the liver cells can disrupt the release of this enzyme into the bloodstream (Muriel, 1998).

ALP is one of the metalloenzymes containing zinc and magnesium in its structure and increasing the amount of zinc in the diet increases the activity of this enzyme (within essential concentration). This enzyme was observed in fish for the first time to determine the amount of zinc required in the diet of channel catfish, *Ictalurus punctatus* (Wilson and Poe, 1974). Besides, a significant decrease in the activity of this enzyme was observed in the plasma of *I. punctatus* and Nile tilapia (*Oreochromis niloticus*) fed with zinc-free diet (Huang *et al.,* 2015). In the present study, it is possible to attribute increase of this enzyme in fish serum following nickel exposure to enhancing effect of this metal in synthesis of ALP. However, in the present study, this increase was not significant in many cases. another research showed that ALP is raised in the serum, liver, and intestine of Nile tilapia following exposure to zinc, cadmium, copper, and lead (Atli and Canli, 2007).

Studies to date have shown that there is a direct linear relationship between serum ALP activity and serum phosphorus concentration. Overall, it is believed that physiological changes resulting from exposure to heavy metals can alter the activity of the enzyme alkaline phosphatase.

Bone is a connective tissue that is constantly changing, and these changes involve three types of cells in bone tissue; Osteoblasts (bone-forming cells), osteoclasts (multinucleated bone-reabsorbing cells), and osteocytes (enclosed within the bone matrix). Osteocytes appear to be involved in the preservation of bone material and the exchange of ions with body fluids. In fact, the number of osteocytes in histopathological observations indicates the amount of metabolic activity of bone

tissue (Aarden *et al.,* 1994). The number of osteocytes in low concentration of nickel treatments was elevated compared to the control treatment while number of osteocytes reduced and osteoclasts increased in high nickel levels. The increase in osteoclast cell density is evidence of an increase in serum mineral phosphorus (Koyama *et al.,* 2002; Mohammadi *et al*., 2018). Therefore, the increased ALP activity, indicates a disruption of bone formation, can itself increase serum phosphorus. It is already shown that number of osteoclasts in *Barbus grypus* with signs of bony deviation increased compared to healthy fish (Malekpouri *et al*., 2015).

In conclusion, nickel within the range used in this experiment appears to be somewhat toxic to fish. Comparing the results obtained for different parameters, it is shown that small amounts of nickel had no significant effect on the metabolic parameters of liver and bone, and intermediate treatments (0.580 and 1.750 mg/L) improved the function of bone and liver. Finally, the highest concentration of nickel treatment significantly reduced the number of parameters, which in a way reflects toxic effects of this element. Therefore, it can be concluded that this element in very small amounts has not played an effective role in the physiological and biochemical processes of carp but high concentrations can show toxic effects. Of course, to reveal such a dual effect, creating treatments with closer concentration intervals and longer experimental periods along with a careful examination of physiological stress parameters should be addressed.

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Research Article

Effectiveness of dietary *Moringa oleifera* leaf powder and extract in the Pacific white shrimp (*Litopenaeus vannamei*)

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Abstract

Moringa oleifera is an important herbal plant with a valuable source of major essential nutrients and nutraceuticals. In this study, we supplemented the diet of *Litopenaeus vannamei* with different levels of *M. oleifera* leaf powder (MLP) and extract (MLE) and evaluated the growth, survival, body and fatty acid composition, hemolymph biochemistry, antioxidant status, and salinity stress resistance after six weeks of feeding. In total, 840 shrimp (2.6±0.02 g) were divided into seven groups including the shrimp fed with the basal diet (control), and the diets containing different levels of MLP [25 (MLP25), 50 (MLP50), and 100 (MLP100) g kg^{-1}] and MLE [0.25 (MLE0.25), 0.5 (MLE0.5), and 1.0 (MLE1.0) %]. The results showed a lower FCR value in MLP25 and MLE0.5 fed shrimp as well as a higher survival rate in MLP100 and MLE0.5 fed shrimp than those of the control group. Dietary MLP enhanced the body contents of lipid and fatty acids (*i.e.*, the pentadecanoic acid and the omega-3 and -6 polyunsaturated fatty acids). The MLP100 diet remarkably enhanced the hemolymph total protein, albumin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels of shrimp. On the other hand, the AST and ALT activities were significantly reduced in the shrimp fed with MLP25, MLE0.25 and MLE0.5 diets. MLP and MLE in the diet of shrimp also led to a significant increase in the hemolymph antioxidant enzymes activity. Moreover, the shrimp fed with MLP50 diet showed a significantly higher survival rate in response to the high salinity stress compared to the control group. In conclusion, the supplementation of both MLP and MLE in the diet of *L. vannamei* showed beneficial effects on the performance of the shrimp farming industry.

Keywords: Shrimp, Moringa, Growth, Fatty acids, Antioxidant capacity

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Introduction

The shrimp farming industry is contributing a major income to several countries in tropical and subtropical countries, especially in Asia (Burford *et al*., 2004, Zhang *et al.*, 2013). The Pacific white shrimp, *Litopenaeus vannamei*, is the most commonly shrimp cultured species in the global economy (Duan *et al*., 2017a). Given the advantages of fast-growing, euryhalinity, high-density culture, etc., *L. vannamei* is considered as the most important economic crustacean species, accounting for 53% of the total crustacean production worldwide (FAO, 2018).

Enhancing the performance of farmed shrimp using natural products is believed to be a beneficial approach to the health management of shrimp aquaculture (Duan *et al.*, 2017b). Using natural and safe feed additives in the shrimp diet could be an eco-friendly approach in shrimp aquaculture to enhance production and prevent disease (Chen *et al.*, 2019). Many herbs and plants and their products are known as important sources of multifunctional curing agents and bioactive compounds (Saini *et al.*, 2016a).

Moringa oleifera (MO) is known as an important herbal plant with a valuable source of major essential nutrients and nutraceuticals (Kunyanga *et al.*, 2013). The leaf of MO is characterized by a rich source of vitamins and minerals (e.g., ascorbic acid, tocopherol vitamin A, calcium, phosphorus, magnesium and potassium), carotenoids and folate, (e.g., β-carotene), and various phenolics and glucosinolates. It also contains low amount of anti-nutritional compounds such as phytic acid, tannins, oxalic acid and lectins (Nouala *et al.*, 2006; Amaglo *et al.*, 2010; Asare *et al.*, 2012; Egwui *et al.*, 2013; Saini *et al.*, 2014a; Saini *et al.*, 2014b; Saini *et al.*, 2014c; Saini *et al.*, 2014d; Karthivashan *et al.*, 2015; Saini *et al.*, 2016b).

The medicinal properties of MO are known as cardiovascular stimulant, antitumor, antipyretic, anti-inflammatory, anti-coagulant, anti-spasmodic, antihypertensive, cholesterol-lowering, antioxidant, anti-diabetic, hepato-protective, antibacterial, and antifungal effects (Anwar *et al.*, 2007). The positive effects of MO on flesh quality and omega-3 poly unsaturated fatty acids (PUFAs) contents have been also reported in animals (Nkukwana *et al.*, 2014; Zhang *et al.*, 2019; Selim *et al.*, 2021).

In aquaculture, the leaf powder and extract of MO have been supplemented to the diet of some fish and shellfish species. The positive effects of MO leaf powder (MLP) on growth performance, antioxidant activity, resistance to starvation stress, immunity system, and lipid metabolism have been demonstrated in Nile tilapia, *Oreochromis niloticus* (Richter *et al.*, 2003; Bbole *et al.*, 2016; Elabd *et al.*, 2019; El-Kassas *et al.*, 2020). Furthermore, MLP enhanced the growth performance in fingerlings of Indian carp, *Labeo rohita* (Hussain *et al.*, 2018). In Bocourti's catfish, *Pangasius*

bocourti up to 100 g kg^{-1} supplementation of MLP did not show any harmful effects on growth, nutrient digestibility, feed utilization, and serum biochemistry (Puycha *et al.*, 2017). The beneficial effects of dietary MLP on antioxidant activity have been also demonstrated in gilthead Seabream, *Sparus aurata* (Jiménez-Monreal *et al.*, 2021). The effect of Moringa leaf extract (MLE) has also been investigated in the diet of farmed shrimp. The MLE showed beneficial effects on growth performance, physiological and immune function, and resistance to *Vibrio anguillarum* infection and ammonia stress in freshwater prawn, *Macrobrachium rosenbergii* (Brilhante *et al.*, 2015; Kaleo *et al.*, 2019). Moreover, the administration of MLE improved growth performance, antioxidant activity, fatty acid composition, and resistance of shrimp *P. vannamei* against *Photobacterium damselae* (Akbary *et al.*, 2021).

Although, the MLE has been administrated in the diet of farmed shrimp, little is known about the effect of MLP on farmed shrimp performance. Therefore, in the present study, different levels of MLP and MLE were supplemented to the diet of the Pacific white shrimp (*L. vannamei*) and the growth performance, body and fatty acid composition, hemolymph biochemicals and antioxidant activity and resistance to salinity stress were evaluated and compared.

Materials and methods

Diet preparation

In this study, the diets with different levels of MLP [25 (MLP25), 50 (MLP50) and 100 g kg^{-1} (MLP100)] and MLE [0.25 (MLE0.25), 0.5 (MLE0.5) and 1.0 (MLE1.0) %] were prepared. A control diet without MO supplementation was also made. The main protein resource formulated in the diets were fish meal, soybean meal, shrimp powder, and rapeseed meal (Table 1). To prepare the diets containing MLP, the leaf of MO was washed and dried at room temperature. Then, the dried leaf was ground into a fine powder. All other ingredients were also powdered and mixed with oil and different levels of MLP and then pelleted (about 2.0 mm in diameter). The pellets were dried to contain nearly 10% moisture, wrapped in plastic bags, and kept at −4°C until consumed by shrimp. To prepare diets containing MLE, the dry powder of MO (100 g) was soaked in 600 mL of ethanol (70%) for 48 h in a percolator. Then, the liquid phase was separated and concentrated several times by filter. The obtained extracts were sterilized and stored in an airtight container at a cooled temperature and then added to the basal diets at the levels described above.

The major chemical and biochemical composition of both MLP and MLE (Table 2) were measured by GC-MS method using a 7890A gas chromatograph with a 5975C Network Mass Selective Detector (Agilent Technologies).

Experimental shrimp and conditions

Following two weeks of acclimation, the shrimp with an initial weight of 2.6±0.02 g were assigned into seven groups (one control and six treated groups), each with three replicates. 40 shrimp were randomly positioned in 300 L circular fiberglass tanks.

Table 1: The ingredients and chemical composition of the experimental diets (g/kg).

Ingredients (g/kg)	Control	MLP 25	MLP 50	MLP 100	MLE 0.25	MLE 0.5	MLE1.0
Fish meal	280	288	297	314	280	280	280
Soybean meal	190	190	190	190	190	190	190
Wheat flour	200	185.5	170	142	199.2	198.5	197
Wheat gluten	96	96	96	96	96	96	96
Corn gluten	134.0	116.5	99.0	64.0	132.3	130.5	127.0
Bentonite	10	10	10	10	10	10	10
Binder	10	10	10	10	10	10	10
Permix (Vitamin & Mineral additives)[1]	20	20	20	20	20	20	20
Fish oil	40.0	39.3	38.7	36.0	40.0	40.0	40.0
Soybean oil	20.0	19.7	19.3	18.0	20.0	20.0	20.0
Moringa leaves powder	0	25	50	100	0	0	0
Moringa leaves extract	0	0	0	0	2.5	5	10
Chemical composition (g/kg)							
Crud protein	424.8	407.5	432.2	420.2	433.9	430.3	423.2
Crud lipid	81.8	95.9	88.0	82.3	81.5	80.2	81.7
Carbohydrate	281.0	298.7	276.1	260.6	266.4	274.3	271.9
Ash	106.6	97.1	100.3	116.7	101.4	104.9	111.2
Dry matter (%)	894.2	899.2	896.6	879.8	883.2	889.7	888.0
Moisture (%)	105.8	100.8	103.4	120.2	116.8	110.3	112.0
Energy (kcal kg^{-1})	18089.6	18542.7	18424.8	17649.9	18041.4	18040.9	17891.4

[1] Permix (Creve Tec shrimp feed concentrate 2%): wheat protein, vitamins minimum value: (inositol, biotin, folic acid, nicotinic acid, panthothenic acid, vit B2 (riboflavin), vit B1 (thiamine), vit B6 (pyridoxine), vit B12 (cyanocobalamine), vit A1000, vit D3, vit K, vit C (L-ascorbic acid), choline, organic trace minerals: (Fe, Cu, Mn, Zn, Se, I), Phosphates, digestibility enhancer, cholesterol.

Table 2: Chemical and biochemical composition of *Moringa oleifera* leaf powder (MLP) and *M. oleifera* leaf extract (MLE).

Parameters in MLE	Value
K (mg L^{-1})	1174.9
Mg (mg L^{-1})	230.00
Ca (mg L^{-1})	36.80
Zn (mg L^{-1})	0.74
Fe (mg L^{-1})	<2.00
Protein (%)	1.24
Stigmasterol (%)	63.05
β-Amyrin (%)	17.75
Stigmast-7-en-3-ol (%)	8.60
4,22-Cholestadien-3-one (%)	5.80
Heptacosane (%)	4.80
Crude protein (%)	25.10
Crude lipid (%)	7.90
Carbohydrate (%)	40.45
Fiber (%)	8.37
Ash (%)	10.90
Dry matter (%)	92.72
Moisture (%)	7.28
Energy (kcal kg^{-1})	1600.15

One-third of the water in each tank was changed every day. Shrimp were fed with the experimental diets at a rate of 6.0–8.0% body weight at 8:00–8:30, 14:00–14:30, and 18:00–18:30. for six weeks daily. The amount of daily feed was readjusted every 2 weeks by

measuring the shrimp total weight in each tank. During the period of feeding trial, the range of temperature, pH, and DO were 30-32±2°C, 7.8–8.5, and >6 mg/L, respectively. After six weeks of the feeding trial, the shrimp from each tank were sampled and the body mass was measured.

Growth indices

Using the following formulae, the shrimp growth indices i.e., weight gain (WG), specific growth rate (SGR), protein efficiency ratio (PER), and feed conversion ratio (FCR) were calculated as follows:

Specific growth rate (SGR %/day)=100×[(ln final body weight–ln initial body weight)/feeding days]
Weight gain rate (WG, %)=100×[(final body weight-initial body weight)/ initial body weight] Feed conversion ratio (FCR)=[feed intake (g) / weight gain (g)]
Protein efficiency ratio (PER)=WG (g)/protein intake (g)
Survival rate (%)=[(initial shrimp number-dead shrimp number)/(initial shrimp number)]×100

Body proximate and fatty acid composition

The whole-body proximate composition of shrimp (three shrimp per tank) was assessed using the AOAC method (Cunniff, 1995). The amount of moisture was measured by drying in an oven (Binder, USA) at 105°C for 24 h. The Crude protein and lipid levels were measured using a Kjeldahl system (Gerhardt, type VAP.40, Germany), and ether extraction in a Soxhlet extractor (Gerhardt, type SE-416, Germany), respectively. The amount of ash was measured using a muffle furnace (Nabertherm, Germany) at 550°C for 8 h.

The fatty acid profile of three shrimp per tank for the control and MLP treatments were evaluated using an adapted method of Lepage and Roy (1984) as formerly described in Pakravan *et al.* (2017). Briefly, the fatty acids were quantified by an Agilent gas chromatograph (Agilent 7890A GC System, USA) using a BP×70 capillary glass column (0.32 mm×50 m, SGE Analytical Science Australia) after esterification and preparation of fatty acid methyl esters.

Hemolymph biochemistry and antioxidant status

Hemolymph was taken from three specimens per tank at the end of the feeding period as formerly described by Niroomand *et al.* (2020). The obtained hemolymph samples were retained in the refrigerator for 1–2 h at 4°C, and then, centrifuged at 4600 ×g for 10 min at 4°C. The biochemical and antioxidant parameters including total protein, cortisol, albumin, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels were determined using the Roche Kits and an automatic blood analyzer (COBAS Integra 400 Plus, Germany). The activities of antioxidant enzymes including glutathione peroxidase (GP_x) superoxide dismutase (SOD), and catalase (CAT) were measured by a

spectrophotometric method using Zell Bio GmbH (Germany) kits as previously described (Rice-Evans and Miller, 1994).

Salinity challenge test

At the end of the experimental period, the shrimp from all treatments were exposed to the salinity stress. For both low and high salinity stress tests, 15 shrimp (9.7±0.16 g) from each experimental group were randomly taken and placed into three 300-L tanks. The shrimp were held off feed 24 h prior to the onset of the stress trial and gradually exposed to both low and high salinity stresses separately at a rate of 5 and 55 ppt, respectively (Akbarzadeh *et al*., 2019). The survival rate of the exposed shrimp was recorded 24 h after exposure and measured as follows:

Survival rate (%)=100×initial shrimp number−dead shrimp number/initial shrimp number

Statistical analysis

The obtained from different parameters were subjected to the normality test (Kolmogorov–Smirnov) and heterogeneity for variance (Leven's test) at the level of $p<0.05$. Then, differences in all the data among the treatments were analyzed using the one-way analysis of variance (ANOVA) followed by the Tukey posthoc test. Values are expressed as mean ± standard deviation (SD). The statistical analyses were conducted by SPSS and the figures were drawn using Sigma Plot software.

Results

Growth performance

The results of growth indices and survival rate of the shrimp fed with different levels of MLP and MLE are shown in Table 3. No significant differences were observed in the initial weight, final weight, weight gain, SGR, and PER among the treatments. However, the FCR value of the shrimp fed with MLE0.5 diet was significantly lower than the control group ($p<0.05$). The survival rate of the shrimp fed with MLP100 and MLE0.5 diets was significantly higher than the control group ($p<0.05$; Table 3).

Table 3: Growth performance and feed utilization of *Litopenaeus vannamei* fed with different levels of *Moringa oleifera* leaf powder (MLP) and *M. oleifera* leaf extract (MLE).

Parameters	Control	MLP 25	MLP 50	MLP 100	MLE 0.25	MLE 0.5	MLE 1.0
Initial Weight(g)	2.53±0.08	2.58±0.01	2.61±0.01	2.49±0.04	2.68±0.02	2.55±0.07	2.50±0.05
Final Weight (g)	9.93±0.53	10.38±0.38	9.54±0.72	9.15±0.37	9.60±0.12	9.62±0.01	10.17±0.41
SGR	3.03±0.19	3.09±0.88	2.86±0.17	2.88±0.06	2.83±0.02	2.94±0.69	3.11±0.04
WG (g)	7.39±0.61	7.80±0.39	6.92±0.71	6.66±0.33	6.92±0.11	7.06±0.07	7.66±0.36
FCR	1.70±0.11 ^{a}	1.46±0.06^{b}	1.73±0.18 ^{a}	1.56±0.03 ^{ab}	1.56±0.06 ^{ab}	1.40±0.05 ^{b}	1.63±0.03 ^{ab}
PER	3.06±0.39	2.77±0.42	2.82±0.20	2.64±0.09	2.70±0.22	2.93±0.16	2.82±0.49
Survival rate (%)	80.83±2.20^{b}	90.83±7.94^{ab}	90.00±1.44^{ab}	96.66±3.33^{a}	91.66±3.33^{b}	100.00±0.00^{a}	83.33±3.00^{b}

Data (mean±SD) with different letters are significantly different among treatments according to the Tukey posthoc test ($p<0.05$). WG: weight gain; SGR: specific growth ratio; FCR: feed conversion ratio; PER: protein efficiency rate.

Proximate body composition

The proximate chemical composition of whole-body of *L. vannamei* fed diets containing different levels of MLP and MLE are presented in Table 4. The MLE 0.25 fed shrimp showed a significantly lower amount of protein compared to other treatments ($p < 0.05$). Moreover, the content of body lipid in the shrimp fed with different levels of MLP was significantly higher than the control group ($p<0.05$), but MLE showed no significant effect on the lipid content of shrimp ($p>0.05$). The MLE0.25 and MLE0.5 treatments showed a significantly lower amount of ash compared to the control group, but the MLE0.5 and MLE1.0 treatments revealed significantly higher amounts of carbohydrates than the control treatment ($p<0.05$). The carbohydrate content of shrimp fed with different levels of MLP was significantly lower than the control group ($p<0.05$).

Table 4: Proximate body composition (g kg^{-1} wet weight) of *Litopenaeus vannamei* fed with different levels of *Moringa oleifera* leaf powder (MLP) and *M. oleifera* leaf extract (MLE).

Parameters	Treatments						
	Control	MLP 25	MLP 50	MLP 100	MLE 0.25	MLE 0.5	MLE 1.0
Protein	198.14 ± 2.11^{a}	195.55 ± 0.67^{a}	202.77 ± 8.18^{a}	201.77 ± 0.18^{a}	177.41 ± 5.32^{b}	196.43 ± 1.58^{a}	200.85 ± 0.69^{a}
Lipid	4.52 ± 0.15^{d}	5.73 ± 0.15^{b}	6.50 ± 0.38^{a}	4.99 ± 0.15^{c}	4.29 ± 0.27^{d}	4.65 ± 0.40^{cd}	4.46 ± 0.12^{d}
Ash	14.16 ± 0.54^{a}	13.96 ± 4.93^{a}	13.94 ± 0.11^{a}	13.58 ± 0.23^{ab}	12.12 ± 0.01^{c}	13.04 ± 0.65^{b}	14.21 ± 0.71^{a}
Carbohydrate	38.45 ± 0.15^{b}	36.47 ± 0.14^{c}	35.03 ± 1.52^{c}	35.14 ± 1.10^{c}	35.33 ± 1.33^{c}	40.19 ± 0.15^{a}	41.42 ± 0.55^{a}
Moisture	744.74 ± 2.66^{b}	748.30 ± 1.15^{b}	741.76 ± 10.48^{b}	745.22 ± 0.54^{b}	770.85 ± 6.93^{a}	745.68 ± 2.05^{b}	739.06 ± 0.72^{b}

Data (mean±SD) with different letters are significantly different among treatments according to the Tukey posthoc test ($p<0.05$). Carbohydrate (CHO) was calculated by the formula: CHO=100-(moisture+crude ash+crude lipid +crude protein)

Fatty acid composition

The composition of body fatty acids in *L. vannamei* fed with diets containing different amounts of MLP and control are shown in Table 5. The contents of C15:0 (pentadecanoic acid) and C17:0 (heptadecanoic acid) were significantly higher in MLP treatments compared to those of the control group. However, the amount of saturated fatty acids (SFA) was significantly higher in the shrimp fed control diet compared to that of MLP treatments ($p<0.05$). The linoleic acid (LA) and alpha-linolenic acid (ALA) fatty acids were significantly higher in MLP100 and MLP25 treatments compared to the control group, respectively ($p<0.05$). Moreover, the amount of docosahexaenoic acid (DHA) was significantly higher in MLP50 fed shrimp compared to the control group ($p<0.05$). The body content of arachidonic acid (ARA) significantly elevated in MLP25 and MLP50 treatments compared to the control group ($p<0.05$). The amount of PUFAs was also significantly higher in both MLP50 and MLP100 treatments compared to the control group ($p<0.05$). The n3/n6 ratio was significantly higher in MLP50 treatment compared to the control group ($p<0.05$).

Hemolymph's biochemical and antioxidant indices

The results of hemolymph biochemical parameters and antioxidant activity of

L. vannamei fed diets supplemented with different levels of MLE and MLP are shown in Table 6. There were significant differences among the experimental treatments (p<0.05). The levels of serum total protein, albumin, and the activities of ALT and AST were significantly higher in MLP100 treatment compared to the control group (p<0.05). On the other hand, the activities of ALT and AST enzymes were significantly lower in MLP25, MLE0.25, and MLE0.5 treatments than the control group. The SOD and GP_x activities were higher in MLP50 and MLP100 groups compared to the control group. Moreover, the shrimp fed with MLE1.0 diet showed a significant increase in the hemolymph CAT compared to the control group (p<0.05). The amount of hemolymph cortisol showed a significant decrease in the shrimp fed with MLP50, MLP100, and MLE1.0 diets compared to the control group (p<0.05).

Table 5: Fatty acid composition of *Litopenaeus vannamei* fed different levels of *Moringa oleifera* leaf powder (MLP).

Parameter	Control	MLP 25	MLP 50	MLP 100
C14:0	0.00±0.00	0.00±0.00	00±0.00	0.00±0.00
C15:0	0.22±0.00^{c}	0.96±0.12^{b}	0.82±0.01^{ab}	1.5±0.34^{a}
C16:0	22.6±0.7^{a}	22.4±0.02^{ab}	20.5±0.71^{bc}	19.5±0.62^{c}
C17:0	1.25±0.01^{ab}	1.08±0.03^{b}	1.6±0.22^{a}	1.3±0.07^{ab}
C18:0	11.01±0.2	10.2±0.1	10.8±0.19	10.56±0.45
C20:0	0.71±0.01^{a}	0.62±0.00^{a}	0.51±0.05^{b}	0.45±0.02^{b}
SFA	35.88±1.02	35.34±0.01	34.32±1.09	33.47±1.52
C16:1n	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
C17:1n	0.27±0.02^{b}	0.49±0.01^{a}	0.63±0.06^{a}	0.5±0.09^{a}
C18:1n-9	27.1±0.67^{a}	23.49±0.00^{b}	22.44±0.31^{b}	22.79±0.96^{b}
C20:1n	1.17±0.03^{bc}	1.33±0.02^{a}	1.25±0.03^{ab}	1.09±0.03^{c}
MUFA	28.57±0.69^{a}	25.32±0.04^{b}	24.32±0.28^{b}	24.38±1.09^{b}
C18:2n-6 (LA)	11.61±0.31^{b}	12.36±0.03^{b}	11.15±0.08^{b}	18.39±3.55^{a}
C18:3n-6	0.00±0.00^{b}	0.15±0.01^{a}	0.00±0.00^{b}	0.00±0.00^{b}
C18:3n-3(ALA)	0.49±0.00^{b}	0.68±0.00^{a}	0.5±0.00^{b}	0.48±0.02^{b}
C20:4n-6 (ARA)	2.87±0.07^{b}	3.36±0.00^{a}	3.21±0.02^{a}	2.63±0.09^{c}
C20:5n-3 (EPA)	10.64±0.24^{a}	9.08±0.01^{b}	11.13±0.19^{a}	9.02±0.34^{b}
C22 :4n-6 (DTA)	0.18±0.01^{ab}	0.22±0.02^{ab}	0.15±0.03^{b}	0.25±0.00^{a}
C22 :5n-6	0.48±0.01^{a}	0.36±0.02^{b}	0.00±0.00^{c}	0.38±0.02^{b}
C22:5n-3 (DPA)	1.08±0.02^{a}	1.14±0.01^{a}	1.15±0.05^{a}	0.84±0.05^{b}
C22:6n-3 (DHA)	8.16±2.4^{b}	11.94±0.02^{ab}	14.02±0.41^{a}	10.1±0.38^{ab}
PUFAs	35.55±1.72^{b}	39.34±0.03^{ab}	41.35±0.81^{a}	42.14±2.62^{a}
HUFAs	21.68±2.09^{b}	24.4±0.04^{b}	28.38±0.63^{a}	21.77±0.82^{b}
Total n-3	20.39±2.13^{b}	22.87±0.06^{b}	26.81±0.66^{a}	20.46±0.8^{b}
Total n-6	15.15±0.41^{b}	16.47±0.09^{ab}	14.53±0.14^{b}	21.68±3.42^{a}
n-3/n-6	1.35±0.17^{b}	1.39±0.01^{b}	1.84±0.02^{a}	1.00±0.2^{b}
DHA/EPA	0.78±0.24^{b}	1.31±0.00^{a}	1.26±0.01^{a}	1.12±0.00^{ab}
ARA/EPA	0.27±0.00^{c}	0.37±0.00^{a}	0.28±0.00^{b}	0.29±0.00^{b}
n-3 LCPUFA	18.8±2.16^{b}	21.03±0.04^{b}	25.16±0.6^{a}	19.13±0.73^{b}
n-3 LCPUFA/ARA	6.59±0.92	6.25±0.02	7.8±0.12	7.25±0.01

Data (mean±SD) with different letters are significantly different among treatments according to the Tukey posthoc test (p<0.05). SFA: saturated fatty acid, MUFA: mono unsaturated fatty acid, LA: linoleic acid, ALA: alpha linolenic acid, ARA: arashidonic acid, EPA: eicosapentaenoeic acid, DHA: docosahexaenoic acid, PUFA: polyunsaturated fatty acid, HUFA: highly unsaturated fatty acids.

Salinity challenge test

The survival rate (%) of *L. vannamei* fed diets containing different amounts of MLP after exposure to low and high salinity stress are presented in Table 7. After 24 h exposure to the low salinity stress, the survival rate did not show any significant differences among all treatments (p>0.05), but the shrimp fed with MLP50 diet showed a significantly higher survival rate in response to the high salinity stress compared to the control treatment (p<0.05).

Table 6: Plasma biochemical and antioxidant parameters of *Litopenaeus vannamei* fed with different levels of *Moringa oleifera* leaf powder (MLP) and *M. oleifera* leaf extract (MLE).

Parameter	Treatments						
	Control	MLP 25	MLP 50	MLP 100	MLE 0.25	MLE 0.5	MLE 1.0
Albumin (mg mL^{-1})	4.65 ± 0.16^{b}	4.36 ± 0.30^{b}	4.87 ± 0.32^{b}	5.02 ± 0.13^{a}	4.93 ± 0.32^{b}	4.47 ± 0.35^{b}	4.65 ± 0.55^{b}
Alanine aminotransferase (ALT) (U ml^{-1})	215.33 ± 12.66^{a}	183.33 ± 3.79^{b}	218.67 ± 21.55^{a}	219.67 ± 16.65^{a}	191.00 ± 4.58^{b}	183.33 ± 11.93^{b}	199.33 ± 8.50^{ab}
Aspartate aminotransferase (AST) (U ml^{-1})	152.67 ± 18.72^{bc}	136.33 ± 4.93^{cd}	170.33 ± 16.26^{ab}	183.67 ± 12.58^{a}	128.00 ± 5.57^{d}	128.33 ± 3.79^{d}	154.67 ± 13.65^{bc}
Cortisol (ng mL^{-1})	23.37 ± 0.61^{a}	24.00 ± 0.36^{a}	20.60 ± 0.79^{b}	20.37 ± 1.33^{b}	23.03 ± 0.40^{a}	22.70 ± 1.11^{a}	20.87 ± 0.65^{b}
Total Protein (mg mL^{-1})	46.00 ± 1.00^{bc}	44.00 ± 1.00^{bc}	47.67 ± 1.53^{ab}	51.00 ± 2.65^{a}	48.00 ± 3.61^{ab}	43.00 ± 1.00^{c}	47.00 ± 3.00^{abc}
Catalase (CAT) (U mL^{-1})	140.00 ± 10.58^{bc}	125.67 ± 7.02^{c}	127.00 ± 10.58^{c}	122.67 ± 5.16^{c}	149.33 ± 2.52^{ab}	149.00 ± 4.58^{ab}	162.67 ± 16.62^{a}
superoxide dismutase (SOD) (U mL^{-1})	57.00 ± 2.00 ab	53.33 ± 1.53^{b}	61.33 ± 5.13^{ab}	64.67 ± 7.37^{a}	57.00 ± 4.36^{ab}	59.33 ± 2.52^{ab}	59.67 ± 4.62^{ab}
Glutathione peroxidase (GPx) (U mL^{-1})	1220.00 ± 65.57^{ab}	1120.00 ± 30.00^{b}	1370.67 ± 82.11^{a}	1361.67 ± 80.36^{a}	1338.33 ± 137.14^{ab}	1226.67 ± 40.41^{ab}	1290.67 ± 79.37^{ab}

Data (mean±S.D.) with different letters are significantly different among treatments according to the Tukey posthoc test (p<0.05).

Table 7: Survival rate (%) of *Litopenaeus vannamei* fed with different levels of *Moringa. oleifera* leaf powder (MLP) and *M. oleifera* leaf extract (MLE) after 24 h exposure to low (8 g L^{-1}) and high (55 g L^{-1}) salinity stress.

Items	Treatments						
	Control	MLP 25	MLP 50	MLP 100	MLE 0.25	MLE 0.5	MLE 1.0
Survival rate (%) at low salinity	73.3±11.6	80.0±0.0	93.3±11.6	80.0±0.0	86.7±11.6	86.7±11.6	73.3±11.6
Survival rate (%) at high salinity	60.0±0.0^{b}	46.7±11.6^{b}	86.7±11.6^{a}	60.0±0.0^{b}	46.7±11.6^{b}	60.0±0.0^{b}	60.0±0.0^{b}

*Data (mean±SD) with different letters are significantly different among treatments according to the Tukey posthoc test (p<0.05).

Discussion

The leaf of *M. oleifera* is a valuable and encouraging source of nutrients for supplementation in aquafeed (Tagwireyi *et al.*, 2017). Given the nutritional value of MO leaf and its extensive availability all over the tropical and subtropical regions, it can be considered as a potential feed ingredient for farmed shrimp feed. Therefore, the present study examined the effectiveness of different levels of MLP and MLE in the diet of the Pacific white shrimp (*L. vannamei*). Our results showed no remarkable effect of dietary MO on the growth indices. However, the supplementation of both MLP and

MLE improved the survival rate of the shrimp. Moreover, the body contents of lipids and fatty acids (*i.e.*, pentadecanoic acid and omega-3 and omega-6 polyunsaturated fatty acids), and the amount of hemolymph biochemical and antioxidant parameters were significantly improved in response to the dietary supplementation of MLP.

After six weeks of the feeding trial, the growth indices of the shrimp were not remarkably affected by dietary MLP and MLE, however, dietary MLE 0.5 showed a significant positive effect on the FCR value. Moreover, both MLP and MLE could significantly improve the survival rate. Previous studies in both freshwater and seawater shrimp farming demonstrated the positive effects of dietary MLE on growth performance. Dietary MLE could improve the growth indices of freshwater prawn, *M. rosenbergii* (Kaleo *et al.* 2019). Similarly, Akbary *et al.* (2021) showed that 1.0 g kg^{-1} MLE significantly enhanced the growth indices of *L. vannamei*. The effectiveness of MLE on the growth performance of shrimp is reported to be attributed to the improved digestive enzymes activity and physiological functions due to the increased energy flows in the host (Kaleo *et al.*, 2019).

Our results also revealed that the supplementation of both MLP and MLE affected the body composition of the shrimp. Interestingly, the shrimp fed with different levels of MLP showed a higher amount of lipids in the body compared to the control group. The enhancement of body lipid content could be attributed to the high amount of lipid in Moringa leaf. It has been known that *M. oleifera* leaf contains more than 7% lipids (Teixeira *et al.*, 2014; Su and Chen, 2020) and this amount is higher than the lipids amounts in other woody plants. Previous studies also showed that dietary MLP increased the body lipid contents in African sharptooth catfish, *Clarias gariepinus* (Idowu *et al.*, 2017), fingerlings of Indian carp, *Labeo rohita* (Hussain *et al.*, 2018), and abalone, *Haliotis asinina* (Reyes and Fermin, 2003). Therefore, dietary MLP can have beneficial effects on farmed aquatic animals by increasing the amount of lipid contents in the body.
Given that the effect of MLE on the fatty acid composition of *L. vannamei* has been already reported (Akbary *et al.*, 2021), in this study, we only evaluated the effectiveness of MLP on the fatty acid contents of the shrimp.

Our results showed beneficial effects of MLP on fatty acid contents in *L. vannamei*. Interestingly, the supplementation of MLP could significantly enhance the content of C15:0 fatty acid (pentadecanoic acid) in shrimp. Pentadecanoic acid has been recently found as a potential essential fatty acid with remarkable health benefits for humans (Venn-Watson *et al.*, 2020). Pentadecanoic acid is believed to provide worthwhile health benefits against several cardiometabolic, liver, and aging-associated conditions (Venn-Watson *et al.*, 2020). Pentadecanoic acid has the antifibrotic, anti-inflammatory, red

blood cell-stabilizing and mitochondrial-reparative properties that can reduce inflammation, anemia, and liver fibrosis (Venn-Watson *et al.,* 2020). Akbary *et al.* (2021) also showed that 1.0 g Kg^{-1} inclusion of MLE in the diet remarkably enhanced the pentadecanoic acid content of *L. vannamei.* On the contrary, dietary MLP showed no effects on the Pentadecanoic acid content of fish such as gilthead seabream, *S. aurata* (Jiménez-Monreal *et al.,* 2021) and finishing pigs (Zhang *et al.,* 2019). Our data also showed that dietary MLP could enhance the contents of both omega-3 and omega-6 PUFAs in the body of *L. vannamei*. Their increment in the MLP-treated shrimp may be related to high levels of the PUFA fatty acids and the high antioxidant contents (*e.g.*, phenolic compounds) in MLP (Lalas and Tsaknis, 2002). The enhancement of PUFA in response to the supplementation of MLE has been reported in *L. vannamei* (Akbary *et al.*, 2021). The results of body fatty acid composition also showed that the dietary MLP increased the n-3/n-6 ratio in the shrimp. Therefore, MLP can be considered a beneficial herbal supplement that increases the omega-3 PUFA contents and increases the quality of the shrimp for consumers. Notably, it is known that over 50 % of fatty acids in *M. oleifera* leaf are unsaturated fatty acids, and α-linolenic acid is the most abundant *unsaturated* fatty acid in *M. oleifera* leaf (Su and Chen, 2020). Therefore, dietary MLP could improve the fatty acid composition of the shrimp due to the presence of unsaturated fatty acids in Moringa leaf. The improvement of fatty acid profile and the increase of omega-3 PUFAs in response to the inclusion of MLP in the diet has been also reported in other animals such as broiler chickens (Nkukwana *et al.,* 2014), pig (Zhang *et al.,* 2019), rabbits (Selim *et al.,* 2021), and goats (Kholif *et al.,* 2019).

In this study, the supplementation of MLP and MLE in the diet of shrimp remarkably improved the hemolymph biochemistry. Our results showed that the levels of total protein and albumin were significantly higher in MLP 100 treatment compared to the control group and other experimental treatments. It has been known that the haemolymph biochemical indicators including the haemolymph protein and albumin contents can be used in determining the health status of the shrimp. In line with our results, dietary MLP significantly enhanced the plasma protein and albumin contents in Nile tilapia (El-Kassas *et al.,* 2020; Elgendy *et al.,* 2021) and other animals (Lu *et al.,* 2016; Meel *et al.,* 2018; Kholif *et al.,* 2019; Afzal *et al.,* 2021). The administration of MLE did not enhance the haemolymph protein and albumin contents in *M. rosenbergii* (Kaleo *et al.,* 2019) which was consistent with our results in *L. vannamei*. However, the addition of MLE to the diet of Nile tilapia, *O. niloticus,* enhanced the levels of plasma total protein and albumin (Shourbela *et al.,* 2020). The positive effects of the dietary MLP on the

plasma protein and albumin contents of the shrimp could be attributed to the high contents of proteins in MLP.

Our data also showed that the activities of ALT and AST were significantly influenced by the dietary MLP and MLE. The serum ALT and AST activities are known as indicators of the health status of hepatopancreas, so that the increased serum ALT and AST activities could be associated with the impaired hepatopancreas cells (Yu *et al.,* 2021). The significant decrease of the serum ALT and AST activities in shrimp fed with MLP25, MLE0.25 and MLE0.5% diets observed in the present study could be related to the protective effects of the MLP and MLE on the shrimp hepatopancreas. In line with our data, a previous study also showed the decrease in activities of ALT and AST in freshwater prawn, *M. rosenbergii* fed with MLE supplemented diets (Kaleo *et al.*, 2019).

The results of this study also showed that the MLP and MLE supplementation improved some plasma antioxidant indices in the shrimp. The positive effects of MLP and MLE on the antioxidant activities of the shrimp could be attributed to the bioactive and biological compounds in Moringa, especially antioxidant substances such as flavonoids (myricetin, quercetin, and kaempferol), phenolic acids (gallic, chlorogenic, and ellagic acid), proanthocyanidins, vitamin E, vitamin C, selenium, zinc, and β-carotene (Makkar and Becker, 1997; Wei and Shibamoto, 2007; Kaleo *et al.,* 2019; Afzal *et al.,* 2021). This positive effect has been already reported in fish and shellfish including *O. niloticus* (Shourbela *et al.,* 2020), *S. aurata* (Jiménez-Monreal *et al.,* 2021), shellfish, i.e., *M. rosenbergii* (Kaleo *et al.,* 2019), *L. vannamei* (Akbary *et al.,* 2021) when fed diets supplemented with MLP and MLE.

In the present study, low and high salinity were chosen as common environmental stressors that are widespread in the shrimp culture systems. Although, *L. vannamei* is a euryhaline shrimp species, however, any changes in the ambient salinity can influence the metabolism, growth, oxygen consumption, feeding rate, molting, and survival (Li *et al*., 2007; Chen *et al*., 2014; Akbarzadeh *et al*., 2019). Our results showed that exposure to low salinity stress did not cause any significant differences in the survival rate of the shrimp. Furthermore, in all feeding treatments, the shrimp could survive more successfully in the low salinity stress compared to the higher one. On the other hand, the survival rate of the shrimp fed diet containing MLP 50 was significantly higher than the control group, when they were exposed to the high salinity stress. It is well-known that the metabolites in Moringa leaf such as proline can improve the plasma membrane functions due to their ability to scavenge reactive oxygen species (ROS), buffer redox potential, and stabilize the membrane. Therefore, the metabolites in Moringa leaf might help the shrimp to mitigate the adverse

effects of the salinity stress (Hassan *et al*., 2021).

In conclusion, the results of the present study showed that the supplementation of both MLP and MLE improved the survival rate of *L. vannamei*. Moreover, the body contents of lipid and fatty acid composition of the shrimp were significantly improved in response to dietary MLP. Both MLP and MLE also improved serum biochemistry and antioxidant activity. Considering the beneficial effects of dietary Moringa on the performance of shrimp observed in this study, up to 100 g kg^{-1} MLP or 1.0 % MLE can be recommended as a supplementation to the diet of farmed shrimp.

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Research Article

Evaluation of yellow mealworm larvae (*Tenebrio molitor*, Insecta, Tenebrionidae) meal as a dietary protein source in Asian Seabass (*Lates calcarifer*) based on growth and some biochemical parameters

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Abstract

This study was conducted to assess the effects of *Tenebrio molitor* larvae meal (TM) in partial substitution of fishmeal (FM), on growth performance, hematological indices, plasma biochemical and antioxidant enzyme activities of Asian Seabass (*Lates calcarifer*). Experiment carried out with 144 pieces of Asian Seabass (mean weight±SE, 54±1.2 g) in a semi-recirculating fish culture system. Fishes were randomly distributed into four groups with three replicates as completely random design. All treatments were fed with iso-nitrogenous and iso-energetic diets at a substitution rate of 0, 20, 40 and 60 % of TM. The experiment was setup for eight weeks and feeding carried out at 3% of body weight/day. Results showed that there is no significant differences (p>0.05) in the growth performance, chemical body composition and protein utilization at the end of the experiment. However, weight gain, lipids and viscerosomatic index showed significant differences among the examined treatments (p<0.05). The red blood cells was not affected by TM diet, but the hemoglobin, hematocrit and mean corpuscular hemoglobin concentration levels decreased with increasing levels of TM (p<0.05). Plasma biochemistry analysis of triglyceride, glucose, cortisol, lysozyme and alkaline phosphatase levels increased significantly with increasing TM in the diet (p<0.05). Increasing the level of TM in fish diets caused an increase in the activity of glutathione peroxidase and catalase, while no significant differences were observed in the activity of superoxid dismutase and malone di aldehyde among the different diets. Overally, this study demonstrates that meal of *T. molitor* can be used as fishmeal replacement up to 40% of diet and is an alternative source of protein in Asian seabass diets.

Keywords: Body composition, Fishmeal replacement, Growth, *Lates calcarifer*, Mealworm, *Tenebrio molitor*

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Introduction

One of the biggest problems in the world is food insecurity due to human population growth and their activities in aquaculture, poultry and ruminants to prepared ingredient to satisfy its high protein requirements (Kohno *et al*., 1986; Matthew, 2009). Animal proteins that represented by fish meal is the most valuable protein source in terms of its containment of essential amino acids and minerals, and despite its use in forming diets, it has some disadvantages, including a high price (NRC, 2011) and lack of sustainability due to the continuous decline of fish stocks (White *et al*., 2004; Panserat, 2009). Therefore, there is an urgent need to find and utilize less expensive and more sustainable protein sources within aquafeeds. Thus, many protein-rich plant sources, such as soybeans have been used in farmed fish diets to replace fish meal (Espe *et al*., 2006; Gatlin *et al*., 2007). However, it was considered a source of concern due to the presence of anti-nutritional factors (Ogunji, 2004; Collins, 2014) that may cause gastrointestinal inflammation (Merrifield *et al*., 2011; Gai *et al*., 2012), high content level of fiber and non-starch polysaccharides, and inadequate fatty acids and amino acids profiles (Gai *et al*., 2012) as well as low palatability of the feed (Papatryphon and Soares, 2001; Gatlin *et al*., 2007).

Recently, the efforts of scientists have turned to use of insect meal as one of the most important available protein alternatives, mostly due to low cost production and high nutritional value. Generally, insects have many advantages; ease to culture on organic matter and other waste materials (Józefiak *et al*., 2016; Józefiak *et al*., 2018), having high feed conversion efficiency (FAO, 2013; Van Huis, 2013), lower levels of greenhouse gases and ammonia emissions (Oonincx *et al*., 2010), and increasing the risk of infection transmission (Van Huis, 2013) and reducing the use of fish meal (Barroso *et al*., 2014). In addition, the diet containing of insect meal are rich in amino acids, fats, vitamins and minerals (Rumpold and Schlüter, 2013; Barroso *et al*., 2014; Nowak *et al*., 2016). Furthermore, the insect larvae can be grown in dense environments (limited spaces) (Makkar *et al*., 2014; Henry *et al*, 2015) as well as fresh and saline water environments (Merritt and Cummins, 1996; FAO, 2013). They are cost-effective in terms of water and energy requirements, there are antifungal agents, and antibacterial peptides in many insects (Nawaz *et al*., 2018).

Recently, most species of insects used in livestock, poultry and aquatic organisms feeds production are *Hermetia illucens* (black soldier fly), *Musca domestica* (common house fly), *Tenebrio molitor* (yellow mealworm), *Bombyx mori* (silkworm) and several locusts (Van Huis, 2013). *T. molitor* meal is considered as a good protein source for fish because of its high protein and essential amino acid content (Rumpold and Schlüter, 2013; Nowak *et al*., 2016). *T. molitor* larvae meal (TM) was used in broiler chickens

(Bovera *et al*., 2015; De Marco *et al*., 2015; Biasato *et al*., 2016) and laying hens (Giannone, 2003; Wang *et al*., 2005). In addition, it has been used in many fish diets as a partial alternative to fish meal; in rainbow trout (*Oncorhynchus mykiss*) (Gasco *et al*., 2014; Belforti *et al*., 2015), African catfish (*Clarias gariepinus*) (Ng *et al*., 2001), common catfish (*Ameiurus melas*) (Roncarati *et al*., 2015), tilapia (*Oreochromis nilotica*) (De Haro *et al*., 2011), Gilthead seabream (*Sparus aurata*) (Piccolo *et al*., 2017) and European sea bass (*Dicentrarchus labrax*) (Gasco *et al*., 2016) and Asian seabass **(***Lates calcarifer* **).** The Asian seabass or barramundi is widely distributed in tropical and subtropical waters of the Pacific Ocean (Katayama and Taki, 1984; Matthew, 2009) and considered as one of the most important cultured fish in the world (FAO, 2006; Glencross, 2006). This species has high nutritional and commercial values and the quality of its firm and flavorful flesh that also grow in brackish and fresh waters (Matthew, 2009). The aim of this study was to assess the replacement of fish meal prepared by yellow mealworm as a dietary protein source for barramondi and determine its effect on growth performance, serum biochemical parameters and liver antioxidant enzymes activity.

Materials and methods

Fish and experimental conditions

This study was carried out at aquaculture facilities in the Isfahan University of Technology, (Isfahan, Iran), equipped with a closed water recirculating system. Asian sea bass (*L. calcarifer*) individuals were purchased from Ramoz Marine Fish Breeding Center (Bushehr, Iran). Before starting the experiment, fish were acclimated from seawater (36 g/L) to saline groundwater (15 g/L) in a circular fiberglass tank (volume of 1 m^3) by gradually decreaseing the salinity over 12 days at a rate of 2 mg/L daily. The chemical composition (mg/L) of used groundwater were borom (2.5), calcium (540.6), potassium (54.7), magnesium (744.8), sodium (3371) and strontium (19). During the two-week acclimatization period, the fish were fed a commercial diet (Dorindaneh Co., Shahrekord, Iran) containing of 12 % moisture, 42 % protein, 18 % lipid, 14.8 % carbohydrate and 10 % ash. A total of 120 fish with initial mean weight of 54±1.2 g (mean±SE) were randomly distributed into 12 recirculating tanks (approximately 300 L; 80 cm in diameter; 70 cm in height), at a density of 10 fish/ tank. The feeding experiment set up for eight weeks and during the experimental period the fish were hand-fed to apparent satiation three times daily (9:00, 13:00 and 17:00). The important water quality parameters were monitored weekly. These parameters including of the water temperature, dissolved oxygen, pH and ammonia content were kept constant at 27°C, 6.5 mg/L, 7.5 and 0.02 mg/L, respectively. The photoperiod of 12:12 (hours light:hours dark) was maintained throughout the experiment. Dead fish were collected and weighed and then

the mortalities were recorded daily.

Fish diets preparation

The dried *T. molitor* larvae and fish waste meal were purchased from a local breeder (Isfahan Province, Iran) and Rizdaneh company, Isfahan). The *T. molitor* larvae was ground into meal an electric blender (A11, IKA, Germany) at nutrition laboratory of Isfahan University of Technology. Then, four isoenergetic and isonitrogenic experimental diets were prepared for Asian seabass based on previous researches (Aquacop-Cuzon *et al*., 1989; Ambasankar *et al*., 2009). These diets were prepared using a commercial meat grinder with a 2 mm screen which were air-dried for 24 h, ground to a suitable size and stored at -2°C in air-tight plastic bags until used. Replacement levels were as follows: a control diet (TM0) without mealworm larvae and three diets in which fish waste meal was partially replaced with TM at 20% (TM20), 40% (TM40) and 60% (TM60). The diets ingredients and chemical composition of TM and FWM diets are presented in Table 1.

Table1: Ingredients (%) and proximate composition (%) of the experimental diets, *Tenebrio molitor* meal (TM) and fish waste meal (FWM).

			Experiment diets			
	FWM	**TM**	**control**	**TM 20**	**TM 40**	**TM60**
Ingredients (g /kg)						
Mealworm larvae			-	4.34	8.48	13.02
Fish waste meal[a]			21.7	17.36	13.02	8.68
Soybean meal			21.7	21.7	21.7	21.7
Wheat gluten			21.7	21.7	21.7	21.7
Wheat flour			21.9	21.9	21.9	21.9
Inactivated baker's yeast			3	3.2	3.5	4
Glumatine [b]			0.5	0.5	0.5	0.5
Lysine			0.5	0.5	0.5	0.5
Methionine			0.5	0.5	0.5	0.5
Salt			1	1	1	1
Molasses			3	3	3	3
Vitamin-mineral premix [c]			1	1	1	1
Vitamin C			0.5	0.5	0.5	0.5
Soybean oil			3	2.8	2.5	2
Proximate composition (g/ kg)						
Dry matter	93.34	97.72	98.32	97.57	98.52	97.5
Crude protein	48.78	48.32	45.87	45.96	45.83	45.72
Lipid	18.73	33.9	8.79	8.86	9.15	9.26
Ash	20.37	3.1	8.42	7.99	6.66	6.72
Carbohydrate[c]	5.56	12.71	34.48	34.94	35.48	36.49
Gross Energy (kcal/kg)	-	-	4128.13	4140.8	4185.6	4231.41

[a] Wastes from the production of fish fillets, and fresh fish supply stores (fish head, visceral by-products)

[b] Glumatine is a manufacturing by-product, which is used as a binder in aquatic food (Karimi *et al*., 2018). [c] Vitamins (mg/kg diet): cholecalciferol, 2,000 (IU/kg diet); retinol, 18,000 (IU/kg diet); menadione sodium bisulphate, 10; α-tocopherol, 35; riboflavin, 25; thiamine, 15; nicotinic acid, 200; Ca pantothenate, 50; pyridoxine, 5; cyanocobalamin, 0.02; folic acid, 10; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
Minerals (mg/kg diet): copper sulphate, 19.6; cobalt sulphate, 1.91; iron sulphate, 200; potassium iodide, 0.78; sodium fluoride, 2.21; manganese oxide, 26; magnesium oxide, 830; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g/kg diet); potassium chloride, 1.15 (g/kg diet); sodium chloride, 0.44 (g/kg diet). (According to the label of vitamin-mineral premix)

* The net energy of the diet Calculated based on carbohydrate= 4.01, protein= 4.01, and lipid= 9.03 (Kellner and Patience, 2017; Tacona, 1990).

[C] Carbohydrate = 100- (protein+ lipid + Ash + Moisture)

Growth performance

At the end of the experiment (eight weeks), fishes were starved for one day and then three fish from each tank were randomly sacrificed through an overdose of anesthetic (tricaine methanesulfonate- MS222: 200 mg/ L**).** The growth performance indices, the protein efficiency ratio (PER), protein productive value (PPV), viscerosomatic index (VSI), hepatosomatic index (HSI) and the body weight gain (BW) were calculated according to the following equations (Li *et al*., 2009):

Feed conversion ratio (FCR) = feed intake (g)/weight gain (g)
Protein efficiency ratio (PER) = weight gain (g)/total protein given (g)
Protein productive value (PPV) = $(W_t \times P_2 – W_0 \times P_1)/ (I_d \times P)$.
Feed intake (FIg/day) =100× total amount of the feed consumes (g)/$[(W_0+W_t)/2]/t$

Where W_0 is the initial body weight (g), W_t is the final body weight (g), P is the crude protein percentage of the diets, P_2 is the crude protein percentage of the carcass at the end of the experiment, P_1 is the crude protein percentage of the carcass at the beginning of the experiment, I_d is the amount of food consumed and t is the experiment period (day). The survival rate was estimated as follows:

Survival rate = (final number of fish/initial number of fish) × 100
BW = final body weight (g) – initial body weight (g)
Weight gain (WG %)=[(final body weight–initial body weight)/ initial body weight]×100
Specific growth rate (SGR %/day) = [(Ln final body weight – Ln initial body weight)/ number of feeding days] × 100

Finally, somatic indices such as hepato-somatic (HSI), viscero-somatic (VSI) and the condition factor (CF) index were calculated as follows:

Condition Factor (CF %) = (weight of fish/ (length of fish)^3) × 100
Hepatosomatic index (HSI %) = (weight of liver/weight of fish) × 100
Viscerasomatic index (VSI %) = (weight of viscera/weight of fish) × 100

Biochemical analyses of diets and fish carcass

At the beginning of the experiment, carcasses of three fish were weighed and kept at freezer at –20°C for initial composition analysis. At the end of the trial, all fishes were starved for one day (to empty the digestive tract). After the biometry of the fishes, the carcasses of three fish were randomly collected in each treatment and then dried, and ground for analyze the approximate chemical composition of the carcass. The proximate analyses of the carcass of the fish, mealworm larvae, fish waste meal and the four experimental diets

were determined according to AOAC (2005) (Table 1). The samples were oven-dried at 105°C for 24 hours to reach a constant weight. Crude protein by using the Kjeldahl method (Kejeltec V40 Auto Analyzer, Bakhshi, Iran), crude lipid by using the Soxhlet extraction method (model 6XI Extraction Unit, Bakhshi, Iran), and ash by incinerating dried samples at 550°C for 4 h, using a Nabertherm muffle furnace (Model:K, Germany) were estimated.

Plasma biochemical parameters

Three fish in each tank were randomly selected and anesthetized using clove powder (200 mg/L) at the end of the experiment. The blood samples were taken from the caudal vein using heparinized plastic syringes, then plasma samples were separated by centrifugation at 3500 rpm at a temperature of 4°C for 10 min, and then it was kept at -20°C for biochemical analyzes. Plasma parameters were measured for concentrations of total protein (TP), triglyceride (TG), cholesterol (CHOL), aspartate aminotransferase activity (AST), alanine aminotransferase activity (ALT), alkaline phosphatase activity (ALK), albumin (ALB), lysozyme, lactate dehydrogenase (LDH), glucose (Glu) and cortisol (Cort) on an automated blood analyzer (Mindray BS 400, Al-Zahra Laboratory Isfahan, Iran) by using of Pars Azmoun Commercial Kits (Karaj, Iran).

Liver antioxidant capacity

Liver antioxidant activity was determined as superoxide dismutase (SOD) (Kono, 1978), catalase (CAT) (Koroluk *et al.*, 1988), the malondialdehyde (MDA) (Placer *et al*., 1966). The glutathione peroxidase (GPx) was measured using the RADOX (UK) Kit.

Statistical analysis

The experiment was carried out by a completely randomized design. The normality and homogeneity of data were assessed prior to any statistical analysis. SPSS 25.0 Software was used for all statistical analysis of the data. Normality of data was evaluated using Kolmogorov-Smirnov test. The One-Way ANOVA was used for significant differences of treatments. Difference between different means was determined by Duncan multiple range test at 95% confidence limit.

Results

Growth performance and biometry

The effects of treated diets during the growth trial are shown in Table 2. There were no significant differences ($p>0.05$) among the groups treated with different levels of TM, neither specific growth rate (SGR), feed conversion ratio (FCR), feed intake (FI), protein efficiency ratio (PER), productive protein value (PPV) nor for the survival rate as compared to the control diet. However, the final body weight (FBW), body weight increase (BW) and weight gain (WG %) showed significant differences among the treatments

(p<0.05), and the weight of the fish grew almost three times at the end of the experiment. In relation to biometric indices, no significant differences (p>0.05) were found in both condition factor (CF) and hepatosomatic index (HSI) when the TM included in the diets (Table 2). On the contrary, viscerosomatic index (VSI) showed significant differences among the treatments (p<0.05).

Table 2: Growth performances, survival rate and somatic indices of Asian seabass (*Lates calcarifer*) fed the experimental diets. Data are means ±SE, n=3.

Growth performance	TM0	TM20	TM40	TM60
Initial body weight (g)	54.65±0.9	55.03±0.79	52.91±0.19	56.12±0.17
Final body weight (FBW) (g)	109.25±6.46^{c}	124.61±3.46^{b}	129.49±5.25^{a}	131.41±2.15^{a}
BW (g)	54.6±5.5^{c}	69.57±4.25 ^{b}	76.58±5.05 ^{a}	75.28±2.6 ^{a}
WG (%)	99.74±8.5^{c}	127.09±5.49^{ab}	144.6±7.29^{a}	119.56±3.86^{b}
SGR (%.day^{-1})	1.25±0.07	1.45±0.3	1.61±0.2	1.23±0.17
FCR	1.06±0.02	1.12±0.13	0.92±0.06	1.01±0.17
FI (g/day)	1.28±0.01	1.45±0.02	1.36±0.01	1.42±0.09
Protein utilization				
PER	2.04±0.04	2.02±0.24	2.13±0.12	1.87±0.36
PPV (%)	17.16±1.05	15.45±3.42	15.74±1.96	15.89±2.87
Survival (%)	83.85±3.55	88.89±0.0	88.89±0.0	81.66±1.66
Biometric indexes				
CF (g.cm^{-3})	1.08±0.01	1.08±0.003	1.1±0.04	1.13±0.01
HSI (%)	1.75±0.25	1.36±0.06	1.9±0.22	1.95±0.01
VSI (%)	7.22±0.18^{b}	7.39±0.11^{b}	8.59±0.36^{a}	8.91±0.41^{a}

Different letters in each raw show significant difference at 95% confidence limit among the experimental treatments ($p<0.05$). Four dietary treatments: TM0: fish waste meal group; TM20, TM40 and TM60: *Tenebrio molitor* larvae meal at 20, 40 and 60% substitution rate of FM groups, respectively.

Chemical composition of carcasses

The results of the carcass proximate composition of the fish fed with TM presented in Table 3. The chemical analysis of carcass showed no significant differences in moisture, dry matter, protein and ash among the treatments (p>0.05). In comparison, there were significant differences in the lipid content (p<0.05) which increased gradually with the increased TM concentration in the feeding groups.

Table 3: Effect of TM meal on proximate composition in carcasses of Asian seabass (*Lates calcarifer*). Data are means ±SE, n = 3.

	TM0	TM20	TM40	TM60
Moisture (%)	71.04±0.81	70.82±0.53	69.68±0.43	70.79±0.1
Protein (g/100 g DM)	70.17±1.18	68.42±1.23	62.68±1.6	64.18±4.23
Lipids (g/100 g DM)	16.25±0.36^{a}	18.13±0.19^{ab}	19.81±0.75^{bc}	20.64±0.19^{c}
Ash (g/100 g DM)	14.05±0.61	11.61±1.96	13.4±0.62	12.06±0.46

Different letters in each raw show significant difference at 5% level among experimental treatments ($p<0.05$). Four dietary treatments: TM0: fish waste meal group; TM20, TM40 and TM60: *Tenebrio molitor* larvae meal at 20, 40 and 60% substitution rate of FM groups, respectively.

Blood indices

Hematological indices of Asian seabass cultured on TM meal diets were mentioned in Table 4. Results showed that there were no significant differences in red blood cells, mean erythrocyte volume (MCV), and mean hemoglobin (MCH) ($p>0.05$). However, there were significant differences in the white blood cells, mean concentration of hemoglobin in red blood cells (MCHC) and hemoglobin content (Hb), and hematocrit (Hct) ($p<0.05$).

Table 4: Effect of TM meal on blood indicators of Asian seabass (*Lates calcarifer*). Data are means ±SE, n=3.

Index	C	TM20	TM40	TM60
RBC (10^6/mm^3)	6.6×10^6	4.1×10^6	6×10^6	5.3×10^6
WBC (10^4/mm^3)	3.4×10^{4b}	4.6×10^{4b}	5.8×10^{4ab}	7.5×10^{4a}
MCV (fl)	63.94±19.93	58.71±5.83	60.87±27.03	58.2±2.90
MCH (pg/cell)	9.66±2.12	18.56±3.25	10.8±3.71	11.53±1.76
MCHC (g/dL)	15.93±1.81^b	31.44±3.84^a	19.63±2.33^b	19.73±2.47^b
Hb (g/dL)	5.76±0.68^{ab}	7.51±0.8^a	4.95±0.56^b	6.07±0.61^{ab}
Hct (%)	32.16±2.58^a	24±0.51^b	25.5±1.33^b	31.16±0.9^a

Different letters in each raw show significant difference at 5% level among experimental treatments ($p<0.05$).

Plasma biochemistry

Triglyceride (TG), glucose, cortisol and alkaline phosphatase (ALK) levels as plasma biochemical parameters were measured in Asian seabass (Table 5). These factors were increased with an increase in TM of the diet. The fishes offered the diet containing the highest TM level (TM60) showed significantly higher TG, glucose and ALK values compared to than those fed the TM-free control diet (TM0). Dietary treatment had no significant influence on any of the other biochemical parameters measured in the present study ($p>0.05$).

Table 5: Effects of TM meal on plasma biochemical indices of Asian seabass (*Lates calcarifer*). Data are means ±SE, n=3.

Index	TM0	TM20	TM40	TM60
Glu (mg/dL)	45.66±11.05^b	42±4.51^b	54.33±8.33^{ab}	77.33±3.92^a
TG (mg/dL)	77.33±7.83^b	117.33±9.49^{ab}	104.66±18.09^{ab}	151±21.65^a
CHOL (mg/dL)	223±7.51	220.33±7.05	222.33±17.24	212.33±8.19
AST (IU/L)	29±3.51	34±7.51	24.66±6.96	30±6.08
ALT (IU/L)	16.66±3.17	18±1.52	18.66±2.18	15.33±3.84
ALK (IU/L)	212.66±13.44^{ab}	253.33±22.15^a	176±14.00^b	264±24.44^a
Alb(g/dL)	1.51±0.04^a	1.48±0.04 ^{a}	1.24±0.07^b	1.28±0.04 ^{b}
TP(g/dL)	4.43±0.26	4.4±0.21	4.16±0.2	4.3±0.25
LDH (IU/L)	472.33±35.48	782±99.41	786±128.57	833.33±143.65
Cort (mg/dL)	1.05±0.31^b	1.47±0.42^b	1.17±0.53^b	2.85±0.17^a

Different letters in lines show significant difference at 5% level among experimental treatments ($p<0.05$).

Antioxidant enzyme activities and immune parameters

The antioxidant capacity in liver of Asian seabass showed that fish fed with TM showed difference in the antioxidant parameters of GPX, CAT and Lysozyme, whereas high values in GPX activity was observed in TM60

treatment (Table 6). On the other hand, no significant differences were observed in the activity of any MDA and SOD ($p>0.05$). In addition, the use of different levels of TM meal increased the lysozyme activities, with highest value 40% TM treatment.

Table 6: Effects of TM meal on oxidative enzyme and immune indices of Asian seabass (*Lates calcarifer*). Data are means ±SE, n=3.

Index	C	TM20	TM40	TM60
MDA (μmol/L)	7.36±0.29	7.56±0.44	7.6±0.51	6.85±0.03
GPX (IU/L)	124.84±8.67^{d}	134.76±8.86^{c}	179.14±65.03^{b}	189.52±3.14^{a}
CAT (IU/L)	1.07±0.22^{ab}	1.67±0.22^{a}	0.85±0.25^{b}	0.51±0.02^{b}
SOD (IU/L)	0.86±0.01	0.92±0.03	0.9±0.02	0.9±0.01
Lysozyme (U/ml/min)	17.87±2.38^{d}	53.67±5.62^{b}	87.4±6.64^{a}	37.15±4.18^{c}

Different letters in lines show significant difference at 5% level among experimental treatments ($p<0.05$).

Discussion

In this experiment, the effects of the partial replacement of FM with TM as a new protein source in Asian seabass feed was tested. The results showed that TM could be a valuable source of protein in Asian seabass at TM20, TM40, and TM60 as a substitute for FM, and also showed that replacement of dietary fishmeal with different levels of TM meal not only had no negative or significant effects on carcasse composition and growth performance parameters such as SGR, FCR, FI, PER, PPV, CF and HSI but also improved WG and SGR. One of the reasons for the growth of fish fed the diets containing TM could be attributed to improvement in the efficiency of nutrient utilization by fish, which might possibly be due to the presence of chitinous materials in the diets (Finke, 2007; Marono *et al*., 2015). The chitinous materials can modulate the gastrointestinal microbiota and potentially impart an improvement in growth efficiency when administered in adequate quantities (Alegbeleye *et al*., 2012). In addition, it has been found that animals' somatic growth is regulated by growth hormone and IGF-I.

Similar to our study, the inclusion of insect meal in rainbow trout diets (Melenchón, 2020), juvenile largemouth bass (Gu *et al*., 2022), blackspot sea bream (Iaconisi *et al*., 2017), did not have any negative effects on growth, protein utilization, WGR, SGR or FCR and the physiological state compared to the control group. On the other hand, Li *et al*. (2022), reported that the inclusion of TM meal in the diets of mirror carp (*Cyprinus carpio*) were significantly improved the FBW, SGR, and PE. In addition, Ido *et al*. (2019), reported the highest FBW, SGR and WG in red seabream (*Pargus major*) fed with 100% FM substituted with defatted TM larvae with no significant differences in the FCR and FI among all fish groups. Besides, the inclusion of TM in rainbow trout diets leads to a decrease in the FCR

alongside a rise in the PER with increasing dietary inclusion levels of TM larvae.

The contradictions in the growth performance and whole-body composition of fish fed with the graded levels of TM can be attributed to one or more factors, including TM factors, fish attributes, dietary factors (TM-compensated FM level and approximate composition of diets) and experimental conditions such as water temperature, salinity, and experimental settings, etc. Thus, optimal FM substitution levels could vary among fishes by insect species, rearing conditions, processing methods of insect meal, and duration of the experimental trials (Osimani *et al*., 2016; Iaconisi *et al*., 2019).

Hematological indices are important indicators for assessing the physiological conditions and health of animals, because these indicators are highly resistant to change (Khadjeh and Peyghan, 2007). For this reason, its results can be used to assess the state of the immune system (Maita, 2007). The results of the present experiment showed that different levels of TM had a statistically significant effect on WBC, MCHC, Hb and Hct in Asian seabass. In agreement with our study, Valipour *et al*. (2019), reported that some indices of hemoglobin, MCH and hematology, such as WBC count and Hct were affected by replacement of fish meal with yellow mealworm. Increased WBC counts may be the result of a protective response of the body during stress (Das *et al*., 2006). Decreased RBC count and a concomitant decrease in Hb and Hct may indicate fish anemia as a result of inhibition of erythrocyte production in hematopoietic organs (Ates *et al*., 2008). The changes of these parameters in relation to different levels of TM in our study did not follow a specific trend and did not indicate a negative effect, although there are not many studies on the effects of insect meal on fish hematological parameters to compare. While the use of insect larvae housefly (*Musca domestica*) as a supplement in diet of African catfish had significant effects on the hematological parameters of this fish (Okore *et al*., 2016). It seems that more studies should be performed to reach a sound conclusion.

Plasma biochemical parameters are important indicators to assess health status and hepatorenal functions (Fazio *et al*., 2019; Abdel-Latif *et al*., 2020) and physiological stress responses of fish fed formulated diets (Dawood *et al*., 2020a, b). In this study, a difference in plasma lipid parameters was observed in Asian seabass which its diet depended on the inclusion of TM in the practical FM-based diet. Whereas, fish fed with diet containing 20%, 40% and 60% TM had significantly higher TG values than fish fed the control diet, while a slight decrease in CHOL was observed in comparison with the control group. Other studies reported that the inclusion of high levels of silkworm pupae (68-90%) in the diets of carp (*Cyprinus carpio*) (Ji *et al*., 2015), TM in the diet of mandarin (*Siniperca scherzeri*) (Sankian *et al*., 2018) and a pre-pupal meal of black soldier fly in

diets of European sea bass (Magalhães *et al*., 2017), causes a significant decrease in plasma CHOL concentrations, which may be attributed to the contribution of chitin that are found in the exoskeletons of insect's meal. In fact, several evidences indicate that chitin and its deacetylated chitosan derivatives can decrease TG in liver and plasma as well as CHOL concentrations. Xia *et al*. (2011) showing significant hypolipidemic and hypocholesterolemic effects in animal and fish models. On the other hand, chitin-chitosan has been suggested to modulate lipid metabolism by interrupting the enterohepatic circulation of bile acid and interfering with digestion and normal absorption of fats in the intestine as well as biosynthesis of fatty acids in hepatocytes (Koide, 1998; Xia *et al*., 2011). In addition, the decreased plasma CHOL level with increased TM levels in Asian seabass diets could be attributed to increased chitin content in those diets, as also demonstrated by previous studies (Li *et al*., 2017; Magalhães *et al*., 2017). Fish plasma glucose levels increase during the stress period, possibly is due to the activity of the hormone catecholamines and the breakdown of glycogen stored in the liver and other tissues (Pottinger, 1998). In the present study, the plasma glucose levels of juveniles were affected by different levels of yellow mealworm. On the other hand, the level of cortisol as a stress hormone was different in treatments and these variations showed a statistically significant difference. According to the results of glucose and cortisol changes, it can be stated that the experimental fish in this study were probably subjected to stress in relation to different diets.

Hepatic enzymes including ALK, AST and ALT, are important indicators for assessing liver disorders in living organisms (Ayalogu *et al*., 2001). Whereas, an increase in its concentrations in the blood plasma indicates damage and necrosis of hepatocytes (Sheikhzadeh *et al*., 2012; Hyder *et al*., 2013; Song *et al*. 2014; Wang *et al*. 2014). The results of the current study showed an increase in the activity of ALT, AST and ALk serums when feeding the Asian seabass on diets containing different levels of TM meal, and therefore, TM meal may cause negative effects on the health of the liver. However, in terms of statistical analysis, AST and ALT were not significantly affected by different levels of TM meal, while ALK showed significant differences in the experimental groups. This result was in agreement with the results of Valipour *et al*. (2019), when feeding juvenile rainbow trout on different levels of yellow mealworm larval meal. Also, increase AST activity was observed in juvenile largemouth bass fed with 66%TM (Gu *et al*., 2022). This is contrary to the study of Li *et al*. (2017), which indicated no effect on the activities of ALT and AST in the serum when feeding juvenile carp with defatted black soldier fly larval meal. Increased serum TP concentrations, including both Alb and Glu are

evidence of immune disorders, impaired kidney activity and liver dysfunction (Banaee *et al*., 2011; John, 2007; El-Kamary *et al*., 2009). In this study, no difference in TP was seen when seabass was fed on diets containing different levels of TM, while a decrease in Alp was found. In agreement with the findings of the present research, the study by Gu *et al*. (2022) showed that the use of 55% and 66% TM in the diet of juvenile Largemouth bass led to a decrease in Alp. Sankian *et al*. (2018) reported no significant differences in TP and Alb in the serum of mandarin fish when using different levels of mealworm diets.

Oxidative stress occurs because of the over-production of reactive oxygen species (ROS), which leads to serious effects on the body of fish such as damage to the DNA in the nucleus of the cell; disruption of the cell membrane, cell wall and cellular proteins; and lipid peroxidation within cell membranes (Pamplona and Costantini, 2011). Radical scavenging enzymes, such as SOD, CAT, and GPX play a role in protecting cells from damage. CAT and GPX scavenge hydrogen peroxide and convert it into the water, while biological function of SOD is the detoxification of the superoxide anions created by partial reduction of O_2 and its transformation to hydrogen peroxide (H_2O_2) (Nordberg and Arnér, 2001). MDA is one of the breakdown products of lipid metabolism and is used as a reliable marker of lipid peroxidation. In our current study, we found the significantly higher activity of CAT in the serum of Asian seabass fed TM20 diet and GPX in serum of fish fed TM40 and TM60. This situation was consistent with research that found high CAT activity in the serum of carp fed with diets with 75% and 100% of defatted black soldier fly larval meal (Li *et al*., 2017) and also in serum of African catfish liver when including riccket meal in their diets (Taufek *et al*., 2016). whereas many of studies confirmed that CAT and SOD activity in juvenile largemouth bass (Gu *et al*. 2022), and GPX activity in mandarin fish (Sankian *et al*. 2018) increased when including different levels of TM meal in their diets. The reason for these results may be attributed to the presence of chitin in the exoskeletons of insects, where sources reported that chitin and its derivatives have antioxidant, antimicrobial and immune-stimulating properties which can positively affect the health of animals (Zhao *et al*., 2010; Ravi *et al*. 2011; Rumpold and Schlüter, 2013; Makkar *et al*., 2014) and prevent harmful effects in various diseases (Khoushab and Yamabhai, 2010; Ngo and Kim, 2014).

Lysozyme is a mucolytic enzyme characterized by the ability to break down the bacterial cell wall by cleaving the 1-4-β-linkages between N-acetylmuramic acid and N-acetylglucosamine in the bacterial cell wall (Saurabh and Sahoo, 2008). In the current study, an increase in serum lysozyme activity was observed in fish fed on graded levels of TM diets with significantly higher values than fish fed

a TM-free diet. The results of a similar study by Sankian *et al*. (2018), showed that TM meal inclusion in mandarin fish feed had positively influenced their humoral immune response and antioxidant defense status, whereas it showed increased activity of lysozyme when TM30 was fed. However, these findings are in contrast to Henry *et al*. (2018), who reported that there were no significant differences in lysozyme activity when European seabass were fed a TM25 meal.

Based on our results, Asian seabass fed on TM diets grew faster compared to those fed a TM-free diet. Therefore, we can consider *Tenebrio molitor* larval meal as a promising candidate for use as an alternative protein source for partial replacement in the diet of Asian seabass. The replacing of 40% of the FM protein with TM did not lead to adverse effects on the growth performance, proximate composition, and antioxidant enzyme activities of Asian seabass**.**

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Research Article

Acute toxicity of hydroalcohlic extracts of *Heracleum persicum* (Golpar) in zebrafish (*Danio rerio* Hamilton)

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Abstract

Heracleum persicum (HP) is an annual herbbelonging to Apiaceae (Umbelliferae) family and traditionally cultivated in Iran for its medicinal properties. Preliminary phytochemical analysis of *H. persicum* extract has shown that it possesses antioxidant properties, which may be attributed that to the presence of furanocoumarin compounds. In an experiment involving *Danio rerio* were, six groups of fish were exposed to different concentration of HA-PA ranging from 470 to 500 mg distributed among six groups in triplicates included G1 (control), G2 (1000 mg/kg), G3 (2000 mg/kg), G4 (4000 mg/kg), G5 (8000 mg/kg) and G6 (16000 mg/kg). The probit value for HA-HP and associated concentrations indicated low toxicity in *D. rerio*. The LC50 of HA-HP for *D. rerio* exposed to various concentrations for 24 hrs was found to be 6020.37 mg/kg with lower and upper bounds of 4477.5 and 8117.9, respectively at a 95% confidence limit. The maximum value of buccal movement rate was observed in animals exposed to 16000 mg/kg of HA-HP measuring between 134.0-192.66 N/min. However, increased doses of HA-HP led to respiratory distress and a decrease in oxygen uptake in fish. Moribund fish were observed at 1000 and 2000 mg/kg with signs of darting swimming, circular movement, and settling on the bottom of the aquarium for up to 12 hrs. The exposure of *D. rerio* to increasing concentration of HA-HP also resulted in the Loss of schooling behavior, which could affect their ability to move in coordination with their peers. Based on these findings, it can be concluded that the LD50 of HA-HP for *D. rerio* after 24 hrs is high indicating it can be used safely as an immunostimulant or for other purposes in fish at lower dosages.

Keywords: Zebrafish, *Heracleum persicum*, Lethal Dosage, Aquaculture

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Introduction

Medicinal plants contain a wide range of natural chemical compounds that possess various pharmacological and therapeutic properties. These compounds are often used in nutritional supplements and culinary preparations. However, the safety of these products must be evaluated to ensure they do not pose any harmful effects on human health. Thus, it is important to assess the toxicity of medicinal plants to determine their potential risks and ensure they are used safely (Modarresi Chahardehi *et al.*, 2020).

Heracleum persicum known as "Golpar" in Iran is an annual herb from the Apiaceae (Umbelliferae) family with various therapeutic properties (Majidi and Lamardi, 2018). Preliminary phytochemical analysis of *H. persicum* extract has revealed the presence of volatile oils, alkaloids, flavonoids, furanocoumarins, terpenoids, triterpenes and steroids (Sayyah *et al.*, 2005). Traditional Iranian literature mentions *H. persicum* as having anti-fungal, anti-convulsant, anti-microbial, anti-oxidant, anti-inflammatory, analgesic, and immunomodulatory activities (Choi and Hwang, 2004; Küpeli *et al.*, 2006). Studies have shown that *H. persicum* possess antioxidant properties, which can help inhibit the normal functioning of cells by trapping free radicals, the main cause of inflammation (Moreno *et al.*, 2020). Consequently, the furanocoumarins present in *H. persicum* have anti-inflammatory effects by inhibiting free radicals (Hemati *et al.*, 2012). that is due to the presence of furanocoumarin compounds (Souri *et al.*, 2004). is the presence of free radical compounds. The herbal plants contain antioxidant compounds that can be trapped free radicals inhibiting the normal functioning of cells. Accordingly, the antioxidant compounds present in this plant (furanocoumarins) cause anti-inflammatory effects by inhibiting free radicals *(*Hemati *et al*., 2010*).* They documented the Effect of the HA-HP (Golpar) on folliculogenesis in female Wistar rats.

On the other hand, the traditional usage of plants/parts in fish food (such as leaves, seeds, bark and roots) have varying potencies in aquaculture (Tewari and Kaur, 2022). Kakoolaki *et al.* (2016) demonstrated that incorporating *Camellia sinensis* into the diet of *Mugil cephalus* enhances their innate non-specific responses, hematological parameters, and growth performance when exposed to *Photobacterium damselae*. Medicinal plants have various biological functions in fish, including reducing stress, antimicrobial activities, boosting immune function, and promoting growth (Rummun *et al.*, 2017). To increase the net profit of fish farming and reduce the costs associated with fish feed, cost-effective plant-based sources may serve as suitable alternatives for inclusion in formulated feed. The use of these natural resources can be highly beneficial in ensuring the sustainable development of aquaculture in terms of environmental, social, and economic efficiency. (Hambrey, 2017; FAO, 2018). With the expansion of

aquaculture practices, there is an urgent need to find alternative sources of fish feed without increasing operational costs. Considering this issue, the use of non-conventional plant sources in the aquatic feed will be of great importance (Tewari and Kaur, 2022). In addition, these plant resources offer several other benefits such as fish pond fertilization, biological treatment, etc., along with improving the farmer's income through integrated farming (Kumar *et al.*, 2017). Due to the widespread use of *H. persicum* fruits as a medicinal plant and Iranian folkloric claims regarding their analgesic and anti-inflammatory effects, it was deemed necessary to evaluate the lethal dosage (LD50) of the hydroalcoholic extract of *H. persicum* fruits in zebrafish, an animal model. The objective of this research was to apply the data obtained to the field of aquaculture. Based on the afore-mentioned results, the aim of this study was determined the physio-toxicity as well as Median Lethal dose (LD50 value) of *H. persicum* extracts in zebrafish over a 24 hrs period. The extract was tested at five concentrations (1000, 2000, 4000 and 16000 mg/kg).

Materials and methods

Animals

As an animal model, wild-type zebrafish (*D. rerio*) of both sexes, approximately 4 months old, were purchased from a private sector in Tehran, Iran. A total of 108 fish were transferred to the laboratory, where the aquarium was designed and set up. The fish were acclimated in a large glass aquarium pre-filled with aerated water, set at a temperature of 28°C using an automatic thermostat, with a pH range of 7-7.5 and a 14:10 light:dark cycle, respectively, for a period of two weeks (Westerfield, 2000). They were fed twice a day The animals were fed twice a day with artemia in the morning and with a commercial ration in the afternoon at the ratio of 3% of body weight.

Herbal preparation and extraction

The fruits of *H. persicum* (HFP) were collected from the suburbs of Shemiran, located in northern Tehran, Iran. Samples were air-dried under natural conditions and powdered using herbarium techniques (Chemicals, 2005). He extraction method was based on the guidelines of the Organization for Economic Co-operation and Development. To prepare the hydroalcoholic extraction of *H. persicum*, 200g of the fruit was powdered and air-dried, then soaked in 1500 mL of a 1:1 EtOH-H2O solution for 48 hours. The combined extract was filtered and evaporated to dryness for 5-6 hours, and then stored in a refrigerator at 4°C until used.

Experimental design

The experimental groups were illustrated in Figure 1. After acclimatization, fish with a weight range of 470 to 500 mg were distributed into six groups, each with six fish in triplicate. The experimental design consisted of six groups: G1 (control), G2 (1000 mg/kg), G3 (2000 mg/kg), G4

(4000 mg/kg), G5 (8000 mg/kg), and G6 (16000 mg/kg), as shown in schematic 1.

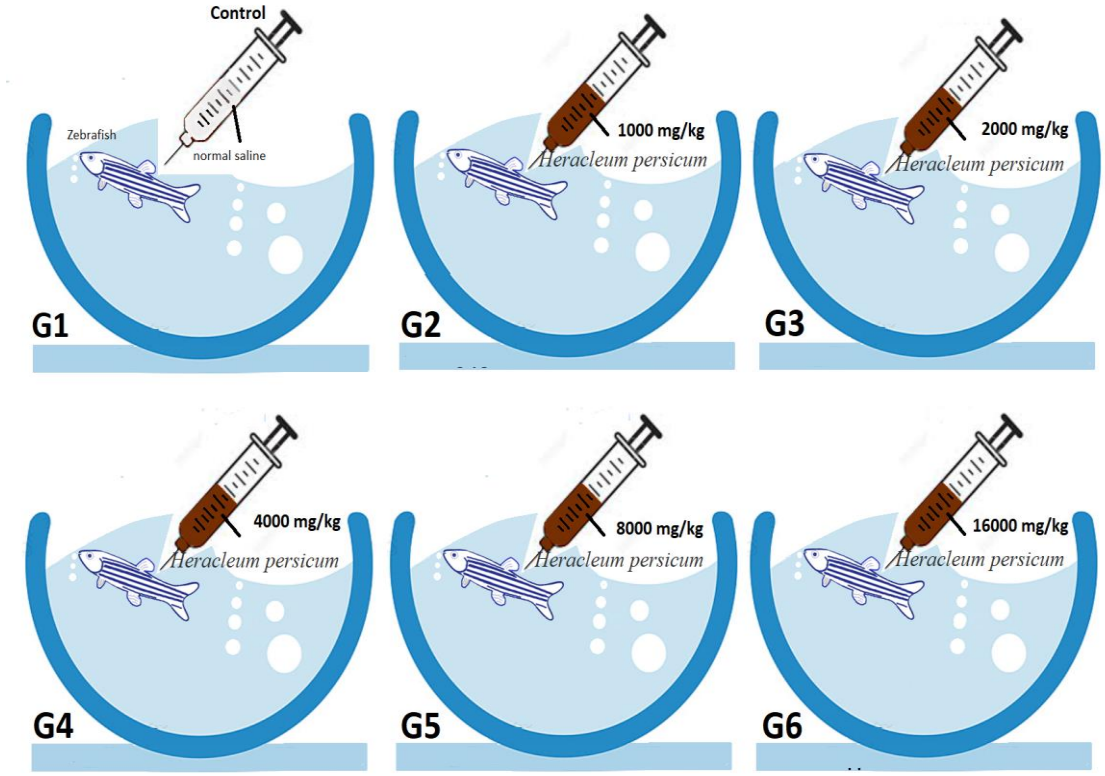

Figure 1: The schematic feature shows the study design of the experiment with 6 fish each aquarium for ip injection of *Heracleum persicum* (Golpar) extract at different dosage into Zebrafish (*Danio rerio*)

HA-HP administration

HA- HP was administered by dissolving it in water using a water-based approach for medicine administration, at the doses mentioned previously. According to this approach, the medicine was directly dissolved in the water based on the volume of the water (Husnul *et al.*, 2015). Therefore, HA-HP was directly dissolved in one liter of water in a jar, and different concentrations were then prepared according to the study protocol.

Dose adjustment

For this trial study, zebrafish with a weight range of 470 to 500 mg were selected. The stock solution of HA-HP was prepared based on the groups specified in the experimental design and calculated in mg/mL. Each zebrafish was administered with a 10 µL volume of the prepared HA-HP working solution at a concentration given in Figure 1, using a 30-gauge syringe (Stewart *et al.*, 2011).

Injection of HA-HP

HA-HP was administered intraperitoneally (i.p.) into the abdominal cavity of the fish (Stewart *et al.*, 2011). Briefly, each zebrafish was sedated by soaking it in cold water until

it presented a lack of locomotion and a drop in respiration rate. The fish was then placed on a wet sponge, and the i.p. injection was carried out according to the aforementioned protocol. The treated fish was then placed in a separate jar pre-filled with clean and aerated water until it had fully recovered.

Median lethal Dosage (LD50 24 h) of plant extract

Acute toxicity of HA-HP fruit extract samples was tested in the zebrafish model (*D. rerio*) as described by OECD guidelines (OECD, 1992). The zebrafish were exposed to the test substance was set for a period of 24 hours. Mortalities were then counted at each hour until the end of experiment. The median lethal dosage (LD50 24 h) of HA-HP extract that caused 50% of fish mortalities was deployed.

Respiratory analysis

The buccal movement rate of fish of each group and replications (3 samples) was calculated as number each minute. The opercular movement rate (OMR) as number(N)/min., which shows the respiratory activity was also measured.

The fish buccal and opercular activity at each minute were totaled twice an hour, during 24 h, in both the control and treated groups. The graphic illustration of the both mean values attained at each dose per time was then designed (Kishore *et al.*, 2022). The video images were made with an inverted microscope (Zeiss, Austria) that was linked to a digital video camera (Canon, Japan) so that the buccal and opercular movement to be seen better (Yaqoob and Schwerte, 2010).

Behavioral analysis and mortality

To test the effects of HA-HP on the control and test groups, physiological reactions were observed and recorded. These reactions were classified into three stages: (1) amplification of movement activity, shock, and contractions in the tail axis; (2) circular swimming and loss of attitude; and (3) tetanus, immobility, relocation of the fish to the bottom of the aquarium, and death. Each zebrafish was assessed individually, and it was considered a dead animal when activity of the buccal or operculum and the reaction to mechanical stimulation could no longer be observed (Souza *et al.*, 2016).

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the differences of LD50 among the groups followed by a post hoc multi-comparison, Tukey test. Data are presented as Mean±SE at 0.05 level of probability. GLM-Univariate followed by Tukey test was used to evaluate buccal and opercular movement rate per minute. Probit confidence limit estimation and LD50 calculation were carried out using SPSS, V.26 for Windows.

Results

Table 1 presents the acute toxicity values of HA-HP in adult zebrafish during a 24-hour exposure period. No mortality was observed in the control group (G1) during the study.

The mortality of D. rerio in G2 and G3 ranged from 0.0-16.6% and 0.0-22.22%, respectively, indicating an increase in mortality in a concentration-dependent and time-dependent manner. This range gradually increased to values of 0.0 - 22.22%, 16.6 - 55.55%, and 44.4 - 100.0%, respectively, in G4, G5, and G6. Among the treatment groups, the LC50 was observed at a 24-hour exposure concentration of 8000 mg/kg in G5 of D. rerio (Table 1).

Table 1: Sets of experimental bioassay data percentage mortality in different dosages and time (*n*:6)

		Time (hours)			
		1	6	12	24
G1	n	0.0±0	0.0±0	0.0±0	0.0±0
	%	0.0±0	0.0±0	0.0±0	0.0±0
G2	n	0.0±0	0.33±0.57	0.66±0.57	1.0±0.0
	%	0±0	5.55±9.62	11.11±9.62	16.6±0.0
G3	n	0.0±0	1.0±0.0	1.0±1.0	1.33±0.57
	%	0.0±0	16.6±0.0	16.6±16.6	22.22±9.62
G4	n	1.0±0.0	1.66±0.57	2.0±0	2.33±0.57
	%	16.6±0.0	27.27±9.62	33.3±0	38.88±9.62
G5	n	1±1.0	2.33±0.57	2.66±0.57	3.33±1.15
	%	16.6±16.6	38.88±9.62	44.44±9.62	55.55±19.24
G6	n	2.66±0.57	4.0±0	5.33±0.57	6.0±0
	%	44.44±9.62	66.66	88.88±9.62	100±0

G1: Control, G2: 1000 mg/kg, G3: 2000 mg/kg, G4: 4000 mg/kg, G5: 8000 mg/kg, G6: 16000 mg/kg, n: number of fish, %: percent of mortality of each group

Figure 2 depicts the probit value for HA-HP and its associated concentrations. Accordingly, the LC50 of HA-HP for D. rerio exposed to various concentrations for 24 hours was 6020.37 mg/kg, with lower and upper bounds of 4477.5 and 8117.9, respectively, at a 95% confidence limit for concentration. This probit model can account for 80.6% of the changes that may be observed in the future (Fig. 2).

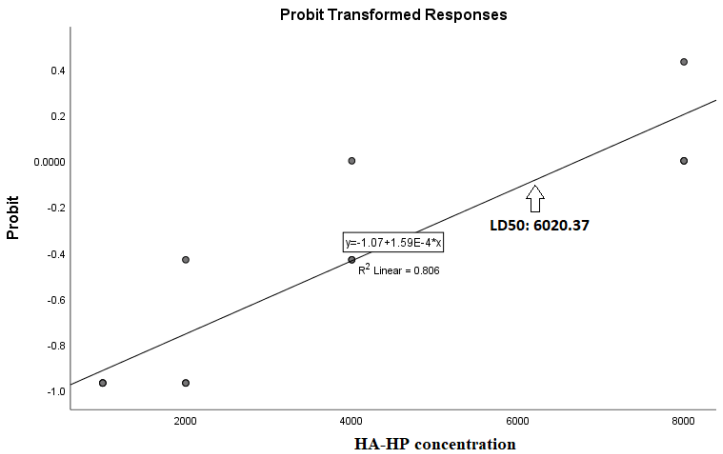

Figure 2: Regression scatter plot with trend line between the of concentration and probit value of HA-HP (mg/kg adult *Danio rerio*) at 24 hrs exposure time (p<0.05)

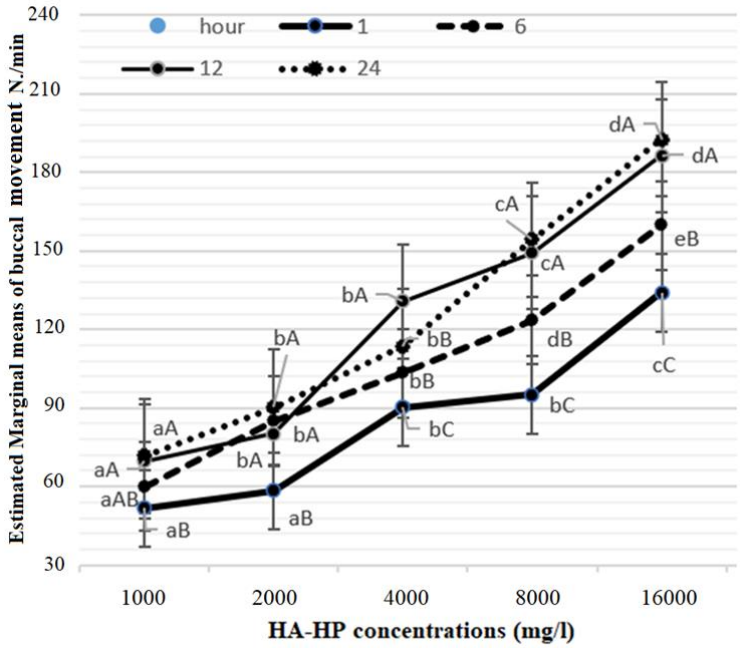

Figure 3: Changes in operculum rate (Number/min) in the adult *Danio rerio* exposed to different concentrations of *Heracleum persicum* (Golpar) during 24 hrs exposure

Figure 3 depicts the estimated marginal mean of buccal movement rate per minute. According to the Univariate GLM test, the partial Eta squared value showed that the model was fitted to the data at 90.7%. The control group showed a buccal movement rate of 40 N/min, which increased in a time-dependent manner. The minimum value of buccal movement ranged from 51.6 (with no significant difference compared to the value of 2000 mg/kg of HA-HP) to 71.66 N/min from 1 to 24 hours of exposure to 1000 mg/kg HA-HP in zebrafish. The maximum value was observed in animals exposed to 16000 mg/kg of HA-HP, with a range of 134.0-192.66 N/min. The buccal movement of animals exposed to 8000 and 16000 mg/L of HA-HP was 149.3 and 186.3 N/min after 12 hours, with no significant difference (p>0.05) compared to animals after 24 hours in the same groups.

Figure 4 shows the estimated marginal mean of the number of opercular movements (OM) per minute. According to the partial Eta squared value, the two-way ANOVA model was fitted to the data at 98.2%. The control group showed an OM rate of 35 N/min, which increased in a time-dependent manner, particularly with increasing HA-HP concentration. The minimum value of OM was observed on day 1, ranging from 51.6 to 71.6 N/min, while the zebrafish were exposed to different concentrations of HA-HP. For the time of 6 hours, this value started with 60.0 N/min at 1000 mg/kg and ended with 159.6 N/min at 16000 mg/kg. The differences in the number of OM per minute were not significant (p>0.05) after 6 hours, either for 1000 or 2000 mg/kg.

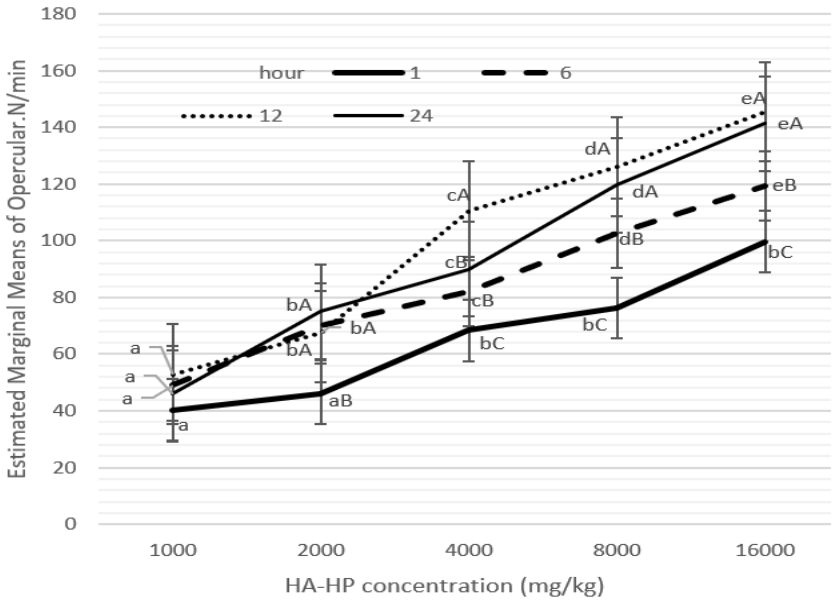

Figure 4: Changes in operculum rate (Number/min) in the adult *Danio rerio* exposed to different concentrations of *Heracleum persicum* (Golpar) during 24 h

Table 2: Clinical sign of *Danio rerio* reaction after exposure to *Heracleum persicum* (Golpar) at different concentrations during 6- hrs interval samplings

C (mg/kg)	Hours	Total number	Stage1	Stage 2	Stage 3	Total	%
0	1-24	18	0/18	0/18	0/18	0/18	0
1000	1	18	1/18	0/18	0/18	1/18	5.55
	6	18	1/18	1/18	1/18	3/18	16.66
	12	17	2/17	1/17	2/17	5/17	29.41
	24	15	2/15	1/15	3/15	6/15	40.0
2000	1	18	1/18	0/18	0/18	1/18	5.55
	6	18	1/18	1/18	1/18	3/18	16.66
	12	17	2/17	1/17	2/17	5/17	29.41
	24	15	3/15	2/15	3/15	8/15	53.33
4000	1	18	1/18	1/18	3/18	5/18	27.77
	6	15	2/15	2/15	5/15	10/15	66.66
	12	10	2/10	1/10	2/10	5/10	50.0
	24	8	1/8	2/8	3/8	6/8	75.0
8000	1	18	1/18	2/18	3/18	6/18	33.33
	6	15	3/15	3/15	4/15	10/15	66.66
	12	11	1/11	2/11	2/11	5/11	45.45
	24	9	2/11	2/11	5/11	9/9	100.0

Table 2 continued:

16000	1	18	1/18	2/18	2/18	5/18	27.77
	6	16	2/16	2/16	4/16	8/16	50.0
	12	12	1/12	1/12	5/12	7/12	58.33
	24	5	0	0	5/5	5/5	100.0

C: Concentration,

The physiological reactions of *D. rerio* to the HA-HP were converted to qualitative statistics and given in Table 2. Accordingly, the clinical signs observed in response to HA-HP was gradually increased so that the maximum percent of clinical cases was 40.0, 53.3 75.0, 100.0 and 100.0%, respectively in groups;1000, 2000, 4000, 8000 and 16000 mg/kg. The moribund fish of G2 was 50% of cases including the fish usually showed darting movement, stressfulness and tail muscle contraction. This value reached 62.5% after 24 hrs in G3, in which the concentration of HA-HP increased two-times more compared with G1. In G4 and G5, approx. 72.2% of cases were dead after 24 hrs and survived fish were suffering from difficulties in movement or respiration. The fish were completely died in G6 24 hrs of exposure. Exposed *D. rerio* exhibited Lack of adequate balance with the increased HA-HP concentrations (1000-4000 mg/kg) and exposure time (6-12 hrs). In control fish, they normally moved and swam all spaces of water column. They eventually moveless at the bottom and dead at higher concentrations (8000-16000 mg/kg) of the HA-HP and exposure time.

Discussion

The present study aimed to analyze the toxicity of *H. persicum* (Golpar) in adult *D. rerio* exposed to different concentrations. Prior to the experiment, the cytotoxicity of HA-HP was tested on artemia larvae and Swiss albino mice, and the results showed an appropriate margin of safety with an LD50 above 2000 mg/kg body weight at an exposure time of 96 hours (Chacha and Mbugi, 2019). Another research showed that the toxicity evaluation with 100 µg⁄mL of the chloroform fraction of HA-HP could induce the highest (97%) mortality rate in *Artemia salina* (Mofasseri *et al.*, 2017) but no description about the dosage per animal weight explained. This finding was not in line with this study showed LD50 of HA-HP for 24 hrs was 6020.37 mg/kg in *D. rerio*.

The buccal and opercular activity rates of 1000-2000 mg/kg at 6 hrs after injection was no significant different with those of 12 and 24 hrs in this study. The rate of buccal movement in the exposed fish decreased with time increased during 96 hrs (Kishore *et al.*, 2022) against this study showed the buccal movement rate increased in time dependent manner. This difference might be due to the former study showed a decrease during 96 hrs and our research

was performed in 24 hrs. They showed that the buccal movement rate was decreased in herbal concentration dependent manner.

Since buccal activity in fish is associated with respiratory activity (Soni and Verma, 2018), the increased dose of HA-HP in zebrafish led to respiratory distress and a gradual decrease in oxygen uptake in fish. The increased opercular activity observed in the treated fish following raised HA-HP could be due to the constant toxic conditions activating sensory inducement to increase opercular movement for appropriate respiratory function of the gills to manage hypoxia caused by the drop in oxygen uptake through the gills (Baruah and Das, 2002). On the other hand, the gradual decreases in oxygen consumption rate in the fish strongly indicates the onset of acute hypoxia under toxicant-induced stress (Vutukuru *et al.*, 2005). A typical resting respiratory rate for adult zebrafish is about 160 number.min^{-1}; while hypoxia may raise respiratory rate to above 300 number. min-^{1} (Jonz and Nurse, 2006). LC50 at 24 hrs of HA-HP exposure of zebrafish in the present study was 6020.37, which was greater than the LC50 of tests conducted with Brine shrimp cytotoxicity assay of extracts for H-HP in disc method (966.44 µg/mL) at 24 hrs (Moshafi *et al.*, 2010). These results suggest that *H. persicum* may act as an immunostimulant, and a lower amount of it can be safely used. In a study evaluating the diet of common carp (*Cyprinus carpio*) enriched with *H. persicum* as a phytoimmunostimulant at three different inclusion levels of 2500, 5000, and 10000 mg/kg, the 5000 mg/kg diet was found to be more effective in enhancing total Ig, lysozyme, and complement activity. (Hoseinifar *et al.*, 2016). LD50 of *H. persicum* seed acetone extract on mice was 1103 (988.2–1245.9) mg/kg with ED50 of 4.15 and 5.35 mg/kg against seizures.

Based on the results of Table 2, The Moribund fish were suffering from HA-HP usually at 1000 and 2000 mg/kg with signs of darting swimming, circular movement, settlement on the bottom of aquarium until 12 hrs. Other studies have exhibited that environmental relevant toxicant in fish can lead lethargy, loss of appetite, anorexia, muscle contraction, stressed swimming, body balance loss, skin darkening, decreased or increased respiratory rate, and finally death (Sahu and Kumar, 2021). Schooling behavior in fish was supposedly initiated on chemical compound and locomotor responses (Pavlov and Kasumyan, 2000), as such the loss of schooling behavior in exposed *D. rerio* in this study that increased concentrations of HA-HP could induce responses in fish could change their ability for coordinated movement within a school. Dislocated ability for spatial recognition such as spatial memory and learning capability particularly in schooling with dreadful implications for fish fitness and survival in the wild have been exhibited following exposure to toxicants (Ward *et al.*, 2008; Jacquin *et al.*, 2020).

Based on the afore-mentioned discussion, it is concluded that LD50 of

HA-HP for *D. rerio* at 24 hrs is great and it can be used as immunostimulant or other usage in fish in a safe margin at low dosage.

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Research Article

Accumulation of heavy metals and detection of resistant-associated genes in *Pseudomonas aeruginosa* in an edible catfish (*Wallago attu*) from Pat Feeder Canal, Pakistan

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Abstract

Seafoods are the main source of animal protein in our daily diet and their consumption has been increased due to its high health benefits over red meats. This study aimed to evaluate the heavy metals accumulation in a freshwater catfish muscle (*Wallago attu*) and the detection of heavy metal resistance genes (HMRGs) in *Pseudomonas aeruginosa* isolated from the fish intestine. *W. attu* (n = 60) was collected from four different sites (Qabula Shakh, Magsi Shakh, Umrani Shakh, and Jamali Shakh) of Pat Feeder Canal, Balochistan. The heavy metals and HMRGs were detected using atomic absorption spectrophotometer and polymerase chain reaction. The concentrations of Cd (0.27±0.001 mg/L), Fe (1.23±0.001 mg/L), and Pb (1.23±0.0005 mg/L) were found to be above the permissible limits of WHO in the samples from Jamali Shakh. Moreover, a strong Pearson's correlation of the metal Cd was observed with Zn, Fe, Cr, Cu, Pb, and Ni. However, Zn has a strong correlation with Fe and Cr; Fe with Cu, Ni, and Cu. Cr with Pb and Cu; Cu with Ni and Pb; while Ni has a strong correlation with Pb and Mn. *P. aeruginosa* was also identified from 41 species out of all fish intestine specimens (68%). Similarly, different heavy metal resistance genes (MRGs) including *czc*A 4 (36.3%), *ncc* 4 (36.3%), *chr*R 2 (18.1%), and *cop*A 1 (9%) were confirmed using PCR. In conclusion, Cd, Fe, and Pb concentrations were higher than the WHO permissible limits. However, other heavy metals (Ni, Zn, Cr, Cu, and Mn) were permissible limits in the fish muscle. The results of this study have shown a correlation between the buildup of heavy metals and the presence of MRGs.

Keywords: Food Safety, Atomic Absorption Spectrometer, Metal Resistance, PCR, Bioaccumulation

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Introduction

There are many different sources of environmental pollutants and heavy metals, such as industrial and agricultural processes, acid rain, and human and animal waste (Facchinelli *et al.,* 2001). Each metal has a minimum threshold of toxicity, which is dangerous to life, and the environment. The heavy metals accumulate in fish body through absorption by the gills, and skin, and become part of the food chain (Alkan *et al.,* 2012).

The fish get contaminated by heavy metals (HMs) that accumulated in the aquatic environment due to the food chain and water contamination (Łuczyńska *et al.,* 2018). However, some of them remain accumulated in fish tissues, while others are eliminated through feces discharge. Fish are directly linked to the aquatic food chain, which can transfer heavy metals contamination to human beings (Araújo *et al.,* 2022). The most common environmental contaminants include mercury (Hg), cadmium (Cd), chromium (Cr), cobalt (Co), lead (Pb), nickel (Ni), copper (Cu), and arsenic (As) (Jaiswal *et al.,* 2018).

The freshwater catfish species, *Wallago attu*, is an important fish used as human food. It consumes leftovers and other organic matters, so it can easily be grown in a normal environment without any special care (Bano *et al.,* 2018). These fish have significant attention because of their economics, nutritional health, quick development, and delicious flavor (Araújo *et al.,* 2022).

Bacteria develop

A tolerance for different amounts of hea -vy metal contaminants. Researchers are paying close attention to metal resistant bacteria in metal-contaminated environments (Pirela *et al.,* 2014). *Pseudomonas aeruginosa* is a normal part of the fish microbiome, but under stressed conditions such as hunger and overpopulation, the bacteria have become extremely opportunistic and harmful, causing severe diseases such as hemorrhagic septicemia, gill necrosis, abdominal distension, splenomegaly, friable liver and congested kidney (Ardura *et al.,* 2013; Dian Fitria *et al.*, 2021).

Balochistan is the largest province of Pakistan and Jaffarabad is one of the urbanized districts of the province, where the Pat Feeder Canal is the primary source of irrigation in Jaffarabad. The canal water is used by the residents for a variety of purposes, including agriculture, drinking, household use, and fish farming. The people of the area catch fish for their food and finance, hence, the contamination of aquatic ecosystems by heavy metals may pose a serious threat to consumers. The purpose of this study was to analyze the relationship between heavy metals contamination in fish and the frequency of heavy metals resistance genes in bacteria living in the fish. Furthermore, this study highlights that metal-resistant bacteria have a high potential in the remediation of metal-contaminated sites, with biosorption being the most promising process.

Material and methods

Sample collection

A total of 60 fish specimens were collected aseptically from the Pat Feeder Canal, Balochistan for heavy metal accumulation and bacterial isolation (Table 1).

Table 1: Biometry of *Wallago attu.*

Sex	Length (cm)	Weight (g)	Mean Length	Mean weight
Male (28)	50-110	1500-4000	89.87	3300
Female (32)	50-130	2000-4500	101	3900
Combine(60)	50-130	1500-4500	95.435	3600

The samples were collected from different active aquaculture sites (Qabula Shakh, Magsi Shakh, Umrani Shakh, and Jamali Shakh) of the district Jafarabad respectively. The collected samples were properly labeled and transported in the cold chain to the Centre for Advanced Studies in Vaccinology and the Biotechnology University of Balochistan and were stored at -4^{o}C for further analysis.

Area Description

Jaffarabad is the second most populated district of Balochistan with 0.64 million population. The district Coordinates are 28°25′N 68°10′E / 28.417°N 68.167°E. The district consists of three tehsils (Dera Allah Yar (Jhat Pat), Ustaa Muhammad and Gandakha. Most of the land is irrigated with Canals because of low annual rainfall which is 50 to 150 mm. The description of the sampling sites along with the longitudinal and latitude coordinates are mentioned in Table 2 and Figure 1.

Table 2: Description of Pat Feeder Canal characteristics for each sampling site

S. No	Sites	Description
1	Qabula Shakh	Located at coordinates: 28.637437, 68.396480 large size canal approximately 3-6 m wide, medium flowing water. Bottom substrate consists of sand mud and silt continuously turbid water.
2	Magsi Shakh	Coordinates: 28.644876, 68.405836 main canal, large size canal approximately 5-8 m wide, medium flowing water. Bottom substrate consists of sand, mud, silt and waste of animals and human continuously turbid water.
3	Umrani Shakh	Coordinates: 28.650619, 68.413432 large canal approximately 6-9 m wide, medium flowing water. Bottom substrate consists of sand, mud, silt, animals, human and agriculture wastes continuously turbid water.
4	Jamali Shakh	Coordinates: 28.660599, 68.427487 large size canal approximately 7-10 m. Medium flowing water. Bottom substrate consists of sand, mud, silt, animals, human and agriculture wastes continuously turbid water.

Heavy metal Analysis

For heavy metals detection, the fish samples were dried and ground to powder form. The dried fish powder (~1g) was suspended in 10ml of Nitric acid (67%), and incubated in a fume hood for 24 hours. Later 4ml Per-Chloric acid ($HClO_4$) was added and heated on a hot plate, cooled and diluted with deionized water to make the volume up to 100ml as proposed by Raka *et al.* (2020). Heavy metal detection was performed by using atomic absorption spectrophotometer (Perkin- Analyst 800 JAPAN) (Malik *et al.*, 2017).

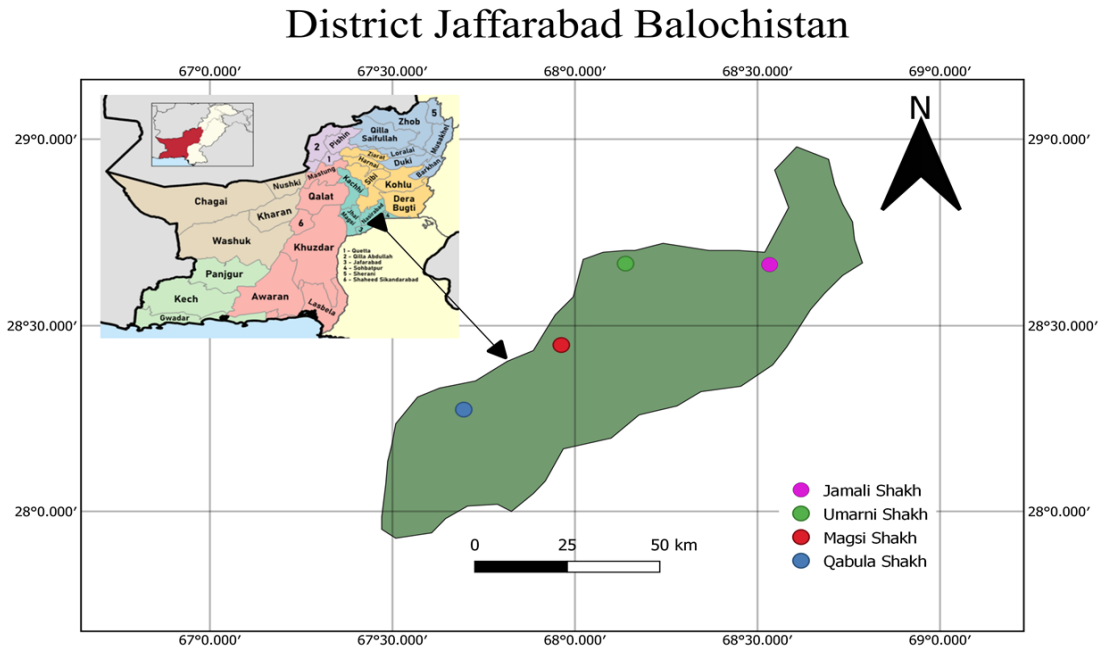

Figure 1: Map showing the location of the study area in district Jafarabad with Sampling Sites.

Microbial analysis

All fish samples were subjected to bacterial culturing and target bacteria isolation. The intestinal content of fish was aseptically removed, diluted with Luria Bertani (LB; Merck) broth, and incubated for 18 hours at 37°C. Initial growth was taken on nutrient agar followed by Cetrimide agar (Merck 1.05284.0500).

Molecular identification

DNA extraction

For genomic identification DNA was extracted through QIAamp (Cat. No. 51304 DNA mini kit) according to the given protocol and instructions.

PCR amplification

A molecular base identification was performed using PCR amplification by targeting 16sRNA, PA-SS-F, and PA-SS-R primers for molecular identification of *Pseudomonas aeruginosa* (Table 3). In total 20 µL PCR reaction mixture 10 µL PCR master mix, 1 µL forward and reverse primer and 5 µL molecular grade water and 3 µL DNA sample was added following (Sukri *et al.*, 2021). Similarly, initial

denaturizing was done at 94°C for 5 minutes while amplifications were carried out for 30 cycles (94°C for 30s, 55°C for 30s and 72°C for 30 s) and a final extension at 72°C for 7 min (SCILOGEX TC1000-G Master Cycler). Amplicons were detected by electrophoresis on 1.5% agarose gel, stained with ethidium bromide and visualized under a gel documentation system.

Table 3: Primers used in identification of *Pseudomonas aeruginosa* heavy metal resistant genes.

Primer	Sequence(5`-3`)	Target length (bp)	Annealing temp (ºC)	References
16sRNA-F 16sRNA-R	AGAGTTTGATCCTGGCTCAG TACGGYTACCTTGTTACGACTT	1500	58ºC	Al-Jailawi *et al*., 2014
PA-SS-F PA-SS-R	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	956	60.9ºC	Al-Jailawi *et al*., 2014
ncc-F *ncc*-R	ACGCCGGACATCACGAACAAG CCAGCGCACCGAGACTCATCA	457	54ºC	Ture *et al.,* 2018
*czc*A-F *czc*A-R	GTTCACCTTGCTCTTCGCCATGTT ACAGGTTGCGGATGAAGGAGATCA	320	58ºC	Kaci *et al*., 2014
*cop*A-F *cop*A-R	CGGTCTCTACGAATACCGCTTCAA GAAATAGCTCATTGCCGAGGCGTT	1,300	58ºC	Bouskill *et al.,* 2007
*chr*R-F *chr*R-R	ATGTCTGATACGTTGAAAGTTGTTA CAGGCCTTCACCCGCTTA	350	55ºC	Ture *et al*., 2018

Identification of heavy metal resistant genes (HMRGs)

Isolation/extraction of plasmid

Pseudomonas aeruginosa isolated from 60 fish samples, were examined for the presence of HMRGs. For this purpose, QIAGEN Plasmid Mini Kit (Cat. No. 12123) was used to extract the plasmids by following the manufacturer's instructions. PCR was done in 20 µL reaction mixture using 10 µL PCR master mix (2X), 1 µL of each of the forward and reverse primer 3 µL plasmid DNA, and 5 µL PCR grade water.

Amplification of HMRGs and Visualization

To amplify genes PCR was carried out as previously mentioned. For each target gene, the primers and annealing temperature are listed in Table 3. Distilled water was used as a negative control (Ture *et al.,* 2018). The final product was visualized in 1.5 percent (w/v) agarose gel.

Statistical analysis

The determined heavy metals values were tabulated and analyzed using SPSS Version 20. Pearson's correlation was used to evaluate the correlation among these metals.

Results

A total of 60 *W. attu* freshwater species were used for the detection of different heavy metals, Ni, Zn, Cr, Pb, Cu, Mn, and Cd from muscles. All three sites (Site 1-3) of Pat Feeder Canal showed significant values for all the selected

heavy metals (Fe, Zn, Cd, Pb, Cu, Ni, Mn, and Cr), under WHO permissible limits. An interesting result was observed in site 4 (Jamali Shakh), where the 3 metals concentrations were found significantly higher for Cd (0.27±0.001 mg/L), Fe (1.23±0.001 mg/L), and Pb (1.23±0.0005 mg/L). However, Zn (3.01±0.0004 mg/L), Cr (0.39±0.0002 mg/L), Cu (0.45±0.0002 mg/L), Ni (0.49±0.001 mg/L), Mn (0.78±0.001 mg/L) were found under WHO permissible limit and person Correlation were found (Fig. 2; Table 4).

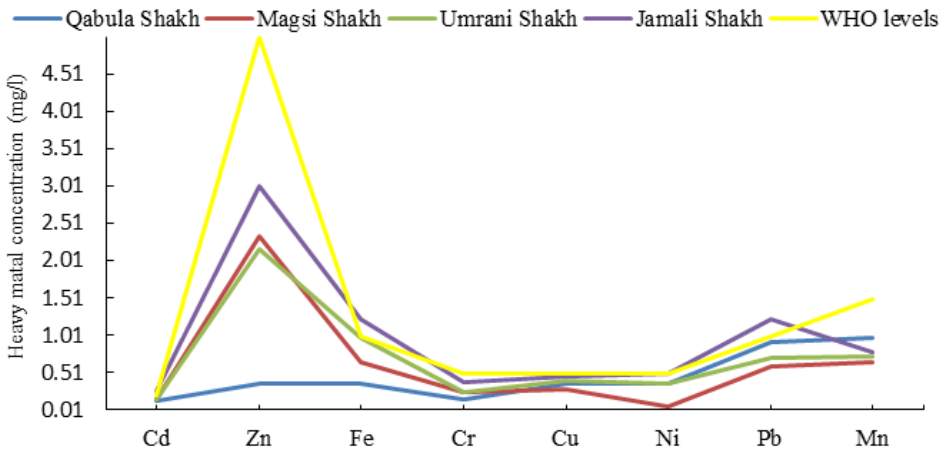

Figure 2: Concentration of different heavy metals in four different sites of Pat Feeder Canal.

Table 4: Mean Concentration (±SD) of heavy metals in *Wallago attu* collected from Pat Feeder Canal

Metals	Cd	Zn	Fe	Cr	Cu	Ni	Pb	Mn
Qabula Shakh	0.13±0.001	0.36±0.0004	0.37±0.002	0.16±0.001	0.36±0.0003	0.36±0.001	0.92±0.0005	0.98±0.002
Magsi Shakh	0.15±0.0004	2.34±0.001	0.64±0.001	0.25±0.001	0.29±0.0006	0.06±0.0004	0.59±0.001	0.64±0.001
Umrani Shakh	0.15±0.0003	2.17±0.0003	0.97±0.0009	0.25±0.0003	0.40±0.001	0.36±0.002	0.71±0.002	0.72±0.001
Jamali Shakh	0.27±0.001	3.01±0.0004	1.23±0.001	0.39±0.0002	0.45±0.002	0.49±0.001	1.23±0.0005	0.78±0.001
WHO	0.2	5	1	0.5	0.5	0.5	1	1.5

Concentration of metals (Mean ± SD microgram/gram (mg/L) in *Wallago attu.*

Samples (60) were processed for heavy metals detection, the data was considered normal according to the central limit theorem. So, Pearson's correlation was used. The results of correlation analysis showed that, Cd has a strong correlation with Zn, Fe, Cr, Cu, Pb and Ni; Zn has a strong correlation with Fe and Cr; Fe has a strong correlation with Cu, Ni and Cu; Cr has a strong correlation with Pb and Cu; Cu has a strong correlation with Ni and Pb; Ni has a strong correlation with Pb and Mn (p<0.01) (Table 5).

Molecular identification of P. aeruginosa and heavy metals resistant genes

All the bacteria, isolated from intestine of fish were confirmed through 16sRNA universal primers amplification with 1500bp (Fig. 3) and using specific gene primers PA-SS-F and PA-SS-R with

956bp (Fig. 4). Out of 60 fish samples, *P. aeruginosa* was confirmed in 41(68%).

Similarly, targeting *czc*A (320), *ncc* (457), *chr*R (350), and *cop*A (1300 bp), respectively to conform the HMRGs in the isolates (Figs. 5-8; Table 6). The *czc*A, *ncc*, *chr*R, and *copA* genes were found in 36.3, 36.3, 18.1, and 9 %, respectively.

Table 5: Correlation of different heavy metals in *Wallago attu* collected from Pat Feeder Canal.

	Cd	Zn	Fe	Cr	Cu	Ni	Pb	Mn
Cd	1							
Zn	0.721**	1						
Fe	0.830**	0.872**	1					
Cr	0.951**	0.900**	0.922**	1				
Cu	0.716**	0.330*	0.742**	0.615**	1			
Ni	0.567**	0.039	0.507**	0.391*	0.954**	1		
Pb	0.797**	0.166	0.477*	0.579**	0.810**	0.845**	1	
Mn	-0.143	-0.782	-0.451	-0.435	0.251	0.529**	0.482*	1

** Correlation is significant at the 0.01 level. * Correlation is significant at the 0.05 level.

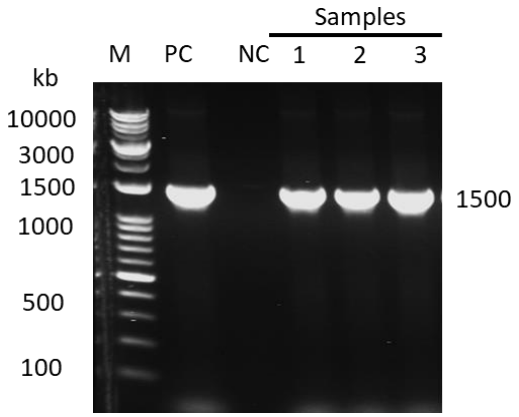

Figure 3: Agarose gel electrophoresis of 16sRNA PCR products, (M) 10 kb ladder: (PC) positive control for *P. aeruginosa*; (NC) negative control; lines 1-3: samples. English.

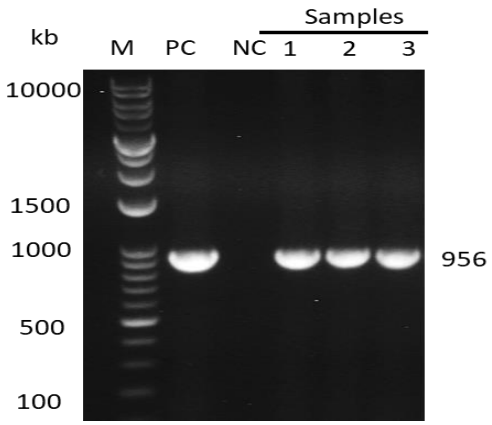

Figure 4: Agarose gel electrophoresis of *PA-SS-F* and *PA-SS-R* PCR products (M) 10 kb ladder: (PC) positive control; (NC) negative control; lines 1-3: samples of *P. aeruginosa.*

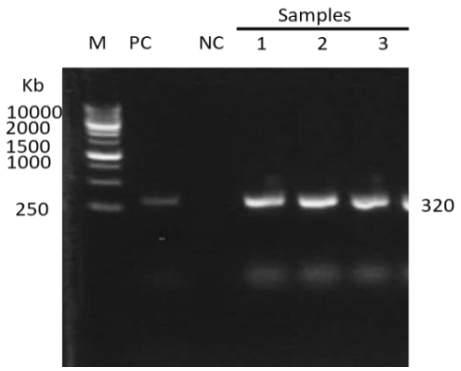

Figure 5: Agarose gel electrophoresis of *czcA* PCR products, (M) 10 kb ladder: (PC) positive control; (NC) negative control; lines 1-3: samples of *czc*A.

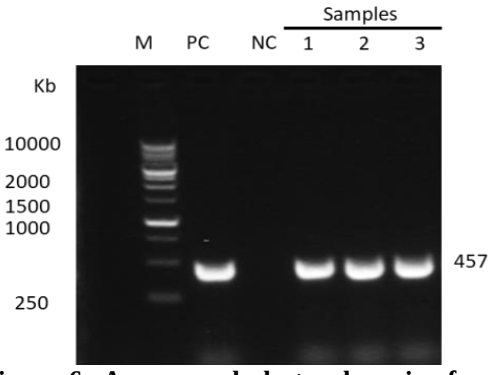

Figure 6: Agarose gel electrophoresis of *ncc* PCR products, (M) 10 kb ladder: (PC) positive control; (NC) negative control; lines 1-3: samples of *ncc.*

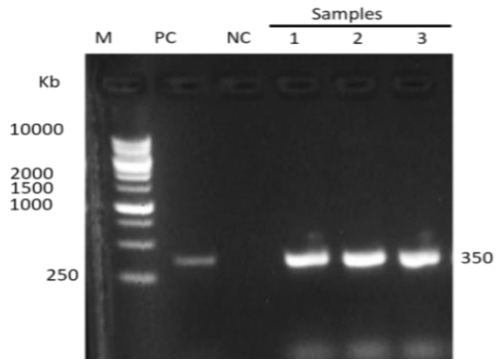

Figure 7: Agarose gel electrophoresis of *chrR* PCR products, (M) kb ladder: (PC) positive control; (NC) negative control; lines 1-3: samples of *chrR*

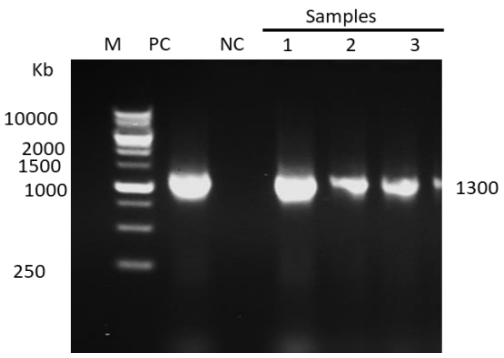

Figure 8: Agarose gel electrophoresis of *cop*A PCR products, (M) kb ladder: (PC) positive control; (NC) negative control; lines 1-3: samples of *cop*A.

Table 6: Heavy metal resistance genes of *Pseudomonas aeruginosa* isolated from *Wallago attu*

Heavy metal resistance gene (%)		***ncc***	***czc*A**	***chrR***	***cop*A**
No of genes		04	04	02	01
Total number of genes	11				
Percentages of genes found		36.3%	36.3%	18%	9%

Percentage (%) of heavy metal resistance genes isolate from *P. aeruginosa.*

Discussion

Freshwater catfish are important due to their commercial value. They are readily available and cheap freshwater food resources and eventually, they are largely consumed by the local and poor communities of the area.

In this study, significantly higher concentrations of Cd (0.27±0.001 mg/L), Fe (1.23±0.001 mg/L), and Pb (1.23±0.0005 mg/L) were found in the fish muscles collected from Jamali Shakh of Pat Feeder Canal. Similar results have been reported by Mahdi *et al*. (2020) from Iran, who reported the high concentrations of Cd, Cu, and Hg in the fish muscles caught from the Oman Sea. Dhaneesh *et al*. (2012) also found the higher concentrations of Cd, Co, and Pb in the fish muscles samples taken from the coastal water of Agatti Island. The outcomes of Mahboob *et al*. (2016) align with the present findings, as they elevated levels of heavy metals (Fe, Cu, Cd, and Pb) in two fish species, *C. carpio* and *W. attu*, collected from the Indus River in Pakistan.

Many researchers reported significant levels of different metals accumulation in the fish tissues (Bawuro *et al*., 2018). The reason behind this phenomenon is the activation of heavy metals through the chelation process involving trace elements and the mixing of sewage water (contaminants) along with the widespread use of pesticides and other chemicals in the area. Many researchers reported that a positive correlation was present among the heavy metals originating from atmospheric changes and anthropogenic activities (Wei *et al*., 2015), seasonal effect (Bawuro *et al.*, 2018), water or soil of the area and trace element (Mahdi *et al.*, 2020). Tariq *et al*. (1994) results are similar to our study; they reported trace elements (Mg^+, K^+, Ca^+, and Na^+) in the fish, and their habitat water and

sediment. This phenomenon can be applied to the current situation of our study area. Many studies investigate that the development of heavy metal resistance in fish which is associated with different bacteria like *E. coli* and Coliforms, *Aeromona* spp, *Vibrio parahaemolyticus* and, *Staphylococcus* spp, because of their prolong exposure to the metals dense habitats (He *et al.*, 2016; Liu *et al.*, 2019; Ture *et al.*, 2021). This bacterial resistance is transferred to other bacteria through vertical and horizontal gene transfers (Liu *et al*., 2016). *Pseudomonas* being prominent resistant bacteria showed resistance, to the diversity of heavy metals, that is why it has been extensively studied under the metals stress conditions due to the presence of HMRGs (Cerdeira *et al*., 2020).

Metal resistances are usually associated with plasmid-borne genes and the transformation of metal resistance genes (HMRGs) from the environments (Wei *et al.*, 2015). Several genes, including *mer*A, *ncc* and *czc*A, sustain the bacteria to tolerate different heavy metals (Ture *et al.*, 2021). In the current study, sixty (60) samples were evaluated, and results showed that *Pseudomonas aeruginosa* contains the majority of heavy metal resistant genes. We found *czc*A (36.3%), *ncc* (36.3%), *chr*R (18%) and *cop*A (9%) gene in *P. aeruginosa*. Bouskill *et al*. (2007) in a similar study confirmed the presence of resistances genes *Cop*A and *ncc*A which are in agreement with the current study. Rashid *et al*. (2021) identified drug resistance in *S. aureus* isolated from a variety of fish in the Gwadar port of Balochistan.

The current study showed a modest presence ratio of *czc*A and *ncc* genes, which is in accordance with the findings of Ture *et al*. (2021). We found *chrR* (18%) and *cop*A (9%) genes, these results concur with those of Chen *et al*. (2019) and Ture *et al*. (2021). Many bacteria are suitable for heavy metal biosorption, as they include a large number of genes related to different metals tolerance (Sukri *et al.*, 2021). Various tolerance mechanisms have been identified in heavy metal resistance genes (Chen *et al.*, 2019).

In conclusion, a significant higher impermissible limit of Cd, Fe, and Pb was found in the muscles of catfish *W. attu* collected from Jamali Shakh of Pat Feeder Canal. However, other metals Ni, Zn, Cr, Cu, and Mn were within permissible limits. Moreover, different HMGs were identified in *P. aeruginosa* isolated from the intestine of the fish. The presence of heavy metals in the fish and the resistant genes are most probably because of the leaching of heavy metals from the surrounding environment to the canal water. Sources identification of this pollution needs a comprehensive study to be conducted on targeted goals. The situation warns of the careful human consumption and regular monitoring for heavy metal concentration in the fish from the area. That can minimize the public health risk associated with the consumption of contaminated fish.

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Research Article

Growth performance and serum immune responses of the common carp (*Cyprinus carpio*) using *Lactococcus lactis* and *Weissella cibaria* as potential dietary probiotics

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Abstract

The present study aimed to investigate the effect of *Lactococcus lactis* and *Weissella cibaria* as potential probiotics on growth performance, some blood and immune parameters, digestive and liver enzyme activity, and intestinal bacterial flora, in common carp (*Cyprinus carpio*) juvenile. Fish (17.00±1.3 g) were divided into 10 treatments. The experimental diets of treatments 1, 2, and 3 were supplemented with *Lactococcus lactis* in doses of 1.5×10^7, 3×10^7, and 4.5×10^7 CFU kg^{-1}, the diets of treatments 4, 5, and 6 were supplemented with *Weissella cibaria* in doses of 1.5×10^7, 3×10^7 and 4.5×10^7 CFU kg^{-1}, these two potential probiotics were equally mixed for preparation the diets of treatments 7,8 and 9 which has been added in doses of 1.5×10^7, 3×10^7, and 4.5×10^7 CFU kg^{-1}. A basal diet (19 $mJkg^{-1}$ of energy and 38% protein) without probiotic was fed to the fish in the control group. Fish were randomly divided into 30 tanks and reared in the water with an average water temperature of 24.5°C. They were fed two times a day at 3% of body weight for 8 weeks. Results showed a significant increase in body weight (about 4 g), specific growth rate, and average daily growth in the most of the probiotic supplemented treatments ($p<0.05$) sespecially in treatments 8 and 5. Also, the highest amount of white blood cells, neutrophil, monocytes, Immunoglobulin M, alternative complement pathway activity (ACH50), lysozyme activity, digestive enzymes, and the lowest amount of liver enzymes (Aspartate aminotransferase and Alanine transaminase) were observed in the groups treated with potential probiotics. According to the results, adding 3 to 4.5×10^7 CFU kg^{-1} of the potential probiotics mixture, or 3×10^7 CFU kg^{-1} *W. cibaria*, could improve the growth performance and health status in common carp.

Keywords: *Cyprinus carpio*, Digestive enzymes, Immune parameters, *Lactococcus lactis*, Probiotics, *Weissella cibaria*

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Introduction

Nowadays, supplementing aquafeeds with probiotics is a new strategy from the nutritional aspect and an alternative remedial agent to overcome antibiotic's adverse influences (Pérez *et al*., 2019, Yeganeh Rastekenari *et al*., 2021, Kahyani *et al*., 2021). Many studies reported the positive effects of probiotics on farmed aquatic species (Irianto and Austin, 2002; Mohapetra *et al*., 2012; Beck *et al*., 2015; Adel *et a*l., 2016; Sayes *et al*., 2018; Mohammadian *et al*., 2019), However, there are still gaps to increase their efficiency for fish culture, which requires continuous research. Probiotic bacteria are live microbial feed supplements that play a beneficial role in the host by alteration in the gut microbial flora (Sayes *et al*., 2018). Probiotics, especially lactic acid bacteria are the major probiotics used in aquaculture (Irianto and Austin, 2002; Mohapetra *et al*., 2012; Hoseinifar *et al*., 2014; Mohammadian *et al*., 2017; Sayes *et al*., 2018) and their positive effects on improving fish immune and growth performance have been proven (Gatesope, 2008; Beck *et al*., 2015; Adel *et al*., 2016; Mohammadian *et al*., 2017; Won *et al*., 2020).

L.lactis and *W.cibaria* are kinds of lactic acid bacteria as natural flora of different species of aquatic animals, and their gens were registered. The genus *Weissella* is a recently classified member of LAB that is isolated from different sources including soil, food products, plants, animals, humans and fish (Fusco *et al*., 2015). Strains of some *Weissella* species are known as opportunistic pathogen present in humans, animals and fish (Costa *et al*., 2015; Fusco *et al*., 2015) but some of them have also been proposed as potential probiotics (Jesus, 2014; Goh and Philip, 2015; Hashemimofrad *et al*., 2016; Adebayo-Tayo *et al*., 2018; Sharma *et al*., 2018; Dey *et al*., 2019). Effects of *L. lactis* and *W.cibaria* on some fish species have also been studied (Jesus, 2014; Shenavar Masouleh *et al*., 2016; Hashemimonfared *et al*., 2016; Munir *et al*., 2016).

Common carp (*Cyprinus carpio*) is the sixth most cultured species in the world with more than 4 million tons of production per year (FAO, 2018). Recognizing appropriate and new strategies to improve production and breeding conditions, would be helpful in the common carp culture. Therefore, in this study, the effect of diets containing probiotic
bacteria *W.cibaria* and *L.lactis* has been studied in common carp to promote some aspects of production industry of this commercial species.

Material and methods

Fish and diets

Common carp juveniles (17.00±1.3 g) were obtained from a local farm in Gilan province. They were randomly divided into 30 tanks (600L, n=10) after a two-week acclimation period. Fish were divided into 9 treatments with a control, each with 3 replications. Water factors including temperature, dissolved oxygen, pH, ammonium, and hardness were measured routinely during the

experiment using an alcohol thermometer (China), Oxygen meter (WTW, Germany), pH meter (AZ8584, China), spectrophotometer (HACH IGS, Germany) and titration (complex metric method) respectively. The average water factors were 24.5° C, 6.1 mg L^{-1}, 7.7, 2 mg L^{-1}, and 212.25 mg L^{-1}, respectively. The potential probiotic bacteria used in the present study were *W.cibaria* (10^{10} $CFUg^{-1}$) and *L.lactis* (10^{10} $CFUg^{-1}$). Bacterial probiotics used in the present study are safe and secure (Soltani *et al*., 2013; Soltani *et al*., 2015; Shenavar masuleh *et al*., 2016; Hashemimofrad *et al*., 2016). They were isolated from *Acipenser persicus* intestine (Soltani *et al.*, 2013) in the international sturgeon research institute and recognized by rRNA 16S gene, and registered in NCBI under code 13 (Shenaver masouleh *et al.*, 2016). Potential probiotic Bacteria powder was prepared from Guilan Science and Technology Park. The diets were prepared by spraying a mixture of 50 ml of sterile physiological serum containing 150, 300, and 450 mg of 2 bacterial strains powder per kg of commercial extrude pelleted diets (Faradaneh Co. 35-38% protein, 4-8% fat, 5-11% moisture, 5-11% ash, 4-7% fiber, and 1.0-1.5 phosphorous) based on the recommended dosages by Yeganeh Rastekenari *et al* (2021) and Ghorbani vaghei *et al.* (2021). Then the prepared feed was placed in a dark and cool place to dry. Prepared feed was placed in the refrigerator (4°C) until the feeding trial (Shenavar Masuleh *et al*., 2016). Fish were fed two times a day at 3% of body weight for 60 days (Hosseini *et al*., 2016).

Growth Performance

Fish weight and length were measured at the beginning and end of the feeding trial to determine the growth performance. Fish were starved for 1 day and anesthetized with clove oil (50 mgL^{-1}) before biometry (Esmaeili *et al*., 2017). At the end of the feeding trial, the percentage of body weight increase (PBWI), condition factor (K), average daily growth (ADG), specific growth rate (SGR) and feed conversion ratio (FCR) were calculated using formulas as below:

$BWI\ (g) = Wt_2 - Wt_1$; $PBWI\ (\%) = [(Wt_2 - Wt_1) / Wt_1] \times 100$; FCR = g dry feed eaten / g live weight gain

$SGR\ (\%\ day^{-1}) = [(LnWt_2 - LnWt_1) / t_2 - t_1] \times 100$ (Merrifield *et al*., 2011); $K = [W / L^3] \times 100$ and $ADG\ (\%) = (Wt_2 - Wt_1) \times 100 / (Wt_1 \times (t_2 - t_1)$ (Bekcan *et al*., 2006)

Where: W= fish weight (g), L=fish length (cm), Ln= natural log, Wt_1= initial weight (g), Wt_2= final weight (g), t_1= first time, t_2 = final time

Blood analysis

At the end of the trial, to measure blood and immune parameters, fish were anesthetized with clove powder (0.5 g L^{-1}) at first. Then blood samples were drawn from the caudal vein and

transferred to two sets of microtubes, one set containing heparin anti-coagulant and the other non-heparinizes. The first was immediately used for hematological examinations and the second was used for Sera separation by centrifugation at 1500g for 20 min (Binaii *et al.,* 2014). The Blood and immune parameters include red blood cell (RBC), white blood cell (WBC), hematocrit (Hct), hemoglobin (Hb) levels, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and differential leukocyte counts (lymphocytes, neutrophils, and monocytes) (Feldman *et al*., 2000), llysozyme activity (Ellis *et al.*, 1990), immunoglobulin M (Amar *et al*., 2000), alternative complement pathway activity (ACH50) (Ortuno *et al*., 1998), amylase (Ross *et al*., 2000), lipase (Shihabi and Bishop, 1971), protease (Bernfeld, 1955) and liver enzymes (alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST) (Borges *et al*., 2004) were measured (Table 1).

Table 1: Details of amounts of potential probiotics in different treatments.

Bacteria Treatment	*L. lactis* (CFU kg^{-1})	*W. cibaria* (CFU kg^{-1})	Mixture of *W. cibaria* and *L. lactis* (CFU kg^{-1})
1	1.5×10^7	0	0
2	3.0×10^7	0	0
3	4.5×10^7	0	0
4	0	1.5×10^7	0
5	0	3.0×10^7	0
6	0	4.5×10^7	0
7	0	0	1.5×10^7
8	0	0	3×10^7
9	0	0	4.5×10^7
10	0	0	0

Bacteriological examination

At the end of the experiment, 3 fish were randomly sampled from each treatment and the total count of bacteria, as well as LABs count, was examined. Fish were anesthetized at first, then the abdominal surface was sterilized with alcohol (70%). Fish anesthetized with clove oil (50 mgL^{-1}) and humanly sacrificed and the intestine was completely separated. Intestinal contents were collected (it was washed three times using sterile physiological serum) and weighed. The contents of the intestine were diluted using physiological saline and the desired dilutions were prepared, then cultured on Tryptone Soy Agar (Merck, Germany) and MRS agar (Man, Rogosa, and Sharpe) (Difco Detroit, MI, USA) to determine the total count of bacteria and lactic acid bacteria, respectively. Plates were incubated for 48-72 hours at 30-35°C and the number of colonies grown on plates were then counted by colony counter (Ringo and Gatesoupe, 1998; Mahious *et al.,* 2006).

Statistical Analysis

Obtained data were analyzed using SPSS software (Version 20) and graphs

were drawn using Excel. First, the normality of the data and homogeneity of variance were checked by Kolmogorov-Smirnov and Levene tests, respectively. To compare blood data and growth coefficients, Two-way analysis of variance and Tukey test was used at 95% confidence level.

Results

Growth performance

The highest amount of body weight gain, percentage of body weight gain, specific growth rate, and average daily growth were observed in treatment 8 and after that in treatment 5, which were significantly different from the control (p<0.05). The data of growth parameters are presented in Table 2.

Table 2: Growth parameters of common carp juveniles fed diets supplemented with different amounts of potential probiotics *L. lactis* and *W. cibaria* after 8 weeks.

Index Treatment	ADG (g)	K	FCR	SGR (%d^{-1})	PBWI (%)	PBW(g)	Final weight (g)
1	0.36 ± 0.01 ^{cd}	0.02 ± 2.58	2.25 ± 0.06 ^{ab}	1.40 ± 0.02 ^{cd}	54.41 ± 0.46 ^{cde}	20.04 ± 0.88 ^{bc}	37 ± 2.12^{c}
2	0.34 ± 0.01 ^{bc}	0.02 ± 2.58	2.16 ± 0.09 ^{a}	1.35 ± 0.02 ^{bc}	53.15 ± 0.36 ^{bc}	18.93 ± 0.56 ^{ab}	36 ± 1.88^{bc}
3	0.33 ± 0.00 ^{ab}	0.01 ± 2.60	2.22 ± 0.05^{ab}	1.30 ± 0.00 ^{ab}	51.70 ± 0.05 ^{ab}	18.53 ± 0.82 ^{ab}	35 ± 1.81^{ab}
4	0.37 ± 0.01 ^{cde}	2.63 ± 0.12	2.26 ± 0.08 ^{ab}	1.43 ± 0.03 ^{cde}	55.00 ± 0.66 ^{cde}	21.00 ± 0.40 ^{cd}	38 ± 1.47^{cd}
5	0.42 ± 0.02 ^{fg}	2.55 ± 0.02	2.13 ± 0.09 ^{a}	1.53 ± 0.04 ^{fg}	57.56 ± 1.02 ^{fg}	23.18 ±0.78 ^{ef}	40 ± 1.74^{ef}
6	0.36 ± 0.02^{cd}	2.57 ± 0.02	2.26 ± 0.05 ^{ab}	1.39 ± 0.05 ^{cd}	54.04 ± 1.16 ^{cd}	20.30 ± 0.96 ^{bcd}	37 ± 1.6^{c}
7	0.40 ± 0.01 ^{ef}	2.61 ± 0.03	2.19 ± 0.03 ^{a}	1.49 ± 0.02 ^{efg}	56.54 ± 0.49 ^{efg}	22.25 ± 0.33 ^{de}	39 ± 1.36^{de}
8	0.43 ± 0.01 ^{g}	2.56 ± 0.05	2.12 ± 0.07 ^{a}	1.57 ± ^{g} 0.02	58.58 ± 0.45 ^{g}	24.30 ± 0.42 ^{f}	41 ± 1.4^{f}
9	0.39 ± 0.02 ^{def}	2.58 ± 0.01	2.14 ± 0.08 ^{a}	1.47 ± 0.04 ^{def}	56.15 ± 0.99 ^{def}	22.07 ± 1.04 ^{de}	39 ± 1.42^{de}
10	0.31 ± 0.02 ^{a}	2.59 ± 0.02	2.43 ± 0.06 ^{b}	1.23 ± 0.04 ^{a}	49.90 ± 1.16 ^{a}	17.06 ± 0.81 ^{a}	34 ± 2.57^{a}
***p*- value**							
W. cibaria	0.001	0.09	0.010	0.010	0.010	0.010	0.010
L.lactis	0.001	0.11	0.060	0.070	0.020	0.010	0.010
L. lactis and *W.cibaria*	0.001	0.10	0.010	0.010	0.010	0.010	0.010

Numbers with different superscripts in the same column are significantly different (p<0.05).

Hematological parameters

According to the results, no significant differences were observed in the number of RBC, Hct, and Hb between treatments (Table 3, p≥0.05).

Table 3: Amount of blood parameters of common carp juveniles fed diets supplemented with different amounts of potential probiotics *L. lactis* and *W. cibaria* after 8 weeks.

Index Treatment	Lymphocytes (%)	Monocytes (%)	Neutrophil (%)	HCT (%)	HB (g/dL)	WBC (mm^3×1000)	RBC (mm^3×1000)
1	79.00±2.00 ^{ab}	5.33±1.15^{ab}	15.33±1.53^{ab}	37.33±1.15	8.20±0.10	5.60±0.46 ^{bcd}	791.67±25.17
2	76.67±0.58^{a}	6.00±1.00^{b}	17.00±1.00 ^{b}	38.67±1.53	8.67±0.32	6.27±0.61^{cd}	819.67±30.44
3	77.00±3.00^{a}	5.33±1.15^{ab}	16.67±1.53^{b}	40.33±2.08	9.00±0.46	6.20±1.18 ^{cd}	868.33±52.52
4	79.67±1.15^{ab}	4.33±0.58^{ab}	15.00±1.00^{ab}	40.33±1.53	8.90±0.26	4.03±0.38^{ab}	859.33±28.04
5	77.33±1.15^{a}	5.33±0.58^{ab}	17.33±1.53^{b}	39.33±1.53	8.83±0.42	5.50±0.72 ^{bcd}	835.33±28.38
6	77.67±1.53^{a}	5.67±1.15^{ab}	15.33±0.58^{ab}	40.33±0.58	8.97±0.21	4.63±0.42^{abc}	855.00±15.62
7	75.00±2.65^{a}	6.00±1.00^{b}	18.33±1.53^{b}	37.67±1.53	8.33±0.40	7.17±0.55^{d}	802.67±29.91
8	79.00±2.00^{ab}	5.00±1.00^{ab}	15.67±1.53^{ab}	38.00±2.00	8.77±0.45	5.50±0.75^{bcd}	837.00±42.51
9	78.67±0.58^{ab}	5.33±0.58^{ab}	15.33±0.58 ^{ab}	38.00±1.00	8.43±0.21	5.67±0.32^{bcd}	809.00±12.77
10	83.33±1.15^{b}	3.33±1.09^{a}	13.00±1.00^{a}	40.00±1.00	8.20±0.10	3.53±0.32^{a}	853.33±20. 21
***p*-value**							
W. cibaria	0.090	0.010	0.010	0.060	0.070	0.010	0.700
L. lactis	0.010	0.010	0.040	0.91	0.001	0.010	0.090
L. lactis & *W. cibaria*	0.000	0.001	0.001	0.100	0.100	0.010	0.110

Numbers with different superscripts in the same column are significantly different (p<0.05).

Also, the lowest number of white blood cells, neutrophils, and monocytes were observed in the control treatment, which was significantly different from most of

the treatments ($p<0.05$). The lowest amount of IgM, ACH50, and Lysozyme activity was observed in the control group ($p<0.05$). In general, the highest amount of immune parameters was observed in treatments 5, 8, and 9 (Table 4).

Table 4: Amount of immune parameters of common carp juveniles fed diets supplemented with different amounts of potential probiotics *L. lactis* and *W. cibaria* after 8 weeks.

Index Treatment	Lysozyme activity (u/mL/min)	ACH50 (U %)	IgM ($mgdL^{-1}$)
1	27.33 ± 0.88^{a}	128.67 ± 2.76^{b}	43.00 ± 0.58^{a}
2	33.33 ± 1.20^{ab}	130.00 ± 1.15^{b}	48.00 ± 3.06^{b}
3	34.33 ± 1.20^{ab}	133.67 ± 1.73^{bc}	44.33 ± 0.33^{ab}
4	26.33 ± 0.67^{a}	123.33 ± 2.85^{a}	45.33 ± 0.33^{ab}
5	38.67 ± 0.33^{b}	138.33 ± 0.67^{c}	55.67 ± 0.33^{c}
6	35.00 ± 1.00^{ab}	133.67 ± 0.88^{bc}	51.33 ± 2.23^{b}
7	26.67 ± 0.88^{a}	141.00 ± 2.08^{c}	55.33 ± 2.33^{c}
8	36.67 ± 2.60^{ab}	141.33 ± 1.33^{c}	57.67 ± 1.76^{c}
9	35.67 ± 2.33^{ab}	141.00 ± 0.58^{c}	56.00 ± 1.53^{c}
10	24.67 ± 0.88^{a}	123.67 ± 1.86^{a}	41.00 ± 0.58^{a}
***p*-value**			
W. cibaria	0.010	0.001	0.001
L. lactis	0.010	0.001	0.001
L. lactis & *W. cibaria*	0.025	0.000	0.000

Numbers with different superscripts in the same column are significantly different ($p<0.05$).

The lowest amount of digestive enzymes as well as, the highest amount of liver enzymes was observed in the control treatment, which showed a significant difference from most other treatments ($p<0.05$). Changes in liver and digestive enzymes are presented in Table 5.

Table 5: Amount of digestive and liver enzymes of common carp juveniles fed diets supplemented with different amounts of potential probiotics *L. lactis* and *W. cibaria* after 8 weeks.

Index Treatment	AST (U/L)	ALT (U/L)	ALP (U/L)	Protease (Umg)	Lipase (U/mg)	Amylase (UmgL)
1	271.67 ± 13.84^{bc}	24.00 ± 1.73^{a}	37.33 ± 1.45^{ab}	37.00 ± 1.52^{ab}	17.00 ± 0.57^{ab}	60.33 ± 0.33^{b}
2	255.00 ± 23.17^{b}	22.00 ± 1.53^{a}	34.67 ± 0.88^{a}	40.66 ± 1.45^{ab}	16.33 ± 0.88^{ab}	5.04 ± 58.33^{b}
3	236.00 ± 21.50^{a}	22.33 ± 1.20^{a}	44.67 ± 2.19^{bc}	40.33 ± 2.84^{ab}	17.00 ± 0.57^{ab}	59.66 ± 2.33^{b}
4	211.00 ± 12.08^{a}	23.67 ± 1.19^{a}	38.33 ± 2.60^{ab}	38.00 ± 0.57^{ab}	16.66 ± 0.33^{ab}	54.66 ± 1.20^{b}
5	225.33 ± 12.67^{a}	23.67 ± 0.33^{a}	48.67 ± 0.33^{c}	40.00 ± 0.33^{b}	18.33 ± 0.57^{ab}	59.33 ± 0.66^{b}
6	235.00 ± 11.02^{a}	24.67 ± 0.88^{a}	42.67 ± 1.20^{b}	45.00 ± 0.57^{c}	20.00 ± 1.15^{b}	60.33 ± 0.88^{b}
7	222.67 ± 18.19^{a}	22.33 ± 0.88^{a}	47.33 ± 2.33^{c}	39.00 ± 3.57^{ab}	14.66 ± 1.25^{a}	62.00 ± 0.57^{b}
8	227.67 ± 19.74^{a}	22.18 ± 1.53^{a}	41.33 ± 0.88^{b}	41.33 ± 2.57^{ab}	18.00 ± 0.57^{ab}	63.66 ± 2.18^{b}
9	246.33 ± 14.67^{a}	21.33 ± 0.88^{a}	47.00 ± 2.65^{c}	40.00 ± 3.60^{ab}	19.66 ± 1.254^{b}	60.00 ± 2.51^{b}
10	312.12 ± 14.04^{c}	29.23 ± 0.33^{b}	64.21 ± 2.19^{d}	30.33 ± 2.60^{a}	12.00 ± 0.57^{a}	40.66 ± 4.09^{a}
***p*-value**						
W. cibaria	0.001	0.031	0.030	0.001	0.001	0.070
L. lactis	0.001	0.041	0.040	0.001	0.003	0.060
L. lactis & *W. cibaria*	0.001	0.001	0.001	0.001	0.001	0.001

Numbers with different superscripts in the same column are significantly different ($p<0.05$).

Bactria flora

The lowest number of lactic acid bacteria grown in the MRS agar medium was observed in the control group, which showed a significant difference with treatments ($p<0.05$) (Fig. 1).

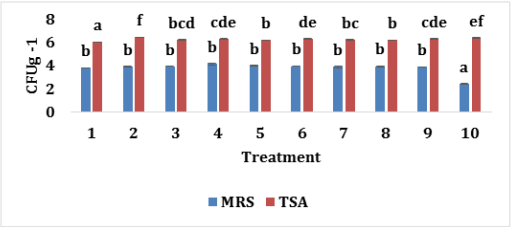

Figure 1: Total count of Bacteria cultured in TSA and MRS agar Interaction p_value (*L. lactis* & *W. cibaria*) for TSA=0.001 and MRS=0.005.

Discussion

In general, the results of the present study indicated the positive effects of two potential probiotics, *W.cibaria* and *L.lactis*, on the growth and immune parameters of the common carp juvenile. The results of growth performance showed that groups fed by the diet supplemented with potential probiotics had better growth performance, especially those in groups 5 and 8.

Enhanced growth performance can be related to an increase in fish appetite due to the stimulation of the digestive system, increase in gastrointestinal efficiency, the population of beneficial microorganisms, and activity of digestive enzymes, also, improvement of intestinal microbial balance leads to better digestion and absorption of feed. Probiotics produce bioactive microbial metabolites such as vitamins, bioactive peptides, organic acids, and fatty acids during fermentation as well as they produce some enzymes (Liu *et al*., 2010) and thus improve the metabolism. Different experiments have shown that probiotics exert their effects through colony formation in the host by secreting growth-promoting nutrients (Bagheri *et al.*, 2008; Mohapatra *et al*., 2012). Irianto and Austin (2002) also stated that adding probiotics to fish feed increase digestive enzyme activity, stimulation of fish appetite, and ultimately increases fish growth. On the other hand, another study reported that biological compounds such as vitamins (especially B vitamins likebiotin and B_{12}), digestive enzymes, proteolytic and peptidolytic enzymes breakdown the indigestible macromolecular compounds by hydrolyzing to peptides and amino acids that could lead to better absorption of nutrients (Abd El-Rhman *et al*., 2009).

Similar to our finding, the positive effects of various probiotics in improving and increasing growth performance have been proven in other studies (Xuxia *et al*., 2010, Beck *et al.*, 2015, Hosseini *et al*., 2016, Hashemi monfared *et al.*, 2016, Nguyen *et al.*, 2017). It should be noted that some bacterial probiotics did not induce desired effects on fish. The reason may be related to type, form, and dose of probiotics, the probiotic carrier, feeding duration, size, and life stage of examined fish (Olsen *et al*., 2001; Mohapatra *et al*., 2012; Yazici *et al.*, 2015).

This is reported that lactic acid bacteria produce compounds such as bacteriocins and thus inhibit the growth of other microorganisms (Vazquez *et al*., 2005) and increase their own population. Lactic acid bacteria can survive effectively in the gastrointestinal tract. They should attach to the intestinal tract to act their probiotic role (Argyri *et al*., 2013; Wang *et al.*, 2014). As a result, according to the positive results obtained after using these potential probiotics, it

seems that the potential probiotic bacteria used in the present study attached suitably.

In the present study, it was found that the number of lactic acid bacteria, was significantly increased in fish that fed diets supplemented with potential probiotics (p<0.05). A significant increase of lactic acid bacteria was reported in Persian sturgeon (*Acipenser persicus*) (Shenavar masuleh *et al*., 2016) and Nile tilapia (Balcazar and Rojas-Luna, 2007) intestines after consumption of probiotics via their diets. It should be noted that studies about the effect of lactococci probiotics on common carp are limited. Feng *et al.* (2019), reported improvement in growth performance and immunity in common carp. No examination was done about the role of *lactococcus* in the microbial balance of the intestine. According to the results, it can be pointed out that the proper colonization of probiotic bacteria was due to the appropriate conditions of stabilization, colonization, and growth in the intestine of common carp.

In the present study, there were no significant changes in RBC number, hematocrit, and hemoglobin levels (p>0.05). In the current research, blood indices did not change as were reported in common carp (Panahi Sahebi *et al*., 2019) and Caspian salmon (*Salmo trutta caspius*) (Hosseini *et al*., 2014). Improper dietary supplements can sometimes cause anemia and reduction of RBC, hemoglobin, and hematocrit, which is usually due to bleeding, hemolysis, or a decrease in RBC production (Hedayati *et al*., 2013), but no negative effect on the hematopoietic process was observed in the present study.

Blood leukocytes such as lymphocytes, neutrophils, and monocytes are parts of the nonspecific cellular immune system. In this study, immune cells were affected by probiotics and the percentage of monocytes and neutrophils in most probiotic-treated fish was significantly more than in the control group. Change in leukocyte number is one of the appropriate indicators that show fish response to various elements like pathogens, etc (Stoskopf, 1993; Nikoskelainen *et al.*, 2003).

It shows that *W. cibaria* and *L. lactis* improved the immune system performance in common carp, especially in combination and the dose of 3×10^7, and 4.5×10^7 CFU kg^{-1}, and 3×10^7 CFU kg^{-1} of *W. cibaria*. Similar to our finding, lactobacilli probiotics increased immune responses and lysozyme activity in Caspian salmon (*Salmo trutta*) (Balcazar and Rojas-Luna, 2007). Besides, an increase in lysozyme activity was reported in rainbow trout fed diet containing *Lactobacillus rhamnous* (Panigrahi *et al*., 2004) and *Pediococcus acidilactici* caused a significant increase in total immunoglobulin and lysozyme activity in *Huso huso* (Ghiasi *et al.*, 2018). The lysozyme levels, especially in serum, reflect the activity of monocytes, neutrophils, and phagocytic cells (Pararat *et al*., 2011). Therefore, in the present study increasing lysozyme along with increasing monocytes and neutrophils can be considered as an

effective factor in the improvement of the immune system of common carp. On the other hand, an increase in lysozyme activity, ACH50, and immunoglobulin, induced by the metabolic activity of probiotic bacteria are the important mechanisms for promoting the immune system in fish (Pourabbasali *et al.*, 2019).

Liver enzymes are known as indicators for biochemical factors in fish under stress (Newaj-Fyzul *et al.*, 2007). Alanine aminotransferase and AST enzymes are two important enzymes that indicate damage (Pascual *et al.,* 2003; Kumar *et al.,* 2011). Sometimes, ALT and AST secretion in the blood increase following the use of oral additives (Mohapatra *et al*., 2012). Similar to our finding, probiotics *Micrococcus luteus* and *Pseudomonas* spp. in Nile tilapia caused a significant reduction in AST and ALT in probiotic-treated fish in comparison to the control (Abd El-Rhman *et al*., 2009), and a reduction in ALP level was observed in Rainbow trout fed diet supplemented with *Saccharomyces cerevisiae* var. *boularadii* (Wache *et al.,* 2006). In another study, AST and ALT levels in Nile tilapia were not affected by probiotics and no significant difference in AST and ALT levels was observed between probiotic and control treatments (Won *et al.,* 2020).Potential probiotics used in the present study are effective with no side effects on common carp juveniles. Since probiotics improve digestion and absorption of nutrients, increase the absorption of vitamins, and improve immune function, these are effective in reducing stress, improving liver function, and consequently reducing liver enzyme levels.

In conclusion, it could be stated that consumption of potential probiotic bacteria *W. cibaria* and *L.lactis*, especially in combination and at a dose of 3×10^7 CFU kg^{-1}, and after that 3×10^7 CFU kg^{-1} of *W. cibaria* and 4.5×10^7 CFU kg^{-1} combination of these two potential probiotic will improves growth performance, the activity of digestive enzymes, intestinal microbial flora, and immune function in common carp juvenile with no negative effect on the liver.

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Research Article

The first report on isolation of a new highly hemolytic toxin, Scatotoxin, from *Scatophagus argus* venomous bonny spines

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Abstract

Scatophagus argus of family Scatophagidae is a venomous fish. Rough spines insulated from scat possessed potent venom composed of several proteins. Envenomation is associated with local necrosis and severe pain. Following our previous report regarding the hemolytic activity of scat crude venom, this work aimed at purification and evaluation of its hemolytic protein, hereafter designated as Scatotoxin. Specimens were collected from coastal waters of the Persian Gulf, Iran. Proteins were extracted from bone tissue by solubilization buffers and subsequently refolded in a refolding buffer. Purification was performed by reverse-phase HPLC method using a linear gradient protocol. To evaluate the hemolytic activity of Scatotoxin, a quantitative microscopic assay was developed using cell counting by which measurement of activity of the least amount of a sample was achievable. Scatotoxin was isolated in 85% acetonitrile. It is an interesting highly hydrophobic protein. Because hemolysis was observed immediately, scatotoxin is considered a very fast-acting hemolytic agent. Scatotoxin indicated as a 72 kDa protein by SDS-PAGE. The amount of 0.5µg crude venom produced 100% hemolysis and HD50 determined at 0.18µg. HD50 for scatotoxin recorded at 0.003µg. High efficiency of both extraction method and microscopic-scale assay led to the reduction of collected specimens and consequently avoiding harmful effects on the Persian Gulf ecosystem. This issue is ethically important due to decreasing the number of samples too. Among the previously reported hemolytic proteins, Scatotoxin is the first report of a highly hydrophobic protein.

Keywords: *Scatophagus argus*, Hemolysis, Scatotoxin, Hydrophobic toxin

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Introduction

Scatophagus argus (Linnaeus, 1766) also known as green scat or spotted scat, belongs to Scatophagidae family, is a fascinating marine fish which is able to live in fresh water too (Feng *et al*., 2020). Its distribution is in Indo-Pacific region in harbors, brackish estuaries, and lower reaches of freshwater streams, frequently among mangroves (Ru *et al*., 2020). Its rough dorsal and anal spines are venomous and capable of imposing lesions. Envenomation appears within 5–10 min as excruciating and persistent local pain, partial paralysis, erythema, itching, swelling and a throbbing sensation that extends to the limbs, disproportionate to the size of injury. The signs are variable, depending on the size of the fish and the quantity of injected venom (Cameron and Endean, 1970). During the past decades, many marine venomous animals were sacrificed to characterization and evaluation of toxicity or biological activities of their venoms. This note is of significant value concerning ethical issues. Furthermore, the yield and quality of venom extraction methods and also cost effectiveness could be important issues in this kind of projects. Therefore, development of a highly efficient extraction method and also a small-scale assay for characterization of venom activity are necessary to decrease the numbers of collected specimens. Venom extraction from bonny spines in venomous fish is laborious because of the spine rigidity and inflexibility. Beside this, low amount of venom is yielded from each specimen. The conventional methods of venom extraction were not practical for green scat due to the abovementioned issues (Endean, 1961; Saunders and Tökés, 1961; Carlisle, 1962). Following our previous report on hemolytic activity of scat venom (Ghafari *et al*., 2013), this study aimed to isolate and purify the hemolytic protein from scat venom. In this regard, an innovative high yield extraction method was implemented and also a new microscopic-scale hemolysis assay was developed.

Materials and methods

Sample collection

Four *S. argus* specimens with medium length of 25cm were collected by trap from coastal waters of Boushehr province, Persian Gulf, Iran. The specimens were transported to Pasteur Institute of Iran by plane, and were kept alive in an aquarium in appropriate conditions.

Ethical issues

Several ethical issues considered in our study according to our previous paper (Ghafari *et al*., 2013). Briefly, clove powder (*Caryophyllus aromaticus*) was used as anesthetizing agent and subsequently the time period for anesthetizing and recovery were shortened too. We avoided decapitating the specimens and after spine removal, the injured location was disinfected. After collection of spines, to avoid killing the fish, they were maintained in a large pool in Pasteur Institute of Iran.

Venom preparation and extraction

The bonny spines were prepared based on Ghafari *et al*. (2013). An innovative method was used for protein extraction from spines based on urea reagent (Rudolph and Lilie, 1996). This idea originated from archeological methods for protein extraction from bony fossils (Cleland *et al*., 2012). This method was developed in our study based on biotechnological methods (Rudolph and Lilie, 1996).

This method consists of two stages, including simultaneous bone demineralization and protein solubilization and refolding processes. In the first stage, bonny structure was demineralized and simultaneously the proteins were extracted from bone tissues by incubating the trimmed spines in a solubilization buffer (containing Urea (8M), NaCl (0.2M), Tris-Cl (50mM), EDTA (2mM) at pH 8.5) at 4°C for 72 hours. In the refolding process, the extracted proteins were refolded by refolding buffer (containing Tris (50mM), NaCl (9.6mM), KCl (0.4mM), EDTA (1mM), Triton (0.5%), Urea (2M) , DTT (1mM) at pH 8) at 4°C for 48 hours. Refolding stage is necessary since structural conformation of the extracted proteins was affected by urea reagent in solubilization stage. Subsequently, filtration (0.2µm- Takara bioscience Co., Japan) and dialysis were performed by an ultra-filter (5kDa cut off- Thermo Co., USA) to remove micro-particles and the released chemical materials, respectively. The sample was then lyophilized by a freeze dryer (Alpha 1-2 LD plus, Martin Christ Gefriertrocknungsanlagen Co., Germany).

Protein determination

Protein concentration was determined by BCA method (Smith *et al.*, 1985) according to manufacturer instruction (iNtRON Biotechnology Co., South Korea). Optical density was measured at 562nm using a microplate spectrophotometer (EPOCH, BioTek Co., USA).

SDS-PAGE

Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out according to standard method (Laemmli, 1970). The venom samples were loaded onto a 12% polyacrylamide gel.

Isolation of hemolytic protein

The extracted crude venom was purified using HPLC system (Knauer Wissenschaftliche Gerate Co., Berlin, Germany) equipped with a C18 column (5µm 100Å - 250 × 4.6mm, Beckman Coulter Co., USA). TFA (0.05%) in ultra-pure water and acetonitrile containing TFA (0.05%), was designated as solution A and B and were used for eluting the fractions. The column was eluted by a linear gradient of solution B from 80 to 95 percent for 45 minutes at 0.5 ml/min. The eluted peaks were monitored at 214nm. The collected fractions were lyophilized by a freeze dryer (Alpha 1-2 LD plus, Martin Christ Gefriertrocknungsanlagen Co., Germany). The lyophilized powder was

solubilized in 200µL ultra-pure water and stored at -20°C.

Micro-hemolytic assay

For visual inspection of hemolysis, a new microscopic assay was developed. Fresh human blood from a healthy volunteer was drawn by venous puncture in heparinized tubes. Plasma and buffy coat were removed by centrifugation (Sigma, 1-14) for 5 min at 664 g at 25°C, and the erythrocytes were washed three times with phosphate-buffered saline (pH 7.4). A suspension of erythrocytes was made at 0.01% dilution in PBS. From this suspension, 10µL RBC placed on a neubauer slide as negative control. A suspension containing RBC (5µL), each of samples (1µL), and PBS (4µL) was used for this assay. This assay performed with distilled water and $CaCl_2$ (2mM) separately to control their effects on the erythrocytes. The results were observed with a microscope (Bell photonic Co., Italy) at 40X magnification and documented using a digital camera (Canon G12 - Japan).

Results

Protein analysis

The extracted venom of *S. argus* was subjected to SDS-PAGE analysis and showed 6 separated bands in the gel. Major and minor protein bands observed between 10 to 250 kDa (Fig. 1).

Protein isolation by RP-HPLC

RP-HPLC of *S. argus* venom on C18 column in a new gradient protocol resulted about 15 fractions during the first 22 min (Fig. 2). The aliquots of each peak were then used for hemolytic activity. We attained three major fractions eluted in about 85-85.83% of acetonitrile (Fig. 3A). SDS-PAGE profile of these fractions showed a 72 kDa protein (Fig. 3B).

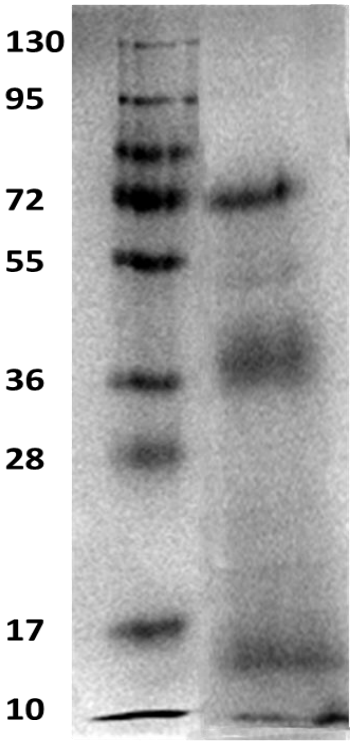

Figure 1: Electrophoretic profile of *S. argus* venom. The venom was analyzed by SDS-PAGE using 12% polyacrylamide gel and stained with coomassie brilliant blue. From left to right: Lane 1. Molecular weight marker (10–250 kDa); Lane. 2 *S. argus* venom.

Morphological evaluation of hemolysis

A microscopic method was performed as hemolytic test and immediate interaction between the erythrocytes and crude venom and also the purified fractions were observed by a microscope at 40X magnification and documented using a digital camera (Figs. 4 and 5).

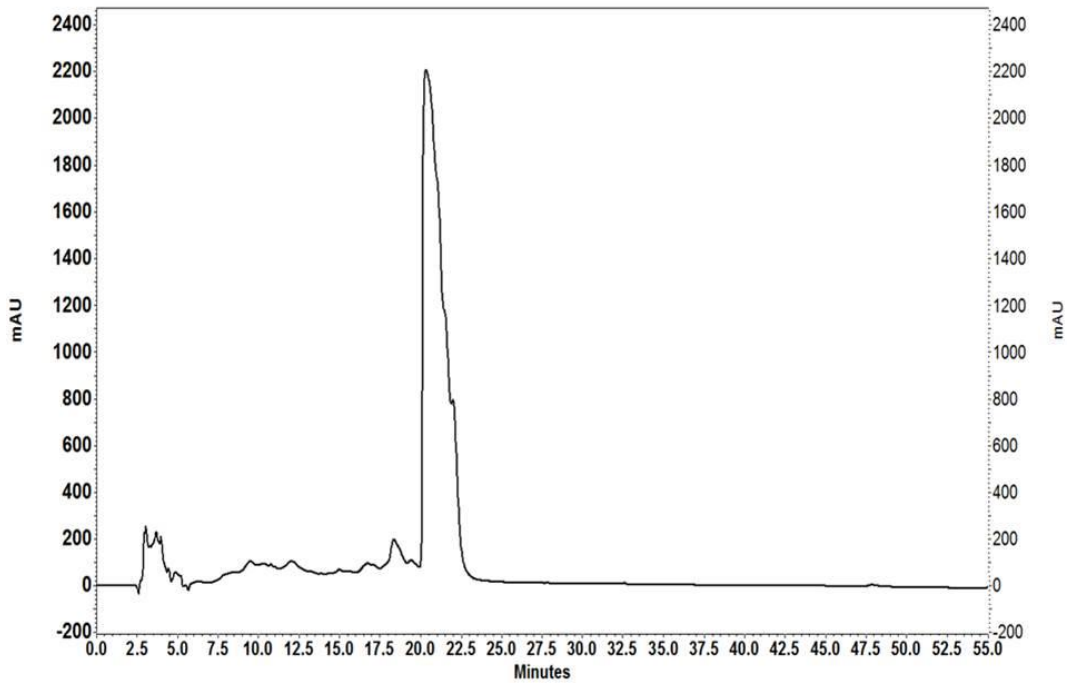

Figure 2: RP-HPLC chromatogram of scat venom. All 15 fractions eluted at high concentration of acetonitrile ranged from 80 to 85.83%.

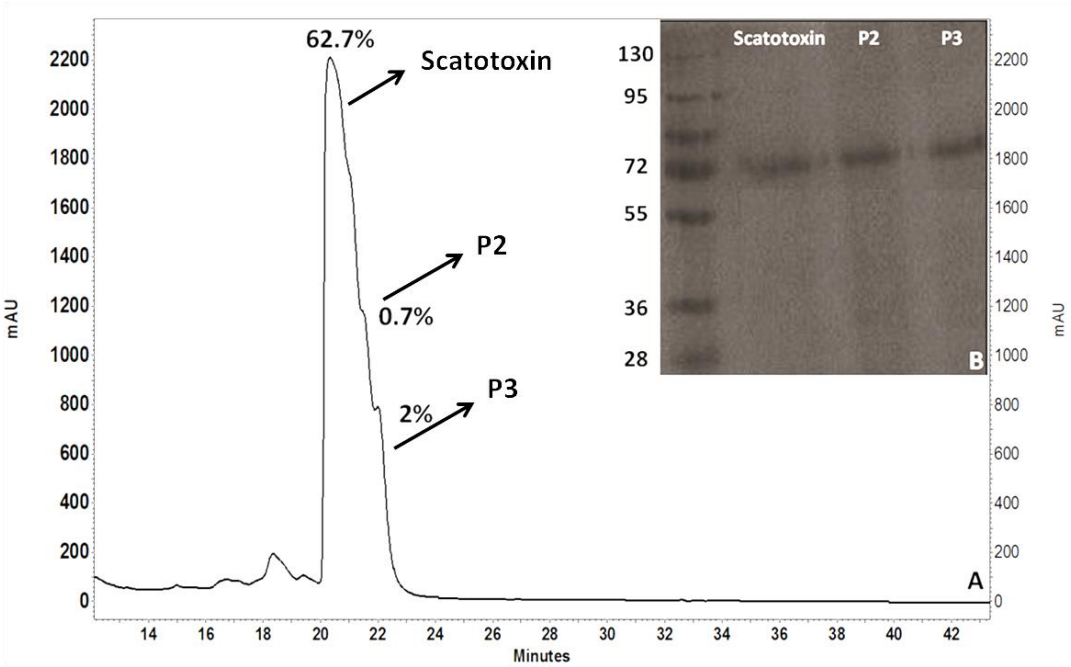

Figure 3: Magnified RP-HPLC chromatogram of scat venom. 3A. Three major peaks were eluted from column at high concentration of acetonitrile ranged from 85 to 85.83%. 3B. The purified fractions were about 72 kDa in SDS-PAGE. Among the peaks, only Scatotoxin had hemolytic activity.

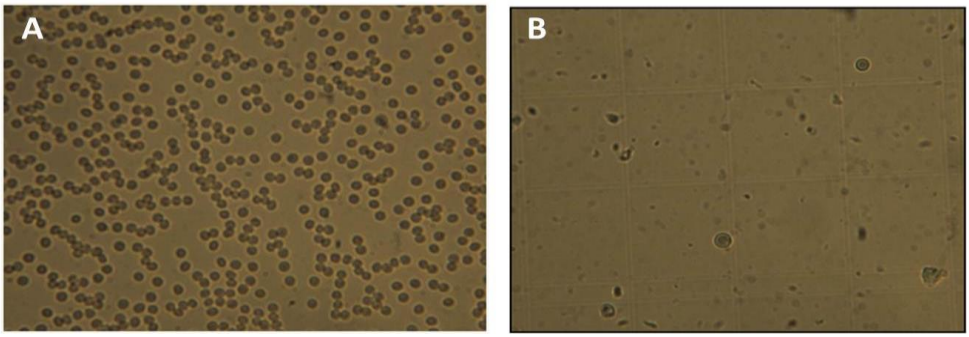

Figure 4: Evaluation of the effect of scat venom on human erythrocytes. Intact erythrocytes before hemolysis assay (4A). Hemolysis of erythrocytes after incubation with scat venom (4B). The results were observed by a microscope (Bell, photonic) at 40X magnification.

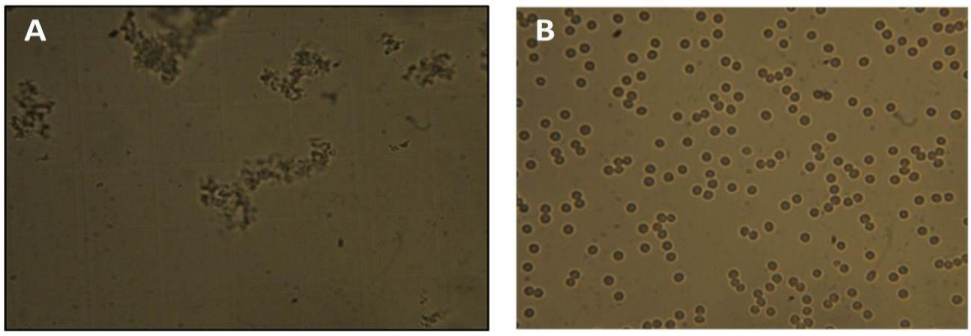

Figure 5: Evaluation of the effect of CaCl2 and distilled water during incubation with erythrocytes. Aggregated erythrocytes after incubation with 2mM CaCl2 (5A). The erythrocytes after incubation with distilled water. No immediate hemolytic effect showed on erythrocytes (5B).

Microscopic scale hemolytic assay

The amount of 0.5µg crude venom produced 100% hemolysis and HD50 identified at 0.18µg. Hemolysis was dose dependent and slope of hemolysis gradually increased up to 0.5µg (Fig. 6). HD50 for Scatotoxin recorded at 0.003µg and hemolysis was dose dependent up to 0.05µg (Fig. 7).

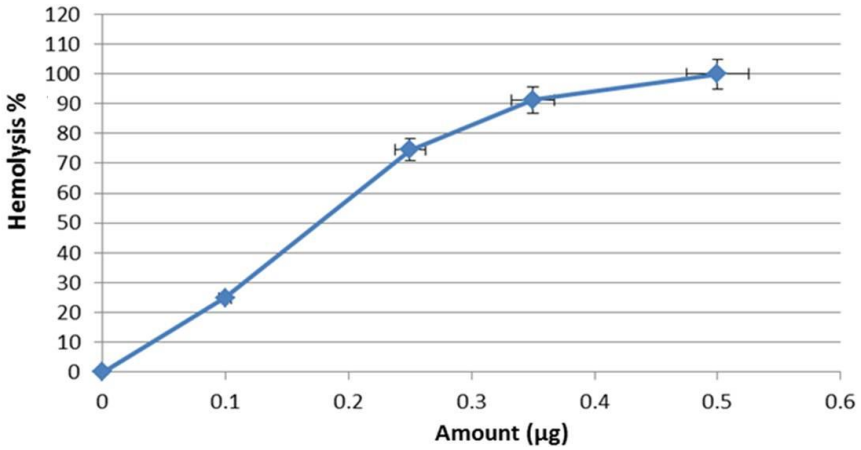

Figure 6: Micro-hemolytic assay for *S. argus* venom on human erythrocytes. The immediate interaction between the erythrocytes (0.01%) and venom was observed by a microscope. The amount of 0.5µg crude venom produced 100% hemolysis and HD50 identified at 0.18µg. Hemolysis was dose dependent and slope of hemolysis gradually increased up to 0.5µg.

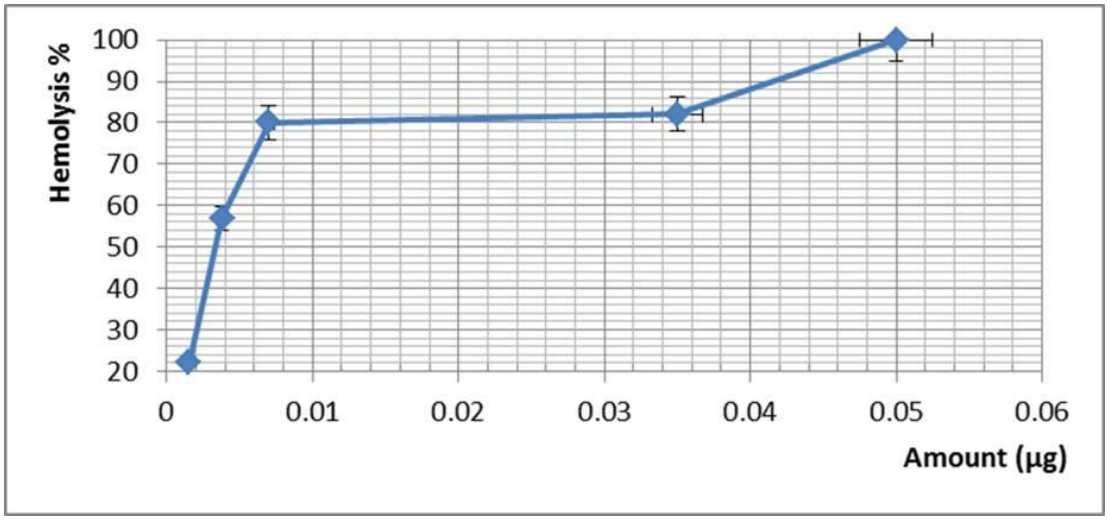

Figure 7: Microscopic hemolytic assay for Scatotoxin on 0.01% human erythrocytes. HD50 recorded at 0.003µg and hemolysis was reached to 100% at 0.05µg.

Discussion

Discovery of bioactive molecules from venomous creatures, especially from marine animals, is of significant value because of their potential to present as a pharmaceutical agent. Among the venomous fish, scat venom has been less considered regarding the characterization of its proteins. Venom extraction from bonny venomous spines is an important step in this kind of research. Alongside this issue, a high yield protein extraction method is critical. Conventional extraction methods use acetone, normal saline, and ammonium acetate which yield low protein levels. Accordingly, in this study, we tried to use an innovative method to improve extraction efficiency regarding quantity and quality.

This new idea triggered by a protein extraction method from bony fossils in archeology using urea reagent (Cleland *et al*., 2012). During demineralization of bonny spines in extraction stage, maintaining the conformation of extracted proteins is necessary for reaching active molecules. Concerning this issue, we used an innovative high yield method for extraction and refolding of proteins from bonny spines. Our reagent was the same as the solubilization buffer that routinely used for solubilizing inclusion bodies containing compact recombinant proteins. For refolding the solubilized proteins, we used a buffer which is the same as the buffer used for refolding of recombinant proteins (Rudolph and Lilie, 1996).

In our previous studies (Ghafari *et al*., 2013; Ghafari *et al*, 2015) the yield of extraction was about 1 to 2 mg protein/specimen while the current method yielded 25 to 30 mg protein/specimen in the same size and weight of collected spines. This amount was approximately 18 fold greater than that of our previous report. In this new extraction method, the proteinaceous nature of crude extract was not affected by the reagents used for extraction. SDS-PAGE confirmed that six separated proteins or peptides in the range of 10-

250 kDa were successfully extracted from spines. *S. argus* crude venom caused hemolysis of erythrocytes suspension at HD50 and HD100 of 0.18 and 0.5 µg, respectively while, Scatotoxin caused hemolysis of erythrocytes suspension at HD50 and HD100 of 0.003 and 0.05 µg, respectively. Hemolysis was dose dependent for both crude venom and hemolytic fraction.

Comparison of HD50 value for melittin, a potent hemolytic peptide from bee venom (Zarrinnahad *et al*., 2018), with Scatotoxin showed that its activity was approximately equal to melittin on human RBCs. Regarding this issue, it can be hypothesized that Scatotoxin may possess anticancer and antimicrobial activity similar to melittin (Mahmoodzadeh *et al*., 2015; Akbari *et al*., 2019). This hypothesis originated from this fact that a hemolytic toxin like melittin invades the membrane of bacteria or cancerous cells and causes necrosis (Mahmoodzadeh *et al*., 2015; Shams Khozani *et al*., 2019).

Hemolysis seems to be an important factor in venom toxicity of marine animals (Moghadasi *et al*., 2020). Almost all piscine venoms possess this activity (Chhatwal and Dreyer, 1992; Garnier *et al*., 1995; Sivan *et al.*, 2007), however some of them possess no hemolytic activity (Memar *et al*., 2016).

Ghadessy *et al*. (1996) reported a protein from a stone fish, Stonustoxin (SNTX), with hemolytic activity. This toxin has a molecular weight of 148 kDa and comprises of two subunits, termed α (71 kDa) and β (79 kDa). Ueda *et al.* (2006) purified a hemolytic lethal factor, Neoverrucotoxin (neoVTX), from a stonefish species *Synanceia verrucosa*. They reported that it is a dimeric protein with a molecular mass of 166 kDa.

Concerning hydrophobicity, some other hemolytic proteins with high hydrophobicity have early been reported from venomous animals including *Hemiscorpius lepturus* scorpion (Borchani *et al*., 2011), *Loxosceles* spp. (Brown spider) venom (Swanson and Vetter, 2006, McDade *et al*., 2010; Lane *et al.,* 2011; Gehrie *et al*., 2013), and *Apis mellifera meda* (Taghizadeh Dezfuli *et al*., 2014; Pashaei *et al*., 2019). According to our results, among 15 fractions that were isolated from crude venom, three major fractions had similar molecular weight of 72 kDa and consequently eluted from the column. For visual inspection of erythrocyte hemolysis, an innovative micro-hemolytic assay was used. The first major peak (scatotoxin) had significant instant hemolytic activity and the other ones were inactive. It is speculated that they are inactive isoforms of scatotoxin. As Scatotoxin eluted at 85% of acetonitrile, it is an interesting highly hydrophobic protein. The area percent of Scatotoxin showed that the hemolytic protein was 71 percent of total proteins in the crude venom. Hemolysis was immediately observed at a maximum time of 30 second thus it can be suggested that scatotoxin is a very fast acting hemolytic agent. This study is pending to monitor in vitro anticancer activity of scatotoxin on several cancer cell lines as a pilot study.

High efficiency of both extraction method and microscopic-scale assay led to reducing the collected specimens and consequently avoiding harmful effects on the Persian Gulf ecosystem. This issue is ethically important due to decreasing the number of samples. Among the documented hemolytic proteins, scatotoxin is the first report of a highly hydrophobic hemolytic protein. In conclusion, our novel extraction method was highly efficient for providing high amount protein from the low numbers of bonny spines while maintaining its biological activity.

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Research Article

Comparative effects of Ovaprim™ and Ovapass™ hormones on some reproductive characteristics of *Schizothorax zarudnyi*

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Abstract

This study was conducted to compare the effectiveness of Ovaprim™ and Ovapass™ hormones to induce spawning in *Schizothorax zarudnyi*. The female (n=48; 1535.12±180.09 g) and male broodstocks (n=53; 752.00±48.30 g) were randomLy allocated in 4 experimental groups including T1: females which received 0.2, 0.5 and 0.5 mL /kg body weight (BW) Ovaprim with 24-h time intervals, T2: females which treated with 0.5 and 0.5 mL /kg BW Ovapass with 12-h time intervals, T3: females were injected with 0.2, 0.5 and 0.5 mL /kg BW Ovapass with 24-h time intervals, and T4: females were injected with a combination of Ovaprim (0.2 mL/kg) and Ovapass (0.5 and 0.5 mL/kg BW) with 24-h time intervals. The amount of injection in the first stage was 10%, the second stage was 90%, and the male fish were simultaneously injected with the second stage of the females at 0.3 mL/kg. The results showed that the highest rate of fertilization was detected in treatments 2 and 4 (93.33% and 92.33%, respectively), which had a significant difference with other groups (p<0.05). No significant differences were observed between the treatments in the indices of relative and working fecundity and dry egg diameter (p>0.05). The lowest and highest latency period was observed in treatments 2 (27.33± 0.66 h) and 1 (36.20±3.77 h), respectively (p<0.05). The results of this study clearly suggested that the Iranian Ovapass hormone is highly effective for *S. zarudnyi* reproduction and might be successfully replace with Ovaprim.

Keywords: *Schizothorax zarudnyi*, Artificial reproduction, Ovaprim, Ovapass, Fertilization

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Introduction

The Hamun mahi, *Schizothorax zarudnyi*, is native to the Sistan basin that is located in the east of Iran (Oveisi and Kavosh 2021) and the species exists only in this region in Iran. In fact, this fish belongs to the Hamun international wetland, which is shared between Iran and Afghanistan (Vekerdy *et al.*, 2006). Several factors such as drought, man-made actions and the introduction of non-native species caused a decline in the population of this fish and it was in danger of extinction (Gharaei *et al*., 2019). In addition to the biological and ecological importance of *S. zarudnyi*, it is delicious and in high demand in the seafood domestic market (Rahdari *et al*., 2013). Therefore, the species could be a potential candidate in the aquaculture industry (Gharaei *et al.*, 2010). The introduction of new species for aquaculture is rapidly expanding due to the development of new technologies for fish breeding. On the other hand, the number of threatened or endangered species is increasing (Arthington *et al*., 2016). There are many ways to protection of native species. Although artificial breeding is not the best solution, there is no other solution under the current conditions when other solutions such as restoring natural spawning grounds or removing non-native fish are not possible (Azari Takami, 2014). In sustainable aquaculture, artificial propagation is the most promising and reliable method to produce high egg quality throughout the year. It involves the use of natural (hypophysation) or synthetic hormones to induce ovulation and spawning in farmed fish (Apollos, 2020; Asadi Eidivand *et. al*., 2022). In captivity, many commercial fish of aquaculture industry exhibit reproductive dysfunctions (Zohar and Mylonas, 2001). So, in these fish hormone therapy is the only way to achieve controlled and reliable reproduction, and not only spawning should be induced in this way, but it is necessary for having good quality gametes and followed having good larvae and fry (Podhorec and Kouril, 2009; Kerdgari *et. al.*, 2022). The reason for the lack of spawning in these conditions is the cessation of the release of LH (luteinizing hormone) from the pituitary (Mylonas *et al*., 1998). The use of hormone therapy causes the release of LH stored in the pituitary and, as a result, advances the final maturation of the oocyte and ovulation (Zohar and Mylonas, 2001). The use of GnRH analogs has been used for hormone therapy of many fish. Of course, in some fish such as carp, catfish, salmon, and some perciforms, there is a strong dopamine system that stops ovulation and sperm emission in captivity (Cabrita *et al*., 2008).

In breeding sectors, aquaculture species are artificially propagated using different hormones, which are produced by synthetic and recombinant methods. There are several reasons for the production of recombinant hormones such as the small number of desired peptides in the production tissues available, and relationship between the structure and function of the hormones is dedicated to removing or replacing one

or more amino acids (Yeganeh *et al*., 2022). For artificial reproduction, synthetic hormones are cheaper and more stable than carp pituitary extract (CPE), which has been used in the past for the reproduction of carp, and therefore have a longer shelf life. Also, these hormones are mostly available in the market in pure form without any reproductive inhibiting agents (Yousefian and Mousavi, 2011). Ovaprim contains combinations of salmon gonadotropin-releasing hormone (sGnRH) and domperidone. The Ovaprim hormone has been used for the artificial breeding of many fish species, such as Indian carps (Cyprinidae) (Mijkherjee *et al*., 2002; Sharma and Singh, 2002; Sarkar *et al*., 2004; Naeem *et al*., 2005 a, b), pike, *Esox lucius* (Khaval *et al.,* 2015) and Longspine scraper, *Capoeta trutta* (Zadmajid, 2016). In Danube bleak (*Alburnus chalcoides*), higher efficiency of Ovaprim than pituitary extract and combination of HCG (Human chorionic gonadotropin) with metoclopramide has been observed (Nosrati *et al*., 2019). Induction of ovulation with Ovaprim in catfish, *Clarias gariepinus* species has been successfully performed (Musa Ahmed and Hagar Talib, 2018). However, there is currently no published data on the use of two types of recombinant hormones (Iranian-made hormone: Ovapass with foreign-made hormone: Ovaprim) in the artificial reproduction of *S. zarudnyi*, but total reliance on Ovaprim hormone means that reproduction of this fish will be disrupted if unavailable. Therefore, the aim of this study was to compare the effect of the hormones Ovapass™ and Ovaprim™ on spawning induction in *S. zarudnyi*. So far, no study has been published about the effects of this hormone on fish.

Materials and methods

Broodstok selection and maintenance

In this experiment, the fish specimens were obtained from healthy broodstocks of Zahak Aquatic Restoration and Genetic Conservation Center (Sistan and Baluchestan province, Iran, 89°30′N, 67°61′E). Before the experiment, 48 female broodstocks (1535.12±180.09 g) and 60 male broods (752.00±48.30 g) were placed separately in rectangular-shaped concrete tanks of running water (3 m^3 volume) of 15-17˚C for 22-24 h (Table 1).

Table 1: Weights and lengths of injected groups (mean ± SD).

	Parameter	Groups			
		1	2	3	4
Female	Weight (g)	1601.33 ± 315.15	1483.33 ± 56.86	1550.00 ± 245.37	1505.83 ± 102.97
	Length (cm)	52.20 ± 0.97	52.60 ± 1.16	49.40 ± 1.24	49.60 ± 1.58
Male	Weight (g)	761.12 ± 29.41	783.59 ± 60.11	725.17 ± 67.12	738.12 ± 36.57
	Length (cm)	24.85 ± 0.63	27.86 ± 1.29	23.12 ± 0.81	24.35 ± 0.57

Females were characterized with soft, swollen belly and pink-red genital. Also, males were characterized by releasing milt upon slight pressure to the abdomen were selected. Prior to injections, the fish were anesthetized with a clove

solution bath at 0.05-0.07 mg/L for 2-3 min (Rahdari *et al*., 2018). The weighing (g), labeling, length measurement (cm), and hormone injection were performed while the fish were unconscious. Following the hormone injection, the specimens (12 females and 15 males) were kept together in circular concrete tanks with a flow-through circuit. Tanks were filled with filtered freshwater and 50% daily water exchanged. During the experiment, the water temperature was 16.4±1.1°C and values for dissolved O_2 and pH were 6.9±0.3 mg/L and 7.5±0.4, respectively. The photoperiod was kept natural (10 h light and 14 h darkness).

Hormones and experimental design

Ovapass™ was purchased from Protein Array Saman (PAS) Company (Tehran, Iran). Its composition is declared similar to Ovaprim™. Ovaprim (each milliliter contains 20 µg Salmon gonadotropin-releasing hormone analog (GnRH) and 10 mg domperidone), purchased from Syndel Laboratories, Ltd., Vancouver, Canada.

Females (n=48) were allocated into four experimental groups. 24 h after adaptation at 15-17°C, four treated groups received hormones via intraperitoneal injections at the base of the pectoral fins as shown in Table 2. The males (n=60) were allocated into four groups and received hormones synchronized to the 2nd female's injection for inducing spermiation (Table 3).

Table 2: The doses of hormones used to induce spawning in female *S. zarudnyi.*

Treatment		Fish No.	Injections dosage/kg BW				Reference
			First	Second	Third	Time interval	
T1	Ovaprim (mL/kg)	12	0.2	0.5	0.5	24h	Rahdari *et al*., 2013
T2	Ovapass (mL/kg)	12	0.5	0.5	-	12h	Recommended by the manufacturer
T3	Ovapass (mL/kg)	12	0.2	0.5	0.5	24h	
T4	Ovaprim (mL/kg) + Ovapass (mL/kg)	12	0.2 Ovaprim	0.5 Ovapass	0.5 Ovapass	24h	

Table 3: The doses of hormones used to induce spawning in male *S. zarudnyi*.

Groups	Treatment	Fish No.	Injection's dosage/kg BW.
1	Ovaprim (mL/kg)	15	0.3
2	Ovapass (mL/kg)	15	0.3
3	Ovapass (mL/kg)	15	0.3
4	Ovaprim (mL/kg) + Ovapass (mL/kg)	15	0.3

To avoid repeated anesthesia and stress to the brood stocks, an arrangement was made to inject the first stage of the female broodstocks immediately after labeling and weighing. The injections were done at the base of the pectoral fin

(Rahdari *et al.,* 2013). To minimize stress in the broodstocks, insulin syringes were used for injection. The male and female brood stocks, which were kept separately in rectangular ponds before the injection, were transferred together to the round pond in the indoor facility, after the injection, for further stimulation. After the second injection, the females were checked every 6 h. Therefore, the fish were anesthetized and the eggs were stripped manually, when ovulation was observed. The spawning rate index (%) was calculated with the following relationship (Billard, 1990):

Spawning rate = (Number of ovulated fish/Total number of injected fish)×100

Collection of Gametes, fertilization, and incubation

During the examination, ripe gamete donors were anaesthetized in a solution containing 0.05-0.07 mg/L clove powder. The females were checked each 8 to 10 h after the second injection. The milt was collected from the male donors by stripping. The ovum was collected from individual female donor by abdominal gently stripped method. Eggs were stripped into a plastic vessel and were fertilized using a "dry method" (Kucharczyk *et al.*, 1997). The eggs were placed on trays and the milt and eggs were mixed slowly with a turkey feather for one min. In general, the ratio of males to females was 3:1 (Gharaei *et al.,* 2019). All spawners were kept one week after gamete collection and all fish were closely monitored to record survival rate.

The fecundity rate was calculated using volumetric technique. This technique uses simple proportionality to estimate total fecundity from a given number of eggs in a known subsample volume and total sample volume value, and then calculates the total number of eggs in the ovary. To determine the relative fecundity, the ratio of the number of extracted eggs to the weight of the fish was calculated (Billard, 1990):

Working fecundity=The total volume of stripped eggs × The average number of eggs in the samples

Relative fecundity=Working fecundity/ total body weight (kg)

For fertilization, a very small amount of water was added so that the sperms become active and fertilization takes place. Then, the adhesion was removed gradually with the water of the hatchery facility. Egg diameter (mm) was measured from 10 eggs from each fish, using an eyepiece micrometer fitted to a dissection microscope. The water was changed over time and after half an hour at most, the adhesion of the eggs was completely removed then left to water

harden for 30 min. Fertilized eggs were incubated in jar incubators (Vase) to start the incubation stage. To determine the percentage of fertilization about 24 h after transferring the eggs to jar incubators, about 100 eggs of each brooder were sampled and after clarification in the clarification solution (1:1:1 methanol+acetic acid+distilled water (Zarei *et al*., 2019)), the stages of embryo growth and development was observed under the loop. Samples with a neural streak were counted to determine the fertilization rate (Pankhurst *et al*., 1996):

Fertilization rate (FR) (%)=Number of fertilized egg/ total eggs×100

Two types of incubators, jar Vase and troughs California, were used for the incubation stages of *S. zarudnyi* eggs. In this way, from the beginning to the egg-hatching stage, a jar Vase was used, and from hatching to post-hatching, and after that, California incubators were used. In each jar incubator, 300 mL of water-hardened eggs were poured and water flow was established. After all the eggs in each jar incubator reached the hatching time, they were transferred to the trough's Californian incubator. The percentage of egg-to-larvae conversion was calculated using the following relationships (Pankhurst *et al*., 1996):

Hatching rate (%)=(Number of viable larvae/ total number of eyed eggs)×100

Larvae Samples were fixed in formaldehyde and external condition were checked by a loop.

Statistical Analysis

Data analysis was done with SPSS 16 software (Chicago, IL, USA). First, their normality was checked by the Kolmogorov-Smirnov test and the homogeneity of variances was checked by Levene's test. Then, differences among the experimental groups were analyzed using One-way variance followed by Duncan's test at a 95% confidence level. Data are represented as mean±standard deviation.

Results

According to our results, ovulation and spawning occurred between 85% and 100% in all groups after hormone injection. There were significant differences between experimental groups in terms of fertilization rate (FR) (Fig. 1), the number of dry eggs per milliliter, spawning success (Table 4), latency period, eyed eggs, and hatching rate ($p<0.05$; Table 5). In this regard, the highest values of FR were observed in T2 ($p<0.05$). Fish of T2 showed the highest spawning rate among all experimental groups, also, there were no significant differences between T2 and T4 ($p>0.05$). As well as, there were no significant differences between experimental groups in terms of working fecundity and relative fecundity (Table 4), the diameter of dry egg and total hormone injected ($p>0.05$; Table 5). Also, the lowest working fecundity and

relative fecundity were observed in T1 (p>0.05; Table 4). Also, the lowest and highest values of total hormone injected were observed in T2 and T1, respectively. The lowest and highest values of the latency period, which is the time interval between the first injection and the time of ovulation, were observed in T1 and T2, respectively (p>0.05; Table 5). The results of the incubation period showed that the time to reach the hatching stage and from hatching to post-hatching was influenced by the water temperature of the incubators.

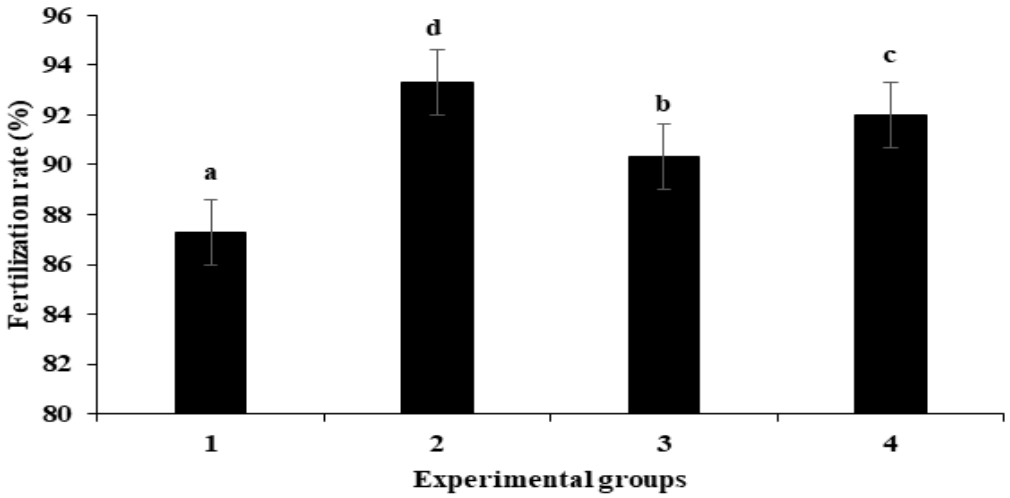

Figure 1: Fertilization rate of different groups injected with Ovaprim and Ovapass hormones (T1: Fish that received 0.2, 0.5 and 0.5 mL/kg body Ovaprim with 24-h time intervals, T2: females treated with 0.5 and 0.5 mL/kg BW Ovapass with 12-h time intervals, T3: females were injected with 0.2, 0.5 and 0.5 mL/kg BW Ovapass with 24-h time intervals, and T4: females were injected with a combination of Ovaprim (0.2 mL/kg) and Ovapass (0.5 and 0.5 mL/kg BW) with 24-h time intervals).

Table 4: The effect of different doses of two types of hormones, Ovaprim and Ovapass, on different parameters of *S. zarudnyi* eggs and larvae (mean ± standard deviation; n=12).

Group	Female brooder weight (g)	Spawning success (%)	Working fecundity	Relative fecundity	The volume of dry eggs per female fish (mL)	The number of dry eggs/mL
1	1601.33± 315.15	85±00.00^{a}	42258.33 ± 10651.89	26504.79 ± 2821.39	153.67 ± 38.73^{b}	275.00±1.00^{a}
2	1483.33± 56.86	100±00.00^{c}	45070 ± 10203.03	30236.04 ± 5803.13	160.00± 36.06^{a}	281.67±1.53^{b}
3	1550.00± 245.37	85±00.00^{a}	52977.22 ± 6191.04	34522.62 ± 4946.76	191.67 ± 23.63^{b}	278.00±1.00^{a}
4	1505.83± 102.97	90±00.00^{b}	50746.67 ± 5306.65	33785.46 ± 3229.86	210.00 ± 55.68^{a}	273.00±1.65^{a}

^{a,b} Different superscripts within a column indicate significant differences (p<0.05). (T1: Fish that received 0.2, 0.5 and 0.5 mL/kg body Ovaprim with 24-h time intervals, T2: females treated with 0.5 and 0.5 mL/kg BW Ovapass with 12-h time intervals, T3: females were injected with 0.2, 0.5 and 0.5 mL/kg BW Ovapass with 24-h time intervals, and T4: females were injected with a combination of Ovaprim (0.2 mL/kg) and Ovapass (0.5 and 0.5 mL/kg BW) with 24-h time intervals).

As the temperature increased, the duration of these periods became shorter and counter. The lowest values of completion of hatching (h) obtained in fish administrated with Ovapass (35±3.2 h; T2) compared to other experimental groups (p<0.05; Table 6). No abnormality was observed in the offsprings of the experimental groups after hormone therapy and spawning.

Table 5: Effect of different doses of two types of hormones, Ovaprim and Ovapass, on different parameters of *S. zarudnyi* eggs and larvae (mean ± standard deviation; n=12).

Group	Diameter of dry egg (mm)	Total hormone injected (mL)	Latency period (h)	Eyed egg (%)	Hatching rate (%)
1	1.67 ± 0.25	1.93 ± 0.36	36.1 ± 3.8^{c}	87.27 ± 0.23^{a}	80.67 ± 0.12^{a}
2	1.48 ± 0.012	1.52 ± 0.98	27.33 ± 0.66^{a}	93.33 ± 0.58^{d}	87.67 ± 4.93^{b}
3	1.61 ± 0.12	1.70 ± 0.19	32.38 ± 2.20^{b}	90.33 ± 0.58^{b}	82.00 ± 1.00^{a}
4	1.69 ± 0.03	1.80 ± 0.15	31.8 ± 1.30^{b}	91.33 ± 0.58^{c}	89.33 ± 0.58^{b}

[a,b] Different superscripts within a column indicate significant differences (p<0.05). (T1: Fish that received 0.2, 0.5 and 0.5 mL/kg body Ovaprim with 24-h time intervals, T2: females treated with 0.5 and 0.5 mL/kg BW Ovapass with 12-h time intervals, T3: females were injected with 0.2, 0.5 and 0.5 mL/kg BW Ovapass with 24-h time intervals, and T4: females were injected with a combination of Ovaprim (0.2 mL/kg) and Ovapass (0.5 and 0.5 mL/kg BW) with 24-h time intervals).

Table 6: Characteristics of water temperature and period of eyed and hatching eggs.

	Group 1	Group 2	Group 3	Group 4
Water temperature until eyed egg period (°C)	16.0±1.3	16.0±1.1	16.8±1.2	17.2±1.0
Eyed egg period (h)	170.0 ± 10.0^{b}	165.0 ± 12.5^{b}	158.5 ± 11.5^{a}	152.6 ± 8.5^{a}
Water temperature from eyed egg stage to hatching (°C)	18.4±1.5	19.5±1.9	17.3±1.1	17.3±1.0
Completion of hatching (h)	48.0 ± 3.5^{b}	35.0 ± 3.2^{a}	52.0 ± 4.3^{b}	52.0 ± 4.1^{b}

[a,b] Different superscripts within a line indicate significant differences (p<0.05).

Discussion

In the present study, a combination of Ovaprim and Ovapass was applied for the first time for inducing spawning in *S. zarudnyi*. *S. zarudnyi* belongs to the Cyprinidae family that has a strong dopamine system (Peter *et al.*, 1986). One of the strong types of evidence for this claim is that the use of pituitary extract and HCG never induces ovulation in the female fish. Therefore, to weaken and eliminate the dopamine system, an anti-dopamine agent (e.g. domperidone, reserpine, metoclopramide or pimozide) must be used (Brzuska, 2021). So far, the Ovaprim hormone has been used for the artificial breeding of this fish (Rahdari *et al*., 2013; Gharaei *et al*., 2019). However, pituitary extract has been effective for inducing spermatogenesis in male *S. zarudnyi*, and even some of its sperm cognitive parameters have been better than Ovaprim. For example, the highest levels of sperm volume, spermatocrit, and sperm density were observed in the treatment that used the pituitary extract (Arabnejad *et al*., 2013).

The efficacy of two synthetic (Ovaprim and Ovatide) for the induced breeding of Indian major carps were compared. The results revealed that

Ovaprim is more effective than ovatide for the breeding of *Catla catla* (Dhawan and Kaur, 2004). Akbari Nargesi *et al.* (2022) showed that two injections of Ovaprim and/or a priming dose of Ovopel (mammalian GnRH analog+metoclopramide, at 18-20 µg and 9-10 mg, respectively) with a resolving dose of Ovaprim were suitable for the artificial reproduction of Rudd (*Scardinius erythrophthalmus*) female breeders, however two injections of Ovaprim recommended for Rudd spawning due to the proper effects and easy application.

The reason for the success of Ovaprim in inducing the spawning of *S. zarudnyi* should be found in the composition of this hormone, which contains dopamine antagonists. In other words, each milliliter contains 20 micrograms of sGnRH (Salmons GnRH) and 10 mg of the anti-dopamine domperidone (Ovaprim^{TM} www.syndel.com). In this study, the effectiveness of Ovapass in inducing spawning of *S. zarudnyi* was compared to Ovaprim. The composition of both hormones is similar, but there are two non-structural differences between them. First of all, Ovapass is an available preparation for fish fry producers. Secondly, the protocol provided for the use of these two hormones by the manufacturing companies is different from each other. This difference becomes important when the duration of hormone therapy until spawning and the amount of hormone used decrease (T2). The frequency of injection is one of the parameters that is important in terms of time, cost and stress to the brooders. In the case of *S. zarudnyi*, at least three consecutive injections are needed for ovulation to occur, while according to the brochure of the manufacturer of Ovaprim, in most carps, only one injection of Ovaprim at the rate of 0.5 mg/kg is enough for ovulation to occur (www. syndel.com). The issue of the necessity of multiple injections in white fish is true for both hormones, Ovaprim and Ovapass, with the difference that the interval between injections for Ovaprim was 24 h, but for Ovapass, it was 12 h, which reduces the time by half, at least in terms of time and cost. The need for one or more injections is related to the characteristics of the fish, such as, in contrast to *S. zarudnyi*, in *Capoeta razii*, the weight of its brooders was less than 100 g (Abdolahpour *et al*., 2021) and/or in *Cyprinus rubrofuscus* with an average weight of 536.7±6 g (Malik *et al*., 2014), spawning occurred only with a single injection of 0.5 mL of Ovaprim per kilogram of body weight. Also, in Indian cyprinid, a single injection of 0.5-2.5 mg/kg of Ovaprim has caused the final treatment (More *et al*., 2010). On the other hand, the total amount of hormone injected into the reproductive system until the induction of ovulation, in treatment 2 (Ovapass), although it did not have a statistically significant difference with other treatments, the amount was 0.4 mL less than Ovaprim, which is practically and economically very important.

Spawning ratio or Spawning success is one of the good indicators to evaluate hormonal effects on ovulation (Szabo *et al*., 2002). In this research, fish of T2

showed the highest spawning rate (100 %) among all experimental groups, which is a very acceptable ratio in fish reproduction.

The observation of high FR in treatments 2 and 4 (93.33 and 90.33 percent) compared to the other two treatments cannot be attributed to the type of hormone, because the composition of Ovaprim and Ovapass is similar and probably other factors such as the type of brooders, sperm quality and inoculation operation have been involved. The fact that the percentage of fertilization did not decrease with Ovapass compared to Ovaprim, indicates the absence of a negative effect of Ovapass. In *Barbus grypus*, the percentage of fertilization in fish injected with Ovaprim was 75-90%, while it was 65-80% in pituitary extract treatment (Ghanemi and Khodadadi, 2017).

In the present study, there was no significant difference in some reproductive parameters, including total hormone injected, fecundity (working and relative), and egg diameter among different treatments, which indicates the comparable efficiency of Ovapass with Ovaprim. Egg quality is defined based on its ability to become an embryo (Bobe and Labbé, 2010). The substances used to induce spawning affect not only the time of spawning but also on the number of fish ready to spawn and the quantity and quality of the obtained gametes (Kucharczyk *et al*., 2007). In perch, *Sander lucioperca*, the quality of eggs collected after induction of ovulation by GnRHs was better than in fish induced with gonadotropic compounds (such as HCG) (Ljubobratović *et al*., 2019). In the present study, Ovaprim and Ovapass were GnRH hormones, therefore it cannot be concluded that hormone therapy had an effect on egg quality indicators such as egg diameter.

The lowest latency period in the present study was related to T2 (27.33±0.66), which used Ovapass. In fish, the latency period depends on various parameters such as water temperature, biological characteristics (species, age and weight), hormone type and injection frequency (Billard, 1990; Yaron, 1995). In grass carp *Ctenopharyngodon idella*, there was no significant difference in the latency period between the pituitary extract injected group and Ovaprim group (Khodabandeh Shelamani *et al*., 2012). In *Aspius aspius*, the latency period in the group that received Ovopel (mammalian GnRH+anti-dopamine metoclopramide) or Ovopel and Ovaprim was 40 h, but in the group that received only Ovaprim it was longer (42-44 h) (Targońska *et al*., 2011). In *Abramis brama*, the shortest latency period was also observed when the pituitary extract was used together with HCG hormone, and the duration of the period was increased with Ovopel (Kucharczyk *et al*., 2007). Since in our study, all treatments were performed on the same species and weight, age and hormone composition were the same. So, the difference between the latency periods of the treatments may be related to the dose and frequency of hormone injections (Rahdari *et al*., 2014).

In conclusion, the artificial spawning of *S. zarudnyi* was enhanced by using stimulating hormones, but Ovapass gave the best results followed by Ovaprim. These stimulating hormones have enhanced reproductive performances. The data of the present study showed that the effectiveness of Iranian Ovapass hormone for inducing spawning of *S. zarudnyi* for reasons such as good performance, cheapness, and easy availability is very suitable and it can be replaced Ovaprim hormone.

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Research Article

Effect of dietary trypsin extracted from the viscera of yellowfin sea bream, (*Acanthopagrus latus*) on growth performance, body composition, and digestive trypsin activity in Sobaity sea bream (*Sparidentex hasta*) larvae

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Abstract

In this study, trypsin was extracted from the viscera of yellowfin sea bream (*Acanthopagrus latus*) and used as an additive in Sobaity sea bream, *Sparidentex hasta*, larvae microdiet. The microdiet was supplemented with the extracted enzyme and its efficiency was evaluated in the growth performance of *S. hasta* larvae. There were five treatments containing zero (control), 1000, 1500, and 2000 IU/kg of the extracted trypsin with 500 IU/kg of a commercial diet of porcine trypsin. *S. hasta* larvae (mean initial weight of 0.021±0.001 g) were fed with experimental diets for 28 days. Fifteen round polyethylene tanks (300 L, a water volume of 100 L) equipped with a water circulation system and an air stone were used for the treatments. The highest body weight gain (BWG), specific growth rate (SGR), daily growth, survival rate, and protein efficiency ratio (PER) were observed in the larvae fed the diet containing 2000 IU/kg trypsin (p<0.05). In addition, the lowest and the highest values of the feed conversion ratio (FCR) vs daily feed intake and trypsin activity were significantly recorded in 2000 IU/kg trypsin and the control groups, respectively (p<0.05). A positive correlation was observed between the increasing levels of trypsin in the microdiets and growth performance (*i.e.* BWG, SGR, daily growth rate, survival rate, and PER). Besides, the dietary addition of external trypsin reduced the values of FCR, daily feed intake, and trypsin activity in the larvae. Overall, trypsin at different doses, particularly at 2000 IU/kg, can significantly improve the performance and feed utilization of *S. hasta* larvae.

Keywords: *Sparidentex hasta*, Weight gain, Protease enzyme, Microdiet, Correlation coefficient.

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Introduction

The development of aquaculture industry, especially marine aquaculture operations, has led to advances in marine fish farming based on the production of resistant and high-quality larvae (Alvarez- Gonzáles *et al*., 2010). An important factor affecting larval quality and survival is the supply of sufficient and essential nutrients. Diets that are used as live or formulated feeds should be able to meet the essential nutritional needs for proper larval growth and to develop maximum physicochemical properties related to digestion and absorption (Kolkovski *et al*., 1993; Yılmaz and İkiz, 2006; Javahery *et al*., 2019; Rachmawati *et al*., 2020; Imaniy *et al*., 2022). In the larval stage of marine fish, the internal organs, in particular the gastrointestinal (GI) tract, do not develop completely at the time of hatching, and it develops during the development period (Nazemroaya *et al.*, 2015b). Hence, initial feeding with microdites requires a high nutrient bioavailability during the early life stages in marine fish larvae, which depends on the proportion and formulation of the microdiets for larval growth and physiological development. In most marine fish larvae, enzymatic activities are significantly lower than the juvenile stage (Hamre *et al*., 2013), resulting in lower larval growth and survival at the onset of feeding with formulated diets (Munilla-Moran *et al*., 1990). Therefore, the complete replacement of live feed with microdiets is ineffective because of problems with the inability of digestion and absorption in the first-feeding stage (Walford *et al*., 1991; Hamre *et al*., 2013). Dependence on live feed is a major problem in the intensive culture of most larval marine fish species (Kolkovski, 2001). This is because the production of live feed requires more than half of the nursery facilities and laborious work as it is an unpredictable process that can be affected by changes in water parameters and does not provide sustainable production (Marte and Toledo, 2015). The onset of external feeding in fish larvae is considered a critical stage associated with high mortality in laboratory and wild populations. After the absorption of the yolk sac, the lack of nutrients in natural conditions reduces larval survival in the first days and weeks of life, which corresponds to the lack of high-quality feed and a proper feeding process in farming systems (Yufera and Darias, 2007). The addition of digestive enzymes as a supplement was reported to result in a 30% increase in the absorption of microdiets (Kolkovski *et al*., 1993).

Fish are processed before their supply for human consumption. Two types of solid and liquid wastes are produced during the processing procedures. The solid waste includes the head, skeleton, fins, tail, skin, and GI tract, and the liquid waste comprises seafood processing wastewater. These wastes are rich in valuable micro- and macronutrients such as proteins, amino acids, bioactive peptides, collagen, gelatin, oils, calcium, and enzymes. Fish waste usually contains 58% protein and 19% ether extract or fat. Moreover,

minerals such as calcium, phosphorus, potassium, sodium, magnesium, iron, zinc, manganese, and copper are found in their compounds (Ramakrishnan *et al*., 2013). The internal organs of aquatic animals are rich in enzymes, exhibiting a high catalytic activity at relatively low concentrations. Enzymes found in fish include pepsin, trypsin, chymotrypsin, and collagenase, which are extracted commercially from the viscera of aquatic animals (Prasertsan *et al*., 2003; Zhao *et al*., 2011).

The reduction of using live feeds during the larval stage is economically important and should be in line with maintaining proper larval survival and growth. Besides, using enzyme supplements in microdiets results in the improved nutrition of marine fish larvae. Some publications reported the effects of diet supplementation with dietary digestive enzymes in yellow perch (*Perca flavescens*) (Kolkovski *et al*., 2000), hybrid tilapia (*Oreochromis niloticus x O. aureus*) (Lin *et al*., 2007), Japanese seabass (*Lateolabrax japonicas*) (Ai *et al*., 2007), African catfish (*Clarias gariepinus*) (Yildirim and Turan, 2010), and Red sea bream (*Pagrus major*) (López-Alvarado, 2013). Enzyme-rich sources are also produced by the aquaculture industry waste. For instance, trypsin was purified from the waste of brown stripe red snapper (*Lutjanus vitta*) (Khantaphant and Benjakul, 2010), silver mojarra (*Diapterus rhombeus*) (Silva *et al*., 2011), zebra blenny (*Salaria basilisca*) (Ktari *et al*., 2012), olive flounder (*Paralichthys olivaceus*) (Kim and Jeong, 2013), merigal carp (*Cirrhinus mrigala*) (Khangembam and Chakrabarti, 2015), and oil sardine (*Sardinella longiceps*) (Khandagale *et al*., 2017). Due to the alkalinity of the larval digestive environment in the early days of hatching, it is possible to increase the activity of alkaline proteases by adding enzymatic supplements. Therefore, the growth and survival of *S. hasta* larvae fed with microdiets supplemented with trypsin extracted from the viscera of *A. latus* were investigated in the present study to evaluate the growth performance, trypsin activity and the regression between supplement enzyme and those parameters.

Materials and methods

Supply of larvae

The larvae were obtained through healthy and wild three-year-old breeders caught from the Persian Gulf. Female and male *S. hasta* broodstocks (mean weights of 2.5±0.98 and 1±0.10 kg, respectively) were spawned at 18°C. The fish were then undergone HCG (human chorionic gonadotropin) hormone therapy for two days in the afternoon to morning in the *marine fish research station of Bandar Imam (Mahshahr, Khuzestan, Iran)*. Active feeding began two days after hatching. On the first day after hatching, the larvae were kept in water containing the microalga, *Nannochloropsis* sp. After active feeding began from the second day until the 8th day after hatching, larvae were fed with a combination of algae and small-size or the S-type rotifer

(*Brachionus plicatiliss*) at a rate of 500,000 cells/mL of the algae and 20 rotifers/mL. From days 9 to 11, 500,000 cells/mL of the algae and 30 large-size L-type rotifers/mL were added to the previous food composition (Nazemroaya *et al*., 2015a, b). To prepare the larvae for manual feeding, microplates without enzyme supplementation with live feed were added to the diet according to the co-feeding protocol (Kolkovski, 2008) from days 12 to 15. The culture facility was equipped with a chlorination system in the water inlet, ventilation, aeration, and central drainage for larval treatments. Fifteen round polyethylene tanks with a capacity of 300 L and a water volume of 100 L were equipped with a water circulation system (from 10-day larvae to the end of the experiment, water exchange was 25% with a flow rate of 850 mL/min, but water was not exchanged before 10 days because of larval size), and an air stone was used for each tank. Seawater with a salinity of 39 ‰ was passed through a sand filter before entry into the chlorination system. A total of 15,000 15-day-old larvae (15 days after hatch: DAH) was randomLy transferred into the tanks (1000 larvae for each replicate) and fed with experimental diets for 4 weeks. The water quality parameters including temperature (22±0.98°C), pH (7.9±0.02), and dissolved oxygen (7.7±0.05 mg/L) were determined daily during the trial. The larvae were randomLy sampled from all replications and treatments at the end of each week, and each tank was daily siphoned to remove all uneaten feeds (if available) and feces. Dead fish were also removed and discarded at each observation.

Preparation of experimental diets

Since micron-sized food preparation facilities are not available in Iran, the experimental feed sample for feeding the larvae was procured from Coppens Co. (The Netherlands) (Advance type, 200-300 microns, Altek Koppens). This feed was specific to marine fish larvae and contained no enzymatic additives, which was selected with a size of 200-300 microns to be suitable for feeding marine fish larvae in the first weeks of active feeding. Protein, fat, fiber, ash, and total phosphorus contents in the prepared food were 56, 15, 0.3, 11, and 1.85%, respectively. The feed also contained stable amounts of such vitamins as A (14000 IE kg^{-1}), D (1300 IE kg^{-1}), E (280 mg kg^{-1}), and C (350 mg kg^{-1}). To add trypsin enzyme supplement extracted from the viscera of yellowfin sea bream, *A. latus*, the dry enzyme powder was mixed with edible oil (sunflower oil) and sprayed on the feed (Kazerani and Shahsavani, 2011). To prevent the adhesion of very fine feed particles, the oil-added feed was air-dried for 2-3 h, powdered again using a stirrer, and then passed through a screen (with mesh size in microns). The same amount of oil without the enzyme supplement was added to the control diet.

Accordingly, experimental dietary treatments (T) consisted of T1 (control) with 30 rotifers/mL without extracted trypsin, T2 containing 500 IU of commercial trypsin (derived from the

porcine pancreas) per kg of diet, and T3, T4, and T5 each containing 1000, 1500, and 2000 IU/kg, respectively, of dietary trypsin extracted from *A. latus* viscera.

The enzyme supplement was a lyophilized powder, with a specific activity of 56.6 IU/mg protein, obtained from trypsin extracted from 15 *A. latus* viscera (with a mean weight of 179.93±93 g and a mean length of 213.67±29 cm) and used in the experimental treatments (Namjou *et al*., 2019). T2 contained a commercial porcine pancreatic trypsin supplement obtained from Sigma-Aldrich (product code T4799).

Fish were fed manually in the tanks 12 h a day every 2 h based on apparent satiety but not during dark hours. The feed was sprayed on the tanks to spread all over the tank surface.

Growth parameters

After recording the weight and mortality of larvae, growth performance was measured using the following formulas:

Body weight gain (BWG, g) = Wt_2 (g)-Wt_1 (g)

Where, Wt_1 and Wt_2 are initial and final weights, respectively.

Specific growth rate (SGR, %/day) = $[(LnWt_2\text{-}LnWt_1)/t_2\text{-}t_1] \times 100$

Where, $LnWt_1$= the normal logarithm of the initial weight, $LnWt_2$= the normal logarithm of the final weight and $t_2\text{-}t_1$= the length of the test period

Daily Growth Rate (GR, g/day) = [Wt_2 (g)-Wt_1 (g)/ ($t_2\text{-}t_1$)]

Where, Wt_1= Initial weight, Wt_2= The final weight, and $t_2\text{-}t_1$= the length of the test period

Feed conversion ratio (FCR) = F(g)/WG(g)

WG= weight gain and F= the amount of food given

Feed intake (FI, %/day) =$100 \times$I (g)/[(Wt_2 (g) + Wt_1 (g))/2$\times$ ($t_2\text{-}t_1$)]

Where, I= total food eaten and $t_2\text{-}t_1$= the length of the test period

Survival rate (SR, %) = $(N_t - N_0) \times 100$ (Ai *et al.*, 2007)

Where, N_0=Number of fish at the beginning of the period) and N_t = Number of fish at the end of the period

Protein Efficiency Ratio (PER) = wet weight gain (g) / protein fed (g)

Trypsin activity (in the extracted enzyme and fish larvae)

It should be noted that about 1 g of the sample was needed for the enzyme analysis (Ramzanzadeh *et al*., 2016). Based on the final weight of the fish, therefore, all fish from each treatment were collected for the enzyme assay and the body composition analysis. To determine trypsin activity, enzyme-extracted samples were first prepared by the defatting process and preparation of

a dry powder, followed by preparing the enzyme crude extract and then purifying the enzyme from the crude extract (Namjou *et al*., 2019). To determine the trypsin activity of larvae at the end of the 4-week experimental period, sampling time was scheduled in the morning prior to feeding, and no feed was added to the rearing tank at night on the day prior to sampling. The small size of larvae makes it difficult to cut the head or spinal cord or to kill them with a sharp blow to the head. Therefore, they were anesthetized moderately with clove powder (200 ppm) (Mirali *et al*., 2013) and then immediately submerged in liquid nitrogen at –196°C. Trypsin (EC 3.4.21.4) activity was measured with *N*--benzoyldlarginine-*p*-nitroanilide (BAPNA) as the substrate. BAPNA (1 mmol/L in 50 mmol/L Tris–HCl, pH 7.5, 20 mmol/L $CaCl_2$) was incubated with the enzyme extract at 37°C. Absorbance was read at 410 nm. After the preparation of samples, the enzyme activity was examined using the following formula (Erlanger *et al*. 1961):

$$\text{Trypsin activity (U/mL)} = \frac{\text{light absorption at 410 nm} * 1000 * \text{the reaction mixture}(ml)}{8800 * \text{reaction time}\ (min) * \text{sample volume}\ (ml)}$$

Where, 8800 (cm^2/mg) is the molar extinction coefficient for ρ-nitroaniline (Erlanger *et al*., 1961).

Carcass analysis

At the end of the fourth week, samples were taken randomLy from all treatments for the carcass analysis. To this aim, the whole larval body was macerated and homogenized due to its small size. Carcass constituents, including protein, moisture, fat, and ash, were analyzed according to the AOAC method (AOAC, 1995).

Data analysis

This experiment was performed based on a completely randomized design with five treatments and three replications. Data normality and homogeneity of variances were examined by Kolmogorov-Smirnov and Levene's tests, respectively. The effects of enzyme supplementation on growth parameters, trypsin activity, and carcass composition in *S. hasta* larvae were evaluated by the one-way analysis of variance (ANOVA) using SPSS 21 software. Significant differences between the means were determined with Duncan's test at a significance level of 5%. Microsoft EXCEL 2016 was also used to determine Pearson's correlation coefficient between data and to draw figures.

Results

According to the results of larval BWG (Table 1), a significantly higher BWG was observed in the treatments fed with trypsin supplement (p<0.05). T5 (0.121±0.003 g) and the control treatment (0.09±0.00 g) presented the highest and the lowest BWG values, respectively. In addition, a positive correlation coefficient was obtained

between increasing trypsin levels and BWG in the experimental treatments (Fig. 1; $p<0.05$, r=0.90). SGR (%day^{-1}) was higher in treatments containing the extracted trypsin than in commercial trypsin and diets without enzyme supplements ($p<0.05$). The highest (7.523±0.79% day^{-1}) and the lowest (6.540±0.002% day^{-1}) SGR values belonged to T5 and the control treatment, respectively. A positive correlation coefficient was found between increasing the extracted trypsin and the increase in SGR (Fig 2; $p<0.05$, r=0.89) (Table 2).

Table 1: Growth performance and survival of *Sparidentex hasta* larvae fed with diets containing different levels of trypsin (commercial trypsin and trypsin extracted from *Acanthopagrus latus* viscera).

Parameter	T1	T2	T3	T4	T5
Initial weight (g)	0.021±0.001 [a]	0.021±0.001 [a]	0.021±0.001 [a]	0.021±0/001 [a]	0.021±0.001 [a]
Final weight (g) 41 (DAH*)	0.110±0.000 [c]	0.128±0.003 [b]	0.123±0.006 [b]	0.132±0.003 [b]	0.142±0.003 [a]
Weight gain (g)	0.09±0.00 [d]	0.108±0.003 [bc]	0.103±0.006 [c]	0.112±0.002 [b]	0.121±0.003 [a]
Specific growth rate (%/day)	6.540±0.002 [d]	7.148±0.079 [bc]	6.989±0.174 [c]	7.247±0/072 [b]	7.523±0.079 [a]
Daily growth Rate (g/day)	0.0034±0.0000 [d]	0.0042±0.0001 [bc]	0.0040±0.0002 [c]	0.0043±0.0001 [b]	0.0047±0.0001 [a]
Feed conversion ratio	1.11±0.003 [a]	0.91±0.018 [bc]	0.95±0.052 [b]	0.88±0.019 [c]	0.80±0.020 [d]
Daily feed intake (%/day)	5.79±0.01 [a]	5.03±0.07 [bc]	5.21±0.21 [b]	4.91±0.08 [c]	4.60±0.08 [d]
Survival rate (%)	95.23±0.21 [c]	95.83±0.42 [b]	96.07±0.23 [ab]	96.10±0.17 [ab]	96.37±0.06 [a]
Protein Efficiency Ratio	0.0017±0.000 [d]	0.0021±0.000 [bc]	0.0020±0.000 [c]	0.0021±0.000 [b]	0.0023±0.000 [a]

Values are represented as mean±STD. Different letters in each row indicate a significant difference among treatments ($p<0.05$). T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively. DAH: days after hatch.

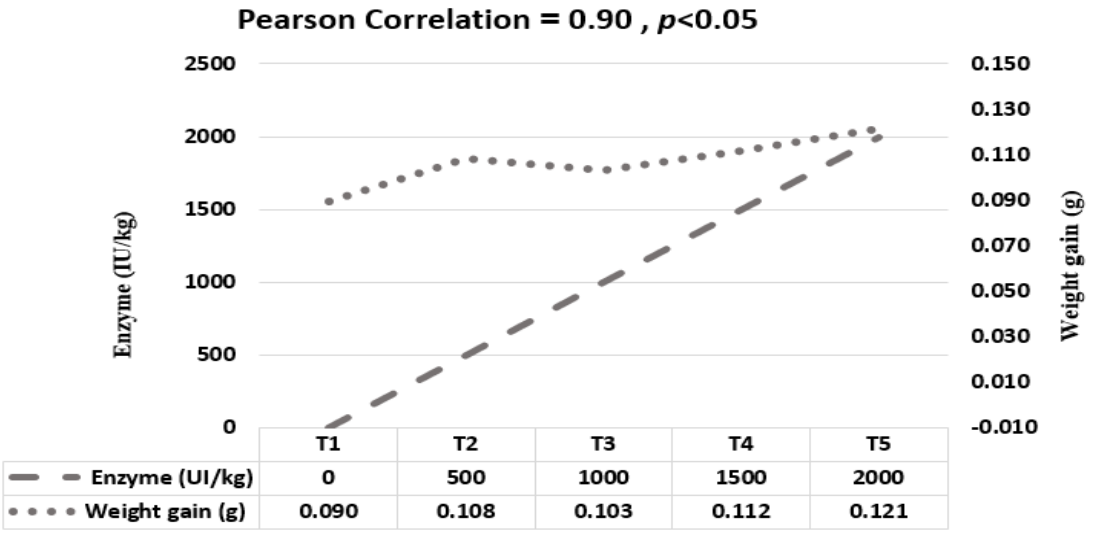

Figure 1: The positive correlation between dietary trypsin levels and body weight gain in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively

Daily growth rates were uppermost in treatments containing enzyme supplements, with T5 (0.0047±0.0001 g day^{-1}) and T1 (0.0034±0.001 g day^{-1}) showing the highest and lowest rates, respectively (p<0.05). There was also a positive correlation coefficient between increasing the trypsin supplement and the daily growth rate of larvae (Fig. 3; p<0.05, r=0.90). The examination of FCR graphs indicated that treatments fed with diets containing the trypsin supplement had significantly lower FCR, with values of 0.8 and 1.1 in T5 and T1, respectively (p<0.05). There was also a negative correlation between FCR and the trypsin increase (Fig. 4; p<0.05, r= −0.89).

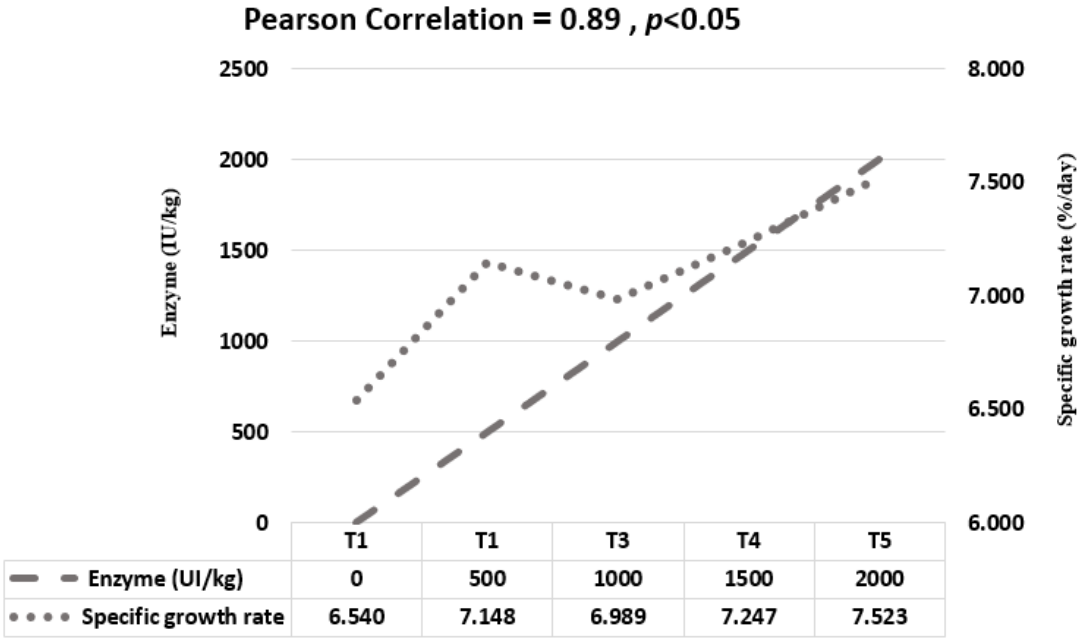

Figure 2: The positive correlation between dietary trypsin levels and specific growth rate in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively.

Table 2: Trypsin activity of *Sparidentex hasta* larvae samples fed with diets containing different levels of trypsin (commercial trypsin and trypsin extracted from *Acanthopagrus latus* viscera).

Parameter	T1	T2	T3	T4	T5
Total activity (IU)	9.80±0.006 ^{c}	10.61±0.012 ^{a}	10.30±0.010 ^{b}	2.72±0.006 ^{d}	0.224±0.001 ^{e}
Specific activity (IU/mg protein)	0.08±0.001 ^{a}	0.06±0.002 ^{c}	0.07±0.002 ^{b}	0.05±0.001 ^{d}	0.01±0.001 ^{e}
Protein content (mg/mL)	2.60±0.006 ^{c}	3.90±0.010 ^{a}	3.20±0.010 ^{b}	1.14±0.001 ^{d}	0.48±0.001 ^{e}

Values are represented as mean±STD. Different letters in each row have a significant difference among treatments (p < 0.05). T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively.

The feed intake levels in T5 and T1 were respectively equal to 4.0±0.08% d^{-1} and 5.79±0.01 % d^{-1} (p<0.05), with a negative correlation between the increasing use of trypsin in the diets of experimental treatments and feed intake levels (Fig 5; p<0.05, r= −0.89). The survival rate was also significantly affected by the dietary trypsin level. Survival rates of 96.37%±0.06 and 95.23%±0.21 were recorded in T5 and T1, respectively, at the end of the experimental period (p<0.05), with a positive correlation coefficient between these two parameters (Fig. 6; p<0.05, r=0.93). Similar results were observed for PER so that T5 with the highest enzyme supplementation had a better PER (0.0023±0.00) than that of the control treatment (0.0017±0.00) (p<0.05). Accordingly, a positive correlation coefficient was obtained between PER and the trypsin increase (Fig 7; p<0.05, r=0.90).

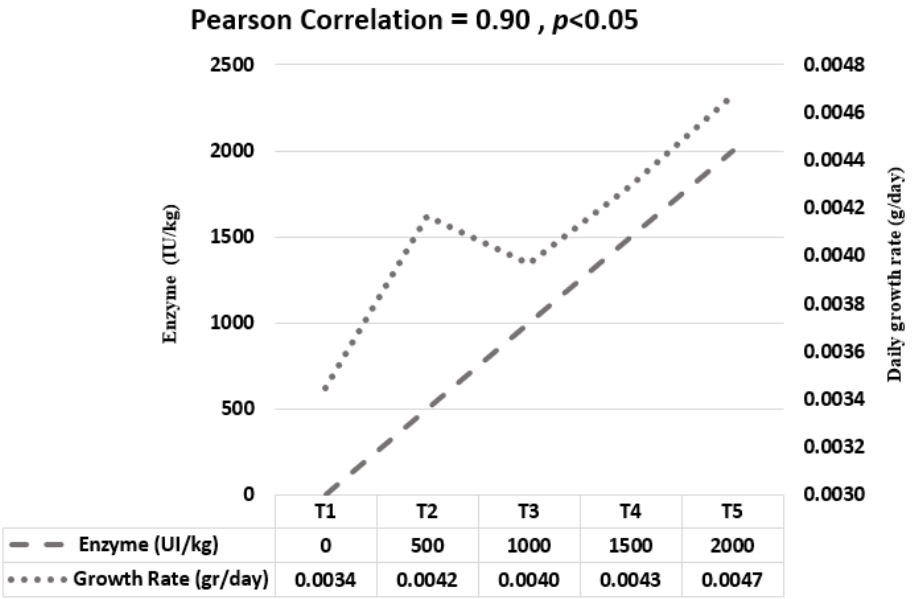

Figure 3: The positive correlation between dietary trypsin levels and daily growth rate in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively.

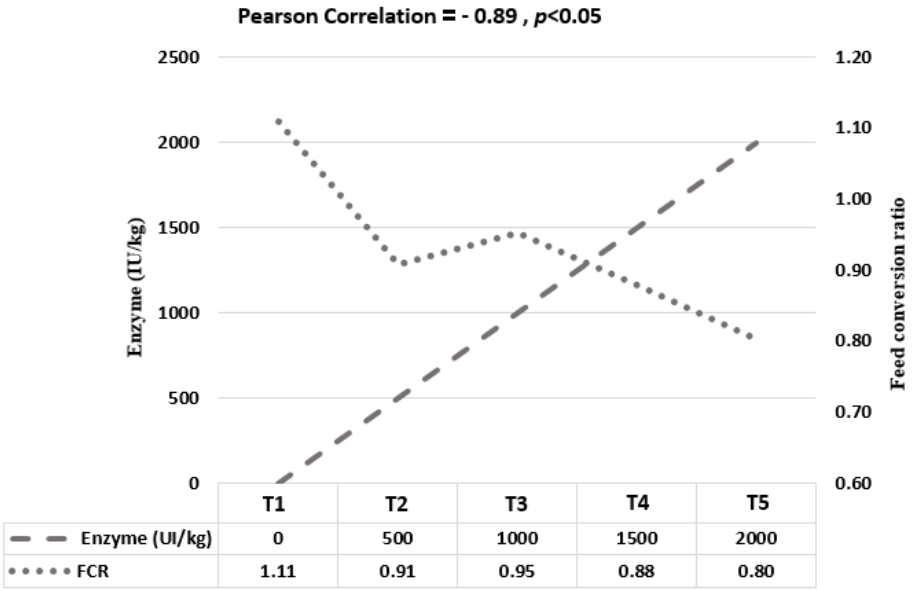

Figure 4: The negative correlation between dietary trypsin levels and feed conversion ratio in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively.

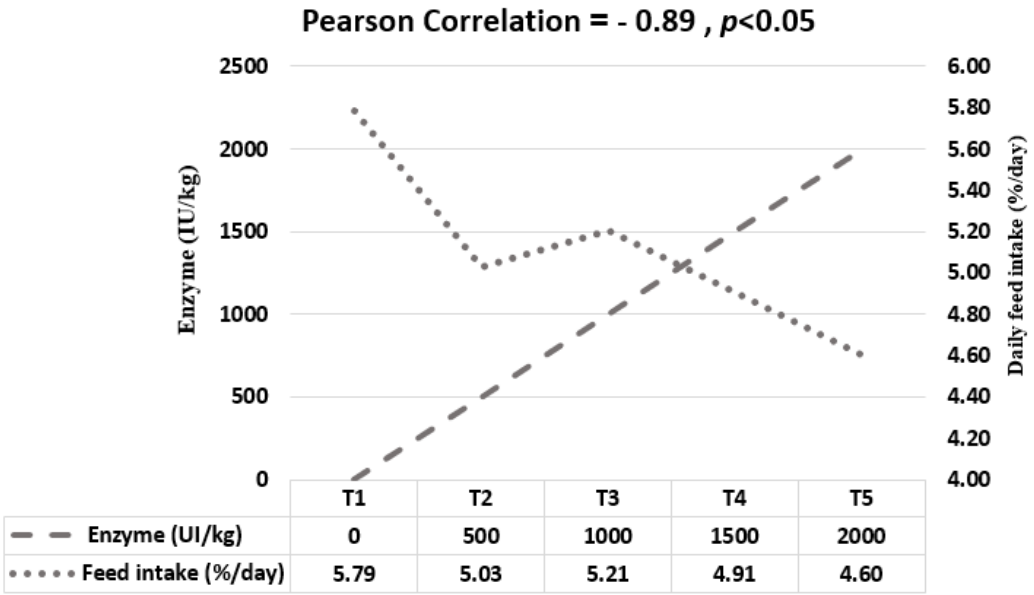

Figure 5: The negative correlation between dietary trypsin levels and feed intake in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively.

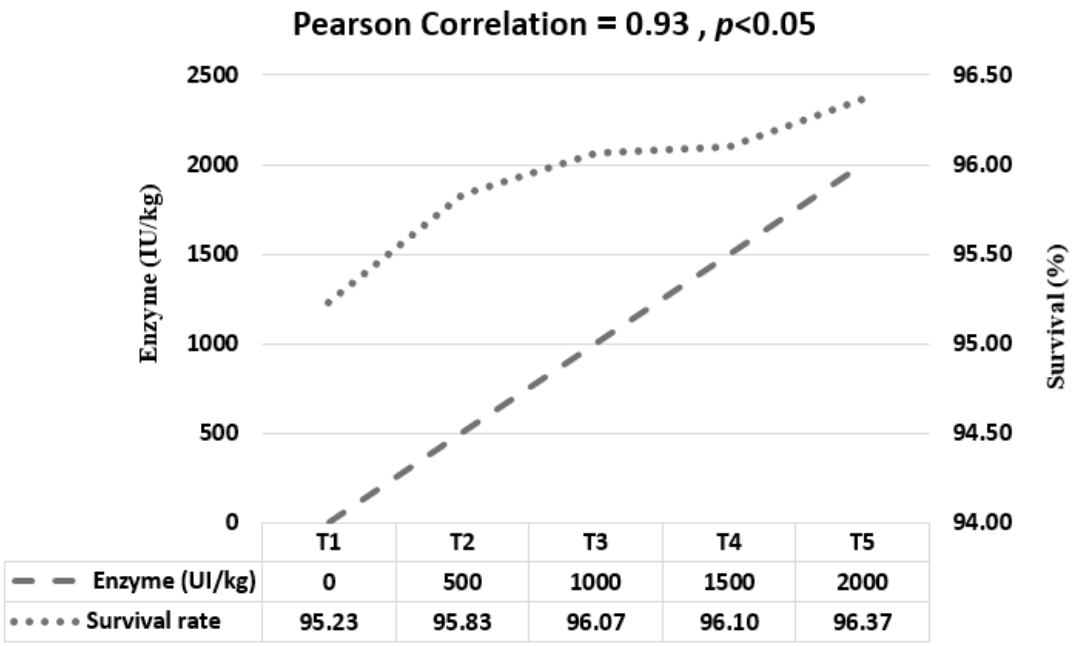

Figure 6: The positive correlation between dietary trypsin levels and survival rate in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively.

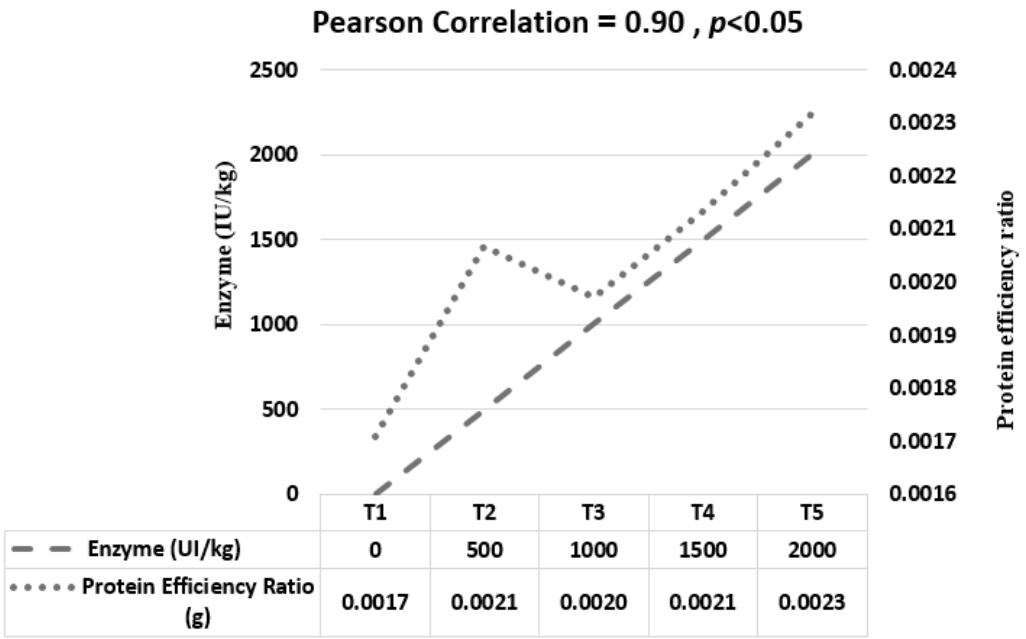

Figure 7: The positive correlation between dietary trypsin levels and protein efficiency ratio in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively.

Measurements of the total activity and specific activity of trypsin in the larval body revealed the lowest levels of 0.224±0.001 and 0.01±0.01 IU/mg protein, respectively, in T5 (p<0.05). Among the other treatments, T2 presented the highest enzyme activity (p<0.05). Similar to the results of

enzyme activity, protein content was lowermost (0.48±0.001 mg/mL) in T5 (p<0.05), with a negative correlation coefficient between total enzyme activity and increasing dietary trypsin supplementation (p<0.05, r= -0.87). Similar results were observed for specific enzyme activity (p<0.05, r= -0.88). In addition, a negative correlation coefficient was observed between dietary trypsin supplementation and protein content in the larval body (p<0.05, r= −0.77).

Based on the results of larval carcass analysis at the end of the 4-week experiment (Table 3), carcass protein contents were different in all treatments (p<0.05), with higher levels in trypsin-containing treatments than in the control fish (p<0.05), as well as in T3, T4, and T5 than those of T1 and T2. T5 and the control treatment contained higher and lower protein levels, respectively than the other treatments (p<0.05). The carcass fat content of *S. hasta* larvae was significantly different in all treatments, with significantly lower levels in trypsin-fed fish than in the control treatment. The highest and lowest fat levels were observed in the control treatment and T4 and T2, respectively (p<0.05). There was no significant difference between T2 and T4 (p>0.05), but these two treatments differed significantly from the other treatments (p<0.05).

Table 3: Carcass analysis of *Sparidentex hasta* larvae fed with diets containing different levels of trypsin (commercial trypsin and trypsin extracted from *Acanthopagrus latus* viscera).

Parameter (% in dry basis)	T1	T2	T3	T4	T5
Crude protein	13.42±0.09 e	15.42±0.04 c	15.90±0.04 b	14.54±0.04 d	16.11±0.01 a
Fat	5.03±0.2 a	4.01±0.10 d	4.24±0.06 c	3.90±0.02 d	4.42±0.05 b
Ash	4.84±0.06 a	4.43±0.01 c	4.30±0.02 d	4.63±0.03 b	4.12±0.06 e
Moisture	76.72±0.10 b	76.15±0.06 c	75.55±0.01 d	76.93±0.08 a	75.35±0.10 e

Values are represented as mean±STD. Different letters in each row indicate significant differences among treatments (p<0.05). T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively.

Carcass ash contents were significantly lower in all treatments containing the extracted trypsin than in the control fish. T5 and the control treatment contained the lowest and the highest ash levels, respectively, among the other treatments. There were significant differences between the results of all treatments (p<0.05). The examination of carcass moisture contents showed that T4 and T5 contained the highest and the lowest levels, respectively. The results of carcass moisture content differed significantly in all treatments (p<0.05).

Discussion

Understanding the condition and function of the GI tract is as important as the determination of nutritional and

environmental needs to specify the hormonal function, food digestion, and absorption processes in the critical stage of larval growth and development. The main feature of this stage is the sources of materials and energy required for larval growth from the yolk sac absorption to external feeding stages. To successfully pass through this stage, all organs related to food capture, digestion, and absorption must be prepared in larvae, and proper food must be available (Yufera and Darias, 2007). Most marine fish larvae within the second and third weeks of life have insufficient digestive capacity for using formulated feed because of their GI tract not fully developed considering structure and function (Nolting *et al*., 2001). Accordingly, the beginning of manual feeding in marine fish larvae is considered after the metamorphic cycle (Kolkovski, 2008). In the present study, adding the extracted trypsin to the diets of larvae resulted in acceptable effects on improvements in growth parameters at the end of the experimental period, in addition to the possibility of feeding after the 15th day of hatching. Moreover, there was also a positive correlation between increasing external trypsin to microdiets and BWG, SGR, daily growth rate, survival rate, and PER, and a negative correlation was found between increasing external trypsin to microdiets, FCR, and daily feed intake. In a study on *S. hasta* larvae for the early replacement of live feed with dry feed, the initiation of feeding with manual feed from day 18 did not negatively affect the growth compared with day 25 (Nazemroaya *et al*., 2015a). It seems that the use of the diet supplemented with extracted trypsin in the present study could help to initiate feeding with manual feed 3 days earlier compared with the last study without negative effects.

In a previous study, the onset of early external feeding with manual feed reduced the growth and quality of larvae and increased the risk of developing abnormal skeletons (Kolkovski, 2008). Although there were adequate levels of digestive enzymes for the digestion of live feed in larvae, these amounts were not sufficient for the digestion of microdites as they contained a higher protein content (60-90% dry matter) while this is only 10% in zooplankton; thus, a high protein content makes the digestion of microdiets difficult for larvae (Kolkovski, 2001). Therefore, the initiation of active feeding with manual food or microdiets was examined with a simultaneous feeding protocol using manual and live feeds concurrently, which enabled the larvae to change faster and more efficiently from live feed to feeding on microdiets. This method leads to more growth and better survival than the method of using only live feed or microdiets. Early feeding of larvae with suitable microdiets provides better nutritional status and acceptance of microdiets by larvae (Kolkovski, 2008).

The use of external enzymes has been suggested as a method for the improvement of larval growth when using microdiets. Different results were observed in some studies by utilizing trypsin and other proteases extracted

from mammals (e.g., pigs and cattle) (Yildirim and Turan, 2010; Kazerani and Shahsavani, 2011).

People Le Ruyet *et al*. (1993) suggested that if manual feeding could be started 15 days earlier in larval sea bass, *Dicentrarchw labrax*, it could replace 80% of Artemia. According to previous studies, however, the early use of manual food led to a weight loss of up to 30% and produced low-quality and poor larvae with skeletal problems. In the present study, the results of trypsin use showed that BWG was higher in all enzyme-fed treatments than in the control, and T5 with the highest trypsin supplementation and the trypsin-free control treatment attained the highest and the lowest BWG, respectively. There was also a significant difference between the results of T5 and the other treatments.

López-ALvarado *et al*. (2013) used pig pancreatin enzyme as the extracted trypsin in larval red sea bream, *Pagrus major*, and found that treatment with the highest level of the enzyme supplement (0.1% of diet) presented the highest SGR and survival rates. In a study on African catfish, *Clarias gariepinus*, increasing extracted trypsin by 0.75 g/kg of the diet increased SGR and PER and produced better FCR in large fish (Yildirim and Turan, 2010). Using commercial complex enzymes containing protease, lipase, and amylase could improve the growth performance of giant gourami (*Osphronemus goramy*) (Imaniy *et al*., 2022).

In general, the function of enzyme supplements can improve growth parameters in two ways. This function can be considered both a supplement to digestive enzymes in larval bodies and an activator of zymogens (Kolkovski *et al*., 1993). Some researchers presented evidence that the addition of external enzymes to fish diets reduced the effect of anti-nutritional factors and improved the yield of dietary amino acids and energy use, thereby improving the growth performance of fish (Lin *et al*., 2007; Soltan, 2009). To explain the relationship between increasing dietary trypsin and elevated survival, increasing the enzyme in the GI tract simplifies the structure of nutrients and provides favorable conditions for increasing the beneficial microbial flora and thus the reduction of pathogens. Enzyme activity in the GI tract produces organic acids, including lactic acid, which enter the structure of pathogenic microbial flora and reduce their development (Ghomi *et al*., 2010). Finally, it should be borne in mind that food efficiency in the growth process of marine fish larvae depends on many internal and external factors. Moreover, the access, identification, and capture of food are influenced by many physicochemical factors, including color, shape, size, movements, and olfactory stimulation at the molecular level (Kolkovski, 2008).

Measurement of trypsin enzyme activity (after day 41 of hatching) revealed that the effect of the commercial enzyme with higher purity and activity was less than the enzyme extracted from the viscera of *A. latus*, which can be due to a variety of reasons. Better growth performance in treatments

receiving more extracted trypsin indicated that trypsin functioned well and improved the digestive process in larvae by binding to available substrates, and therefore its activity was minimal at the end of the experimental period (Caruso *et al*., 2009). Additionally, enzymes develop during the developmental period in marine fish larvae depending on the species, temperature, and the type of available food (Kolkovski, 2008). In *S. hasta* larvae, a continuous change in the relative activity of proteases from alkaline to acidic during larval development indicates the fact that alkaline proteases are not the main enzymes of protein digestion, and trypsin levels will decrease from day 40 onwards with the development of the GI tract by the presence of pepsin (Nazemroaya *et al*., 2015b). It can, therefore, be concluded that the decreased trypsin enzyme activity in the larvae that received more extracted trypsin resulted in an elevated larval development and growth process, as well as a decrease in trypsin levels with the stomach development. The results of the correlation between enzyme activity in the larval bodies and increasing the extracted trypsin cannot be considered a negative function of the extracted trypsin. The negative correlation between the total and specific activities of trypsin in larval bodies with increasing the extracted trypsin might indicate the increased gastric development in the larvae.

In a similar study on juvenile yellow perch (*Perca flavescens*) fed with diets containing 1% of enzyme supplements (bombesin and pancreatin), the specific activities of trypsin and chymotrypsin enzymes did not change significantly compared to other treatments, which was attributed to the development of the GI tract and the enzyme secretion process (Kolkovski *et al*., 2000).

The use of enzymes in fish food can not only affect growth and reduce nutritional costs but also increase protein levels in the carcasses of produced fish. In vertebrates, digestion generally takes place in two ways, through enzymes produced by the food host and the other through enzymes secreted by the bacterial flora in the GI tract (Kar and Gosh, 2008). Besides improving diet efficiency, the secretion of enzymes facilitates the digestion process and ultimately increases protein retention, leading to increases in nutrients, including carcass protein. In addition to improving the digestion of protein compounds, the activity of such enzymes as protease leads to the better absorption of these compounds and thereby increases the carcass crude protein content (Ghobadi *et al*., 2009).

The carcass chemical composition indicated that the protein content was significantly higher in the body of larvae that received the extracted trypsin than in the extract-free treatment, which increased with rising dietary levels of the extracted trypsin. Using the extracted enzyme yielded better results even at low levels than the commercial enzyme in terms of carcass protein content in larvae. In contrast, fat and ash contents were higher in the bodies of larvae with

no trypsin supplementation and even in those fed the commercial enzyme than in the other treatments.

The elevated carcass protein was in line with the improvement of growth parameters, which might eventually be due to the increased levels of enzymes responsible for dietary protein digestion, resulting in more protein retention in larval carcasses. In a study on the African catfish, *C. gariepinus*, protein content was measured at 21.75% by increasing the enzyme supplementation by 0.75 g/kg diet in large fish, which was higher than treatments with less and without enzyme supplementation (Yildirim and Turan, 2010).

In conclusion, utilizing enzymes extracted from the viscera of the marine fish, *A. latus*, at 1000 and 1500 IU/kg of the diet yielded similar results to using the commercial enzyme at a level of 500 IU/kg of the diet. In addition, better results can be achieved by increasing 2000 IU/kg of the diet, thus producing *S. hasta* larvae with better growth, reduced production costs, and larval survival improvement. Therefore, this study demonstrates that unvalued marine fish waste can be used to produce valuable products with proper management to improve the growth of fish larvae and reduce environmental pollution by affecting feeding efficacy. It can be concluded that the supplementation of manual feed with enzymes can be used to start manual feeding at a younger age and reduce the period of feeding with live feeds.

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Research Article

Replacement effects of soybean meal with sesame seed cake on growth, biochemical body composition, and economic efficiency of *Cyprinus carpio* formulated diet

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Abstract

A 90-day feeding trial was run to evaluate the effect of replacement of soybean meal (SBM) with sesame seed cake (SSC) at 0, 25, 50, 75, and 100% in the isonitrogenous (30% crude protein) and iso-lipidic (8% crude lipid) experimental diets on growth, nutrient and economic efficiency, and biochemical body composition of juvenile *Cyprinus carpio*. Common carp with an average initial weight of 83.09±0.06 g with a density of 30 numbers were randomly fed experimental diets in 15 tanks (300 L) with the flow-through system. The results showed that survival and growth rate, visceral indices, and proximate biochemical composition of the whole body and the fillet except protein did not significant between treatments. The feed intake, feed conversion ratio and protein efficiency ratio, calcium and phosphorus, nitrogen retention efficiency, nitrogen wastage except for SSC25, and phosphorus wastage except for SSC25 and SSC50 were not significantly affected by the dietary treatments. Economic conversion ratio of diets decreased, whereas profit index increased with increasing levels of dietary SSC. Reduced cost of SSC100 was about 35.10%. The number of white blood cells was significantly higher in SSC0 than in other experimental treatments. Hemoglobin was higher in SSC75 than in SSC0. Among serum parameters, cholesterol was significantly lower in SSC0 treatment than in SSC25 treatment. Comparable growth performance, nutrient wastage, economic efficiency, and some blood factors indicated that SBM could be replaced by 75 to 100% SSC in the formulated diet of *C. carpio* juveniles.

Keywords: Replacement, Sesame seed cake, Soybean meal, Body composition, Economic efficiency, *Cyprinus carpio*

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Introduction

Common carp (*Cyprinus carpio*) is widely cultured in Iran due to its nutritional value, market demand, and economic value (Modaberi *et al*., 2022). The protein requirement in the diet of omnivorous fish is often met by soybean meal (SBM) (Roy *et al.,* 2014; Lawal *et al.,* 2016; Olude *et al.,* 2016). Among the reasons for the widespread use of SBM in aquafeed are its protein content, high digestibility and almost balanced amino acid profile (Abdel-Warith *et al.,* 2019; Biswas *et al.,* 2019; Arriaga-Hernández *et al.,* 2021). Due to the increasing demand and dependence of the food industry on SBM, its use and accessibility in aquafeed are limited. Consequently, increased demand has also led to higher prices (Guo *et al.,* 2011; Lawal *et al.,* 2016; Dernekbaşı *et al.,* 2017). As a result, the cost of farmed fish increases with the increase in the price of plant protein input in the diet. Therefore, it is necessary to find eco-friendly and economically feasible plant protein sources to replace SBM (De Boer *et al.,* 2014). Among plant protein sources, oilseeds are more important than legumes in fish nutrition. Sesame seed (SS) (*Sesamum indicum* L.) has comparable nutritional value to other protein sources of oilseeds, including SBM and other legumes (Wei *et al.,* 2022). Sesame seed cake (SSC) is obtained by mechanical pressing of SS (Onsaard *et al.,* 2010). This cake consists of about 35-45% protein. The amino acid composition of SSC, except for the lower lysine content and the higher methionine content, is similar to that of SBM (Dernekbaşı *et al.,* 2017). SBM contains anti-nutritional factors such as protease, lipase, and α- amylase inhibitors, lectins, tannin, phytic acid, saponin, antivitamins, beta-conglycinin, estrogenic isoflavones, phytohemagglutinin, and low sulfur amino acid such as methionine and cysteine (Hekmatpour and Mozanzadeh, 2021) that may have negative impacts on hematological indices, growth performance and feed utilization (Francis *et al.,* 2001; Gatlin III *et al.,* 2007). The SSC almost lacks anti-nutrient factors, except oxalate and phytate (Wei *et al.,* 2022). Sesame oil seed also contains zinc, iron, copper, and vitamin B6 for the production and function of red blood cells. In addition, this oilseed contains antioxidants, including vitamin E and ligands, and is a good source of lecithin, selenium, magnesium, calcium, and phosphorus (Sauvant *et al.,* 2002; Nang Thu *et al.,* 2011). This oil seed is cultivated in 16 provinces of Iran. As a locally produced crop, it is easily available at a lower cost compared to SBM. The price of imported soybean meal: sesame seed cake is 3:1.

It was reported that low levels (25-50%) of SBM replacement with sesame seed meal (SSM) did not decrease growth and nutrient efficiency of African Catfish (*Clarias gariepinus;* (Jimoh and Aroyehun 2011; Lawal *et al.,* 2016). Information on the overall performance of omnivorous fish in response to dietary replacement of SBM with SSC is limited. Based on growth, feeding efficiency, nutrient apparent

digestibility coefficient, and body composition of omnivorous species, 25% (Hasan *et al.*, 1997; Ray 1999; Olude *et al.*, 2016) to 50% (El-Saidy *et al.*, 2009; Roy *et al.*, 2014) was reported as the optimum replacement level of fish meal with SS meal. In carnivorous fish, replacement of plant protein sources with SS meal at levels less than 20% (Fagbenro *et al.*, 2013; Dernekbaşı *et al.*, 2017); 20-50% (Fagbenro *et al.*, 2010a; Jimoh and Aroyehun 2011; Jimoh *et al.*, 2014) and above 50% (Enyidi *et al.*, 2014; Lawal *et al.*, 2016) did not show negative effects on fish performance. Replacing SBM with SS meal did not adversely affect hematological parameters of *Clarias gariepinus* (Fagbenro *et al.*, 2010b; Lawal *et al.*, 2016). This nutritional trial was designed to evaluate the growth performance, nutrient and economic efficiency, body composition, blood parameters responses of *C. carpio* to dietary SBM replacement by SSC.

Materials and methods

Formulated diets preparation

Five isonitrogenous (300 g kg^{-1} crude protein) and isolipidic (80 g kg^{-1} crude lipid) experimental diets were formulated, using WUFFFDA 2.0 software, in which 0 (SSC0 or the control diet, where poultry by-product meal (PBM), tuna by-product meal (TBM) and SBM were the main protein sources), 25 (SSC25), 50 (SSC50), 75 (SSC75), 100% (SSC100) of SBM were replaced with SSC. The proximate analyses of the diets are given in Table 1. Fish oil, tuna by-product meal, PBM and the other feed ingredients were provided by the Beyza Feed mill company. Starch and gelatin were cooked separately and blended with the ingredients to produce a homogeneous mixture in a Hobart-type mixer. Oils and water were then added and thoroughly mixed. Pellets (3.0 mm diameter) were produced using a pelletizing machine (CPM, model CL series; USA), air-dried (Iran khodsaz oven) at 40 ºC for 48 h to about 10% moisture, sealed in plastic bags and stored frozen (−20ºC) before use in the feeding trial (Table 1).

Feeding trial

This study was carried out in the South Iranian Aquaculture Research Center, Ahvaz, Iran (SIARC). Four hundred and fifty healthy juveniles of common carp (mean body weight 83.27±0.1 g, mean±SD), produced at the hatchery of SIARC, were randomly distributed into fifteen 300 L cylindrical polyethylene tanks with a flow-through system (1 L min^{-1}). Fish were acclimated to the experimental condition for 2 weeks before the onset of the feeding trial. Water temperature ranged between 22.17 and 24.79 ºC (mean of 23.48±1.2ºC) during the experimental period. The average dissolved oxygen and pH values were 6.8±0.4 mg l^{-1} and 7.37±0.2, respectively. The photoperiod was based on natural environmental fluctuations (12-hour light and 12 hour dark) throughout the experiment. Triplicate groups of fish were hand-fed one of the experimental diets to visual satiation thrice daily (08:00 h, 13:00 h and 17:00 h) for 90 days.

Sampling methods

Before starting the feeding test, the weights of 20 fish were measured to the nearest 0.1 g and whole body was kept at -80 ºC until the biochemical composition analysis. At the termination of the feeding trial, 24 h after the last feeding, fish in each tank were anesthetized with anaesthetic2-phenoxyethanol (0.5 mL L^{-1}; (Zahl *et al.,* 2012; Utne-Palm and Smith 2020) and individually weighed to the nearest 0.01 g (Wang *et al.,* 2015; Yaghoubi *et al.,* 2016).

Blood samples were taken from the caudal vein of four fish (per replicate) using a 2.5 mL heparin syringe (Heparinsodium 5000 IU mL^{-1}, Alborz Daru, Iran). The extracted blood sample was aliquoted into two parts (one for measuring blood factors, and the other for separating serum) in the microtubes. Separating serum of the blood sample was done by centrifuging at 3000g for 10 min at 4ºC and stored in Eppendorf tubes at −80 ºC until further serum biochemical analysis (Carobene *et al.,* 2016). Three fish from each tank were sacrificed with the high levels of anesthetic (4 mL L^{-1}). The visceral contents of the fish were isolated on dry ice, and the total visceral contents were weighed to the nearest 0.01 g and then weighed separately for liver and visceral fat. The fillet was removed from the rest of the carcasses. In addition, three fish were isolated from each experimental tank to analyze the biochemical composition of the whole body. Fillets and whole body were transferred in labeled bags to a freezer at -80ºC until further analysis (Qin *et al.,* 2016).

Biochemical composition of diets and body

Proximate biochemical composition of ingredients, diets, whole body, fillet, and liver was measured based on AOAC, 2005 methods. Dry matter was determined using a moisture analyzer (AM B5 0, ADAM, UK) and gravimetric calculation. Using the Kjeldahl method and device (BÜCHI, Auto-KjeldahlK-370, Switzerland), after acidic digestion of the sample, distillation, and titration with 4% boric acid, the amount of nitrogen obtained was multiplied by 6.25 for protein content calculation. Total lipid was measured by Soxhlet (Barnstead/ Electrothermal, UK) using petroleum ether with a boiling point of 40-60ºC as a solvent. Raw dietary fiber after acidic and alkaline digestion of the sample by the raw fiber extractor (VELP® Scientifica, Italy) and placing the dry sample in the furnace at 550ºC for four hours and calculating the amount of sample lost in the furnace (Finetech, Shin Saeng Scientific, South Korea) was calculated. Ash of ingredient, diet, whole body, and fillets were measured using a furnace at 550ºC for 8 hours. Calcium and phosphorus content were measured on the ash obtained from items and then measured by titration and spectrophotometer (at a wavelength of 700 nm), respectively (AOAC, 2005).

Blood parameters measurement methods

Hemoglobin concentration was measured by the cyanomethahemoglobin method at a

wavelength of 540 nm using a commercial kit (Zistshemi, Iran; (Houston 1990). The red blood cells (RBC) were determined optically with a Neubauer chamber using the Natt and Herrick (1952) solution as diluent. Hematocrit (Hct) was estimated by the micro-hematocrit method (Brown, 1988). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to the following formula (Ellis and Campbell, 2007):

MCV= (Hct/ RBC)×1

MCH=Hem/RBC

MCHC g dL−1= (Hb/Hct)×100

Serum biochemical parameters were analyzed using an auto-analyzer (Mindray BS-200, China) with commercial clinical investigation kits (Pars Azmoon Kit, Tehran, Iran). Biochemical measurements were carried out for glucose, total protein (TP; (Morris *et al.,* 1996), albumin, triglyceride and total cholesterol (CHO; (McGowan *et al.,* 1983). Globulin was calculated by subtracting the albumin values from the total plasma protein (McClatchey, 2002).

Standard formulae were used to assess growth performance and morphometric indices: weight gain (WG;), daily growth rate (DGR); viscera somatic index (VSI), intraperitoneal fat ratio (IPF), hepatosomatic index (HSI), condition factor (CF), survival rate (SR); feed utilization: feed intake (FI), feed conversion ratio (FCR), the protein efficiency ratio (PER), retention efficiency of nitrogen (NRE), phosphorus (PRE), waste output of total nitrogen (NW) and total phosphorus (PW); economic indices: economic coefficient ratio (ECR), reduced cost (RC), profit index (PI) were calculated as follows:

WG (g)= (Wt−W0) (Iqbal *et al.*, 2022)

DGR= (Wt−W0)/t (Zaikov *et al.*, 2008)

CF (gcm−3)=100×W_t/L_s^3 (Wang *et al.*, 2015)

VSI(%)= 100 ×W_v/W_t (Wang *et al.*, 2015)

IPF (%) =100 × W_{pf}/W_t (Yaghoubi *et al.*, 2016)

HSI(%) =100 ×W_l/W_t (Wang *et al.*, 2015)

SR (%)=100×Nt/N0 (Slawski *et al.*, 2011; Iqbal *et al.*, 2022)

FI (% day−1)=100×I/[(W0+Wt)/2×t] (Wang *et al.*, 2015)

FCR=I/(Wt−W0) (Wang *et al.*, 2015)

PER=(Wt−W0)/(I×CNf) (Yaghoubi *et al.*, 2016)

NRE (%)=100×(Wt×CNt−W0×CN0)/(I×CNf) (Wang *et al.*, 2015)

PRE(%)=100×(Wt×CPt−W0×CP0)/(I×CPf) (Wang *et al.*, 2015)

NW [g N (kg fish gain)−1]=1000×(I×CNf)×(1- NRE)/[(Wt−W0)×6.25] (Wang *et al.*, 2015)

PW [g P (kg fish gain)−1]=1000×(I×CPf)×(1-PRE)/(Wt−W0) (Wang *et al.*, 2015)

ECR =FCR×Cost of feed (Piedecausa *et al.*, 2007)
RC=100-(100×(ECR diet/ECR Control diet)) (Hernández *et al.*, 2014)
PI=Cf (kg)/CN (Hernández *et al.*, 2014)

Where I (g) is the total amount of the eaten experimental diets on a dry matter basis ; W0(g) is the total initial body weight and Wt (g) is the total final body weight; Ls (cm), Wv (g), Wpf and Wl(g) are the final body length, viscera weight, intraperitoneal fat weight and liver weight; t (day) is the duration of the feeding trial; Nt is the number of fish at the end of the feeding trial and N0 at the start; CNt, CPt, and CN0, CP0, are the final and initial levels (%) of crude protein, phosphorus; CNf, CPf, (%) are the crude protein, phosphorus contents of the test diets. Cf is the value of fish; CN is the cost of feed. Cost of feeds were presented in Table 1

Data analysis

The data are presented as means±standard error of the mean calculated from three replicates. The data of each parameter were tested for normality and homoscedasticity by applying Brown-Forsythe and Welch tests respectively (Iqbal *et al*., 2021). A one way analysis of variance (ANOVA) was performed with diet as the independent variable. A Tukey's HSD test was used for post hoc after a significant ANOVA (p<0.05). Data were analyzed using SPSS ver.25.0 (Chicago, Illinois, USA) and figures were prepared using Microsoft Excel 2010. Quadratic Regression analysis was done to model relationship between the measured parameters and the SBM replacement level. The result is a regression equation that can be used to predict the maximum and minimum SBM replacement levels. The equation has the form:

$y=ax\ 2+bx+c$

Where $a\neq 0$, $x= -b/2a$

Results

Experimental diets

The sesame seed cake used in this experiment was higher in crude lipid (8.6%), crude fiber, phosphorus, and calcium content than SBM. The crude lipid, crude fiber, phosphorus, and calcium contents of SSC were 7.4, 1.75, 1.5, and 2.7 times higher than in SBM, respectively. With increasing level of replacement of SBM with SSC, the dietary crude fiber was significantly increased (p<0.05, Table 1). Dietary phosphorus content was significantly higher in SSC75 and 100 than in other experimental diets (p<0.05). Dietary calcium content was significantly higher in SS75 and 100 than in SSC0 (p<0.05).

Survival, production, and nutrient utilization

The juvenile common carp remained in good health throughout the feeding trial and neither deformity nor disease was observed. Percentage SR without significant differences was 95 in SSC0 to 100% in SSC75 (p>0.05). FBW, WG, DGR and SL did not show significant differences between treatments

(p>0.05). In SSC treatments, the FI, FCR and PER did not show a significant difference with SBM-based treatment (SSC0; p>0.05; Table 2). The value of FCR in treatments containing SSC was lower than in the control treatment. The value of PER was higher in SSC treatments than in SSC0. Significantly higher NRE was observed in SSC25 in comparison with SSC0 (p<0.05). The NW in SSC treatments was lower than in the control treatment (SSC0).

Table 1: Formulation of the experimental diets were fed by juvenile *C. carpio* for 90-day

	SSC0	SSC25	SSC50	SSC75	SSC100
Dietary ingredients (g. kg^{-1})					
Tuna by-product meal	70	70	70	70	70
Poultry by-product meal	120	120	120	120	120
Soybean meal	350	262.5	175	87.5	0
Sesame seed cake	0	87.5	175	262.5	350
Wheat middlings	200	200	200	200	200
Corn meal	90	90	90	90	90
Barely meal	85	85	85	85	85
Fish Oil	25	25	25	25	25
Sunflower oil	25	20	12	5	0
Corn Starch	0	5	13	20	25
Vitamin Premix[a]	10	10	10	10	10
Mineral Premix[b]	15	15	15	15	15
Stay-C	5	5	5	5	5
Betaine	5	5	5	5	5
Dietary Proximate composition (% Dry-weight basis)					
Dry matter	93.65±0.58	93.18±0.22	92.90±0.58	92.62±0.55	92.34±0.63
Crude protein	29.60±0.14	29.97±0.17	29.34±0.28	29.70±0.26	29.47±0.37
Crude lipid	7.38±0.26	8.36±0.09	7.81±0.30	8.48±0.77	7.86±0.57
Crude fiber	2.61±0.06[e]	3.12±0.04[d]	3.62±0.08[c]	4.13±0.10[b]	4.64±0.05[a]
Ash	7.57±0.17	7.34±0.16	7.72±0.05	7.73±0.04	7.79±0.01
Phosphorus	1.50±0.07[b]	1.55±0.16[b]	1.65±0.09[b]	2.23±0.12[a]	2.50±0.09[a]
Calcium	1.92±0.05[b]	2.05±0.03[ab]	2.22±0.11[ab]	2.45±0.21[a]	2.49±0.06[a]

Composition of ingredients (% Dry-weight basis):
Tuna by product meal: 60.52% crude protein, 9.42% crude lipid, 13.09% ash, 1.52% calcium, 3.95% phosphorus
Poultry by-product meal: 55.62% crude protein, 23.38% crude lipid, 4.11% ash, 3.9% calcium, 6.62% phosphorus
Soybean meal: 42.12% crude protein, 1.16% crude lipid, 7.04% ash, 0.79% calcium, 0.73% phosphorus
Sesame seed cake: 40.32% crude protein, 8.6% crude lipid, 7.04% ash, 2.12% calcium, 1.13% phosphorus
Tuna by-product meal, poultry by- product meal, soy bean meal and the other feed ingredients were provided by the Beyza Feed mill company. a) Vitamin premix: Vit. A, 2000 IU/Kg; Vit. D3, 800 IU/Kg; Vit E, 88 IU/Kg; Vit K, 3 mg/kg; Vit C, 200 mg/kg; Vit B1, 12 mg/kg; Vit B2, 14 mg/kg; Vit B5, 70 mg/kg; Vit B3, 50 mg/kg; Vit B6, 12 mg/kg; Vit B9, 3 mg/kg; Vit B12, 0.016 mg/kg; Vit H2, 0.14 mg/kg. Damloran Pharmaceutical Company, Broujerd, Iran. b) Mineral Premix: Selenium, 0.168 mg/kg; Iron sulfate, 20 mg/kg; Copper sulfate, 2 mg/kg; Calcium iodate, 2 mg/kg; Zinc oxide,33.2 mg/kg; Cobalt, 0.336 mg/kg; Manganese oxide, 16.8 mg/kg. c) SSC.CP: The portion of sesame seed cake protein in crude protein; d) SSC. TDP: The portion of sesame seed cake protein in total dietary protein.

A significantly lower NW was observed in SSC25 and SSC50 treatments (p<0.05). Significant differences were not detected in PRE between fish fed

SCC0 and other dietary treatments (p>0.05). A significantly lower PW was measured in SSC25 and SSC50 than in SSC0 (p<0.05). According to the quadratic regression, 47.81–56.34% replacement of SBM by SSC could result in the highest FBW (Table 2; Fig. 1), PER (Fig. 2) and the lowest values of PW (Fig. 3).

The price of experimental diets presented in Table 1. The value of ECR decreased with increasing levels of SSC. RC of SSC100 was about 35.10% in comparison with the control diet. PI increased with an increased replacement level of dietary SBM.

Table 2: Growth performance and feed utilization of juvenile *C. carpio* and economic indexes of experimental diets and quadratic regression between experimental diets and FBW and PER and PW

Indices	SSC0	SSC25	SSC50	SSC75	SSC100
Growth performance					
IBW (g)	83.09±1.21	83.29±1.13	83.44±1.12	83.00±1.12	83.28±1.17
FBW (g)	169.99±2.70	179.27±3.00	177.87±0.81	173.95±3.63	170.96±2.27
WG (g)	86.90±1.56	95.99±3.98	94.43±2.87	90.95±1.10	87.68±2.78
DGR (g.day^{-1})	0.97±0.05	1.07±0.07	1.05±0.06	1.01±0.04	0.97±0.04
SL (cm)	15.78±0.17	15.57±0.21	15.45±0.22	15.71±0.18	15.28±0.21
SR (%)	95.56±4.44	97.78±2.22	97.33±3.85	100.00±0.00	97.78±2.22
feed utilization					
FI (%.day^{-1})	1.26±0.01	1.22±0.01	1.22±0.00	1.26±0.02	1.24±0.02
FCR	1.66±0.05	1.50±0.05	1.52±0.02	1.64±0.04	1.59±0.07
PER	2.01±0.06	2.23±0.07	2.19±0.02	2.13±0.05	2.10±0.08
NRE (%)	28.40±2.28^{b}	36.10±1.44^{a}	33.57±1.42^{ab}	29.82±82^{ab}	29.72±3.32^{ab}
PRE (%)	16.55±1.75	20.0±2.68	21.99±0.69	18.85±2.06	19.55±0.42
NW (%)	56.94±1.81^{a}	45.92±1.03^{c}	48.45±1.04^{bc}	53.32±1.00^{ab}	53.46±2.53^{ab}
PW (%)	13.83±0.29^{a}	11.92±0.40^{b}	11.85±0.10^{b}	12.46±0.34^{ab}	12.75±0.07^{ab}
Dietary price (Rial)	166805	156680	146255	135930	125805
Economic indexes					
ECR	19389.63	16002	14630.76	14092.52	12053
RC	0	8.76	17.59	26.43	35.10
PI	2.75	2.81	3.12	3.49	3.96
Quadratic regression					
	R^2	B1	B2	Percentage of replacement	Regression P-value
FBW	0.795	-30.926	29.574	Xmax=47.81	0.002
PER	0.784	-0.789	0.889	Xmax=56.34	0.002
PW	0.8185	5.806	-6.454	Xmin=56	0.002

A different superscript in the same row denotes statistically significant differences (p<0.05).

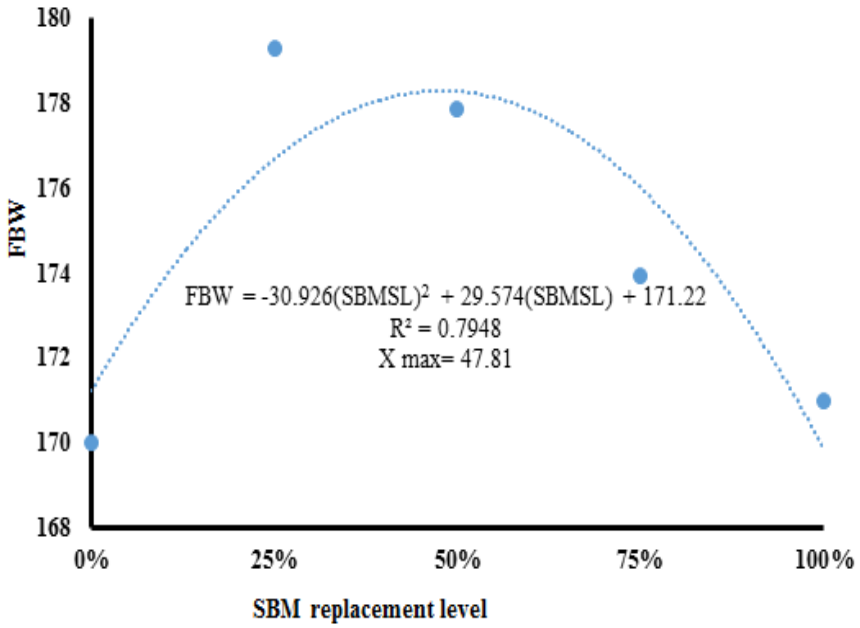

Figure 1: The relationship between final body weight (FBW) and dietary SBM replacement level (SBMSL) with SSC in juvenile common carp diet.

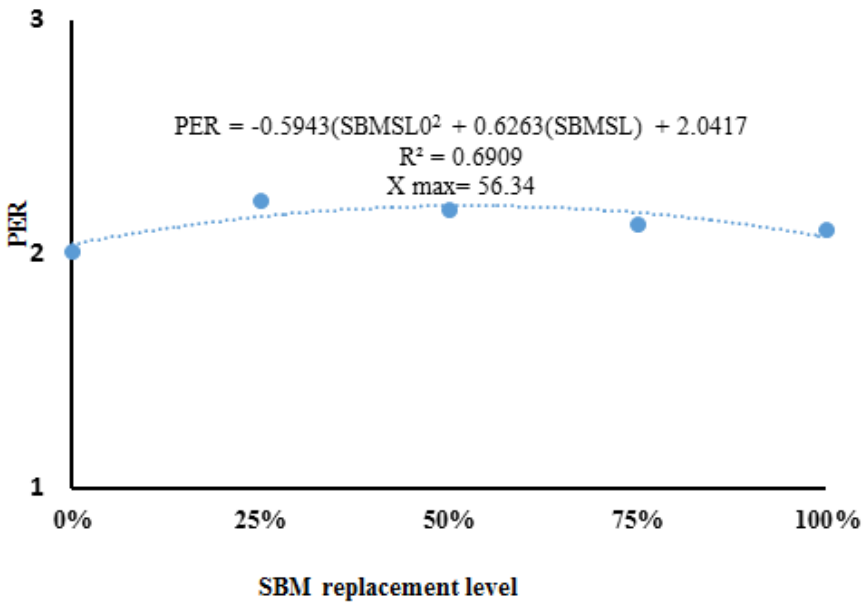

Figure 2: The relationship between protein efficiency ratio (PER) and dietary SBM replacement level (SBMSL) with SSC in juvenile common carp diets.

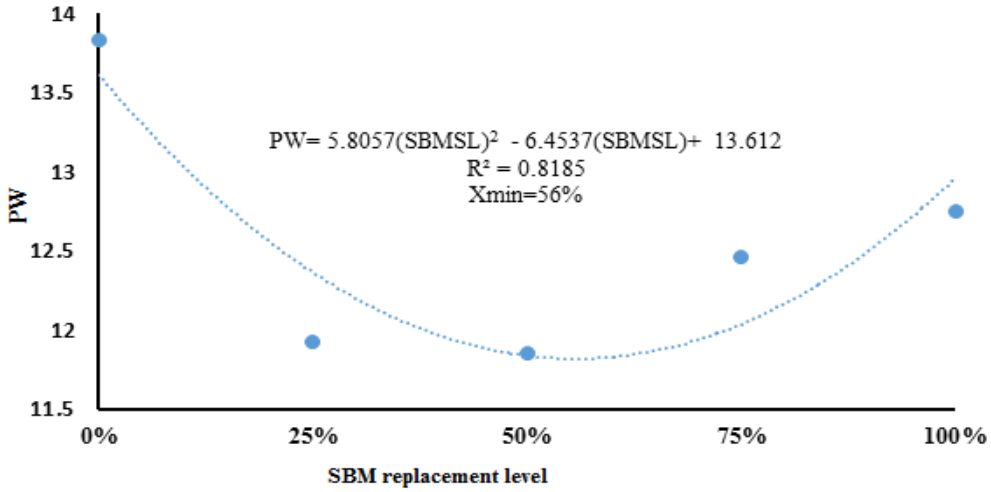

Figure 3: The relationship between phosphorous wastage (PW) and dietary SBM replacement level (SBMSL) replacement with SSC in juvenile common carp diets.

Body composition

The value of VSI, IPF, HSI, and CF did not differ significantly between experimental treatments. IPF and HIS were higher in SSC treatments than in the control treatment. HIS varied from 1.04 in SSC0 to 1.54 in SSC75. IPF ranged from 0.64 to 0.92. The whole body proximate biochemical composition such as moisture, protein, lipid, ash, and calcium, did not show significant differences between treatments (p>0.05; Table 3). Common carp's total protein, lipid, and ash content ranged from 42.42% to 45.89%, 27.65 to 31.31, and 14.73 to 15.72, respectively. Phosphorus content was significantly higher in SSC75 than in SSC100 (p<0.05).

Table 3: Morphometric indices, whole body, fillet and liver proximate composition of common carp fed the experimental diets (means± SE, n=3).

Diets	SSC0	SSC25	SSC50	SSC75	SSC100
Morphometric indices					
CF(%)	1.84±0.04	1.81±0.05	1.79±0.06	1.82±0.05	1.75±0.05
VSI	19.76±1.28	17.44±1.67	18.58±1.05	15.07±2.25	18.49±1.05
IPF	0.64±0.21	0.84±0.25	0.92±0.32	0.68±0.19	0.72±0.20
HSI	1.04±0.32	1.06±0.24	1.43±0.21	1.54±0.36	1.32±0.32
Whole body proximate composition					
Moisture	66.02±1.98	63.01±1.08	62.20±0.88	64.31±0.87	63.77±0.29
*Crude Protein	45.89±0.86	45.14±0.34	42.42±0.56	42.50±0.50	42.76±1.55
*Crude Lipid	27.64±1.39	27.85±1.41	29.03±1.68	31.13±1.58	30.20±1.3
*Ash	15.72±0.21	15.55±0.26	14.73±0.16	15.22±0.55	15.14±0.23
Calcium	1.73±0.21	1.80±0.11	1.80±0.32	2.08±0.32	1.57±0.14
Phosphorus	2.02±0.05^{ab}	1.89±0.09^{ab}	1.97±0.04^{ab}	2.06±0.06^{a}	1.79±0.02^{b}
Fillet proximate composition					
Moisture	67.40±0.95	65.94±2.45	64.60±1.66	65.77±3.16	68.94±0.17
*Crude Protein	53.96±0.83^{b}	55.27±0.73^{ab}	54.98±1.20^{ab}	57.62±0.72^{a}	54.96±0.75^{ab}
*Crude Lipid	33.52±1.50	33.69±1.45	34.03±1.27	34.30±1.10	34.32±1.45
*Ash	3.25±0.25	3.18±0.22	3.21±0.13	3.44±0.29	3.53±0.12
Liver proximate composition					
Moisture	71.78±2.61^{a}	61.12±1.44^{ab}	66.70±4.30^{ab}	58.71±0.62^{b}	66.17±0.86^{ab}
Crude Lipid	43.67±1.05^{b}	50.70±0.84^{a}	46.07±0.39^{b}	51.73±1.07^{a}	44.85±0.91^{b}

* % of dry weight. A different superscript in the same row denotes statistically significant differences (P<0.05).

Moisture, lipid, and ash of fish fillets fed different levels of SSC did not show significant difference with fish-fed control diet (p>0.05). Compared to the control treatment, significantly higher fish fillet protein was observed in SSC75 (57.62±0.72%; p<0.05). The Lipid content of the liver was significantly higher in SSC25 and SSC75 than in other treatments (p<0.05). The liver moisture was significantly higher in the control treatment than in SSC75 (p<0.05).

Blood parameters

There was no significant difference between the treatments in RBC, Hct, MCV, MCH, and MCHC (p>0.05; Table 4). The RBC was higher in SSC50, 75, and SSC100 than in the control treatment. The highest percentage of hematocrit was recorded in SSC100 (p>0.05). The amount of hemoglobin was significantly lower in the control treatment (9.31±0.58 g. dL^{-1}) than in the SSC75 (11.95±0.71 g.dL^{-1}). The hemoglobin and hematocrit percentage in treatments containing SSC were higher than in the control treatment. Serum albumin, globulin, total protein, glucose, triglyceride (TG), calcium, and phosphorus, did not significantly differ between experimental treatments (p>0.05; Table 4). Serum CHO was significantly lower in SSC0 than in SS25 (p<0.05). The serum calcium and phosphorus did not significantly differ between treatments (p>0.05). The serum phosphorus was lower in SSC0 than in other treatments.

Table 4: Blood and serum biochemical parameters of juvenile *C. carpio* fed the experimental diets (means ± SE, n = 3).

Diets	SSC0	SSC25	SSC50	SSC75	SSC100
Hematological indices					
RBC	1884166.67± 98122.21	1879166.67± 56858.51	2015000.00± 77689.16	1955000.00± 69788.64	2033333.33± 83014.54
Hct (%)	35.92±1.04	39.0±0.88	37.75±0.82	36.17±1.00	39.83±1.51
Hem*	9.31±0.58^{b}	9.53±0.28^{b}	10.17±0.21^{ab}	11.95±0.71^{a}	10.84±0.39^{ab}
MCV	195.93±10.65	210.12±9.30	190.11±7.54	186.02±4.42	201.98±15.38
MCH	44.95±2.79	50.08±2.12	52.75±3.41	60.65±4.37	53.59±5.55
MCHC*	26.55±2.55	26.63±1.43	27.37±0.99	32.50±2.79	26.82±1.05
Serum biochemical composition					
Albumin*	1.11±0.18	1.25±0.19	1.15±0.07	1.187±0.25	1.24±0.19
Globulin*	1.86±0.47	2.42±0.16	1.96±0.29	2.22±0.43	1.90±0.13
Total protein*	2.96±0.29	3.67±0.08	3.11±0.22	3.40±0.26	3.14±0.22
Glucose**	83.67±9.17	82.00±12.50	78.33±8.33	79.33±10.90	93.00±9.29
Cholesterol**	115.00±4.36^{b}	133.00±1.53^{a}	125.00±5.77^{ab}	126.00±3.06^{ab}	124.33±2.91^{ab}
Triglyceride**	229.00±11.27	230.67±19.24	210.00±6.66	218.00±12.86	217.67±14.91
Calcium**	8.97±0.23	9.30±0.36	8.87±0.58	9.63±0.27	8.80±0.47
phosphorus*	4.97±0.61	7.40±0.78	6.73±1.56	6.47±0.34	5.97±0.18

unit *(g dL1) and ** (mg dl^{-1}). RBC: Red blood cell, Hct: Hematocrit, Hem: Hemoglobin, MCV: Mean corpuscular volume, MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration, A different superscript in the same row denotes statistically significant differences (p< 0.05).

Discussion

In the present study, based on feed intake, it could be confirmed that the use of SSC did not have a negative effect on feed attractiveness. Also, the bioavailability of nutrients was not affected by the attractiveness of the experimental diets. In our study, changes

in PER showed an analogous trend to GP. The existence of a positive relationship between PER and GP was attributed to the opportunity for nutrition to the satiation, which ensures that the fish receives adequate food (Zhou *et al.*, 2010). In our trial, treatment with lower NRE was consistent with treatment with lower GP. Quadratic regression analysis of growth and feed performance against soybean meal replacement level revealed that around 50-60% (47.81-56.34%) replacement level resulted in maximized feed utilization and growth performance. Increasing protein absorption and decreasing its excretion leads to higher GP (Green and Hardy 2002). Kumar *et al.* (2020) and Arriaga-Hernández *et al.* (2021) suggested that the reduction in GP and NE of fish fed high levels of SBM could be attributed to high level of anti-nutritional factors such as beta-conglycinin, phytate, saponins, lectins and indigestible carbohydrates, trypsin inhibitor, lower protein digestion and lower amino acid absorption. The results of other study, showed the effect of trypsin inhibitor on reducing NRE, and increasing metabolic NW (Kader *et al.*, 2012). Kumar *et al.* (2020) observed higher crude protein content and NRE in fish fed dietary SBM contains lower trypsin inhibitors, lectin, oligosaccharides, and carbohydrate. The researchers reported that at low levels of plant protein sources, physiological mechanisms in fish could offset the negative effects of anti-nutritional substances (Jimoh and Aroyehun, 2011). In our trial, the phosphorus content of dietary SSC was 1.5 times that of SBM. Treatments containing SSC showed higher phosphorus, PRE, GP and lower PW than SBM-based treatment.

The dietary SSC helps reduce phosphorus pollution of the aquatic environment by improving the phosphorus bio-availability, increasing PRE, and reducing the demand for phosphorus supplementation in aquafeed (Yoo *et al.*, 2005). The researchers have cited that amino acid deficiency in SBM is not a growth-limiting factor, but a lack of phosphorus bio-availability reduces the growth rate (Brown *et al.*, 1997; El-Sayed and Tacon 1997). In several studies, the use of high levels of SBM and its products significantly reduced the dietary phosphorous digestibility and PRE (Yoo *et al.*, 2005; Biswas *et al.*, 2019). A decrease in phosphorus efficiency, NRE, and PRE were observed in fish fed high levels plant protein levels, which may due to high levels of phytic acid (Plaipetch and Yakupitiyage, 2014). Guo *et al.* (2011) reported that the phytic acid content of SSC was less than half that of SBM. Removal of phytin with microbial phytase from soy protein increased PRE and NRE (Plaipetch and Yakupitiyage, 2014; Biswas *et al.*, 2019).

Morphometric indices of *C. carpio* did not significantly differ between dietary treatments. In other studies, lower levels of SBM replacement (50-60%) with plant protein sources did not affect the visceral indices of Nile tilapia (Guo *et al.*, 2011) and hybrid tilapia, *O. niloticus* × *Oreochromis aureus* (Yue and Zhou, 2008). In the present study,

the liver moisture was lower, but liver lipid, whole body lipid, fillet lipid content, and HIS was higher in SSC treatments than in SBM-based treatment. Dernekbaşı *et al.,* (2017a), cited that the increase in body lipid at high levels of SS meal could be due to the higher lipid content of SS meal than SBM. The crude fiber content of SSC was almost twice that of SBM. The treatments with higher crude fiber had higher HSI, liver, whole body, and fillet lipid. The main part of the polysaccharides extracted from sesame seed includes arabinose, glucose, xylose, galactose, and mannose with trace amounts of rhamnose. The occurrence of xylose, glucose and fucose suggested the possibility of xyloglucans (Ghosh *et al.,* 2005). Improved growth performance and feed utilization in kutum (*Rutilus frisii* kutum) fed dietary galactooligosaccharide and xylooligosaccharide as fermentable fiber were reported by Kumar *et al.* (2008) and Hoseinifar *et al.* (2013). The fermentable fraction of the dietary fiber enhanced the production of metabolites such as short chain fatty acids (Yarahmadi *et al.,* 2014; Mirghaed *et al.,* 2018). Fillet protein was significantly higher in fish fed SSC75 diet than in fish fed SBM-based diet. Dernekbaşı *et al.* (2017), reported that the body protein content increased with increasing sesame seed meal level in replacement with soybean meal. The researchers attributed the decrease in body protein to a decrease in PER and NRE (Kader *et al.,* 2012; Dernekbaşı *et al.,* 2017) and lack of complete absorption of dietary protein in the body (Egerton *et al.,* 2020).

The RBC, Hem, Hct, MCH, and MCHC value were higher in the SSC treatments than in the SBM-based treatment. Similar to our observations, higher blood indices were reported in fish fed diets containing lower levels of SBM (Zheng *et al.,* 2012; Lawal *et al.,* 2016; Viana *et al.,* 2019). Higher Hem and RBC are indicators of the health (Ye *et al.,* 2019) and higher oxygen-carrying capacity in fish fed this level of nutrients (Nazir *et al.,* 2021). It could also be stated that the total SBM replacement with SSC did not result in symptoms of diet-induced anemia (Demir *et al.,* 2014; Grant 2015; Nazir *et al.,* 2021). Decreased blood factors in fish fed SBM diet were attributed to the effects of ANs such as phytic acid (Hassaan *et al.,* 2018), which may lead to iron mal-absorption in fish intestines (Barros *et al.,* 2002; Kasiga 2018; Ye *et al.,* 2019). SBM contains lectin, phyto-hemagglutinin, and saponin (NRC 1993; Francis *et al.,* 2001), which have a high capacity for clotting and hydrolysis of red blood cells (Lim and Lee 2009; Lim *et al.,* 2011). Sesame seed is almost devoid of the above ANs (Wei *et al.,* 2022). The serum protein and NRE were higher in SSC treatments than in the control treatment. Similar to the present study, high levels of dietary soy products reduced serum protein level (Xu *et al.,* 2012; Shamna *et al.,* 2017; Nazir *et al.,* 2021). No significant difference in serum albumin and globulin content of dietary treatments could indicate that the alternative plant protein source did not

affect the content of some components of the intrinsic defense mechanism such as total protein, albumin, and globulin (Magnadóttir 2006; Shahsavani *et al.,* 2010; Soltanzadeh *et al.,* 2016). The absence of significant differences in serum glucose content, showed that fish were not affected by replacing two plant protein sources and are in good and stress-free conditions (Svoboda *et al.,* 2001; Wagner and Congleton 2004). It also indicates that the sesame seed cake did not affect energy metabolism (Zhou *et al.,* 2005; Soltanzadeh *et al.,* 2016). The serum phosphorus in fish-fed SSC diets was higher than in the fish-fed SBM-based diet. Similarly, it was reported that plasma phosphorus decreased due to the reduced availability of phosphorus in a diet containing high soy content (Lim *et al.,* 2011; Yaghoubi *et al.*, 2016). Most phosphorus in soy is associated with phytic acid and is unavailable (Liener 1994; Yaghoubi *et al.,* 2016). The results showed no significant differences in serum calcium between treatments. Half of all plasma calcium is combined with plasma protein (Andreasen, 1985); it seems that the absence of significant differences in plasma protein levels may lead to a stable plasma calcium concentration between experimental treatments. In the present feeding trial, compared to the SBM-based treatment (350 $g.kg^{-1}$ SBM), the treatments containing SSC, had higher serum cholesterol, HSI, and IPF. Similar to our observations, it was reported that soy products reduced the activity of fat-degrading enzymes and interrupted the absorption of bile acids and total cholesterol in fish diet (Makino *et al.,* 1988), consequently reducing serum total cholesterol levels in European seabass (*Dicentrarchus labrax*; Dias *et al.,* 2005). This is mainly due to the fat-reducing effect and high levels of estrogenic iso-flavones, phytates, saponins, phytosterols in the soy products which may interfere with various stages of fat digestion, including emulsion, hydrolysis, binding of fatty acids to micelles, reabsorption and re-esterification in intestinal cells (Setchell and Cassidy, 1999; Biswas *et al.,* 2019).

In conclusion, juvenile *Cyprinus carpio* fed SSC diets showed higher blood indices, serum phosphorus, phosphorous retention efficiency, growth performance and lower phosphorous wastage than fish fed SBM-based diet as a consequence of lower ANs such as phytic acid in the diets. Also SSC treatments had higher crude fiber, as well as higher lipid of liver, whole body, and fillet, serum cholesterol, HSI, and IPF as a consequence of the higher fermentable fraction of the dietary fiber and due to the fat-enhancing effect and lower levels of some ANs such as estrogenic iso-flavones, phytates, saponins, phytosterols in the SSC. Comparable growth performance and measured physiological responses indicated that SBM could be replaced by 75 to 100% SSC in the formulated diet of *C. carpio* juveniles.

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Research Article

Effect of 17α-methyl testosterone, tamoxifen, and letrozole on growth performance and sex reversal of rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Sex reversal and producing a monosex population is one of the most preferred growth promotion techniques of rainbow trout culture. Thus, the effects of 17α-methyltestosterone (2 mg/kg), tamoxifen (2, 20, and 100 mg/kg), letrozole (2, 20, and 100 mg/kg), and a combination of tamoxifen (100 mg/kg) and letrozole (100 mg/kg) on growth, masculinization and serum steroid content of rainbow trout were investigated in this research. Ethanol-dissolved chemicals were sprayed on commercial trout diet, and ethanol was evaporated overnight. The fish were fed the treated diet for two months and afterward, they were fed a commercial diet for six months. Results showed that 17α-methyltestosterone reversed the sex of rainbow trout effectively. The proportion of males, intersex, and females in this group were 76.67%, 10%, and 13.33%, respectively. In contrast with 17α-methyltestosterone, using tamoxifen and letrozole showed no effect on sex reversal of rainbow trout. Growth performance was adversely affected by all chemical-treated diets. However, compensatory growth occurred during first month after ending treatment period.

Keywords: Masculinization, Rainbow trout, Sex reversal

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Introduction

Sex determination is strongly related to genes in higher vertebrates; consequently, it is challenging to reverse the sex in these vertebrates. Conversely, in fish sex differentiation process can be easily affected by internal and external conditions (Piferrer *et al*., 1994). Thus, for sex reversal in fish, oral administration of steroid hormones is used by aquaculturists.

Fish sex is separated into genotype, determined by the genes responsible for gonads formation, and phenotype, the appearance of ovary or testis. The genotype of sex, related to genes, is determined at the time of fertilization, whereas, phenotype differentiation, the appearance of male or female sex organs, occurs in embryonic and larval development stages. This process is changed with administration of exogenous androgens and estrogens. Because monosex population of some cultured species, having a higher growth rate in comparison with mixed sexes, it is preferred by aquaculturists. For example, male tilapia, female common carp, and female rainbow trout are preferred for culture.

Monosex populations can be achieved either by directly treating fish with hormones to produce preferred sex or by indirect method with two steps; in the first step, embryos/ larvae/ juveniles are treated with androgens or estrogens to produce neo male (XX♂), neo female (XY♀, ZZ♀), or super male (YY♂) populations. In the second step, these sex-reversed fish are used as breeders for producing all female or all-male populations (Hoga *et al*., 2018). Similar to humans, sex determination system in rainbow trout is XX/XY type; thus, to obtain all-female population, XX males are produced by administration of androgens in the first step, and these sex-reversed males are used for mating with normal females in the second step. Among androgens, 17α-methyltestosterone (MT) is numerously used for producing neo male population of teleost fishes (Asadi Eidivand et al., 2022).

In addition, effects of chemicals such as tamoxifen (TM) and letrozole (LZ), with anti-estrogenic activities, on sex differentiation process and sex reversal have been studied in many species of vertebrates (Singh and Srivastava, 2015; Alijani *et al*., 2022**)**. It is well demonstrated that TM and LZ suppress estradiol production in mammals (Bhatnagar *et al*., 2001; Simpson *et al*., 2002) and fish (Kwon *et al*., 2000; Sun *et al*., 2007; Singh *et al*., 2012). Based on acting mechanisms and pathways, there are two groups of anti-estrogenic chemicals. Chemicals, including TM, directly affect actions of estrogens because they inhibit the binding of estrogens to estrogen receptors. Additionally, some studies indicated that masculinization effect of TM is related to suppression of ovary-type P450arom mRNA expression (Kitano *et al*., 2007; Hulak *et al*., 2010). In contrast to the first group, chemicals like LZ act indirectly by inhibiting the excretion of estrogens. In this process, LZ inhibits aromatase activities, the enzyme that plays a crucial role in turnover of estrogens (Sun *et al*.,

2011). Consequently, ovarian development is suppressed, and sex differentiation leads to development of testis (Singh and Srivastava, 2015).

However, the efficacy of TM and LZ is yet to be evaluated in rainbow trout. Thus, effects of tamoxifen (a receptor blocker), letrozole (an aromatase inhibitor), and 17α-methyltestosterone on growth, masculinization, and serum steroid content of rainbow trout were investigated in this study.

Material and methods

Fish and rearing system

All-female larvae of rainbow trout before yolk sac uptake were transferred to Urmia University by nylon bags containing water and oxygen (1:3 ratios) from Rashekan rainbow trout hatchery. After two days of adaptation, the larvae were randomly distributed among 27 tanks, 125 individuals per 25-liter tank (three replicates per treatment). Water flow rate of 1.3-2.9 l/m and an aeration system were established for each tank in a flow-through system. Water temperature, pH, and dissolved oxygen values were 13.1-15.3°C, 7.85-8.04, and 7.96-8.22 mg/L, respectively. The fish were kept in these conditions for 2.5 months. Afterward, all fish were weighed, counted, and transferred to 200-liter tanks. Water flow rate of 5.7-6.4 l/m and an aeration system were established for each tank in a flow-through system. Water temperature, pH, and dissolved oxygen values were 14.8-15.6°C, 7.94-8.08, and 7.11-7.84 mg/L, respectively (Table 1). Under these conditions, the fish were raised for 5.5 months.

Table 1: Physico-chemical factors of the rearing water during experimental period.

Treatment	Temperature (°C)		pH		DO (mg/L)		water flow rate (l/m)	
	1-75 daf	76-240 daf	1-75 daf	76-240 daf	1-75 daf	76-240 daf	1-75 daf	76-240 daf
C	14.3±0.75	15.1±0.26	7.96±0.08	7.99±0.05	8.07±0.11	7.53±0.25	2.13±0.63	6.07±0.23
M	14.1±0.77	15.3±0.29	7.94±0.08	7.98±0.05	8.11±0.13	7.69±0.31	2.06±0.65	5.95±0.25
T2	14.2±0.72	15.1±0.24	7.93±0.09	8.04±0.04	8.05±0.10	7.59±0.24	2.10±0.62	6.01±0.31
T20	14.3±0.70	15.2±0.22	7.96±0.08	8.00±0.04	8.08±0.11	7.42±0.20	2.03±0.59	6.21±0.20
T100	14.3±0.73	15.0±0.28	7.97±0.07	7.98±0.05	8.01±0.14	7.61±0.27	2.25±0.63	6.17±0.26
L2	13.9±0.69	15.3±0.30	7.91±0.09	7.97±0.06	8.05±0.10	7.54±0.21	2.08±0.66	6.13±0.28
L20	14.1±0.74	15.1±0.25	7.96±0.09	8.04±0.04	8.07±0.11	7.52±0.19	2.33±0.60	6.05±0.23
L100	14.3±0.74	15.2±0.27	7.92±0.08	8.01±0.05	8.13±0.09	7.46±0.25	2.16±0.62	6.01±0.22
T/L100	13.9±0.71	15.1±0.20	7.90±0.07	7.98±0.04	7.97±0.15	7.71±0.32	2.19±0.65	5.99±0.24

Data represents mean ± standard deviation; treatments include C: control, M: 17α-methyltestosterone, T2: 2 mg tamoxifen/kg feed, T20: 20 mg tamoxifen/kg feed, T100: 100 mg tamoxifen/kg feed, L2: 2 mg letrozole/kg feed, L20: 20 mg letrozole/kg feed, L100: 100 mg letrozole/kg feed, and T/L100: 100 mg tamoxifen and 100 mg letrozole/kg feed; daf: days after initiation of feeding.

Experimental feed

The alcohol evaporation method was used to prepare the chemical-treated diets (Navarro-Martín *et al*., 2009). 17α-methyltestosterone (Aburaihan Pharmaceutical Co., Tehran, Iran), tamoxifen (Iran Hormone Pharmaceutical Co., Tehran, Iran), and letrozole (Soha Pharmaceutical Co., Tehran, Iran) after dissolving in 95% ethanol, were sprayed on the commercial trout diet (Faradaneh Co., Tehran, Iran). 95% ethanol was added to the diet of the control group without the chemicals. Alcohol of the diets was evaporated at room temperature overnight then stored at 4°C in refrigerator. There were nine treatments (with triplicates) containing 0 (control), 2 mg MT/kg feed (M), 2, 20, and 100 mg TM/kg feed (T2, T20, and T100, respectively), 2, 20, and 100 mg LZ/kg feed (L2, L20, and L100, respectively), and combination of 100 mg TM/kg feed and 100 mg LZ/kg feed (T/L100). The fish were fed with treated diets for two months after initiation of the exogenous feeding.

Sampling and measurements

A portable multimeter (WTW, Multi 3630 IDS, Weilheim, Germany) was used to record rearing water temperature, pH, and dissolved oxygen. At the end of the experiment, body weight of all fish from each tank were measured. Weight gain was calculated using the formula "Weight gain=Final weight–Initial weight". The formula "SGR (%day^{-1})=100×(lnWt-lnW0)/t" was applied for calculating specific growth rate (SGR), where Wt and W0 are final and initial weight, respectively, and t is growth time in days (Gisbert and Williot, 1997). Growth retarding rate was calculated using the formula "GRR=(Wc–Wt)/Wc", where Wc and Wt represent mean body weights of control and experimental groups, respectively. For calculating GRR values, means of replicates were used (Shen *et al*., 2015). GSI values were measured using the formula "GSI=(gonad weight/body weight)×100".

For hormonal and histological analyses, 30 fish from each treatment were sampled randomly at the end of the experimental period. The fish were anesthetized with 500 mg/L carnation, and the blood of six specimens of each group was pooled. The samples allowed clotting in serum separator tubes for two hours at room temperature. The samples were centrifuged at 1000 rpm for 15 min at 4°C to obtain serum and stored at -80°C in freezer. Commercial kits (Monobind Inc., Lake Forest, USA) and microplate reader (BioTek, Synergy HT, USA) were used to estimate the serum 17β- estradiol and testosterone.

The fish were dissected after collecting blood. Gonads were weighed and fixed in Bouin solution. Standard histological techniques and microscopic analyses were used for sex determination of experimental fishes (Shen *et al*., 2015).

Statistical analysis

SPSS 22 was used for all statistical analyses of data. Levine's Test was used for Homogeneity of variances testing,

and Shapiro-Wilk test was used for normality of distribution testing. One-way ANOVA was used to analyze the mean values, then Duncan's *post-hoc* Test was used to separate significantly different groups. All analyses were performed at α=0.05 (Irani and Noori, 2020).

Results

Growth and survival

Values of body weight in the control group were significantly more than T/L100 group 15 days after initiation of the experiment (Fig. 1). In contrast, there were no significant difference among the control, M, T100, L100, and T/L100 groups 60 days after initiation of the experiment (at the end of the hormonal treatment period). Similar results were observed 15 days after ending the hormonal treatment period. There was no significant difference among all groups in the rest of the experimental period (Fig. 2).

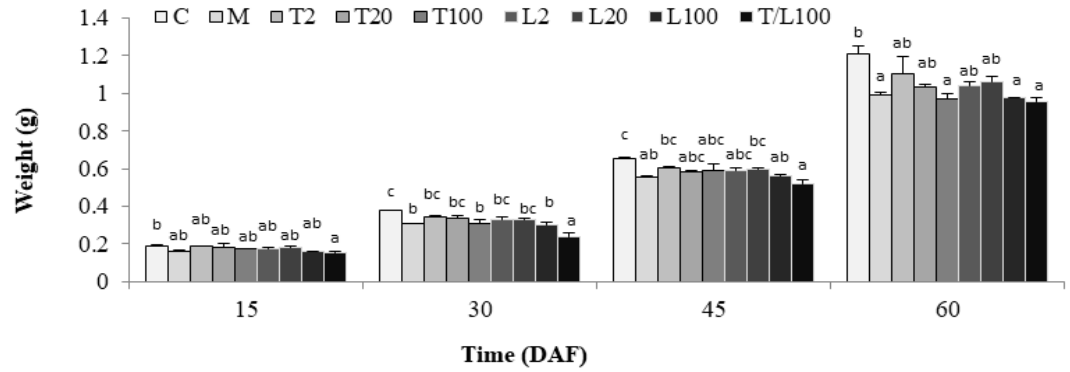

Figure 1: Mean values of rainbow trout body weight during hormonal treatment period. Error bars show standard deviation. Different superscripts represent significant differences among treatments, one-way ANOVA, α<0.05, C: control, M: 17α-methyltestosterone, T2: 2 mg tamoxifen/kg feed, T20: 20 mg tamoxifen/kg feed, T100: 100 mg tamoxifen/kg feed, L2: 2 mg letrozole/kg feed, L20: 20 mg letrozole/kg feed, L100: 100 mg letrozole/kg feed, and T/L100: 100 mg tamoxifen and 100 mg letrozole/kg feed; DAF: days after initiation of feeding.

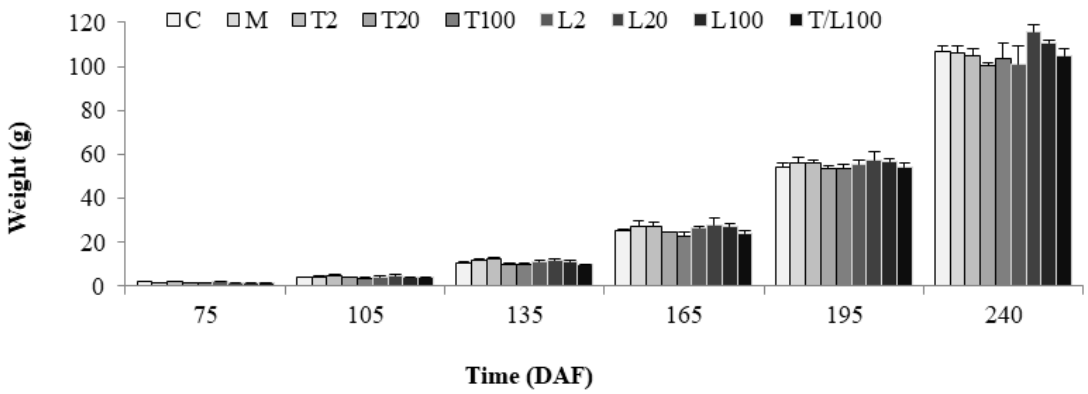

Figure 2: Mean values of rainbow trout body weight after hormonal treatment period. Error bars show standard deviation. C: control, M: 17α-methyltestosterone, T2: 2 mg tamoxifen/kg feed, T20: 20 mg tamoxifen/kg feed, T100: 100 mg tamoxifen/kg feed, L2: 2 mg letrozole/kg feed, L20: 20 mg letrozole/kg feed, L100: 100 mg letrozole/kg feed, and T/L100: 100 mg tamoxifen and 100 mg letrozole/kg feed; DAF: days after initiation of feeding.

Growth suppression, especially in M, T100, L100, and T/L100 groups, started 15 days after initiation of the hormonal treatment. Growth retarding rate increased during the first month, while it was almost constant during the second month (Fig. 3). Compensatory growth occurred 45 days after ending the hormonal treatments, as there were no significant difference in the growth performances among the groups from 105 DAF onwards.

SGR values were low on 15 DAF and increased afterward (Fig. 4). The values decreased gradually from 60 DAF onwards. There were significant differences between the control and T/L100 groups on 15 and 30 DAF.

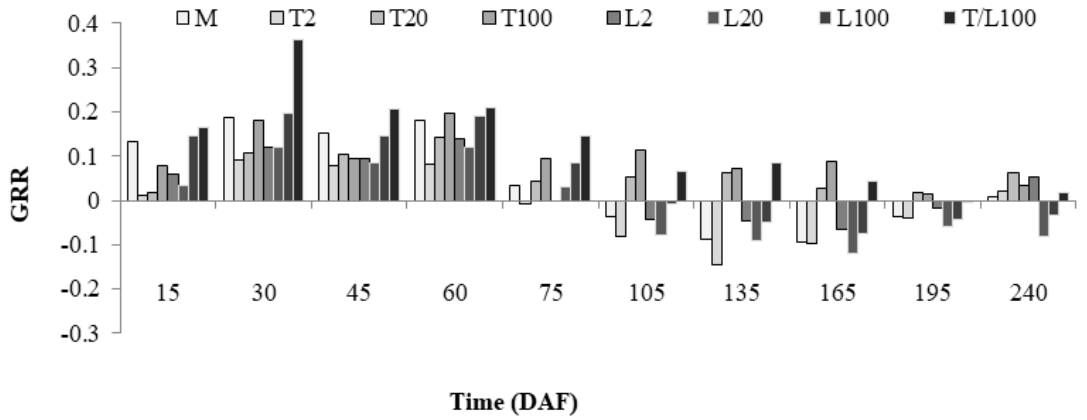

Figure 3: Growth retarding rate of rainbow trout body weight during experimental period, M: 17α-methyltestosterone, T2: 2 mg tamoxifen/kg feed, T20: 20 mg tamoxifen/kg feed, T100: 100 mg tamoxifen/kg feed, L2: 2 mg letrozole/kg feed, L20: 20 mg letrozole/kg feed, L100: 100 mg letrozole/kg feed, and T/L100: 100 mg tamoxifen and 100 mg letrozole/kg feed; DAF: days after initiation of feeding.

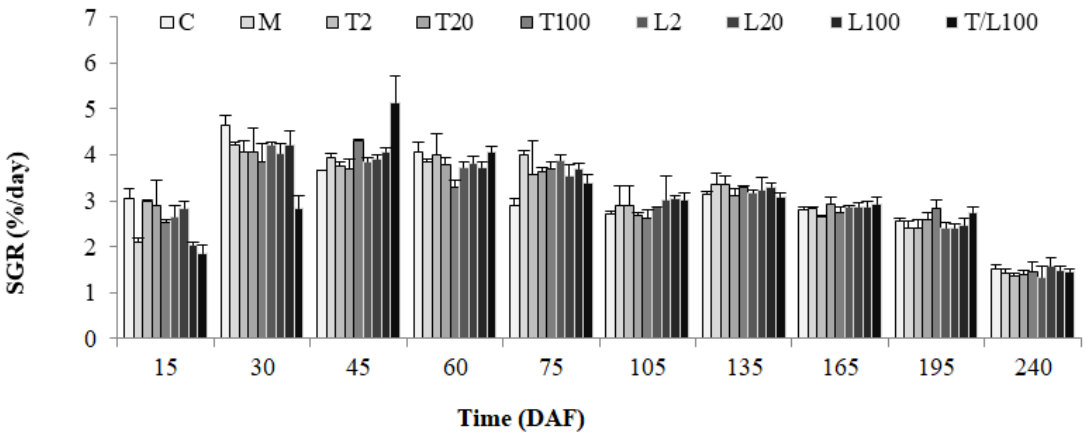

Figure 4: Mean SGR values of rainbow trout during the experimental period; error bars show standard deviation. C: control, M: 17α-methyltestosterone, T2: 2 mg tamoxifen/kg feed, T20: 20 mg tamoxifen/kg feed, T100: 100 mg tamoxifen/kg feed, L2: 2 mg letrozole/kg feed, L20: 20 mg letrozole/kg feed, L100: 100 mg letrozole/kg feed, and T/L100: 100 mg tamoxifen and 100 mg letrozole/kg feed; DAF: days after initiation of feeding.

With exception of 45 DAF, lowest FCR values were observed in control group during the hormonal treatment period (Fig. 5). Values of M, T100, L100, and

T/L100 groups were higher than those of other groups during this period, whereas there were no significant difference among the experimental groups from 75 DAF onwards.

Sex reversal and GSI

Histological examination of gonads showed that sex reversal occurred only in the group treated with 17α-methyltestosterone. Proportions of males, intersex, and females in this group were 76.67%, 10%, and 13.33%, respectively.

Mean GSI values in males of group M were significantly more than those in other groups. In contrast, the values in females of this group were significantly lower than those in other groups (Fig. 6). There was no significant difference among the rest of the groups.

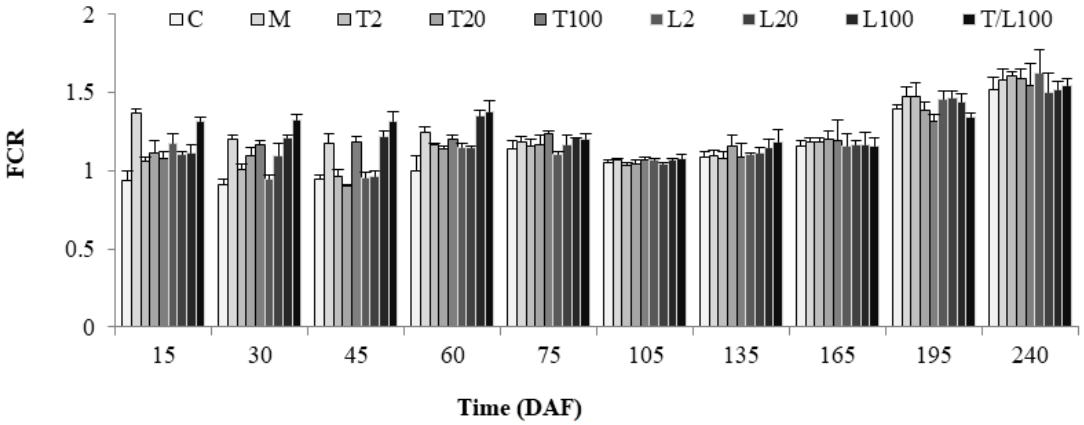

Figure 5: Mean FCR values of rainbow trout during the experimental period. Error bars show standard deviation. C: control, M: 17α-methyltestosterone, T2: 2 mg tamoxifen/kg feed, T20: 20 mg tamoxifen/kg feed, T100: 100 mg tamoxifen/kg feed, L2: 2 mg letrozole/kg feed, L20: 20 mg letrozole/kg feed, L100: 100 mg letrozole/kg feed, and T/L100: 100 mg tamoxifen and 100 mg letrozole/kg feed; DAF: days after initiation of feeding.

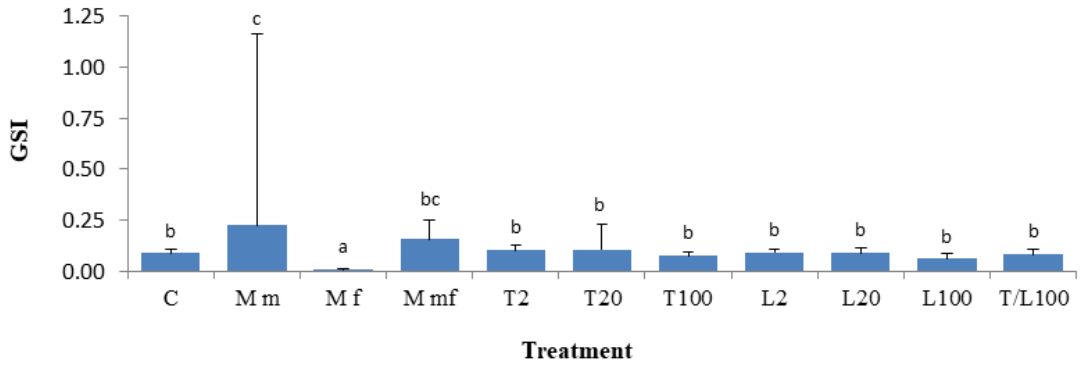

Figure 6: Mean GSI values of rainbow trout during the experimental period. Error bars show standard deviation. Different superscripts represent significant differences, one-way ANOVA, α<0.05; C: control, M: 17α-methyltestosterone, T2: 2 mg tamoxifen/kg feed, T20: 20 mg tamoxifen/kg feed, T100: 100 mg tamoxifen/kg feed, L2: 2 mg letrozole/kg feed, L20: 20 mg letrozole/kg feed, L100: 100 mg letrozole/kg feed, and T/L100: 100 mg tamoxifen and 100 mg letrozole/kg feed; DAF: days after initiation of feeding.

Steroids

Tamoxifen and letrozole caused no significant change in both testosterone and estradiol of experimental groups in comparison with the control group, whereas sex-reversed males in the 17α-methyltestosterone treated group showed significantly higher and lower concentrations of testosterone and estradiol, respectively, compared to other groups. Testosterone concentration in intersex fish was also significantly higher than that in females of all groups (Fig. 7).

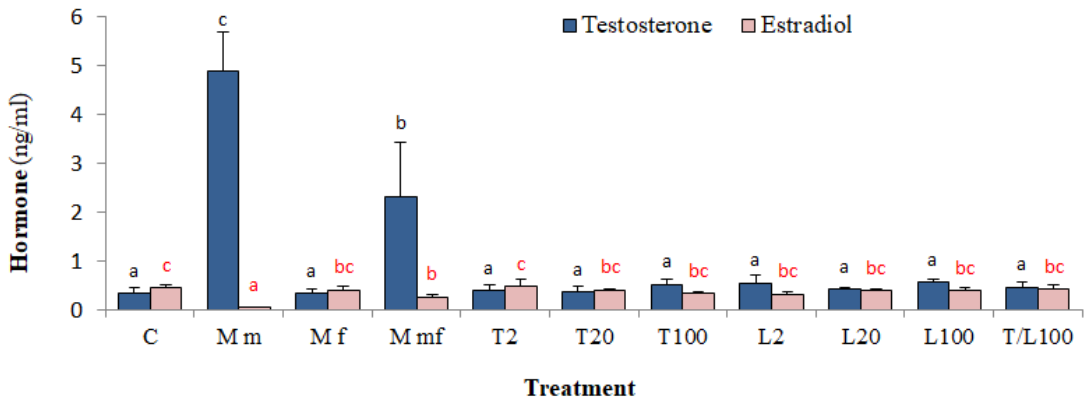

Figure 7: Testosterone and estradiol values of rainbow trout during the experimental period. Error bars show standard deviation. Different superscripts represent significant differences, one-way ANOVA, α<0.05; C: control, M: 17α-methyltestosterone, T2: 2 mg tamoxifen/kg feed, T20: 20 mg tamoxifen/kg feed, T100: 100 mg tamoxifen/kg feed, L2: 2 mg letrozole/kg feed, L20: 20 mg letrozole/kg feed, L100: 100 mg letrozole/kg feed, and T/L100: 100 mg tamoxifen and 100 mg letrozole/kg feed; DAF: days after initiation of feeding.

Discussion

Oral administration effects of 17α-methyltestosterone (2 mg/kg), tamoxifen, (2, 20, and 100 mg/kg), letrozole (2, 20, and 100 mg/kg), and a combination of tamoxifen (100 mg/kg) and letrozole (100 mg/kg) on growth, GSI, steroids, and sex reversal of rainbow trout were investigated in this study. The hormones have been used for sex reversal of several farmed species to produce a monosex population. There are several economic advantages to rear the most profitable gender, which possesses more growth characteristics (Taranger *et al*., 2010; Singh, 2013; Hoga *et al*., 2018). 17α-methyltestosterone (MT) is numerously used for producing neomale population, and all-female population can be produced with these neomale breeders (Piferrer, 2001). In addition, chemicals like tamoxifen and letrozole, with anti-estrogenic activities, have been used for sex reversal in mammals, birds, amphibians, reptiles, and fishes (Singh and Srivastava, 2015**)**.

In the current study, growth performance was significantly affected by all administrated chemicals during two-month treatment period, and suppression of growth occurred, especially at 17α-methyltestosterone and high dosages of both tamoxifen and letrozole

administrations. Compensatory growth occurred one month after the chemical treatment period. Similar findings were reported by Shen *et al*. (2015) for use of 17α-methyl testosterone in yellow catfish *Tachysurus fulvidraco*. In contrast, they achieved different results in use of letrozole, as low dose (20 mg/kg) of letrozole enhanced growth performance, while in higher doses (50 and 100 mg/kg) growth was not affected (Shen *et al*., 2015). Betancur *et al*. (2014) reported promotion of growth performance in low-dose of LZ (25 mg/kg) in treated red tilapia. This effect could be observed until one month after the treatment period. In their study there was no significant difference among growth values of the control, 17α-methyltestosterone (60 mg/kg), and high dosage (100 mg/kg) of letrozole-treated groups.

These findings indicate significant differences in effects of hormones and other chemicals on growth performance of farmed fish, which could be due to species-specific characteristics. However, these adverse effects were not permanent, and usually compensatory growth occurred during the first month after ending chemical treatment period.

In this study, GSI values in tamoxifen and letrozole-treated groups were not affected, as there were no significant differences between the above-mentioned groups and the control group. Similarly, sex differentiation of rainbow trout was not affected by oral administration of tamoxifen and letrozole, whereas GSI values and sex differentiation of the 17α-methyl testosterone-treated group were significantly influenced as GSI values of males were significantly more than those of the rest. While, in females of the group M, GSI values were significantly lower than those in other groups, which means that the ovary development was suppressed by 17α-methyl testosterone administration.

In contrast to our results, tamoxifen and letrozole showed significant effects on sex reversal of warm-water fishes, like common carp, catfish, and tilapia. A study of tamoxifen and letrozole influences on common carp (*Cyprinus carpio*) and Nile tilapia (*Oreochromis niloticus*) by Singh and Sirvastava (2015) indicated that 100 mg letrozole/kg feed made about 79% masculinization in common carp, and 88% masculinization in Nile tilapia, while the same dose of tamoxifen brought about 63% and 78% masculinization, respectively (Singh and Sirvastava 2015). Oral administration of letrozole (20, 50, and 100 mg/kg feed) produced about 75-83% males in yellow catfish (*Tachysurus fulvidraco*) that were significantly higher than male ratio in the control group, while 17α-methyl testosterone did not affect sex reversal of yellow catfish (Shen *et al*., 2015).

Thus, non-effectiveness of tamoxifen and letrozole on sex reversal in this study might be a result of rainbow trout physiology because this fish is considered a cold-water fish with different physiological requirements in comparison with warm-water fishes.

Monosex population of some cultured species like rainbow trout, having higher

growth rate in comparison with mixed sexes, is preferred by aquaculturists. Thus, 17α-methyltestosterone is commonly used for masculinization of this species. In the current study, this hormone effectively changed sex proportion of rainbow trout. In addition to 17α-methyltestosterone, use of chemicals with anti-estrogenic activities (tamoxifen and letrozole) for sex reversal, has been attempted in some ornamental and warm-water fishes. In contrast with 17α-methyltestosterone, tamoxifen and letrozole showed no effect on sex reversal of rainbow trout in this study. Growth performance was adversely affected by all chemical-treated diets. However, compensatory growth retardation occurred during first month after ending the treatment period.

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Research Article

Effect of dietary *Ganoderma lucidum* extract on growth performance, blood biochemical parameters, and antioxidant status in juvenile beluga (*Huso huso*)

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Abstract

In the present study, the dietary effect of *Ganoderma lucidum* extract (GLE) on growth performance, antioxidant status, and some blood biochemical parameters was investigated in *Huso huso*. The fish were divided into four groups and fed with different concentrations of 0, 0.5, 1, and 2 g/kg GLE for 6 weeks. The results showed that the growth indices including weight gain and specific growth rate were increased significantly compared to the control group. However, the food conversion rate was significantly decreased in 1 and 2 g/kg GLE-supplemented groups. An increasing trend of serum total protein and IgM was observed in the groups fed with GLE, while the fish fed with 1 and 2 g/kg GLE showed significant differences compared to the control group (p<0.05). The serum glucose level decreased in the groups fed with GLE compared to the control group, however, it was not significant. The triglycerides and cholesterol levels were significantly reduced in 1 and 2 g/kg GLE added groups compared to 0.5 g/kg GLE and control group (p<0.05). An increase in the serum total antioxidant capacity was observed in GLE-supplemented groups, which significantly raised in 1 and 2 g/kg GLE (p<0.05). Serum malondialdehyde decreased in the groups fed with GLE, which significantly reduced in 2 g/kg GLE compared to the other groups (p<0.05). In conclusion, dietary GLE showed a proper effect on growth performance, antioxidant capacity, hypolipidemia, and immunity in *H. huso*.

Keywords: Lingzhi mushroom, Sturgeon, Growth, Blood biochemistry, Antioxidant enzymes

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Introduction

The increasing demand for seafood has caused a remarkable development in the aquaculture industry and therefore, aquatic health management is critical for farming fish species. In the meantime, different stressors, pathogens, and environmental pollutants lead to disease outbreaks in fish farms. Immune and antioxidant systems play an essential role in combating different stresses and diseases in fish (Galina *et al*., 2009). Using bioactive compounds as nutritional supplements in the fish diet seems to boost the immune and antioxidant systems against stressors (Amar *et al*., 2004; Martinez-Álvarez *et al*., 2005; Hoseinifard *et al*., 2018).

Ganoderma lucidum commonly known as 'Reishi' or 'Lingzhi' is a medicinal fungus with several interesting compositions such as polysaccharides, terpenoids, nucleotides, steroids, fatty acids, proteins, flavonoids, alkaloids, antioxidants, glycopeptides, vitamins, and minerals, which triterpenoids and polysaccharides are the major components (Zjawiony, 2004; Paterson, 2006; Liu *et al*., 2016). *G. lucidum* has been widely used as an oriental mushroom for centuries to improve some disorders like hypertension, bronchitis, immunological disease, anorexia, hepatitis, and cancer (Boh *et al*., 2007; Zhao *et al*., 2016).

Because of the great properties and multifunctional ingredients of *G. lucidum*, it has a high potential to treat several diseases and use in the nutraceutical and pharmaceutical industries (Li *et al*., 2013; Stojković *et al*., 2014).

A few researches have been reported on the effect of *G. lucidum* as a nutritional supplement in aquatic animals. Dietary *G. lucidum* polysaccharides increased immune inflammatory response and antioxidant enzyme activity in (*Cyprinus carpio*) against CCl4, which caused hepatocyte lesions (Liu *et al*., 2015). *G. lucidum* polysaccharides revealed the desirable effect on the survival and growth performance of *Ctenopharyngodon idella* (Chithra *et al*., 2016). Yin *et al*. (2009) reported *G. lucidum* extract can increase the immune system status of common carp (*Cyprinus carpio*) against *Aeromonas hydrophila*. In other research, *G. lucidum* extract enhanced the survival rate, growth performance, digestive enzymes, and antioxidant activities in giant freshwater prawn (*Macrobrachium rosenbergii*) (Mohan *et al*., 2016). *G. lucidum* extract showed beneficial effects on growth and health status as well as antioxidant enzymes stimulation in the red hybrid Tilapia (*Oreochromis* sp.) (Wan *et al*., 2021). *G. lucidum* polysaccharides increased both specific and non-specific immunity as effective immunostimulants against *Vibrio harveyi* in pearl gentian grouper (*Epinephelus* sp.) (Zhang *et al*., 2022).

The effect of *G. lucidum* extract as a dietary supplement on sturgeons has not been reported so far. Sturgeons are valuable fish species belonging to the Acipenseridae family. Unfortunately, conservation threats have endangered sturgeons critically (Carmona *et al*.,

2009). The culture of these highly endangered species, especially the great sturgeon (*Huso huso*), can relieve the pressure on the sturgeon populations in the Caspian Sea (Hoseinifar *et al*., 2011). The great sturgeons or beluga are suitable fish species for aquaculture because of their valuable caviar and meat (Mohseni *et al*., 2008, Yadolahi et al., 2022).

In aquatic animals, stress happens in the conditions of water physicochemical changes, nutritional deficiencies, water pollution, xenobiotics, and diseases (Hwang and Lin, 2002; Yeganeh Kari *et al.,* 2022). Therefore, using the proper dietary supplements which can improve growth performance, and increase the immune and antioxidant systems may enhance the survival of fish in culture (Trichet, 2010; Taleghani *et al*., 2019). The present study aimed to evaluate the dietary effect of *G. lucidum* extract (GLE) on growth performance, some serum biochemical parameters, and antioxidant status in beluga (*H. huso*) juvenile.

Materials and methods

Experimental setup

120 healthy juvenile beluga (*H. huso*) with an average weight of 34.63±4.77 g were collected from the Culture and Breeding Center of Shahid Rajaei (Sari, Iran). After two weeks adaptation period, ten fish were randomly distributed into separate tanks as four groups with three replications. Each tank with 500 cm^3 size and 250 L volume of water was supplied with an inlet water flow rate of 2.47 L min^{-1}. Based on the previous studies (Chithra *et al.,* 2016; Mohan *et al.,* 2016), the groups received different concentrations of 0, 0.5, 1, and 2 g/kg GLE in the diet. Ingredients of the diet were mixed well with GLE and then made into pellets. The composition of experimental diets is shown in Table 1.

Fish were fed 4% of body weight four times a day (Adel *et al*., 2016) for 6 weeks. Water physicochemical parameters of fish tanks were checked daily during the experimental period including temperature 25±1°C, dissolved oxygen 6.5 mg/L and 7.2−7.4 pH.

Table 1: Dietary ingredients and proximate composition.

Ingredients (g/kg)	Ganoderma lucidum extract (g/kg)			
	0	**0.5**	**1**	**2**
Fish meal	460	460	460	460
Soybean oil	58	58	58	58
Wheat flour	150	150	150	150
Soybean meal	100	100	100	100
Meat meal	90	90	90	90
Cellulose	2	1.5	1	0
Vitamin mixture[a]	35	35	35	35
Mineral mixture[b]	25	25	25	25
Fish oil	60	60	60	60
Binder	20	20	20	20
Ganoderma lucidum extract	0	0.5	1	2
Proximate composition (%)				
Dry matter	91.3	91.5	91.2	91.5
Crude protein	39.6	39.5	39.7	39.7

Table 1 continued:				
Crude lipid	16.9	16.9	16.8	16.7
Ash	10.0	9.8	10.1	10.2
Crude fiber	2.5	2.6	2.5	2.3
Moisture	8.0	7.9	8.0	8.2
NFE[c]	22.3	22.7	22.1	22.6
Gross energy (kcal g^{-1})	3.6	3.6	3.6	3.6

a Unit/kg of mixture: vitamin A, 1,600,000 IU; D3, 400,000 IU; E, 40 IU; K3, 2000 mg; H2, 240 mg; B1, 6000 mg; B2, 8000 mg; B3, 12,000 mg; B5, 40,000 mg; B6, 4000 mg; B9, 2000 mg; B12, 8000 mg; vitamin C, 60000 mg; inositol, 20,000 mg; BHT, 20,000 mg. [b]Unit/kg of mixture: mineral: Fe, 26,000 mg; Zn, 12,500 mg; Se, 2000 mg; Co, 480 mg; Cu, 4200 mg; Mn, 15,800 mg; I, 1000 mg; choline chloride, 12,000 mg. [C] Nitrogen-free extracts (NFE) = dry matter - (crude protein + crude lipid + ash + fiber).

Ganoderma lucidum extract

The fruiting bodies of *G. lucidum* were provided from Iran Ganoderama (Karaj, Iran). The *G. lucidum* specimens were cut into small pieces and mixed to obtain powdered samples for extraction. The GLE was performed according to the procedure described by Taofiq *et al*. (2017). Briefly, the powder of *G. lucidum* was extracted in a Soxhlet apparatus using ethanol. Eventually, the dried ethanolic extracts were obtained by rotary evaporator under reduced pressure (Stuart RE 300, UK).

Growth parameters

The initial and final body weight and length of each fish in different groups were measured at the beginning and the end of the experiment. The weight gain (WG), length gain (LG), specific growth rate (SGR), condition factor (CF), and feed conversion ratio (FCR) were calculated as follows:

WG (g) = final weight (W_2, g) - initial weight (W_1, g)
LG (cm) = final length (L_2, cm) - initial length (L_1, cm)
SGR (%) =100 (ln final weight− ln initial weight) / number of days
CF (%) = 100× final weight (g) / final length $(cm)^3$
FCR = feed intake (g)/weight gain (g)

Serum biochemical parameters

At the end of the experiment, six fish from each group were randomly sampled. Blood samples were collected from the caudal vein and transferred to the non-heparinized microtube for serological examination. The serum samples were separated by 1006 ×g centrifuging for 10 min. The serum biochemical parameters including total protein, albumin, immunoglobulin G (IgM), glucose, triglyceride, cholesterol, aspartate aminotransferase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) were analyzed by commercial kits (Pars azmoon, Iran) using an auto-analyzer (Cobas Mira plus, Germany).

Antioxidant and oxidative stress analysis

Serum total antioxidant capacity (TAC) was measured using commercial kit (Teb Pazhouhan Razi (TPR), Iran) according to the manufacturer protocol.

Serum malondialdehyde (MDA) was evaluated based on a thiobarbituric acid reaction by commercial kit (Teb Pazhouhan Razi (TPR), Iran).

Statistical analysis

Statistical analysis was performed using SPSS version 22. Data were analyzed using a One-way test of variance (ANOVA) to compare means. Duncan's test was performed to analysis of significant differences among groups. The statistical significance level was $p<0.05$. Data are presented as mean± standard error (SE).

Results

Growth and feed utilization indices

In this study, the growth indices including WG, LG, and SGR were increased significantly in the groups supplemented with GLE compared to the control group ($p<0.05$). The highest increase was observed in 2 g/kg GLE. However, it was not significantly different from the group fed with 1 g/kg GLE ($p>0.05$). FCR decreased in GLE supplemented-group with significant differences in 1 and 2 g/kg GLE compared to the other groups ($p<0.05$). There was no significant difference in CF of all groups ($p>0.05$; Table 2).

Table 2: Growth performance of *Huso huso* fed diets supplemented with *Ganoderma lucidum* extract for 6 weeks.

Growth parameters	*G. lucidum* extract (g/kg)			
	0	0.5	1	2
W_1 (g)	34.21 ± 4.9	34.87 ±4.7	34.9 ±4.9	34.57 ±4.6
L_1 (cm)	19.67 ±1.2	19.80 ±1.1	19.29 ±1.09	19.62 ± 1.0
W_2 (g)	89.27 ± 3.7^a	96.67 ±3.5^a	108.51 ± 3.8^b	110.93 ±4.4^b
L_2 (cm)	27.34 ±0.3^a	28.45 ±0.4^a	29.64 ±0.4^b	30.22 ±0.3^b
WG (g)	55.06 ±0.9^a	61.79 ± 1.2^b	73.69 ± 1.5^c	76.36 ± 1.0^c
LG (cm)	7.84 ±0.16^a	8.64 ± 0.13^b	10.35 ± 0.34^c	10.60 ± 0.27^c
SGR (%)	1.03^a ± 0.00	1.10 ±0.02^b	1.22 ± 0.02^c	1.26 ±0.01^c
CF	0.43 ± 0.01	0.41± 0.00	0.40 ±0.01	0.41± 0.00
FCR	1.70 ± 0.03^a	1.62 ± 0.03^a	1.50 ±0.03^b	1.47 ±0.01^b

Data are presented as mean ± SE. Different letters above the values indicate significant difference among groups ($p<0.05$). W_1, initial weight; L_1, initial length; W_2, final weight; L_2, final length; WG, weight gain; LG, length gain; SGR, specific growth rate; CF, condition factor; FCR, Feed conversion ratio.

Serum biochemical parameters

The results of this study showed an increasing trend of serum total protein in the groups supplemented with GLE, which significantly raised in 1 and 2 g/kg GLE compared to control group ($p<0.05$).

Also, the IgM level increased in GLE-supplemented groups. The lowest and highest IgM levels were observed in control group and 2 g/kg GLE-supplemented group, respectively. Meanwhile, the amount of albumin in different groups was not significantly different ($p>0.05$; Fig.1).

The amount of glucose in the groups fed with GLE showed a decreasing trend. However, there was no significant difference among the groups ($p>0.05$). The triglyceride level was reduced in GLE-supplemented groups and showed a significant difference in 1 and 2 g/kg GLE groups compared to 0.5 g/kg GLE and control group ($p<0.05$). The lowest

cholesterol level was observed in 2 g/kg GLE, which was significantly different compared to 0.5 g/kg GLE and the control group (p<0.05), however, it was not significantly different from 1 g/kg GLE (p>0.05; Fig. 2).

There were no significant differences in the amount of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in all groups (p>0.05; Table 3).

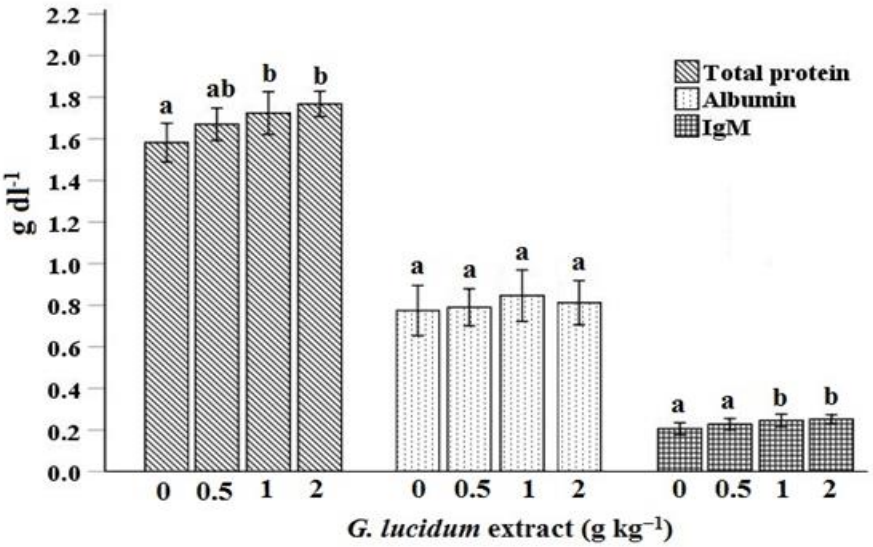

Figure 1: Serum biochemical parameters including total protein, immunoglobulin M (IgM), and albumin in *Huso huso* fed diet supplemented with *Ganoderma lucidum* extract for 6 weeks. Data are presented as mean±SE. Different letters above the bars indicate significant difference among groups (p<0.05).

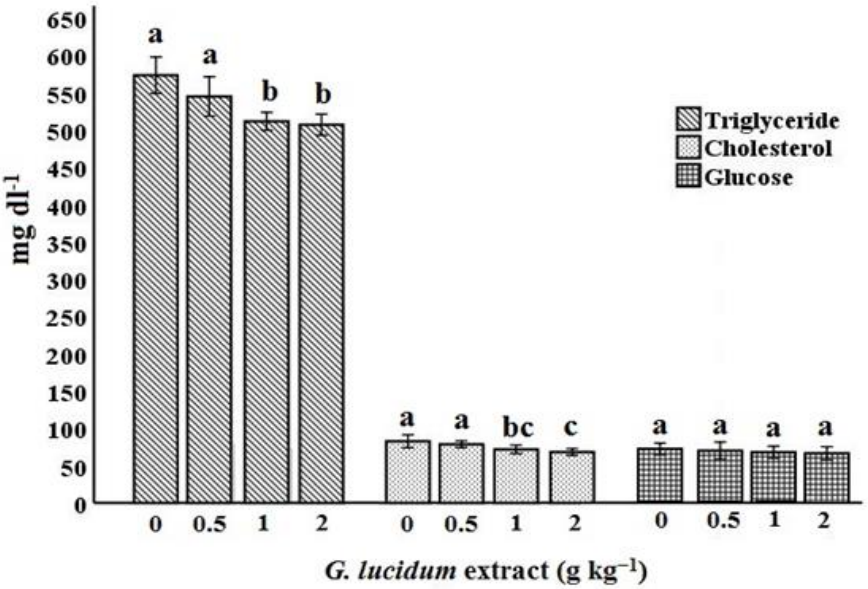

Figure 2: Serum biochemical parameters including glucose, triglycerides, and cholesterol in *Huso huso* fed diet supplemented with *Ganoderma lucidum* extract for 6 weeks. Data are presented as mean ± SE. Different letters above the bars indicate significant differences among groups (p<0.05).

Antioxidant and oxidative stress analysis

In the present study, serum TAC was affected by GLE. The highest TAC level was observed in 2 g/kg GLE, which was significantly different from 0.5 g/kg GLE and control group ($p< 0.05$). In contrast, serum MDA decreased in the groups with dietary supplementation, which was significantly lower in fish fed with 1 and 2 g/kg GLE compared to the other groups ($p< 0.05$; Table 3).

Table 3: Serum metabolic enzymes and antioxidant status in *Huso huso* fed diets supplemented with *Ganoderma lucidum* extract for 6 weeks.

Parameters	*G. lucidum* extract (g/kg)			
	0	**0.5**	**1**	**2**
AST (UL^{-1})	426.6±22.95	443.6±37.79	471.0± 32.48	431.6±23.91
ALT (UL^{-1})	12.6±1.07	11.2±1.24	11.6±1.02	12.0±0.70
ALP (UL^{-1})	265.4±16.86	250.4±8.68	255.0±14.36	245.0±0.05
TAC ($\mu M\ l^{-1}$)	116.70± 1.49^{a}	121.28± 1.27^{a}	138.66±1.51^{b}	143.57± 2.84^{b}
MDA ($\mu M\ l^{-1}$)	9.72±0.37^{a}	8.96±0.45^{a}	6.24±0.39^{b}	5.01±0.29^{c}

Data are presented as mean ± SE. Different letters above the values indicate significant difference among groups ($p<0.05$). AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; TAC, total antioxidant capacity; MDA, malondialdehyde.

Discussion

Ganoderma lucidum contains beneficial biological compounds such as polysaccharides and triterpenoids. Recently, more attention has been paid to the therapeutic effects of *G. lucidum* (Wachtel-Galor *et al*., 2004; Paterson, 2006). This study showed an increase in the WG, LG, and SGR values as well as a decrease in the FCR in the groups fed diets supplemented with GLE. These results indicate the appropriate effect of GLE on the growth performance of fish during the experimental period. The ability to enhance the growth performance of GLE is due to the effect of immune stimulation caused by *G. lucidum*, which regulates immunity and prevents disease. In the study of Chithra *et al*. (2016) *G. lucidum* polysaccharides caused a significant increase of body weight and SGR in grass carp (*Ctenopharyngodon idella*) (Mohan *et al*., 2016).

Also, Mohan *et al*. (2016) reported an increase in the digestive enzymes including protease, amylase, and lipase in giant freshwater prawn (*M. rosenbergii*) fed with *G. lucidum* polysaccharides. The dietary supplementation with *G. lucidum* polysaccharides increased the secretion of digestive enzymes and enhanced the absorption of nutrients from the gastrointestinal tract and finally, improved the growth performance of *M. rosenbergi* (Mohan *et al*., 2016).

In the present study, the amounts of total protein and IgM increased with the increment of GLE in the diet. Total protein includes albumin and globulins. Globulins are the main constituents of serum protein that makeup immunoglobulins which are essential in the immune response. Serum

immunoglobulin level is a substantial indicator of immune status. B lymphocytes that originate from the anterior part of the kidney, spleen, and anterior part of the heart become the cells that secrete plasma cell antibodies and produce immunoglobulins (Yu *et al*., 2008; Yildiz *et al*., 2009). The increase of immunoglobulin in this study indicates an improving effect of GLE on the immune system.

This study showed hypoglycemia in the groups that supplemented with GLE, however, this reduction was not significantly different among the groups. *G. lucidum* facilitates the inflow of calcium to pancreatic cells by releasing insulin which leads to hypoglycemia (Zhang and Lin, 2004). In the present study, triglycerides and cholesterol were reduced in the GLE-supplemented groups, which significantly decreased in 1 and 2 g/kg GLE compared to 0.5 g/kg GLE and control group (p<0.05).

Triglycerides and cholesterol are important lipid metabolism biomarkers (Chen *et al*., 2014). Serum cholesterol reduction could be due to plant sterols (Fremont *et al*., 2000, Avci *et al.*, 2006), which are being considerably supplemented to the diet for preventing hypercholesterolemia and hyperlipidemia (Rubel *et al.*, 2011). The results of this study confirm former researches that reported hypolipidemia caused by *G. lucidum* (Kabir *et al*., 1988; Berger *et al*., 2004).

In this study, an increase in TAC was observed with a significantly different in 1 and 2 g/kg GLE compared to 0.5 g/kg GLE and control group. TAC protects biological molecules against oxidation (Yousefi *et al*., 2019). Antioxidants reduce oxidative stress by scavenging reactive oxygen (Lee *et al*., 2009). TAC indicates total antioxidant capacity, which illustrates antioxidant ability to resist oxidants (Taheri Mirghaed *et al*., 2018; Yousefi *et al*., 2019).

The result of this study indicated that GLE reduces the concentration of serum lipid peroxidation which showed its effect by decreasing serum MDA. Lipid peroxidation is an adverse event that leads to oxidation of unsaturated fatty acid, because of antioxidant system failure (Yilmaz, 2019). MDA which is produced by lipid peroxidation cause oxidative stress. Therefore, MDA is a biomarker used to evaluate lipid peroxidation and oxidative stress (Hwang *et al*., 2013;Taheri Mirghaed *et al*., 2018; Yousefi *et al*., 2019).

It can be concluded from this study that dietary GLE improved growth performance and antioxidant capacity in *H. huso*. Moreover, results revealed that GLE possesses hypoglycemic, hypolipidemic, and immunostimulatory properties.

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Short Communication

Oncogenic papillomavirus-like particles in Angelfish (*Pterophyllum scalare*) from Iran

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Introduction

The incidence of neoplasia is not a specificity of human tissues and is not even restricted to mammals. Notably, it is a common occurrence in piscine species -the largest group of vertebrates- with over 27,000 species (Claver and Quaglia, 2009). Compared to mammals, malignant neoplasms with or without metastasis are seldom reported in fish (Hugh Ferguson, 2006; Grizzle and Goodwin, 2010), which could be due to a different antitumor immunity in fish (Bubanovic and Najman, 2005). Fish neoplasms are often benign (da Rocha *et al.*, 2017). In general, benign neoplasms are well-differentiated neoplasms that represent a slow rate of growth and have been demonstrated as a cohesive expansive mass that is often delineated by a fibrous capsule (Cotran *et al.*, 1999). Cutaneous neoplasm -also known as skin tumor- is a rare and frequently benign soft tissue neoplasms, however, recurrence is common in cases of incomplete surgical excision (Groff, 2004). The most recent WHO classification for skin tumors (Gershon, 2021) categorizes fibromas and their

precursor lesions, totally benign, based not only on their clinical and histomorphological characteristics but also on their molecular profile and genetic fingerprint. Fibromas are characterized as discrete, raised, homogeneous, encapsulated, white to pale tan or pink, firm to hard tumors of variable size with a narrow (pedunculated) or broad base that may be firmly attached at the base or may appear as a loosely attached tag of tissue and may not always be covered by epithelium. It is rarely identified in fishes (Ferguson, 1989, Roberts, 2001). Papilloma generally is a benign neoplasm of epithelioid cells that both higher and lower vertebrates could engage with (Peters and Watermann, 1979; Hedrick *et al.*, 1990; Rahmati-Holasoo *et al.*, 2015). It is known as the most prevalent skin neoplasms in wild and reared fish species and their appearance features are described as discrete, soft to firm, slightly to prominently raised , Single or multiple, papillary growths with a presence as flat to nodular cutaneous plaques (Roberts, 2001; Chong, 2022). Papillomaviruses as infectious agents of the family *Papillomaviridae* suspected in association with several of these proliferative diseases in a wide range of vertebrates, alike in piscine species(López-Bueno *et al.*, 2016; Tisza *et al.*, 2020; Labella *et al.*, 2019; Surján *et al.*, 2021; Kraberger *et al.*, 2022). It is worth mentioning that, an odontomas/fibromas retroviral-induced tumor (Rahn *et al.*, 2004; Claver and Quaglia, 2009) and a fibroma tumor previously recognized in *Pterophyllum spp* (Coffee *et al.*, 2013; Vergneau-Grosset *et al.*, 2017). Given that the detections of various types of viruses in fish tumor lesions were severely reported (Anders and Yoshimizu, 1994). To our knowledge, this is the first report of oncogenic virus detection that has a striking resemblance to papillomavirus particles associated with fibropapilloma tumor of angelfish in Iran.

Materials and methods

Specimen collection

This study was performed on 100 specimens of 1 to 2 years angelfish (*Pterophyllum scalare*) (72% male and 28% female) belonging to an ornamental fish reproduction farm in Gilan province, which delivered alive to "Virology laboratory for fish, of Inland waters aquaculture research center, Bandar Anzali, Gilan, Iran" with visible lips hyperplasia, for diagnostic examinations. To perform a necropsy, fish were anesthetized with clove (*Syzgium aramaticom*) powder which was diluted in water (0.7 g. L^{-1}). The routine tissue collection was performed from the main organs (lips, liver, kidney, and spleen) for histopathological examinations.

Physicochemical assessment

After observing the abnormal signs in fish, the water samples of the studied reproduction farm were immediately collected. The collection assortment,

preservation, and analysis of the main ions of water samples were carried out through standard methods explained by the EPA (Nelson, 2003).

Histopathological examinations

Tissues were excised and placed in 10% buffered formalin solution in conical tubes. After 24 hours, formalin was refreshed. Tissues were dehydrated in ethanol gradient of 50, 70, 90, and 100 percent of concentration and cleared with chloroform then embedded in paraffin. The sections of 5 µm were prepared from tissue samples using a rotary microtome(Leitz 1512, Germany) and stained with hematoxylin and eosin (H&E) and also Masson's trichrome (MT). Afterward, sections were examined by light microscope (Nikon Ci-L plus, Japan) according to the standard procedures (Bancroft and Gamble, 2008).

Electron microscopy

Sections were prefixed with 2.5% glutaraldehyde (TAAB laboratories-3 Minerva, Calleva park, Aldermaston, Berks, RG78NA, England–EM grade) in 0.1 M phosphate buffer saline (PBS, pH 7.2) for 2 h at 4°C. The electron microscopy procedure was performed in Rastak Lab (Tehran, Iran) as follows: prepared sections were washed three times in the PBS (10 min for each time). After washing, they were post-fixed in 0.5% osmium tetroxide (TAAB laboratories-3 Minerva) in the same buffer at room temperature for 1 h. After washing in the PBS for three times (10 min for each time), the samples were dehydrated in ascending alcohol series, acetone, acetone-resin mixture (50/50) and finally embedded in TAAB embedding resin (TAAB laboratories-3 Minerva) and polymerized in 60°C for 48 h. Fifty nm ultra-thin sections were then prepared by Lika Ultracut R (Lika, Wetzlar, Germany), placed on 300 mesh copper grid and double stained with 20% uranyl acetate (BDH Laboratory Chemicals Division, England, No. 0148860) in pure methanol (E. Merck, D-6100 Darmstadt) for 45 min and in Reynolds solution (lead nitrate and sodium citrate; Reynolds, 1963) for the same time. Finally, the samples were examined with the transmission electron microscope (EM208S, PHILIPS, Netherlands) at the accelerating voltage of 100 kV.

Results and discussion

Physiochemical characteristics of water

The water source of the surveyed ornamental farm was obtained from deep wells. As mentioned in Table 1, the consequences of hydrochemical assessments of water samples, declared the normal condition for examined water samples, in a way that neither endocrine-disrupting nor health-threatening rate of measured parameters resulted (Table 1).

Macroscopic and microscopic examinations

Grossly, abnormal cutaneous hyperplasia was clearly observed in all 100 freshwater angelfish, with the

characteristics of smooth prominent or cauliflower-like swellings, consistency, relatively crisp and brittle lips. Furthermore, there weren't any signs of existing abnormalities on the other parts of the skin. It should be mentioned that no abnormal swelling or metastases were detected in the internal organs during the necropsy. The diameter of the nodular tumors varied between 0.5 cm × 0.5 cm to 1cm × 1 cm. The color of the investigated tumors varied from light pink to cream. In addition, tumors were in constant contact with the surrounding skin tissue (Fig. 1).

Table 1: Consequences of hydrochemical assessments of water samples

Parameter	Value	Parameter	Value
Temperature (°C)	21	K^+ (mg/L)	1.97
Electrical conductivity (ms/cm)	0.71	Total hardness (mg/L)	246
pH	8.10	Cl^+ (mg/L)	147
Turbidity (F.T.U)	28	Nitrite (NO_2^-) (mg/L)	0.001
Dissolved Oxygen (mg/L)	11.3	Nitrate (NO_3^-) (mg/L)	0.037
Ca^{2+} (mg/L)	61	Sulfate (SO_4^{2-}) (mg/L)	30.9
$Mg2^+$ (mg/L)	23	Phosphate (PO_4^{3-}) (mg/L)	0.055
Na^+ (mg/L)	25.5	K^+ (mg/L)	1.97

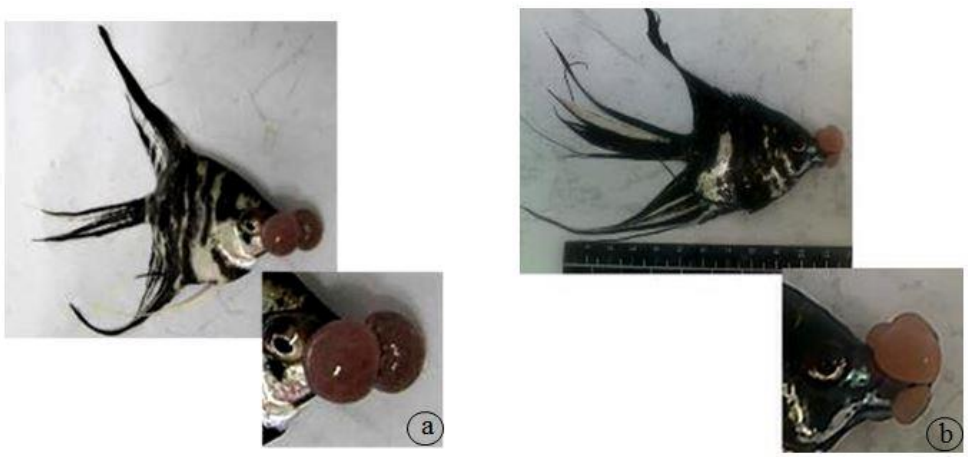

Figure 1: Gross pathology of *Pterophyllum. scalare*. (a, b) represent typical epidermal fibropapilloma on the jaws.

In the microscopic study, prominent squamous cell hyperplasia was observed with increasing epidermal thickness. The vacuolization and necrosis of cells were clear in the malpighian layer. In the dermis layer, the proliferative fibroblasts were seen in a way that the arrangement of fibroblast cells was bundled. Additionally, thickness, folding and swelling, and vascularization were also observed. The Hypodermis contained thick edematous fibrovascular stroma and fibroblastic cells were severely proliferated, which was considered as the tumor stem. Mason trichrome-specific staining showed blue collagen fibers that resulted in fibroblast activities. The tumor cells showed in reddish-brown (Fig. 2).

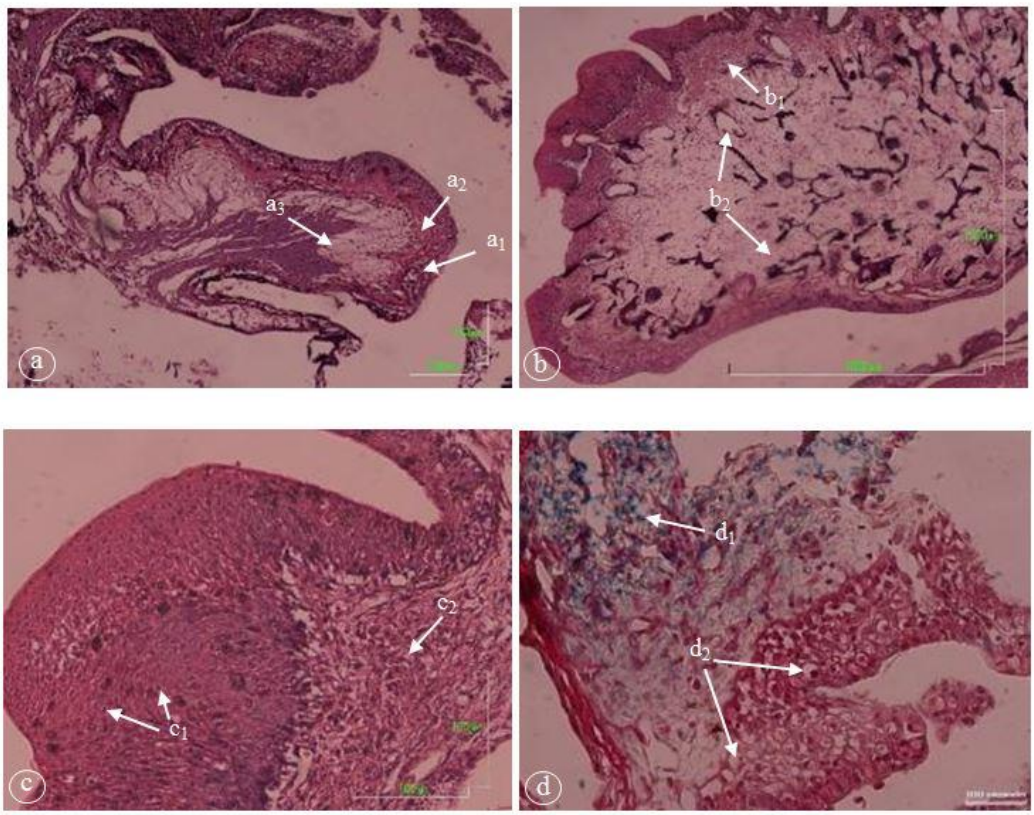

Figure 2: (a) A fibropapilloma tumor; (a_1) the hyperplasia of epidermis that vacuolization of epidermal cells showed with arrow; (a_2) the swelling and folding of abnormally proliferated dermis; (a_3) the stem of tumor, fibroblasts proliferated intensely (H&E, Bar: 100 µm). (b_1) vacuolization of malpighian cells; (b_2) The dermis area was severely edematous vascularization and budding was also observed (H&E, Bar: 1000 µm). (c) Hyperplasia of squamous cell carcinoma, the thick fibrovascular stroma of epithelium; (c_1) the swollen nuclei of activated fibrocytes which indicates collagen production and tumor appearance; (c_2) necrosis and abnormal proliferation of malpighian layer (H&E, Bar: 100 µm). (d) Hyperplastic mass of lips skin; (d_1) green-blue points illustrate collagen fibers, (d_2) Conjunctival squamous papilloma and epithelioid cells with pleomorphic nuclei and vacuolated cells were observed (MT, Bar= 100 µm).

Virus morphogenesis

Examination of ultrathin sections of virus-infected tissues by transmission electron microscopy (TEM), showed aggregation of virus particles. In some infected cells, virions existed within both the cytoplasm and nuclei (Fig. 3a). Similar to the virions of the family *Papillomaviridae*, studied virions were seen as non-enveloped and capsomeres arranged in icosahedral symmetry which could be observed as hexagonal two-dimensional arrays in the size range of approximately 50 to 70 nm (Fig. 3d).

In this study, histological analyses demonstrated that the lesions in *P. scalare*, which are mainly observed in the skin of fish in spring, are cutaneous hyperplasia that represented characteristics of both fibroma and papilloma, which is called fibropapilloma. Therefore, this study is the first report of fibropapilloma of neoplastic transformation in tissues of angelfish in Iran.

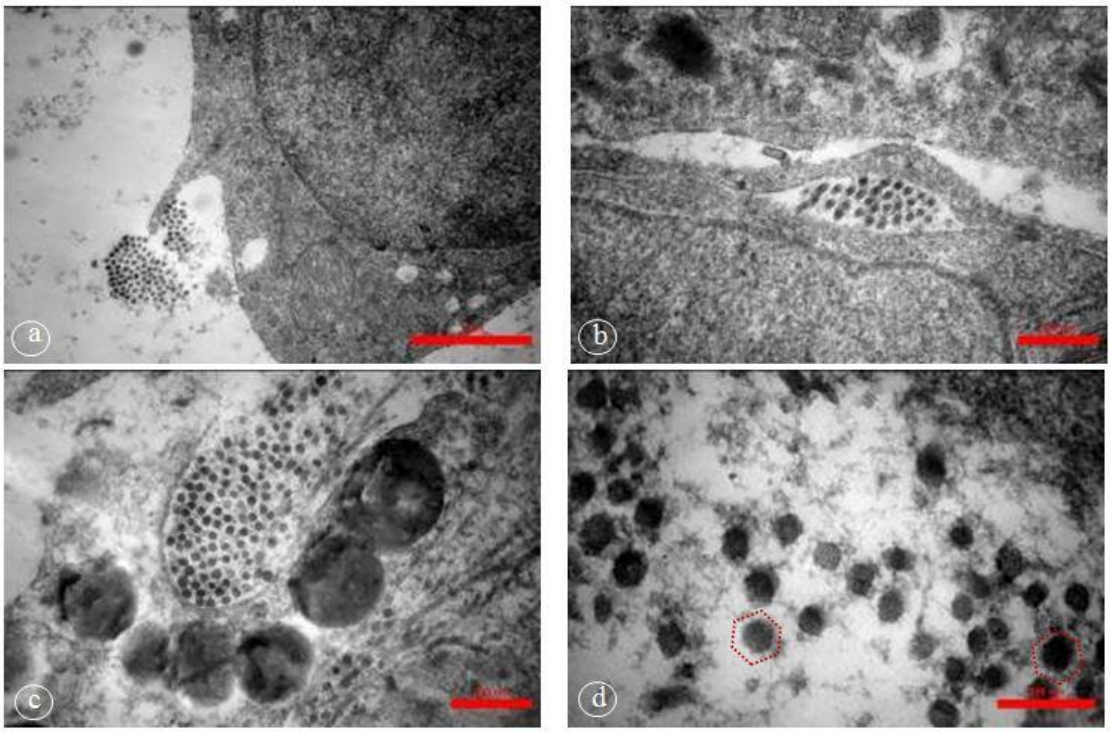

Figure 3: Electron micrograph of the cytoplasm and nuclei of enlarged cells. Many Inclusions are present in the cytoplasm that contain densely staining virus-like particles; (a to c) the aggregations of virus-like particles presented in various magnifications; scale bars refer 1µ, 500 nm, and 500 nm respectively. (d) the two-dimensional hexagonal characteristics of some papilloma virus-like particles outlined with red dashes; Bar=200nm

The most revealed reason for tumor manifestation in fish are infectious agents. Due to the visibility and ease of sampling, virus-associated tumors of the skin have been studied extensively (Bowser and Casey, 1993). Considering that thirteen proliferative neoplasms have been identified as diseases associated with the presence of retroviruses or retrovirus-like particles in fish species (Quackenbush *et al.*, 2010), based on provided electron micrograph evidence of this research, the main features of *Retroviridae* family including spherical envelope, diameter ranging between 100 to 200 nm, heterogeneous morphologies and polymorphic capsids of mature ones and distinct doughnut shape morphology of immature ones which capsomeres covered by the surface membrane (Zhang *et al.*, 2015) was not observed. Conversely, the electron microscopic observations of the present study suggested the presence of papillomavirus-like particles in examined angelfish. Papilloma in *P. scalare* is histologically similar to the papilloma seen in Brown bullhead (*Ameiurus nebulosus*) and White sucker (*Catostomus commersonii*) (Smith, 1989; Poulet *et al.*, 1994; Premdas *et al.*, 1995). Although the papilloma in the present study showed different developmental stages, none of the specimens examined were invasive or metastatic, indicating that the tumors were benign.

Inflammation in papilloma can be a progressive factor for papilloma growth (Smith, 1989; Premdas *et al.*, 1995). In the present study, no leukocytes were observed in the histological sections of angelfish. To prevent neoplasms development in the aquatic environment, filtration, frequency of water exchanges, disinfection, and the employment of broodstocks free of oncogenic agents are recommended.

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