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Effect of Different Levels of the Dietary *Psidium guajava* L. Leaves Meal and Extract on Growth Performance, Feed Utilization, Body Composition, Immune-Related Genes Expression, and Health Status of the Thinlip Grey Mullet (*Liza ramada*) Fish

Mohamed M. Toutou[1], Mohamed Ahmed Abd allah Zaki [2], Asmaa Ibrahim Mohamed Abd EL-Monem[2], Ayaat Mohamed Elmaghraby[3], Salah Mahmoud Abdel-Rahman[3], Essam Menshawy Nasr Abd Rabou[2]

[1]Fish Nutrition Laboratory, Aquaculture Division, National Institute of Oceanography and Fisheries NIOF, Alexandria, Egypt
[2]Animal production Department, Faculty of Agriculture, Alexandria University
[3]Department of Nucleic Acid Research, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technological Applications, Alexandria, Egypt
[*]**Corresponding Author: mtoutou50@yahoo.com**

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ABSTRACT

Seven isonitrogenous (25.12% crude protein) were formulated to evaluate the effects of using different levels (0, 1, 2, and 4%) of guava (*Psidium guajava* L.) leaves meal (GLM) and their extracts (GLE) at the same levels as dietary supplements on growth performance, feed utilization, body composition, health status, and immune-related genes expression in the thinlip grey mullet (*Liza ramada*) fish. The results showed significant differences in growth performance; where, fish fed on 4% GLE recorded the highest values in final body weight (FBW), average daily weight gain (ADG), specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER), except for protein productive value (PPV), which recorded the highest value on 2% GLE. However, significant differences were noticed in energy utilization. Significant differences were noticed in body composition data between all treatments. The crude protein content in fish was decreased in all treatments compared to the control group. The differences between all biochemical parameters of serum were significant. The highest values of urea, creatinine, ALT, and AST were recorded in the control group when compared with all treatment groups. In terms of total protein, albumin, and globulin, the lowest values were recorded in the control group. Significant differences were observed in both amylase and lipase activities, with all treatments showing higher levels compared to the control group. In terms of both interleukin-1β and hepcidin genes expression, they were significantly decreased by about 99.9 in all treated groups (1, 2, and 4%) compared to the control group (0%). In conclusion, the treatments of fed guava leave meal and their extracts in *Liza ramada* have positive effect to improve the health status of the fish compared to the control.

INTRODUCTION

The global production of the mullet was 284,469 tons, which is approximately 13.95% **(FAO, 2020)**. The importance of the mullet is due to the fact that it is one of the

most important fish species in mariculture programs in numerous Mediterranean and Atlantic countries **(Carbonara *et al.*, 2022)**. Although, it is the second in fish aquaculture in Egypt, the thinlip grey mullet (*Liza ramada*) constitutes the majority of the aquaculture harvest of the mullet in Egypt **(GAFRD, 2020)**. A major challenge in the mullet farming is to produce a high quality and quantity product at a low cost while maintaining its nutritional value **(Toutou, *et al.*, 2023)**. Hence, one of the most important steps in developing and implementing a profitable and sustainable culture practice for this species is to formulate a nutritionally balanced and cost-effective commercial feed **(Abo-Taleb *et al.*, 2021)**. Due to the intensification practices in global aquaculture, fish are often confined in small volumes, which can result in outbreak diseases. In this context, the use of antibiotics is very usual. Chemotherapy is widely used to prevent and treat disease outbreaks, but it has multiple negative effects on the environment and human health **(Mishra *et al.*, 2022)**. Thus, looking for natural substance able to reduce the use of the antibiotics is imperative. Therefore, natural alternatives such as the inclusion of plant materials in fish feed formulation do not only have antimicrobial potential but also have other properties, such as digestive stimulant, anti-inflammatory and antioxidant that can benefit humans and other animals **(Emara *et al.*, 2020; Zhu, 2020; Kurian *et al.*, 2021)**. In recent years, immune-stimulant plants or their by-products have been used in aquaculture feed as an antibiotic's alternative. These plants could help develop more sustainable aquaculture all over the world. There is a great interest at present in the study of medicinal plants among them, guava (*Psidium guajava* L.). Guava is an important food crop and medicinal plant in tropical and subtropical countries and is widely used. The extracts and metabolites of this plant, especially those in the leaves and fruits, possess beneficial pharmacological activities **(Pimpley and Murthy, 2021)**. For its chemical components and its pharmacological and clinical uses, a number of metabolites have been shown to possess beneficial biological activities mainly belonging to phenols, flavonoids, carotenoids, terpenoids, and triterpenes **(Kumar *et al.*, 2021)**. There are many other important functions also attributed to guava leaves, including antioxidant, antimicrobial, anti-hypertensive, anti-inflammatory, anti-neoplastic, hepatoprotective, anti-allergic, anti-toxic, antispasmodic, cytotoxic, cardio protective, and anti-ulcer activities and supports its traditional uses **(Ceballos-Francisco, *et al.*, 2020; Giri *et al.*, 2020, Nhu *et al.*, 2020)**. Today, guava leaves are still used as laxatives and remedies for cold and cough, in addition to treating diarrhoea, dysentery, wounds, vomiting, gastrointestinal problems, and diabetes **(Poolsawat *et al.*, 2020)**. For this reason, we attempted to extrapolate the beneficial effects of guava (*Psidium guajava* L.) to aquaculture fish **(Poolsawat *et al.*, 2020)**. The available information on the natural aromatic plants as feeding attractants for farmed aquatic marine species, from percepective of nutritional studies, are very scarce **(Amulejoye *et al.*, 2020; Nhu *et al.*, 2020; Owolabi *et al.*, 2021)**. Therefore, the objective of this study was to investigate and evaluate the use of guava (*Psidium guajava* L.) leaves meal with different levels and their extracts as dietary supplements on growth

performance, feed utilization, body composition, health status, and the immune-related genes response of the thinlip grey mullet (*Liza ramada*) fish.

MATERIALS AND METHODS

Fingerlings and experimental design

This experiment was accompanied by cooperation between the Fish Nutrition Laboratory, Alex (N.I.O.F.), and Animal Production Department, Faculty of Agriculture Alexandria University, Egypt. The thinlip grey mullet fingerlings were obtained from a private fingerlings farm in Kafr El-Sheikh Governorate, Egypt. This experiment was carried out in El-Max Station for Applied Aquatic Research, Alexandria Branch (NIOF). After acclimation for two weeks, a total of 225 thinlip grey mullet fingerlings with an average weight of 10.2± 0.2g fingerlings^{-1} were randomly stocked in 21 glass aquarium (200L), representing seven treatment groups with three replicates each. The experimental glass aquaria were cleaned and supplied with marine water and aeration. A flow-through system was used in this trial to renew water quality. Water quality parameters were measured daily, including temperature, pH (Jenway Ltd., Model 350 pH meter), and dissolved oxygen (Jenway Ltd., Model 970 dissolved oxygen meter). According to the standard method described in **APHA (1998)**, mean values (± standard deviation) of water temperature (20± 2 ºC), salinity (37.5± 0.5ppm), dissolved oxygen (7± 0.2mg/ L) and pH (7.9± 0.1) were daily recorded. The fingerlings were fed the experimental diets till satiation for six days per week covering 12 weeks.

Preparation of the plant leaves extracts

Fresh guava leaves were dried at 40°C in a vacuum oven for 72h ground to pass a 1.0mm screen. Ten gram of plant materials were weighed in beaker and covered with aluminium foils then extracted by 100ml of ethanol (80:20, v/v) in water bath at 50°C for 4h as described by **Abeysinghe et al. (2007)**. Extracts were filtered by Whitman filter paper No, 42 (125mm), and then ethanol was removed using rotary evaporation at 40°C (Jobling laboratory Division, UK) and dried with a freeze drier. Moreover, it was stored at 4°C for assay.

Extraction of tannins

In laboratory, 250mg of dried (finely ground) plant material was taken in a glass beaker of approximately 25ml capacity. Subsequently, 10ml of aqueous acetone (70%) was added, and the beaker was suspended in an ultrasonic water bath and subjected to ultrasonic treatment for 2min in ice. The contents of the beaker were then transferred to centrifuge tubes and subjected to centrifugation for 10min at approximately 3000g at 4^{o}C. The supernatant was collected and kept on ice for the analysis of total phenol (TP), total tannin (TT) and condensed tannins (CT) according to the method of **Makkar (1993) and Porter (1986)**, respectively.

Experimental diets and feeding regimens

The formulated feed was prepared in the fingerlings nutrition laboratory (NIOF). Iso-nitrogenous (~25% crude protein) and iso-caloric (~442 kcal/ 100g gross energy, GE) experimental diets were prepared, the proximate chemical analyses of the formulated feeds (on D.M. basis) are presented in Table (1). The guava leaves meal and their extracts at the same levels (0, 1, 2, and 4%), respectively, were used as dietary supplements in the diets of the *Liza ramada* fish for 84 days. It should be noted that the 0.0 level is the control. The ingredients were blended with water to make a paste for each diet. The pastes were pelleted at 0.5mm pellets during the experiment. The feed was dried in an oven that was thermostatically regulated at 50°C for 36 hours and was then stored in plastic bags at −20°C until use. Seven experimental diets were formulated as follows: D1 is control, D2 is 1% GLM, D3 is 1% GLE, D4 is 2% GLM, D5 is 2% GLE, D6 is 4% GLM and D7 is 4% GLE. During the experiment, the total quantity of feed consumed by the fingerlings in each aquarium was estimated, and the feed intake of each fingerling was calculated accordingly.

Performance growth rates and feed-utilization efficiency

At the end of the experiment, the fingerlings were harvested, counted, and weighed. The performance growth rates and feed-utilization parameters were determined as follows: Weight gain (g) (WG)= FW – IW (Where, FW= final fingerlings weight (g) and IW= initial fingerlings weight (g)); specific growth rate (SGR%/ fingerlings/ day)= 100*[(Ln FW) - (Ln IW)]/ no. of experimental days; fingerlings survival (%)= 100* [final number of fingerlings/initial number of fingerlings]; daily feeding rate (%/ fingerlings-weight/ day)= 100* [FI/ FW] (Where, FI= total actual feed intake (g); feed conversion ratio (FCR) based on DM= FI (g) as dry weight/ WG (g); protein efficiency ratio (PER)= WG (g)/ protein intake (g); and protein productive value (PPV%)= 100* [protein gain (g)/ protein intake (g)].

Proximate chemical analyses of feed and fingerlings

At the beginning of the experiment, a sample of the tested fingerlings (approximately ten fingerlings) was randomly collected and preserved for initial body chemical composition. At the end of this experiment, nine fingerlings from each treatment were sampled to determine their proximate chemical compositions. Specimens of the tested feeds, and fingerlings samples were subjected to proximate chemical analyses of moisture, crude protein, crude lipid, crude fiber, and ash contents according to the **AOAC (2007)**.

Table 1. Feed ingredients (%) and proximate chemical analysis (%) on DM basis of the experimental diet used in the experimental diets

Item	Experimental No.[1]						
	D1	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**
Feed ingredients (100g1)							
Fish meal (68%)	10	10	10	10	10	10	10
Corn gluten	10	10	10	10	10	10	10
Soybean meal	25	25	25	25	25	25	25
Wheat bran by product	22	22	22	22	22	22	22
Yellow corn	10	9.17	9.9	8.34	9.8	6.67	9.6
Rice bran	15	15	15	15	15	15	15
Premix[2]	2	2	2	2	2	2	2
Plant oil[3]	6	6	6	6	6	6	6
Guava leaves meal (GLM)	-	0.83	-	1.66	-	3.33	-
Guava leaves extract (GLE)	-	-	0.1	-	0.2	-	0.4
Proximate chemical analysis (%) on DM basis							
Dry matter (DM)	97.2	97.3	97.2	97.2	96.7	96.8	96.8
Crud protein (CP)	25.1	25.2	25.1	25.1	25.2	25.1	25.0
Ether extract	11.5	11.2	11.5	11.5	11.5	11.6	11.4
Ash	11.2	12.3	11.2	11.2	12.1	11.7	12.4
Crude fibre	4.90	4.86	4.90	4.90	4.92	4.88	4.96
Nitrogen free extract (NFE)	47.3	46.44	47.3	47.3	46.28	46.72	46.24
Gross energy (kcal/100g DM)[4]	444.5	438.9	444.52	466.7	440.9	443.08	438.7
P/E ratio[5]	56.47	57.42	56.47	56.96	57.16	56.65	56.99

[1]Diet 1 (control diet without GLM or GLE); diets 2, 3, and 4 were containing 1, 2, and 4 % GLM and diets 5, 6 and 7 were containing 1, 2, and 4% GLE, respectively. [2]Premix composition: Each 1.0kg contains Vit A (400000i.u.), Vit D3 (100000i.u.), Vit E (230mg) Vit K3(165mg), Vit B1 (300mg), Vit B2 (80mg), Vit B6 (200mg), Vit B12 (1.0mg), Vit C (650mg), niacin (1000mg), methionine (3000mg), Choline chloride (10000mg), folic acid (100mg), biotin (2.0mg), pantothenic acid (220mg), magnesium sulphate (1000mg), copper sulphate (1000mg), iron sulphate (330mg), zinc sulphate (600mg), cobalt sulphate (100mg), calcium carbonate up to (1000g). [3]plant oil (a mixture of lin seed oil and soybean oil in a ratio of 1:1). [4]Gross energy (GE) was calculated as 5.64, 9.44 and 4.11kcal/ 100g for protein, lipid and NFE, respectively (**NRC, 1993)**. [5]P/E ratio= protein to energy ratio mg crude protein/Kcal gross energy.

Blood samples for haematology

The blood samples from six fish of the different groups were collected by suction from the caudal peduncle blood vessels. Whole blood samples were collected in small plastic vials.

Serum constituents

Blood samples were collected and transferred to centrifuge tubes and allowed to clot at room temperature. Serum was then separated by centrifugation at 3000rpm for 20 minutes. The serum was stored at -20ºC until further analysis. Serum total protein (g/ dl) was determined using the biuret test according to **Henry and salon (1974)**. Albumin content was determined according to **Doumas *et al*. (1972)**. The globulin content (G) was estimated by subtracting the albumin content (A) from total protein content. Urea, creatinine enzymatic (mg/ dl), activities of aspartate aminotransferase (AST, U/ l), and alanine aminotransferase (ALT, U/ l) were determined by the colorimetric method according to **Reitman and Frankel (1975)**. Serum lipase was determined by the enzymatic colorimetric assay according to **Panteghini *et al*. (1991)**. Amylase was determined by the method of **Winn-Deen *et al*. (1988)**.

Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA from liver tissues (n= 3 fish organs/ group) was isolated using Genozol Tri RNA Kit (Geneaid) according to the manufacturer protocol. The extracted RNAs from different samples were quantified and qualified (purity) using NanoDrop Spectrophotometer. Total RNAs samples were normalized (same concentration) to avoid any false increase in gene expression levels. Using TOPreal^{TM} One-step RT qPCR Kit (*SYBR Green with low ROX*), gene expression of hepcidin and interleukin-1β (IL-1β) (target genes) and β-actin (reference gene) were quantified by real-time PCR System (BioRad) with the use of specific primers sequences (forward/reverse) 5′-GCAATGCTGAATGCCTTCAT-3′/5′-GCTTCTGCTGCAAGTTCTGA-3′ for hepcidin gene (**Accession no.: MH674371.1/NCBI/GeneBank**), 5′-ACCAGCTGGATTTGTC AGAAG-3′/5′- ACATACTGAATTGAACTTTG-3′ for IL-1β gene **(Byadgi *et al.*, 2016)** and 5′- CCACGAGACCACCTACAACA-3′/5′- CTCTGGTGGGGCAATGAT-3′ for β-actin gene **(Bangcaya, 2004)**. The qRT-PCR was performed in a reaction mixture of 10µl using 0.5µl TOPreal^{TM} One-step RT qPCR enzyme mix, 5µl TOPreal^{TM} One-step RT qPCR reaction mix (2X), 0.5µl forward and reverse primers (10pm), 0- 3.5µl water (PCR grade) and 50ng RNA template. qRT-PCR program was applied as one cycle of cDNA synthesis at 50°C for 30 min, one cycle of enzyme mix activation at 95°C for 10min and followed by 45 cycles of denaturation at 95°C for 5sec., annealing at 60°C for 30sec and extension at 72°C for 30sec. The specificity of real-time PCR amplification was validated by analysis of melting curve, which ensured that only one PCR product was specifically amplified at the target size. The calculated expression analysis was done using $2^{-\Delta\Delta Ct}$ method **(Livak & Schmittgen, 2001)**, where the fold change ($2^{-\Delta\Delta Ct}$) = 1 (control group), <1 = down regulated and >1 = up regulated.

Statistical analysis

The data collected on the investigated were analyzed with one-way analysis of variance (ANOVA) using the SPSS (version 16/statistical package) to evaluate the

divergences among the tested treatments. The differences among the individual experimental treatments were assessed using Duncan's multiple range tests at the $P \leq 0.05$ level.

RESULTS

Growth performance and feed utilization

Growth performance and feed utilization parameters: initial body weight (IBW), final body weight (FBW), weight gain (WG), average daily weight gain (ADG), specific growth rate (SGR), food conversion ratio (FCR), protein efficiency ratio (PER), protein protective value (PPV), energy utilization (EU) and survival rates (SR) are presented in Table (2). The results showed that no significant differences were noticed for fish fed the experimental diets in final weight (FW), weight gain (WG), specific growth rate (SGR), food conversion ratio (FCR) and protein efficiency ratio (PER). However, significant differences were recorded in energy utilization (EU), and fish fed on D7 (4% GLE) recorded the highest values in all parameters except protein protective value (PPV), which recorded the highest value by D5 (2% GLE). It should be noted that all fish in all treatments showed good survival rates (SR) and recorded no significant differences ($P \leq 0.05$).

Table 2. Growth performance and nutrients-utilization of *Liza ramada* fed on different levels of leaves meal and their extract of *Psidium guajava* for 84 days

Indicator	Treatments (mean ±SE)						
	D1	D2	D3	D4	D5	D6	D7
IBW g/fish	10.3±0.02	10.2±0.03	10.3±0.01	10.23±0.0	10.3±0.03	10.27±0.02	10.30±0.01
FBW g/fish	35.2±0.33	34.1±0.34	34.8±0.32	34.7±0.31	35.3±0.34	35.1±0.33	36.9±0.30
WG g/fish	24.9±0.33	23.9±0.35	24.5±0.33	24.5±0.33	25.07±0.3	24.9±0.36	26.6±0.35
ADG g/fish/d	0.30±0.004	0.28±0.005	0.29±0.006	0.29±0.001	0.30±0.003	0.30±0.004	0.31±0.004
SGR %/d	1.5±0.01	1.4±0.02	1.4±0.04	1.4±0.011	1.5±0.011	1.5±0.011	1.52±0.011
FCR	1.95±0.02	2.03±0.022	1.99±0.023	1.99±0.024	1.94±0.026	1.96±0.022	1.84±0.023
PER	2.02±0.02	1.91±0.026	1.97±0.028	1.99±0.022	2.05±0.027	1.99±0.027	2.12±0.027
PPV %	52.9^{b}±1.15	44.72^{c}±1.16	51.34^{b}±1.1	46.95^{c}±1.17	57.66^{a}±1.19	47.44^{bc}±1.1	44.70^{c}±1.18
EU%	49.1^{b}±1.82	50.29^{b}±1.8	61.04^{ab}±1.9	53.36^{ab}±1.5	57.82^{ab}±1.5	58.72^{ab}±2	64.45^{a}±1.8
SR %	96.6±1.02	96.67±1.0	96.67±1.3	96.67±1.01	96.67±1.0	96.67±1.2	100.00±1

- Only means with different superscript letters are significantly different ($P \leq 0.05$). SE, is standard error.

Body chemical composition

The results of the body chemical composition data on dry weight basis for fish fed the experimental diets are summarized in Table (3). The significant differences ($P \leq 0.05$) were noticed among all treatments in dry matter, crude protein (CP), ether extract (EE) and ash contents. The highest values of moisture content were recorded by fish fed on D7 (fish fed on 4% GLE). Crude protein content for fish was raised in treatments D2, D4 and D5, while, in treatments D3, D6 and D7, it was decreased compared to the control group (D1). The highest value of the ether extract content was observed in D7 (4% GLE), while the highest value of the ash content was noticed in fish fed on D2 (1% GLM).

Table 3. Body chemical composition of *Liza ramada* fed on different levels of leaves meal and their extract of *Psidium guajava* for 84 days

Item	Initial	D1	D2	D3	D4	D5	D6	D7
Moisture %	71.76±0.31	63.50^{c}±0.32	65.37^{b}±0.29	63.50^{c}± 0.20	65.57^{b} ±0.35	61.98^{d}±0 .29	65.44^{b} ±0.34	66.69^{a} ±0.34
Body composition on dry matter (%)								
Crud protein (CP)	61.85±0.51	52.4^{b}±0.50	56.41^{a}±0.52	52.21^{b}± 0.55	57.53^{a} ±0.55	55.96^{a}±0 .48	51.85^{b} ±0.53	52.32^{b} ±0.50
Ether extract	19.62±0.60	33.20^{b}±0.60	27.84^{c}±0.61	34.86^{ab} ±0.62	29.24^{c} ^{d}±0.60	30.56^{c}±0 .61	33.50^{b} ±0.62	35.40^{a} ±0.64
Ash	**18.52±0.28**	**14.29^{b}±0.27**	**15.49^{a}±0.27**	**12.36^{c}± 0.26**	**12.61^{c} ±0.28**	**12.87^{c}±0 .26**	**14.17^{b} ±0.26**	**11.94^{c} ±0.28**

Only means with different superscript letters are significantly different ($P< 0.05$) , Mean ±SE, is standard error.

Biochemical blood parameters

The results of the biochemical blood parameters such as urea, creatinine, ALT, AST, total protein, albumin, and globulin are presented in Table (4). Significant differences in all biochemical parameters were noticed. The highest values of urea, creatinine, ALT, AST were noticed in the control group (D1), but the lowest values of urea, ALT, AST were observed in D7 (4% GLE), except creatinine which recorded the lowest value in D6 (4% GLM). On the other hand, the highest values of albumin content were recorded by D7 (4% GLE). In terms of total protein, albumin and globulin, the lowest values were recorded in the control group (D1). While, the highest values of total protein and globulin were noticed in D2 (fish fed on 1% GLM), except albumin which recorded the highest value in D7 (4% GLE). Concerning the serum amylase and lipase contents, significant differences were observed. Both amylase and lipase recorded higher values in all treatments (D2- D7) than the control group (D1), and the highest value was noticed in fish fed D7 (4% GLE), as shown in Fig. (1).

Hepcidin and interleukin-1β genes expression

Hepcidin and interleukin-1β immune-related genes expression in fish fed the experimental diets containing different levels of leaves meal and their extracts of guava were significantly down-regulated compared to the control group. The interleukin-1β gene expression was significantly decreased by about 99.9, 99.9, 99.7, 39.3, 99.9 and 99.7% in all treated groups of 2D, D3, D4, D5, D6 and 7D, respectively, compared to the control group (D1). Additionally, the hepcidin gene expression was significantly decreased by about 99.9% in all the treated groups (D2- 7D), except for D5 which was 40.1% compared to the control group (D1), as shown in Fig. (2).

Table 4. Biochemical blood parameters of the thinlip grey mullet fed on different levels of leaves meal and their extract of *Psidium guajava* for 84 days

Item	D1	D2	D3	D4	D5	D6	D7
Urea (mg/dl)	17.16^{a}±0.41	15.33^{b}±0.38	18.66^{a}±0.45	14.66^{b}±0.40	13.66^{b}±0.46	14.33^{b}±0.37	14.17^{b}±0.40
Creatinine(mg/dl)	0.62^{a}±0.39	0.63^{a}±0.29	0.47^{c}±0.27	0.51^{b}±0.38	0.54^{b}±0.30	0.42^{d}±0.26	0.45^{c}±0.30
AST (u/l)	242.0^{a}±6.03	212.7^{b}±6.03	234.7^{a}±5.0	185.7^{c}±6.04	187.8^{c}±6.1	181.0^{c}±6.3	166.7^{d}±5.83
ALT (u/l)	8.67^{a}±0.20	7.00^{bc}±0.09	7.33^{b}±0.23	6.00^{bc}±0.23	6.67^{bc}±0.25	6.33^{bc}±0.18	5.97^{c}±0.22
T. protein (g/dl)	3.18^{d}±0.07	3.89^{a}±0.09	3.49^{cd}±0.09	3.68^{c}±0.08	3.37^{cd}±0.11	3.71^{b}±0.08	3.58^{c}±0.10
Albumin (g/dl)	1.69^{d}±0.31	1.93^{b}±0.33	1.78^{bc}±0.28	1.92^{b}±0.29	1.72^{d}±0.30	1.83^{c}±0.28	1.96^{a}±0.34
Globulin (g/dl)	1.49±0.15	1.96^{a}±0.17	1.71^{b}±0.19	1.76^{b}±0.16	1.65^{c}±0.15	1.88^{ab}±0.11	1.62^{c}±0.17

Only means with different superscript letters are significantly different ($P \leq 0.05$), Mean ±SE, is standard error.

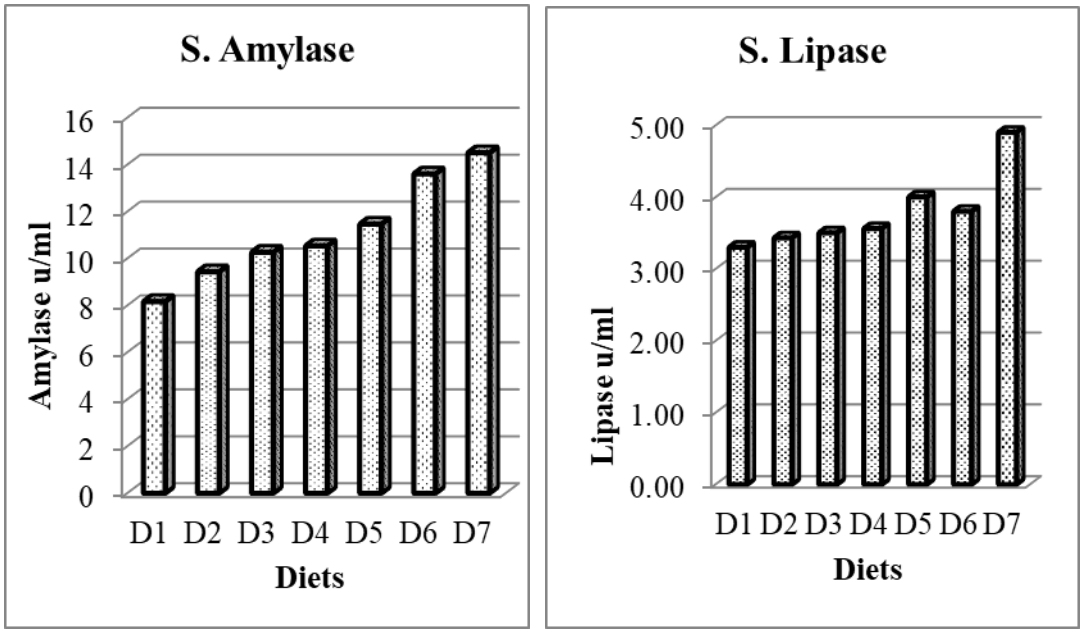

Fig. 1. Serum amylase and lipase enzymes (u/ ml) of *Liza ramada* fed on different levels of leaves meal and their extracts of *Psidium guajava*

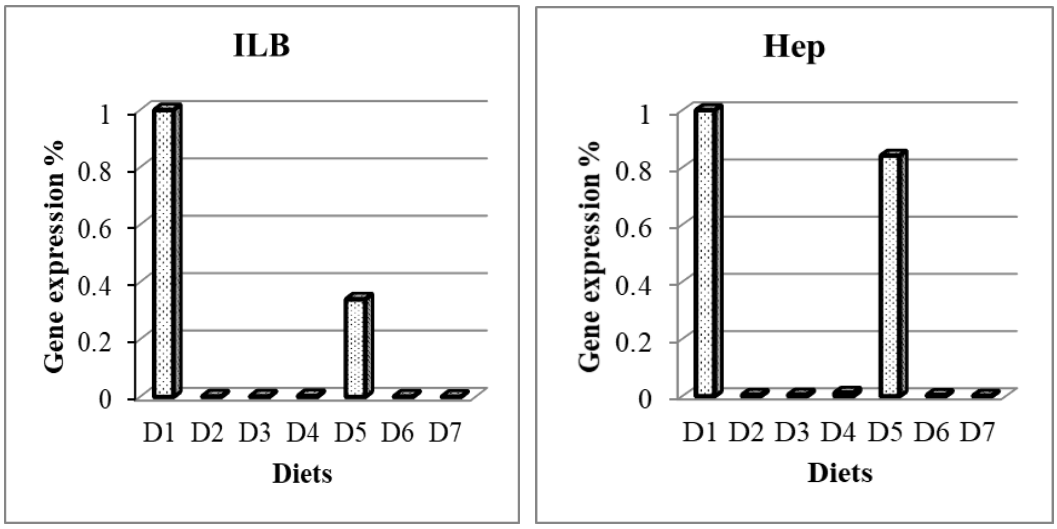

Fig. 2. Gene expression of IL-1β and Hep in the *Liza ramada* fish fed the experimental diets values are expressed as means ±standard deviations

DISCUSSION

Among plants considered to have such potential is the guava (*Psidium guajava* L.). There are many important functions attributed to guava leaves, in the current study, although no significant differences were recorded in growth performance parameters, these parameters were improved in treatments compared to control group, and the significant differences were noticed in protein protective value (PPV) and energy utilization (EU). The current results agree with those of **Effendi *et al.* (2022)**, who recommended the addition of guava leaves into common carp feeds to improve growth and feed conversion ratio (FCR) in *O. niloticus* fed *Psidium guajava* leaf extract supplemented diets. Additionally, **Olusola and Olorunfemi (2017)** assessed the effects of varying degrees of guava leaves on *clarias gariepinus*. Furthermore, **Giri *et al.* (2015)** and **Fawole *et al.* (2018)** stated that, guava leaf dietary supplementation dramatically improved the development performance and nutrient utilization in the *L. Rohita* fingerlings.

In terms of the proximate chemical composition, the crude protein, ash, and moisture content of the fish were significantly altered after the feeding trial. However, our data showed that the ether extract content increased in all treated fish ($P \leq 0.05$) compared to the control group, except in D2 (1% GLM). These results partially agree with the findings of **Fawole *et al.* (2022)**.

Haematological values in current study showed that the fish were in good condition, as most of the parameters were within the desirable ranges for fish culture, and significant differences in all biochemical parameters were recorded. The highest values of urea, creatinine, ALT and AST were noticed in he control group compared to the treated fish. On the other hand, the improvement was noticed in the amylase and lipase contents

in treatments compared to the control group, consistent with findings from **Mello *et al.* (2013)** and **Nwanna *et al*. (2013)**.

Serum creatinine is an important indicator of renal health because it is an easily measured byproduct of muscle metabolism that is excreted unchanged by the kidneys. If the filtration in the kidney is deficient, creatinine blood levels rise. Therefore, creatinine levels in blood and urine may be used to calculate the creatinine clearance, which correlates with the glomerular filtration rate. The elevation in the urea level in the infected fish may be due to gill dysfunctions, as the urea depended totally for excretion on the gills **(Yang & Chen, 2003)**.

On the other hand, our current results noticed decreases in urea and creatinine in the treated fish compared with control group. The presence of many bioactive molecules in medicinal plants may explain the health promoting activity that is attributed to different chemical compounds such as phenolic acids, flavonoids, tannins, alkaloids, terpenoids, saponins, glycosides and others. The presence of such bioactive plant components explains the enhancement of immunological parameters in fish fed medicinal herbs **(Ghosh *et al.*, 2019)**. This attribute can alter enzyme profiles or activity levels **(Debnath *et al*., 2007)**.

The current results match with the result conveyed by **Abdel-Tawwab and Hamed (2020)**. The authors recorded that the elevation in total protein and its derivatives in the blood of fish supplemented with dietary guava leaves extract indicates to the immune-stimulatory on the Nile tilapia. The results accomplished from this trial are similar to those of **Fawole *et al*. (2016)**. A feeding trial was conducted to assess the haemato-immunological response of the *L. rohita* fingerlings fed guava leaves extract infected with *Aeromonas hydrophila*. The results recorded that total protein level increased in all the treatments group compared with the control group. Similarly, the albumin and globulin content in the treatment groups were significantly higher compared with the control.

The results of the present study revealed that the activities of AST and ALT enzymes decreased significantly in the guava leaves extract fed groups compared to the control, where the higher activities were recorded in the control fed fish. Similar results were reported in the *L. rohita* **(Giri *et al*., 2017)**. These data are in parallel with the data concluded by **Omitoyin *et al*. (2019)**. There was a significant reduction recorded in AST and ALT values for fish fed guava leaves extract fortified diets. The fact that the enzyme activities were reduced showed that the extract protects against hepatic damage. Furthermore, **Ghaffar *et al*. (2020)** reported that the serum biochemical parameters (ALT and AST) are reliable and gold standard clinical chemistry markers to know the hepatic injury. The significantly higher values of these enzymes might be due to the damage in the membranes of hepatocytes, which led to an increase in the drainage of these enzymes into serum **(Abarike *et al*., 2020)**. However, the presence of metabolites such as tannins, alkaloids, and saponins in the leaves of the *P. guajava* extracts might be responsible for

cell membrane protection and stability against free radical mediated toxicants **(Gobi *et al*. 2016)**. These results coincide with the obtained results.

Digestive enzymes are one of the cornerstones of digestion for the net efficiency of the whole digestive process mainly depends on the type and functional characteristics of these parameters **(Pujante *et al.*, 2017)**. Degradation of nutrients in the digestive tract of fish largely depends on available enzymes. Hence, determining the digestive enzyme activity is of a potential interest to obtain and complete valuable information concerning fish digestive physiology. The digestive enzyme activity varies within species. It is influenced by biotic (size, age and origin) and abiotic. Medical plants and their derivatives have been reported to increase the secretion of the digestive enzymes **(Ahmadifar *et al*., 2021)**.

In current study, the values of lipase and amylase in treated fish groups were significantly increased compared to the control group. The dietary supplementation with dried leaves of *Psidium guajava* (GLP) increased the lipase activity in the hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) compared to the control group levels although the increase was only statistically significant in the group fed GLP. These results concur with previous studies conducted in different aquatic species, reporting significant increases in serum and mucus protease activity after dietary supplementation with guava leaves **(Yin *et al*., 2014; Gobi *et al*., 2016; Ceballos-Francisco *et al*., 2020)**.

The present outcomes may be attributed to the bioactive GLE compounds, which have potential immune stimulating properties **(Harikrishnan *et al*., 2011; Cuevas *et al*., 2013)**. Similar results have been observed in the Indian major carp **(Giri *et al.*, 2015)**, the Mozambique tilapia **(Gobi *et al.*, 2016)**, and the common carp **(Hoseinifar *et al.*, 2019)**, where dietary supplementation with guava leaves powder or extracts enhanced various physiological parameters. In addition, the transfer of nutrients from food to the body increases, and this affects fish development positively.

Guava leaves also contain essential oils, which improve feed quality, productivity and health. Essential oils mixed with feed can stimulate the central nervous system, which ultimately results in an increase in appetite and the consumption of nutrients **(Olusola & Olorunfemi, 2017)**. The presence of essential oils can stimulate the production of digestive juices that produce a suitable pH for digestive enzymes, such as pectinase **(Weli *et al*., 2018)**. At the same time, there is an increase in the activity of digestive enzymes, the real effect of this mechanism being the improvement of energy conversion and digestion of food substances and a positive influence on the metabolism of nitrogen, amino acids, and glucose **(Yu *et al*., 2009)**.

According to **Omitoyin *et al*. (2019)**, phenols, tannins, and saponins from guava stimulate the secretion of digestive enzymes, thereby affecting the growth and absorption of nutrients by the fish. The growth augmenting effects observed in the leaf extracts groups could be ascribed to the systemic bioavailability of the bioactive principles

present in the leaf's extracts, which appears to enhance digestive enzyme function, thereby improving nutrient uptake more efficiently through the wall of the digestive tract.

The modifications in the gene expressions almost mirror the growth performance trend and biochemical parameters described earlier. These results have provided further evidence supporting the nutrigenomic principle that nutrients in a feed formula are dietary signals which may affect the expression of the immune regulatory genes. The guava leaves extract (GLE) was found to be effective in enhancing a nonspecific immune response and growth performance and in reducing the *V. parahaemolyticus* infection in the white shrimp **(Abidin *et al*., 2022)**.

The identification of genes related to immunity in fish and the determination of their expression patterns have received a great attention. Mostly, the increase in the expression of immune genes is usually considered as a sign for immune stimulation or enhanced immune response **(Abo-Al-Ela, 2018)**. In the current study, the gene expression of hepcidin and ILB were significantly up-regulated in the control group, whereas the genes in the treated groups were down-regulated.

The cytokine interleukin-1β (IL-1β) is a key mediator of the inflammatory response. It is essential for the host-response and resistance to pathogens, it also exacerbates damage during chronic disease and acute tissue injury **(Lin & Edelson, 2017)**. Il1β is well-characterized cytokine; it plays an important role in the cellular responses to immunological challenges, infection, and inflammation **(Tassakka & Sakai, 2004)**. In fish, il1β is constitutively expressed in several tissues, such as spleen, head kidney, and liver, and higher expression has been detected in the spleen **(Lu *et al*., 2012; Bo *et al*., 2015)**. Several studies on dietary supplementation with guava leaves observed an enhancement of the innate immune response in the *P. monodon* serum and hepatopancreas **(Yin *et al*., 2014)**, as well as in the serum of the *L. rohita* and skin mucus of the *C. carpio* fingerlings **(Giri *et al*., 2015; Hoseinifar *et al*., 2019; Giri *et al*., 2020; Abidin *et al*., 2022)**. The results accomplished in the current trial are similar to those of **Giri *et al*. (2015)**, who reported a decrease in the production of IL-1β in the *C. carpio* fingerlings treated with guava leaves **(Hoseinifar *et al*., 2019)**. The administration of the *P. guajava* leaves significantly modulated some immune-related enzymes (protease, antiprotease and peroxidase) in the skin mucus of the hybrid tilapia. In addition, the bacterial load after the *Vibrio harveyi* infection in skin, spleen, and liver significantly reduced in fish supplemented with guava leaves **(Ceballos-Francisco *et al*., 2020)**.

Fish have developed many defence mechanisms against a variety of pathogens; one of these defences is antimicrobial peptides (AMPS). That are considered one of the major defence lines and involved in the innate immune responses. Among the AMPS is hepcidin, which is one of the family of defence proteins; it has proved to be a vital effector molecule in the response of the innate immunity. The anti-pathogen activity of hepcidin involves varied modes of action, such as limiting iron availability. For pathogens, it affects the membrane integrity, hence increasing the membrane

permeability and destroying the pathogens **(Hal *et al*., 2022)**. Hepcidin is known to be upregulated to limit the circulating iron that is necessary for infectious pathogens, thereby increasing iron retention in macrophages **(Rishi *et al*., 2015)**. Iron restriction during infection (iron competition) is recognized as one of the general innate defence mechanisms against many bacterial and viral pathogens **(Lee *et al*., 2022)**. It is possible that this complexity is more common in fish than we thought, and it provides a clear evidence for fish innate molecules, as HDPs are much more specific and evolved in contrast to the classical immunity described in mammals **(Serna-Duque *et al*., 2022)**. Normally, the low levels of hepcidin are found in healthy specimens, while the presence of a pathogenic organism causes their up-regulation **(Hoseinifar *et al*., 2019)**. Thus, the expression of hepcidin is considered as an indicative of its role in the direct killing of pathogens and underlines its function as a component of the innate defense mechanism **(Wang *et al*., 2009)**.

However, although the use of plant products is considered safe by most consumers and sector operators, it is necessary to support this assumption with an experimental evidence. In addition to immune-response enhancement, our study also provides an evidence that dietary supplementation with guava leaves could effectively regulate the expression of certain cytokine-related genes.

CONCLUSION

The current study showed the superiority of the treatments, in which the fish of the *Liza ramada* were fed guava leaves meal and their extracts compared to the control, to improve the health status. Finally, in the present research, we demonstrated that the leaves meal and the extracts obtained from guava have beneficial effects on the fish health. These results indicate that guava leaves meal and their extracts are low-cost options with significant potential benefits if used routinely.

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