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Effect of *Mentha arvensis* enriched diet to promote the growth and immune response of *Clarias batrachus* against *Aeromonas hydrophila* challenge

ABSTRACT

The study was conducted to investigate the effects of fish fed diet *Mentha arvensis* extract on growth performance, non-specific immunity and expression of some immune-related genes and resistance to *Aeromonas hydrophila* in *Clarias batrachus*. Five diets were formulated with 0,1,2,3, and 4% of *M.arvensis* leaf extract. The results indicated that, compared to the control groups, 2-4% dietary inclusion increased growth and feed consumption. In the dietary inclusion of 3-4% *M. arvensis* extract groups were increased relative on weight gain, specific growth rate, RBC, WBC, total hemocyte counts, total protein, globulin than control. Fed diet supplements with 3% mint-extract increased the total protein, WBC and globulin and phagocytic indexes and lysozyme activity increased at the 2, 3 and 4% of mint groups relative to the control. The PCR analysis showed that *TNF*, *IL-1, MyD88,* and *TLRs* were increased in the 2-4% fed diet *M. arvensis* extract groups than the control. These results suggest that 3% of *M.arvensis* extract significantly influences the immunomodulatory activity and immune-specific genes of *C. batrachus*.

Keywords: Growth performance; Wild mint; Walking cat fish; Serum biochemical parameters; Innate immune-related genes;

1. Introduction

The walking catfish, *Clarias batrachus,* is a bottom dweller, freshwater and omnivorous fish, among Asia's most prominent cultivated fish species (Narra,2017). *Aeromonas hydrophila* is utmost commonly come upon bacterial pathogen. To reduce mortalities, chemicals and antibiotics are used to prevent and treat infection, increasing environmental pollution and enhancing antibiotic-resistant pathogens. Natural immunostimulants could be a potential alternative to other detrimental chemical supplements and allow sustainable aquaculture (Baba et al.,2018; Sattanathan et al.,2020a). Mint extracts (*Mentha piperita*) showed positive effects on growth, hemato-biochemical factors, and increased resistance to various bacterial infections in tambaqui (*Colossoma macropomum*) (Ribeiro et al., 2016), Caspian brown trout (Adel et al.2015a), *Yersinia ruckeri* (Adel et al.,2016), *Labeo rohita* (Sattanathan et al., 2020b), *Rachycentron canadum* (Wu et al.2016), *Rutilus kutum* (Adel et al.,2015b) and *Lates calcarifer* (Talpur,2014). As a result, it is critical to reduce inflammation and boost immunity in animals by balancing pro- and anti-inflammatory cytokine production by sufficient nutrition and/or supplements (Baba et al.,2018). The effects of some feed additives, such herbs, prebiotics, probiotics, and synbiotics, on the immune system have been examined (Hoseinifar et al.,2016; Khalil et al.,2017). However, to the best of our knowledge there is no information on the effects of *A.hydrophila* infection on the fish expression of innate immune gene profiles in *C.batrachus*. Thus, the work was to investigate the effects of dietary inclusion of *M.arvensis* on growth performance, mortality rate, hematological parameters and immune responses in the *C.batrachus*, challenged with *A. hydrophila*.

2 Materials and Methods

2.1. Herbal collection and diets

The experimental sample of the *M.arvensis* herbal was collected from a local market and washed in distilled water. The herbal extract was air-dried, and then the dark was made into

powder with nylon mesh. *M.arvensis* powder was mixed in a 1:5 ratio with 90% ethanol for 48 h using a shaker. The *M.arvensis* powder was shaken for 48 h while being combined in a 1:5 ratio with 90% ethanol. The filter paper was used to separate the ethanol from the powder mixes, which was done in a rotary evaporator at 30-45°C; the extract of *M.arvensis* was stored at -20°C till usage (Adel et al.,2015b). The commercial fish-fed diet (Growel-feeds, India, containing 8% ash, 40% protein, 6% fat, 3% fiber, and 12% moisture) was ground into a powder and combined to the appropriate concentration to produce five experimental diets with varying compositions. The nutritional source of fish was pleased by the diets according to NRC (2011). The obtained pathogenic bacteria, *A.hydrophila* (MTCC1739; IMTECH, Chandigarh, India), followed by the culturing duration of 24h in the tryptone soya broth (TSB) at 37°C and the glycerol-containing culture was kept at -20°C until required.

2.2. Experimental Design and Pathogen

Healthy *C.batrachus* (average weight 9±2.4g) were purchased from local fish farm in Tamil Nadu, India. The fish were kept in a 500-L rectangle fibre tank a 24°C for two weeks to get used to the lab environment. Three hundred fishes were randomly split into five groups in each triplicate after two weeks of acclimatization to commercial fish-fed diets, as follows: 0%: Control (basal diet), 1%: Diet+1g/kg *M.arvensis*,2%: Diet+2 g/kg *M.arvensis*, 3%:Diet+3g/kg *M.arvensis*, and 4%: Diet+4 g/kg *M.arvensis* for 8-weeks feeding trial.

The *A. hydrophila* were cultured overnight in LB broth under shaker with 200 rpm. The bacterial culture medium was centrifuged to the pellet cells and the cells were washed thrice with 1XPBS. The cell counting was done and suspension was diluted to get a cell density of 1 x 10^7 cells/mL. Fish was given anesthesia in a 150mg/L buffered MS-222 solution (Sigma), experimental groups 1 to 4% were challenged intraperitoneally (IP) injected with 100 μL of PBS containing *A.hydrophila* (1x10^7cells/mL) on the zero day. The control group fishes were injected with 100 μL PBS. The respective pellets diets were provided twice a day throughout

the experimental time. During the experiment, water parameters were measured, including dissolved oxygen level of 5.2±0.14 to 6.5±0.43mg/L, temperature level at 25 to 30°C, and pH range of 6.95 to 7.80, ammonia 0.12±0.01 to 0.15±0.02 mg/L and nitrate 0.022±0.01 to 0.024±0.02mg/L.

2.3. Sampling Collection

Each experimental sample group was randomly selected at weeks 2,4,6, and 8 post challenge with *A.hydrophila*. The challenged fish were then given a 150 mg/L buffer solution while sedating (MS-222,Sigma-Aldrich,USA). Each sample's blood was collected from the caudal vein using a 24-gauge syringe needle. The collected blood was split between tubes that had been heparinized and non-heparinized. The non-heparinized blood samples were centrifuged at 3500xg (RCF) for 15 min to properly extract the serum after 2h of incubation at room temperature. Before usage, the serum was separated and kept at -20°C. Tissue samples from the spleen and head of the kidney were collected, promptly added to the TRI® reagent (Invitrogen, USA), and then stored at -20°C until use.

2.4. Hematology

Giemsa staining was used to stain the collected blood cell, and its estimated value was based on its morphological characteristics. By using a hematocytometer, the diluted blood cells were utilized to calculate the erythrocytes (1:1000 in PBS) and leucocytes (1:100 in PBS) (Blaxhall and Daisley,1973). Using a commercial estimate kit (Liquichem Total Protein/Albumin kit, Recorders and Medicare Systems Pvt. Ltd., India), the total protein and albumin components of the serum were determined using the BCG technique and the Biuret method (Lopez et al.,2015), respectively.

2.5. Growth Performance

The following parameters were measured with being deprived of feeding for 24 h before weighing and sampling and at the end of the feeding trial.

Weight gain(W_g) = W_f (g)–W_i (g)x100

Specific growth rate(SGR) =100 [Ln W_f (g)–Ln W_i (g)]/T

Fed conversion ratio(FCR) = F_i(g)/W_g(g)

Where W_i is the initial weight, W_f is the final weight, and T is the number of days in the feeding period.

2.6. *Phagocytic activity*

The phagocytic activity of leucocytes was analyzed by a modification of early procedure (Lopez et al.,2015). The phagocytosis index was determined to mean the number of yeast cells engulfed by positive phagocytes multiplied by the percentage of phagocytosis using the following formula (Sönmez et al.,2015). The microscopic method determined the phagocytic activity:

Phagocytic activity(%) = (number of phagocytic cells/Total number of cells)×100.

2.7. *Lysozyme activity*

A turbidimetric test was used to measure the lysozyme activity (Mandalakis et al.2021). In a 0.5M sodium phosphate buffer, 10-50μL of test serum and 0.5-1mL of micrococcus lysodeikticus suspension (Sigma) was added and mixed. Using an OD at 530 nm spectrophotometer, the samples were measured. As a benchmark, egg white lysozyme was employed.

2.8. *Respiratory burst activity and Complement activity*

The respiratory burst activity of blood leucocytes was confirmed by nitro blue tetrazolium reagent (Mandalakis et al.2021). The complement activity (Mandalakis et al.,2021) was measured by hemolysis unit mL^{-1} using sheep red blood cells (HiMedia,India) as targets. In the complement activity, the volume of complement producing 50% hemolysis (ACH50 unit/mL) was determined.

2.10. *RNA extraction and cDNA synthesis*

Following the manufacturer's instructions, total RNA was extracted from each group's spleen samples (n = 6) using the TRI® reagent (Sigma, India). About 0.2 U of DNase was used to separate RNA samples. On a Nano-drop system, the quantitative and qualitative analysis of RNA was determined at 260 and 280 nm (A260/A280). In accordance with the instructions provided by the manufacturer (Bangalore Genei, Karnataka, India), 1 ug of RNA sample was added to a 20L reaction of the Prime Script™ RT Reagent Kit for cDNA synthesis.

2.11. Real-time PCR analysis

Quantitative real-time PCR (ABI 7500, Applied Biosystem, USA) analysis was used to amplify gene expression profiles using the pairs of carp-specific primers for *GAPDH*, *IL-1β*, *TNF- α*, *TLR-2*, *MyD88* (**Table 1**) in specific genes obtained from NCBI. All the primers were commercially purchased from Bangalore Genei, Karnatka, India. A total of 20μL of the real-time PCR reaction were used, consisting of 2 μL of cDNA, 0.5 μL of forward and reverse primers (both at 100 mM), 10 μL of 2x SYBR Green qPCR Master Mix, and 5 μL of water. The thermal analyzer cycle at 95°C for 5 mins, followed by 40 cycles at 95°C for 10s, 56°C for 15s, and 72°C for 15s. The relative gene expression levels target gene was normalized to *GADPH* and analyzed using the $2^{-\Delta\Delta}$CT method (Schmittgen and Livak, 2008).

2.12. Statistical analysis

All the data were statistically analyzed by one-way analysis of variance, using SPSS (version 19). Tukey's tests were used to compute a significant comparison between the treated groups. The mean and standard deviation data (n=6) are presented as follows, and P values under <0.05 were regarded as significant.

3. Results

The present study evaluated the survival, growth rate, and weight gain of infected fish that were increased with *M.arvensis* extracts fed diet compared with control groups (Table 2). The survival rate (96.2, 96.8 and 97.6%) was seen in all fish groups with a fed diet. The weight gain

and growth rate were noted in 3 and 4% of *M.arvensis*-enriched diets. The feed conversion rate was noted as 3 and 4% with *M.arvensis* concentrated diets treated fish group. In the control groups, 1% *M arvensis* fed diet; fish showed a similar effect of feed conversion rate.

The experimental results revealed that the total number of RBC and WBC were increased by *M.arvensis* extracted fed diet in a dose-dependent manner (Table 3). The RBC was increased with 3% of fed diet *M.arvensis* supplement compared with control on weeks 6 and 8. The WBC level progressively increased after 6-8 weeks in *C.batrachus* fish-fed diet supplementation with 3 and 4% of *M.arvensis* extracts. Similarly, globulin increased in 3 and 4% *M.arvensis* supplemented fed diets, which was not found in the 1 and 2% supplementation diet compared to the control group. The administration of supplemented-fed diets at 1-2% treatment did not increase the total protein and globulin for 2-4 weeks. The total protein was significantly increased in fish fed with 3-4% *M.arvensis* fed diets (Table 3), 3 and 4g/kg of dry matter of *M.arvensis* extract, respectively.

The phagocytic activity in *M.arvensis* supplemented fed diets gradually increased (Fig. 1A), and a significant difference was observed in the phagocytic activity after 2 weeks. Significantly phagocytic activity was observed in infected fish fed diet with 3 and 4% of *M. arvensis* extract treated experiments after 2 weeks. In the beginning, the treated and control group indicated the normal level. The respiratory burst was increased in all treatment groups of *M. arvensis* supplemented fed diets evaluated against the control group (Fig.1B). The greatest respiratory burst was observed in the fish-fed diet with 3 and 4% of *M.arvensis* extracts. All four-dose treatment groups increased the respiratory bust in 6-8 weeks compared to the control group. The overall experimental results of the lysozyme activity in serum showed a significant enhancement compared with a control group (Fig.1C). The enzyme production reached statistically significant with 3 and 4% of *M.arvensis* extract formulated diet from 6-8 weeks. *M. arvensis* extract-supplemented fish group showed a significant increase in the complement

activity throughout the experiment than the control fish group (Fig.1D). It was significantly higher with 3 and 4% of the fed diet on 6-8 weeks. However, no prominent differences were found in the complement activity between 1-2% of the fish and the control groups.

The relative mRNA expression of *IL-1β, TNF-α, TLR2* and *MyD88* of an immune gene in the spleen were analyzed by the quantitative-PCR method and significantly expressed on 2-8 weeks (Fig.2). The level of *IL-1* transcription was significantly greater after the second week of mint extract fed diets (3 and 4%) treated fish (Fig.2A). Expression of *TNF-α* in fish mint treated 2 and 3% was significantly higher at 8 weeks post treatment as compared to the control (Fig. 2B-D). Expression of *TLR* and *MyD88* in fish mint treated 3% was significantly higher at 6 to 8 weeks post treatment as compared to the control (Fig.2C-D). Mint fed diet of fish to showed a time-depend induction of *TNF-α*, *TLR* and *MyD88* gene transcription was up-regulated than the control group (Fig.2B-D).

4. Discussion

The main compounds of *M.Arvensis* were discovered to be alkaloids, flavonoids, polyphenols, tannins, cardiac glycosides and eugenol when it was analyzed (Malik et al.,2012). The recent study showed that the 34 compounds and other minor substances were examined, such as piperitone (1.32%), methone (5%), neomenthyl acetate (5.18%) and isomenthone (5.24%) and menthol 77.94% (Makkar et al.,2018). Among them, menthol, p-menthone, isomenthone and neo-menthol were major and commercially valued. Also, the study demonstrated that the mint and its ingredients have diverse biotic activities and anti-inflammatory properties (Makkar et al.,2018).

The immunological response to infections is now being studied and broadly beneficial when used as dietary additive of marine polysaccharides from seaweed (Liu et al.,2020). Usage of natural goods or newly created natural chemicals has improved fish's immune defense and survival against infections. Recently, many plant extracts like, sage, mint and thyme oils,

196 *Allium stipitatum* powder, *Taraxacum officinale*, flower extract and *Berberis vulgaris* fruit
197 extract have been discovered and provided in fish feed (Sönmez et al.,2015; Shekarabi et al.,
198 2022; Shekarabi et al.,2022). The growth act, survival proportion, and immune function
199 against *V.harveyi* disease resistant increased with the supplementation of peppermint feed
200 (Adel et al., 2015a). On the other hand, *M.spicata* did not respond to growth level and
201 antioxidant activity in juveniles fish (Sönmez et al.,2015). The experiment assay analyzed the
202 effect of *M. arvensis* extract on the growth performance, existence, non-specific/specific
203 immune response, and disease resistance of *C.batrachus* against *A. hydrophila*. The results of
204 *M.arvensis* extract-treated fish survival and growth performance were at high-level (Adel et
205 al.,2015b).

206 In the immune system, blood parameters are a basic tool to indicate the physiological
207 condition, health status and disease tracking, the feed supplement or anti-nutritional elements
208 concentration based on enhance the hematological and biochemical profiles (Abasali and
209 Mohamad,2010). The WBC is one of the important factors in preserving against chemicals and
210 pathogens (Abasali and Mohamad, 2010). In *C.batrachus*, the hematological parameters such
211 as WBC, RBC, cumulative protein and globulin were slightly greater in the infected fish-fed
212 diet at 3 and 4% *M. arvensis* extract treatment groups, which was similar to other case studies
213 (Adel et al.,2015a; Adel et al.,2016; Abasali and Mohamad,2010). The WBC number was
214 increased with a 3% fed diet of the *M.arvensis* fish group at 6 weeks. The RBC response was
215 observed in *M.arvensis* extract-treated fish group after 4th week. Similar results reported that
216 increased RBC and Hb parameters in other studies (Paknejad et al.,2020). The ability of the
217 fish immune system to better inhibit the bacterial population in fish bodies (Adel et al., 2015a).
218 The levels of globulin and albumin in the fish serum total protein were thought to be associated
219 with a more active innate immune response. Diverse humoral components in serum proteins

engage phagocytosis activity and initiate the host natural immune response to fish infection (Adel et al., 2015a).

The peptide levels of globulin indicate the potential innate immune function in the blood. In addition, *M.arvensis* extract enhanced the other immunomodulatory factors such as phagocytic index and respiratory burst during infection periods. Fishes were given a diet containing a variety of herbal extracts, the fish's rudimentary innate immune systems' phagocytic and respiratory burst responses were boosted (Sattanathan et al.2020b). Likewise, a study reported that the *C.auratus* against *A.hydrophila* supplemented with azadirachtin (Kumar et al.2013). The superoxide anion is generated during respiratory bursts by phagocytes in banana shrimp (Liu et al.,2020), a toxic form of oxygen, and also observed in herbal-treated fish (Jian and Wu,2004). In the fish immune system, phagocytosis is one of the essential cellular responses confirmed by different kinds of herbal extracts (Chi et al.,2016).

The potential non-specific immune mechanism of alternative complement activity is preserved against microorganisms such as bacterial, fungal, viral and parasitic in fish (Jian and Wu, 2004). Lysozyme is a significant part of the non-specific immune system in fish, which can hydrolyze bacterial cell walls (Adel et al.,2015a; Adel et al.,2016). The rise in lysozyme enzyme level suggests promoting various humoral factors which can protect against the host infection (Chi et al.,2016). In this study, the herbal extract significantly increased the lysozyme activity after 2 weeks. This experiment's results agree with the prior research with some know antimicrobial activities (Chi et al.,2016; Liao et al.,2021). The obtained results suggest that *M. arvensis* taken as a supplement may activate the antimicrobial defenses of *E.malabaricus*, which may affect phagocytic activity, the production of reactive oxygen species, and serum lysozyme activity. One of the main sterilizing strategies for removing bacteria in teleost's has been discovered as a complement's bacterial activity (Liao et al.,2021). Our experiment result showed that supplementing the fed 1-4% *M.arvensis* diet after 2 weeks increases the

complement activity. Although complement activation is often beneficial for fish, extended activation may have negative consequences, including immunosuppression (Awad and Cerezuela,2015). In these hypothesis parameters, evidence in fish with mint treatment might be elevated against pathogens and increase non-specific immunity.

However, the immune-related gene expression against bacterial infection is limited in *C. batrachus*. In the present study, *M.arvensis* extract was responsible for the genes related to the immune system, such as *TNF-α, IL-1β,* and *MyD88*, in the infected *C.batrachus* treated with 3 and 4% of fed diets after 4 weeks. Our results correlated with previous studies showed that the fish feed diet supplemented with *Olea Europea L.* (Baba et al.,2018), *S. platensis* (Ragap et al.,2012), *T foenum-graceum* (Awad and Cerezuela,2015*)* extracts will exhibit *IL-1β, IL-8*. The *M. arvensis* extracts enhanced cytokines, and also found that the plant extracts improved *TLR2* and *MyD88*. Similarly, previous study has noted that the translation of immune responses (Adel et al., 2015a) fish by mint extracts. Thus, the study hypothesized and the result suggested that the *M. arvensis* extracts can enhance the immune response of *C. batrachus* during bacterial infection.

5. Conclusions

In conclusion, our reports suggested that the suitable dose of fed diet supplementation of *Mentha arvensis* extract leads to increased survival rates during bacterial infection. Slightly increased levels of hematological factors such as WBC, RBC, globulin, and complementary revealed the hematological effect of *Mentha arvensis* extract. The genes responsible for immunity, such as *TNF-α*, *IL-1β*, and *MyD88*, could be used as potential indicators and increase the level of gene expression. Our results indicated the possible immune-stimulatory and pro-inflammatory role was noted 3% of *Mentha arvensis* enriched diet in *Clarias batrachus*. Further research on the specific fraction of *Mentha arvensis* should be conducted to

understand better the effect of immunomodulatory activity and immune-specific genes of *Clarias batrachus*.

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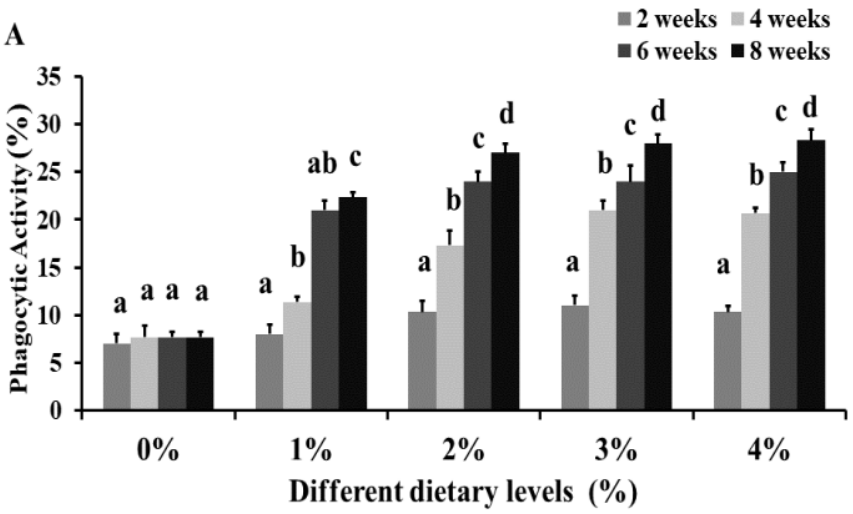
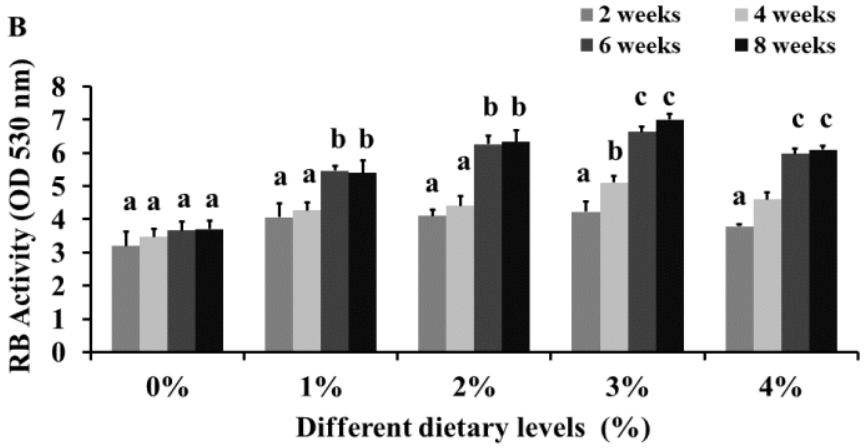
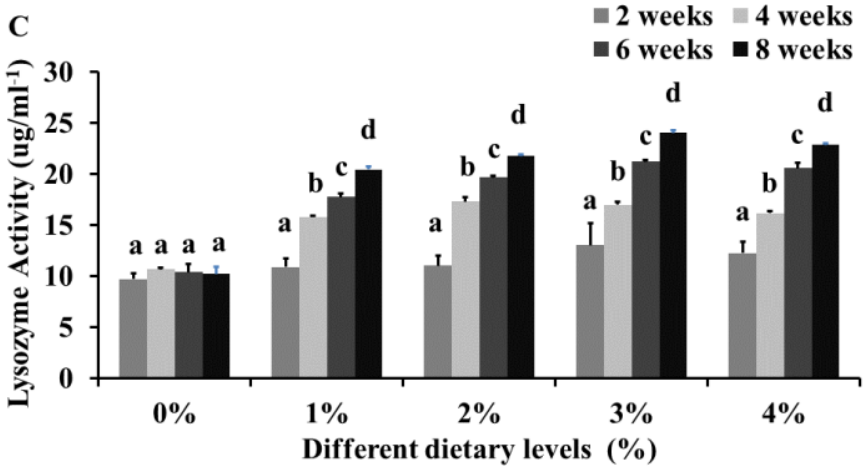

A
2 weeks
4 weeks
6 weeks
8 weeks
Phagocytic Activity (%)
0%
1%
2%
3%
4%
Different dietary levels (%)

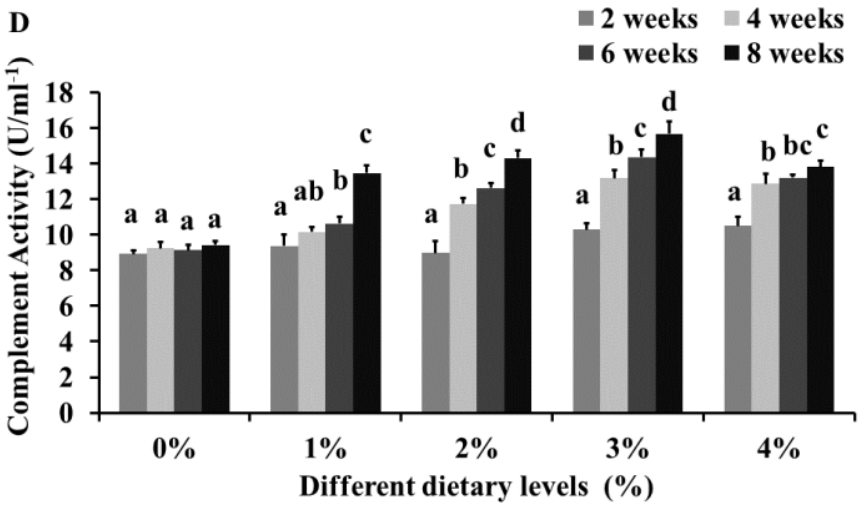
386


B
2 weeks
4 weeks
6 weeks
8 weeks
RB Activity (OD 530 nm)
0%
1%
2%
3%
4%
Different dietary levels (%)

387


C
2 weeks
4 weeks
6 weeks
8 weeks
Lysozyme Activity (ug/ml-1)
0%
1%
2%
3%
4%
Different dietary levels (%)

388



389

390 **Figure 1.** The (A) phagocytic activity, (B) respiratory burst (RB), (C) lysozyme activity and
391 (D) complement activity in *C.batrachus* after treatment of *M.arvensis* extracts. Data are
392 represented as mean ± standard deviation (n=6). Significant differences are indicated by
393 different letters on treated and control groups ($p<0.05$). Means 0,1,2,3 and 4% were basal diet
394 with infection plus 0,1,2,3 and 4g/kg of dry matter of *M.arvensis* extract, respectively.

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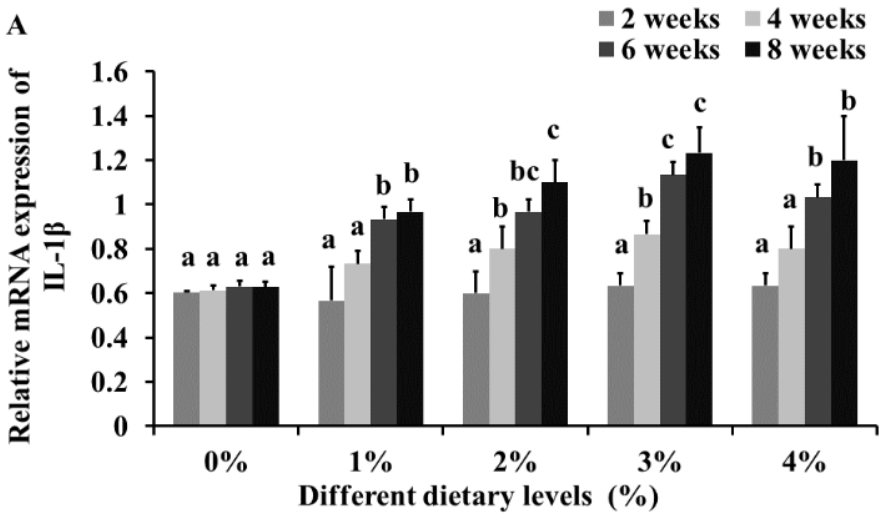
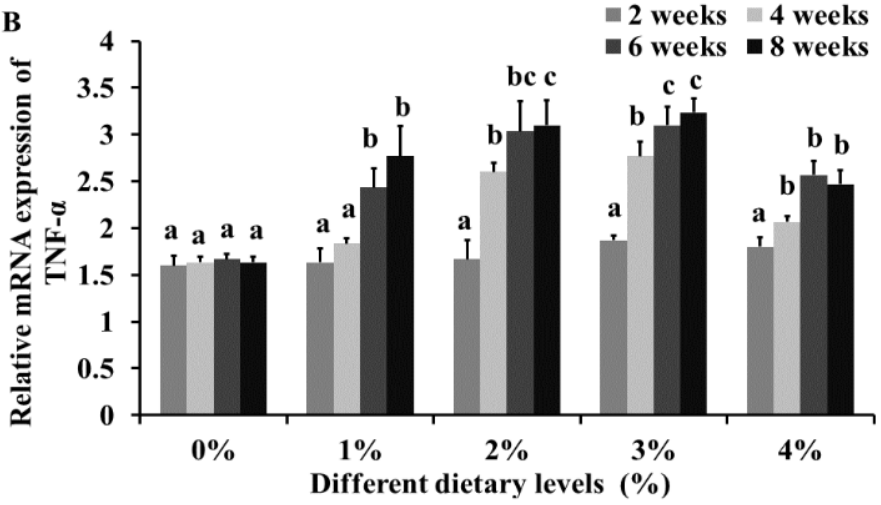
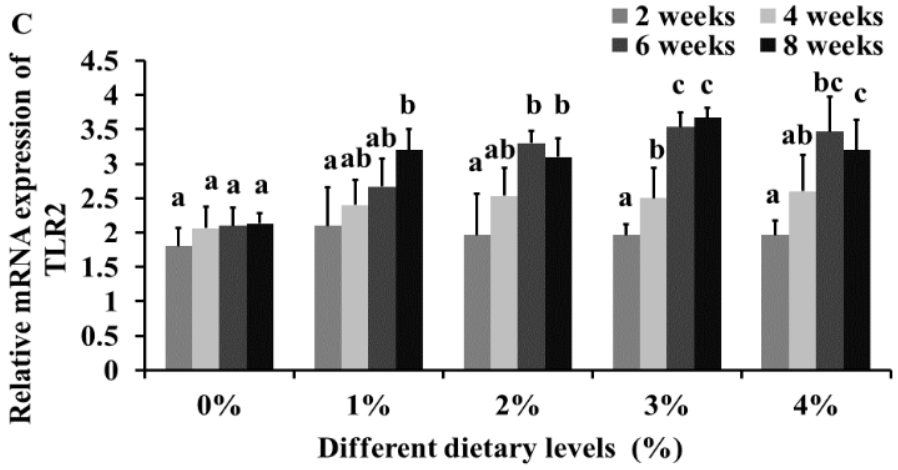

A
2 weeks
4 weeks
6 weeks
8 weeks
Relative mRNA expression of IL-1β
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a a a a
a a b b
a b bc c
a b c c
a a b b
0%
1%
2%
3%
4%
Different dietary levels (%)

397


B
2 weeks
4 weeks
6 weeks
8 weeks
Relative mRNA expression of TNF-α
4
3.5
3
2.5
2
1.5
1
0.5
0
a a a a
a a b b
a b bc c
a b c c
a b b b
0%
1%
2%
3%
4%
Different dietary levels (%)

398



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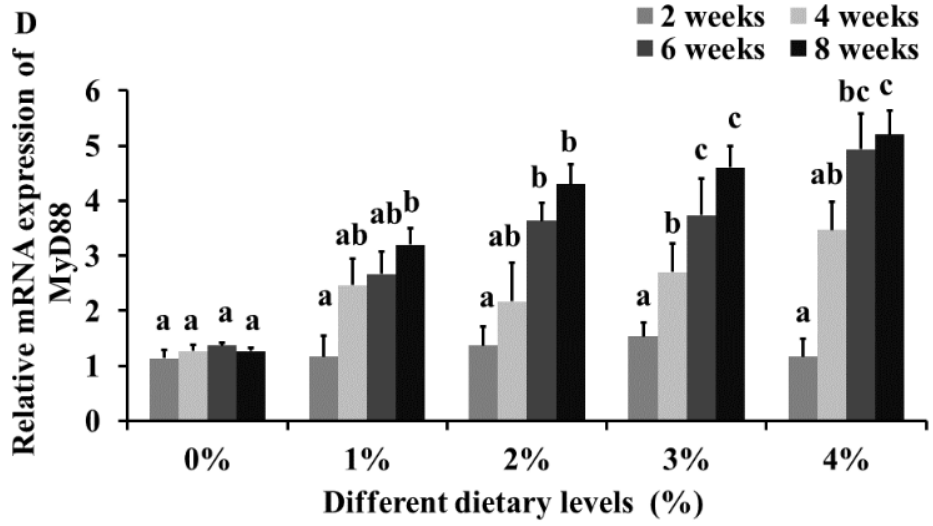

400

401 **Figure 2.** Innate immune genes of (A) *IL-1β,* (B) *TNF-α,* (C) *TLR* and (D) *MyD88* expressions
402 in *C.batrachus* after treatment of *M. arvensis* extracts. Data are represented as mean ± standard
403 deviation (n=6). Significant differences are indicated by different letters on treated and control
404 groups ($p<0.05$). Means 0%, 1%, 2%, 3% and 4% were basal diet with infection plus 0,1,2,3
405 and 4g/kg of dry matter of *M.arvensis* extract, respectively.

406
407

Table 1. Oligonucleotide primers used in the analysis of mRNA expression by real-time PCR

Primers	Nucleotide Sequences (5'-3')	Size (bp)	TM	Optimum Annealing Temperature (◦C)	Primer Efficiency (%)	Slope	R2	Pearson's coefficient	Acc. No.
GAPDH	F-TGTCCCAACTCCCAATGTGT R-CTGCAGCCTTAACCACCTTC	95	74.5905	59	110	-1.195	0.981	0.991	KC414932.1
IL-1β	F-TGAGAATGTGATTGAAGAGACCA R-AAGACAAGGTTGTGCAGTGC	88	68.1894	61	103	-3.166	0.994	0.953	JQ309137.1
TNF-α	F-CGCTGGTTTCCAACAGTTCT R-CTCGTTGCCCTCCAGTTTTA	83	72.5533	60	97	-3.384	0.998	0.974	KM593875.1
TLR-2	F-GCGAAGAGGACACACCTAGA R-AGATGCTTCAACAGGAACGC	113	67.2567	58	95	-3.031	0.981	0.980	KC907861.1
MyD88	F-GATGGTCAAACGCCAGAGAC R-CGCACAGCTTCAGGTTGTAA	118	73.2412	60	108	-3.144	0.992	0.951	JQ990986.1

Table 2. Growth rate, food conversion and specific growth rate of *C.batrachus* fed a diet with different levels of *M.arvensis* extracts for 8 weeks.

Parameters	Diets[1]				
	0%	**1%**	**2%**	**3%**	**4%**
Initial Weight (g)	9.27±0.00	9.25±0.03	9.24±0.02	9.26±0.03	9.27±0.02
Final Weight (g)	9.41±0.17^{a}	10.25±1.05^{ab}	10.41±0.51^{ab}	11.68±0.08^{c}	11.47±0.81^{c}
Weight gain (g)	13.66±17 ^{a}	100.33±10^{ab}	117±53^{ab}	242.16±60^{b}	219.33±80^{b}
Specific growth rate (%)	1.48±0.22^{a}	1.71±0.34^{ab}	1.85±0.11^{ab}	1.92±0.08^{b}	1.98±0.24^{b}
Feed conversion ratio	2.18±0.19^{a}	2.13±0.39^{a}	1.97±0.20^{a}	1.97±0.28^{a}	1.92±0.15^{a}
Survival rate (%)	66.66±5.77^{a}	73.33±5.77^{ab}	73.33±5.77^{ab}	80±0.00^{b}	76.66±5.77^{ab}

^{a,b,c}Means within the same row with different superscript letters are significantly different ($P<0.05$).

Data are presented as mean±standard deviation. [1]Means 0,1,2,3 and 4% were commercial fish-fed diet with infection plus 0,1,2,3 and 4g/kg of dry matter of *M.arvensis* extract, respectively.

Table 3. Hematological and biochemical parameters in *C.batrachus* fed with *M.arvensis* extracts against *A.hydrophila*.

Parameter	Weeks[1]	Diets[2]				
		0%	**1%**	**2%**	**3%**	**4%**
RBC (million/m^3)	0	2.12±0.03^{abc}	2.12±0.03^{abc}	2.14±0.05^{abc}	2.18±0.01^{abc}	2.18±0.21^{abc}
	2	2.22±0.12^{bcd}	2.22±0.12^{abc}	2.25±0.16^{abc}	2.22±0.06^{abc}	2.26±0.10^{abc}
	4	2.16±0.08^{abc}	2.16±0.0^{abc}	2.23±0.07^{abc}	2.74±0.17^{abcde}	2.75±0.18^{abcd}
	6	2.14±0.08^{abc}	2.14±0.08^{abc}	2.54±0.26^{abcd}	2.85±0.01^{abc}	2.90±0.05^{cde}
	8	1.98±0.27^{abc}	1.98±0.27^{abc}	2.53±0.12^{abcd}	3.67±0.19^{g}	3.33±0.49^{de}
WBC (Per uL)	0	4320.03±5.64^{a}	4327.60±10.10^{bc}	4325.77±9.86^{abc}	4322.90±7.57^{ab}	4321.93±3.02^{ab}
	2	4324.50±0.61^{abc}	4329.60±7.28^{bcd}	4332.53±1.68^{cde}	4342.03±2.37^{efg}	4339.50±2.56^{efg}
	4	4326.60±6.98^{abc}	4331.97±0.75^{cde}	4332.07±2.00^{cde}	4343.47±1.58^{efg}	4342.10±0.00^{efg}
	6	4326.47±3.72^{abc}	4332.40±7.75^{cde}	4340.10±4.06^{efg}	4349.50±3.22^{ghi}	4343.77±1.32^{efg}
	8	4327.87±3.02^{bc}	4335.97±4. 69^{def}	4345.57±2.70^{efg}	4353.53±2.55^{g}	4345.53±4.71^{efg}
Hematocrit (%)	0	31.10±0.00^{ab}	31.24±1.16^{ab}	31.13±0.61^{ab}	31.51±0.32^{abc}	31.25±0.74^{ab}
	2	30.87±1.45^{ab}	31.26±0.21^{ab}	31.68±0.19^{abc}	32.54±0.16^{bcd}	31.50±0.19^{ab}
	4	30.50±1.22^{a}	31.49±0.19^{ab}	31.66±0.26^{abc}	33.56±0.21^{def}	32.59±0.40^{bcd}
	6	29.67±0.15^{a}	31.63±0.56^{abc}	32.58±0.21^{bcd}	34.38±0.30^{de}	34.57±0.52^{de}
	8	30.17±1.25^{a}	32.57±1.18^{bcd}	33.79±0.02^{de}	34.90±1.00^{e}	34.67±0.33^{e}
Hemoglobin (g/dL)	0	8.23±0.07^{ab}	8.69±0.22^{ab}	8.79±0.06^{ab}	9.99±0.53^{abc}	8.15±0.24^{ab}
	2	8.34±0.07^{ab}	8.79±0.26^{ab}	9.49±0.47^{abc}	8.67±0.22^{bcd}	8.45±0.20^{ab}
	4	8.28±0.16^{a}	8.69±0.08^{ab}	9.98±0.01^{abc}	9.97±0.43^{cdf}	8.97±0.62^{bcd}
	6	8.25±0.03^{a}	9.04±0.17^{abc}	9.57±0.40^{bcd}	11.06±0.39^{df}	9.96±0.06^{df}
	8	8.21±0.03^{a}	9.57±0.23^{bcd}	9.80±0.12^{df}	10.87±0.51^{d}	10.64±0.40^{d}
Total protein	0	1.26±0.14^{a}	1.27±0.10^{a}	1.27±0.07^{a}	1.29±0.08^{a}	1.27±0.09^{a}

(mg/dL)	2	1.20±0.15[a]	1.27±0.01[a]	1.26±0.03[a]	1.36±0.09 [a]	1.25±0.13[a]
	4	1.25±0.18[a]	1.25±0.21[a]	1.27±0.15[a]	2.65±0.26[bc]	3.06±0.10[c]
	6	1.27±0.13[a]	2.21±0.17[b]	2.26±0.15[b]	3.74±0.17[d]	3.75±0.17[d]
	8	1.27±0.15[a]	2.24±0.24[b]	2.28±0.06[b]	3.95±0.35[d]	3.78±0.25[d]
Albumin (mg/dL)	0	1.21±0.10[a]	1.23±0.15[b]	1.27±0.22[abc]	1.29±0.17[abcd]	1.25±0.11[abc]
	2	1.25±0.15[abcd]	1.28±0.15[abc]	1.68±0.20[abcdef]	1.64±0.14[abcdef]	1.67±0.16[abcdef]
	4	1.36±0.19[abcde]	1.67±0.20[abcde]	1.81±0.17[cdef]	1.76±0.13[abcdef]	1.77±0.12[abcdef]
	6	1.57±0.22[abcdef]	1.79±0.47[abcde]	1.82±0.15[def]	2.58±0.26[g]	1.91±0.11[f]
	8	1.45±0.20[abcde]	1.89±0.30[abcde]	1.86±0.15[def]	2.99±0.04[g]	2.46±0.10[g]
Globulin (mg/dL)	0	1.27±0.06[ab]	1.29±0.07[abc]	1.24±0.16[a]	1.23±0.09[a]	1.26±0.10[ab]
	2	1.32±0.11[abcd]	1.32±0.07[abcd]	1.33±0.11[abcd]	1.37±0.11[abcde]	1.39±0.03[abcde]
	4	1.36±0.21[abcd]	1.37±0.19[abcd]	1.37±0.10[abcde]	1.61±0.19[abcde]	1.60±0.13[abcde]
	6	1.40±0.14[abcde]	1.39±0.06[abcd]	1.39±0.07[abcde]	1.73±0.11[e]	1.69±0.15[de]
	8	1.41±0.17[abcde]	1.40±0.09[abcde]	1.45±0.16[abcdef]	1.67±0.09[cde]	1.66±0.14[bcde]

[1] Means different time period (weeks). Data are represented as mean±standard deviation (n = 6).

[a,b,c]Means within the same row with different superscript letters are significantly different ($P<0.05$). [2]Means 0,1,2,3 and 4% were commercial fish-fed diet with infection plus 0,1,2,3 and 4g/kg of dry matter of *M.arvensis* extract, respectively.

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33 Ramy M. Shourbela, Waleed N. El-Hawarry, Medhat El-Fadadney, Mahmoud A.O. Dawood. "Oregano essential oil enhanced the growth performance, immunity, and antioxidative status of Nile tilapia (Oreochromis niloticus) reared under intensive systems", Aquaculture, 2021
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