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Possible effect of hala extract (*Pandanus tectorius*) on immune status, anti-tumour and resistance to *Yersinia ruckeri* infection in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The possible effect of dietary administration of hala extract (*Pandanus tectorius*) on rainbow trout (*Oncorhynchus mykiss*) immune status as well as its effect as an anti-tumour agent was studied. Fish were divided into 4 groups before feeding with commercial diet (0%, control; 0.5%, 1% and 2% of hala extract) for 2 weeks. The effect of diet on the humoral immune parameters, ie total protein, myeloperoxidase content, antiproteases, lysozyme and bactericidal activities were studied. Also, the effect of the diets on the expression of some immune-related genes in rainbow trout head-kidney (*TNF, LYZ2, IL-8* and *CD-4*) as well as tumour suppressor gene (*WT-1a*) was investigated. At the end of the feeding trial fish groups were challenged with *Yersinia ruckeri*. The results demonstrated enhancement in all the immune parameters in fish fed hala extract diets compared to control fish especially with the highest dose (2%) which recorded the highest significant increase ($p < 0.05$) in some parameters (total protein, myeloperoxidase content, antiproteases, and bactericidal activities) compared to the control. The results obtained from challenge with *Y. ruckeri* revealed reduction in the mortalities in fish groups fed with 1% and 2% doses of hala extract. Feeding with hala extract provoked upregulation in all immune-related genes. Again, the highest dose of hala extract showed a significant upregulation in *WT1a* expression ($p < 0.05$). The current study suggest that the hala extract, especially the highest dose, could be considered a good food additive to improve the immune status, resist tumour formation and to resist or control infectious diseases of rainbow trout.

1. Introduction

Aquaculture is considered to provide a valuable source of essential protein required for human health. However, the intensive and extensive aquaculture industry is subject to disease outbreaks [1]. However, controlling fish diseases by antibiotics and chemotherapeutics has caused the development of drug resistant pathogens in addition to accumulation of residues in environment and fish tissue and subsequently in humans [2]. On the other hand, medicinal plants provide a promising, alternative method for resisting and/or controlling fish diseases [3]. The world tends to use medicinal plants for treatment not only because they are cost effective, biodegradable, and safe but also for long lasting effects than the synthetic drugs provide and which have faster recovery rates [4]. It is worth mentioning that many medicinal plants have antioxidant properties which delay or prevent oxidative damage, therefore playing a vital role in disease prevention [5].

Previous studies showed a marked enhancement of the fish immune system after administration of different parts of medicinal plants (roots, leaves, seeds, and flowers). Moreover, various levels of immune response depended upon plant concentrations, time and method of administration [3]. For example, dietary supplement with three doses of fenugreek seeds improved the immune status of gilthead seabream (*Sparus aurata* L.) especially with the highest dose (10%) [6]. Moreover, common carp (*Cyprinus carpio*) showed enhancement in immune parameters after administration of a diet supplemented with a 2% dose of *Achillea wilhelmsii* leaf extract [7]. Interestingly, catfish (*Clarias gariepinus*) injected with 50 mg kg^{-1} of leek leaf extract showed an increase in humoral immune response one month post-injection [8].

Hala tree (*Pandanus tectorius*) belongs to the family Pandanaceae that comprises around 600 members [9]. This tree was initially cultured in Asia and extends to tropical northern Australia and Pacific islands of Oceania [10]. It contains triterpenoids and flavonoids [11], thus it was

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successfully used in folk medicine in many countries. In Kiribati, the leaves are used as therapy for cold, influenza, asthma, hepatitis, boils and cancer, while the roots are used to treat haemorrhoids. Fruits, flowers and aerial roots are used to treat digestive and respiratory disorders in Hawaii. While, In Palau, roots and leaves are used to alleviate stomach cramps and vomiting, respectively [10].

Previous studies recorded antioxidant, antibacterial, anticoagulant, anti-inflammatory, hepatoprotective, antidiarrheal, anticonvulsant, diuretic and anti-cancer activities for leaf extract [10,12]. However, there has been no investigation carried out on the effect of hala leaf extract on the immune system of fish. Thus the current study was carried out to investigate the possible effects of dietary supplement of hala leaf extract on rainbow trout immune response either in serum or in cell by examine the expression of some immune- related genes (*TNF, LYZ2, IL-8* and *CD-4*) in head kidney as well as study its effect as anti-tumour agent by examination of the expression of *WT-1a* gene (tumour suppressor gene).

2. Materials and methods

2.1. Preparation of plant extract and diets

Hala (*Pandanus tectorius*) leaves were collected from a local market in Saudi Arabia. About one kilogram of dried powder of hala was extracted using 95% alcohol (Merck, Germany) by percolation till exhaustion (4 × 4 l) and filtered off by filler paper. The combined filtrates of the plant were evaporated under reduced pressure and low temperature using rotator evaporator. The obtained residue (250 g) was used in preparing the diets. Four different concentrations were prepared; commercial diet non-supplemented (0%, control), commercial diet supplemented with 0.5 g (0.5%), 1 g (1%) and 2 g (2%)/100 g of hala extract.

2.2. Fish, experimental design and sampling

Rainbow trout (*Oncorhynchus mykiss*) of average weight 18 ± 1 g were obtained from a commercial fish farm in Scotland, and acclimatized in aerated free flowing freshwater (14 ± 2 °C). During acclimatization, fish were fed three times daily with a commercial diet (Biomar). Fish were distributed randomly into 4 groups each with 30 fish (10 per replicate) and fed for 14 days with 0.5 g (0.5%), 1 g (1%) and 2 g (2%)/100 g of *hala* extract. Controls were fed with commercial diet only to examine the possible mode of action and effect on immune status. Blood was collected from fish anaesthetised using. 3- amino benzoic acid ethyl ester; Sigma-Aldrich, Basingstoke, U.K.) by syringe before transfer to Vacuettes without heparin (Greiner, Stonehouse, U.K.) and left to clot for 2 h at 4 °C, prior to centrifugation (1600 g, 25 min, 4 °C), and stored at - 20 °C until use. The fish were then sacrificed using an overdose of the above anaesthetic.

2.3. Humoral immune parameters

2.3.1. Lysozyme activity

Serum lysozyme activity was measured according to Ref. [13]. Briefly, 60 µl of serum was added to 2 ml of a suspension of *Micrococcus lysodeikticus* (0.2 mg ml^{-1}) in a 0.05 M sodium phosphate buffer (pH 6.2) and absorbance was measured at 530 nm after 0.5 and 4.5 min on a spectrophotometer. A unit of lysozyme activity was defined as the sample amount causing a decrease in absorbance of 0.001 min^{-1}.

2.3.2. Total protein content

Total protein was measured by Bradford assay using bovine serum albumin (BSA) as the standard. Briefly, 2 mg ml^{-1} solution of BSA was prepared and serial dilutions made with phosphate buffer saline (PBS). Around 20 µl of each dilution was added to 1 ml of Bradford reagent (Sigma-Aldrich) before incubated at room temperature for 15 min. The standard curve was prepared by measuring the absorbance of each sample at 595 nm verses the sample concentration. Serum samples were diluted (1: 100) in PBS before 20 µl of each serum dilution was added to 1 ml of Bradford reagent. After incubation for 15 min, the absorbance of the unknown samples was taken and plotted onto the standard curve to obtain the total protein content for each sample [14].

2.3.3. Antiproteases activity

The serum anti-trypsin activity was measured according to Lange, Guđmundsdottir [15]. Thus, 20 µl of standard trypsin solution (Sigma-Aldrich, 5 mg ml^{-1}) was incubated with 20 µl of serum for 10 min at 22 °C. Subsequently, 200 µl of 0.1 M PBS (PH 7.2) and 250 µl of 2% azocasein solution (Sigma-Aldrich, 20 mg ml PBS^{-1}) were added. The mixture was incubated for 1 h at 22 °C before stopping with the addition of 500 µl of 10% (v/v) trichloro acetic acid (TCA). Then, the mixture was incubated for 30 min at 2 °C before centrifuging at 6000 × *g* for 5 min. About 100 µl of the supernatant was transferred to a 96 microwell flat bottom plate containing 100 µl of 1 N NaOH well^{-1}. The absorbance was read in the ELISA reader at 410 nm. Positive control (100%) was prepared by replacing the serum with buffer. For a negative control, buffer replaced both serum and trypsin. The percentage inhibition of trypsin activity was calculated by comparing with a positive control sample.

2.3.4. Myeloperoxidase content

The myeloperoxidase content of serum was measured according to Ref. [16]. Briefly, 50 µl serum was diluted with 135 µl of Ca^{+2} and Mg^{+2} free HBSS (Sigma-Aldrich) in flat-bottomed 96-well plates. Then, 50 µl of 20 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB, Sigma-Aldrich) and 5 mM H_2O_2 (Sigma-Aldrich) were added (both substrates of peroxidase). The reaction was stopped by adding 50 µl of 4 M sulphuric acid (H_2SO_4) after 2 min. The absorbance was read at 450 nm by ELISA reader. Blank sample was without serum.

2.3.5. Bacterial culture and bactericidal activity

Yersinia ruckeri was identified and obtained from Heriot Watt University before inoculation into TSA media (Oxoid) for 48 h at 25 °C. The culture was centrifuged for 10 min at 3000 × *g* at 4 °C and the pellet resuspended in 0.9% of saline. The bacterial suspensions were counted using a hemocytometer slide at a magnification of 400× on a light microscope.

Serum bactericidal activity was done according to Kajita, Sakai [17] using *Yersinia ruckeri.* Briefly, 100 µl of serum was mixed with 100 µl of bacterial suspension, before incubation for 1 h at 25 °C. A blank control was also prepared by replacing serum with sterile PBS. The mixture was then diluted with 0.05 M sodium phosphate buffer, PBS (pH 6.2) at a ratio of 1:10. Around 50 µl of mixture was plated onto the nutrient agar plates and incubated for 48 h at 25 °C before the number of colonies was counted.

2.4. Challenge with Yersinia ruckeri

All challenge experiments were done under a UK Home Office Project License (held by A.R. Lyndon) and a UK Home Office Personal License (held by D. A. Austin) and approved by the Heriot-Watt University Ethics Committee.

Yersinia ruckeri was grown in nutrient broth (Oxoid) for 24 h at 25 °C. The culture was centrifuged at 3000 × *g* for 10 min at 4 °C, before the supernatants were discarded, and the pellets resuspended in 0.9% (w/v) saline. The challenge test was carried out on fish by intraperitoneal injection with 0.1 ml volumes containing 10^3 cells/fish, as preliminary experiments had determined this to be the LD80 for the challenge strain of *Y.* ruckeri. Mortalities were recorded for up to 10 days [18].

Table 1
Primers used for real-time PCR.

Gene name	Primer sequences (5'- 3')	Products size	GenBank number
Cluster of differentiation (CD4)	GCCCTGCAGAGGACAAATCT TACAAAGGCCACTGGAGCTG	171	NM_001124539.1
Lysozyme II (LYZ2)	TCCAGATCAACAGCCGCTAC GATTCCGTTCGGGTCCAACA	149	NM_001124716.1
Interleukin 8 receptor (IL-8)	CGGTGCCGTCATATTCCTGT GGGTCAGGGACTGTTGACTG	110	NM_001124279.1
Beta-actin (β -actin)	ATGGGCCAGAAAGACAGCTACGTG CTTCTCCATGTCGTCCCAGTTGGT	186	AJ438158.1
Tumour necrosis factor TNF	CAAGAGTTTGAACCTCATTCAG GCTGCTGCCGCACATAAAG	130	NM 001124374
Wilms' tumour suppressor 1a (WT-1a)	ATGTTCAGCAACGCACCCTA GAACTGGGAGGAGTGGTGTG	129	NM_001124294.1

2.5. Gene expression by real time PCR

Total RNA was extracted from the head kidney and liver using RNA extraction kit (Applied biosystem, UK). The quantity of RNA was measure using nanodrop. Around 1 μg of RNA was used in Reverse transcription by using cDNA kit from Fermentas (York, UK).

The expression of *TNF, LYZ2, IL-8, CD-4, β -actin*, and *WT-1a* genes (Table 1) in head kidney were analysed by real-time PCR machine (ABI PRISM 7500 instrument, Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Mixture (comprised of 10 ml of SYBR Green supermix, 5 ml of primers (0.6 mM each) and 5 ml of cDNA template) were incubated for 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. Gene expression was corrected by the reference gene, β-actin in each sample. The primers used are shown in Table 1. Gene expression of the samples compared to the controls was calculated according to the following equation:

$$Ratio = \frac{(E_{t\,\arg et})^{\Delta C}\, T\ t\ \arg et\,(control - sample)}{(E_{EF1\alpha})^{\Delta C} T\ t\ \arg et\,(control - sample)}$$

2.6. Statistical analysis

Data were expressed as fold increase (mean ± standard error, SE), obtained by dividing each sample by the mean control value. Values higher than 1 express an increase while values lower than 1 express a decrease in the indicated gene. Data were analysed by one-way analysis of variance (ANOVA). When differences were found among treatments, Tukey's test was used to compare means by Minitab statistical software (Minitab, Coventry, UK). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Humoral immune parameters

In Lysozyme activity and Myeloperoxidase content (Figs. 1 and 2), the highest significant values ($p < 0.05$) were reported in fish groups fed with 0.5% and 2% of hala extract with respect to the values found in the control group (0%). Although all doses of hala extract showed highly serum total protein compared to the control (0%) none of them showed significant differences compared to the values found in control group (Fig. 3). The antiprotease activity (Fig. 4) was increased in specimens fed with 0.5% or 2% doses of hala extract (compared to control), although only in the group fed 2% dose the increases were statistically significant, compared to the values found in control ($p < 0.05$). Regarding bactericidal activity (Fig. 5), all groups fed with hala extract showed high activity compared to the control group (especially with 2% dose) but without significant differences.

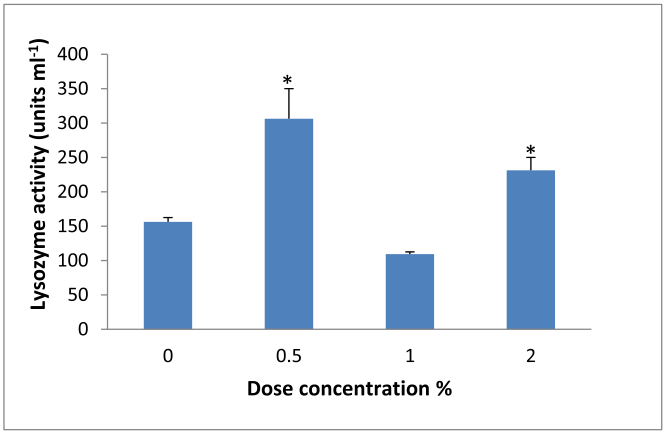

Fig. 1. Serum lysozyme activity of rainbow trout fed for 2 weeks with dietary hala extract supplemented doses: 0% (control), 0.5%, 1% and 2%. Data are presented as mean ± S.E. Asterisk represents significant difference from control $p \leq 0.05$. Bars = mean ± S.E.

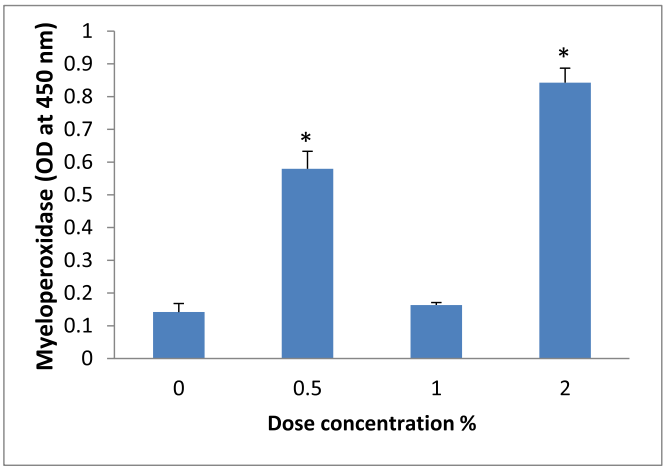

Fig. 2. Serum Myeloperoxidase content of rainbow trout fed for 2 weeks with dietary hala extract supplemented doses: 0% (control), 0.5%, 1% and 2%. Data are presented as mean ± S.E. Asterisk represents significant difference from control $p \leq 0.05$. Bars = mean ± S.E.

3.2. Challenge with Yersinia ruckeri

Fish groups fed with diets supplemented of hala extract for 2 weeks resulted in reduction in mortalities after challenge with *Y. Ruckeri* (Fig. 6). The resistance to *Y. ruckeri* infection was increased in fish groups fed for 2 weeks with 2% and 1% doses of hala extract where the survival percent was 26.67% and 21.43%, respectively, compared to

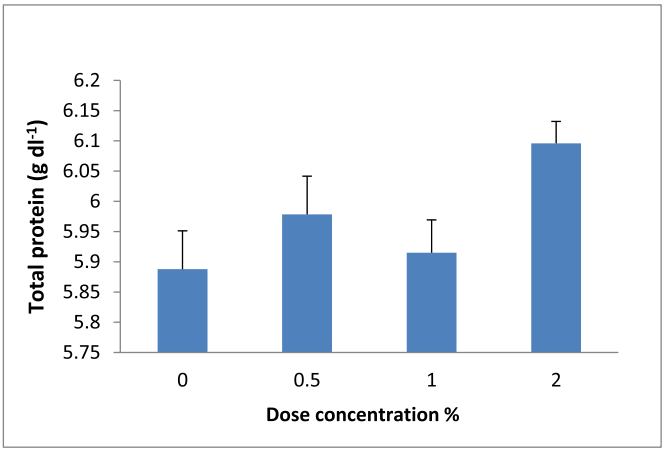

Fig. 3. Serum total protein of rainbow trout fed for 2 weeks with dietary hala extract supplemented doses: 0% (control), 0.5%, 1% and 2%. Data are presented as mean ± S.E. Asterisk represents significant difference from control $p \leq 0.05$. Bars = mean ± S.E.

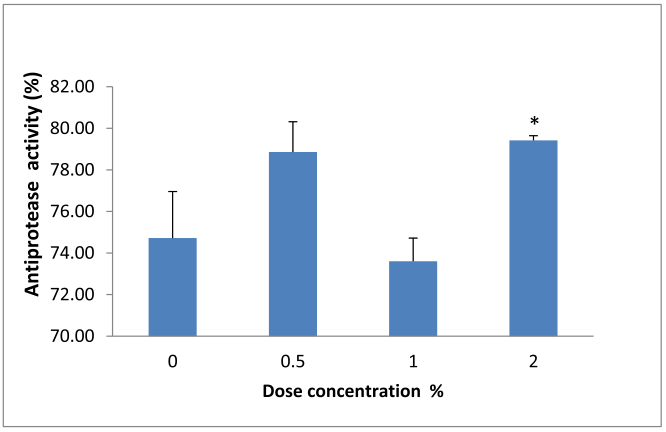

Fig. 4. Serum Antiprotease of rainbow trout fed for 2 weeks with dietary hala extract supplemented doses: 0% (control), 0.5%, 1% and 2%. Data are presented as mean ± S.E. Asterisk represents significant difference from control $p \leq 0.05$. Bars = mean ± S.E.

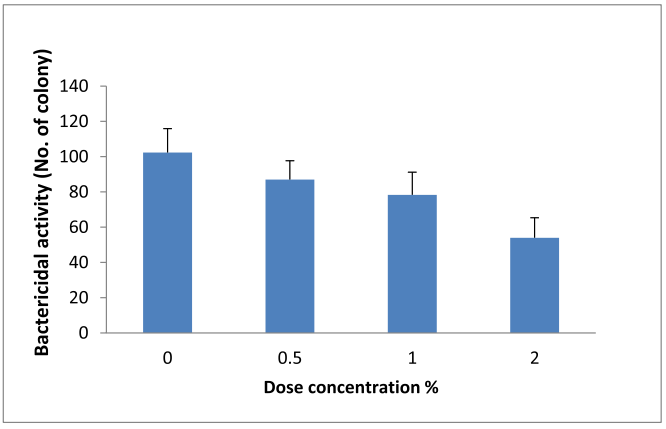

Fig. 5. Serum bactericidal activity of rainbow trout fed for 2 weeks with dietary hala extract supplemented doses: 0% (control), 0.5%, 1% and 2%. Data are presented as mean ± S.E. Asterisk represents significant difference from control $p \leq 0.05$. Bars = mean ± S.E.

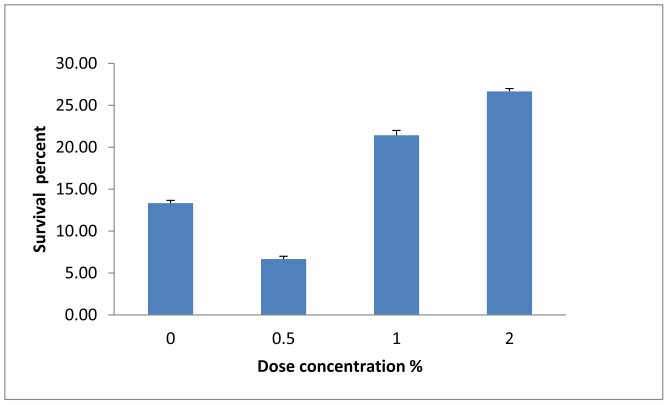

Fig. 6. Survival of rainbow trout fed for 2 weeks with dietary hala extract supplemented doses: 0% (control), 0.5%, 1% and 2% followed by challenge with *Yersinia ruckeri*. Data are presented as mean ± S.E. Asterisk represents significant difference from control $p \leq 0.05$. Bars = mean ± S.E.

control group (33.3%). However, the difference was not significant.

3.3. Gene expression

The results revealed an increase in the expression of immune related genes (*TNF, LYZ2, IL-8* and *CD-4*) in the head kidney of rainbow trout after administration of diets enriched with 0.5% and 1% of hala extract for 2 weeks compared to the expression recorded in the control group (Fig. 7). Interestingly, the lowest dose (0.5%) showed the highest expression compared to other groups and control group while only being statistically significant ($p < 0.05$) for *TNF* and *IL-8* compared to the control.

Moreover, the results showed significant up- regulation in *WT-1a* gene of head kidney ($p < 0.05$), in fish fed with 1% and 2% doses of hala extract compared to control. The highest expression was recorded in the dose of 2%.

4. Discussion

Lysozyme is an important non-specific immune parameter which plays a vital role in fish defence mechanisms against diseases. It is responsible for opsonin, and thus activates the complement system and

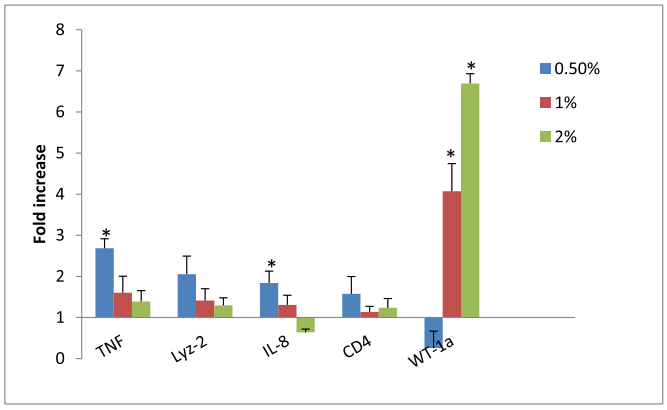

Fig. 7. Expression of immune related genes (TNF, LYZ2, IL-8 and CD-4) and tumour suppressor genes (WT-1a) in the head kidney of rainbow trout fed dietary hala extract supplemented doses of 0.5%, 1% and 2% for 2 weeks. Data are expressed as fold increase (mean ± standard error, SE), obtained by dividing each sample value by the mean control value at the same sampling time. Values higher than 1 express an increase while values lower than 1 express a decrease in the indicated gene. Asterisks denote significant differences between control and treatment groups ($P < 0.05$).

phagocytes [19]. Our results revealed a significant enhancement in serum lysozyme activity of the fish group fed 0.5% and 2% of hala extract, respectively, for 2 weeks compared to the control group (0%). This could be attributed to a dose dependent effect of hala extract on rainbow trout. Similar observation was reported in lysozyme activity of rainbow trout fed for 2 weeks with 0.5% and 1% of tetra (*Cotinus coggyria*) [20], and 1% & 2% of black cumin oil (*Nigella sativa*) [21]. Also, the highest activity depended mainly on the dose administration.

Myeloperoxidase is an important enzyme expressed mainly in neutrophils, which have the ability to produce hypochlorous acid from one of the oxidative radicals (H_2O_2) [22]. This process has a great benefit to kill invading microorganisms [23]. The results showed an increase in myeloperoxidase content in all treatment groups with hala extract, especially in those fed with 0.5% and 2% where a significant value ($p < 0.5$) was recorded compared to control group. Similar to our study, myeloperoxidase was increased significantly in rainbow trout fed for two weeks with 1% and 2% of lupin (*Lupinus perennis*), mango (*Mangifera indica*), and nettle (*Urtica dioica*) [3]. Also, the highest myeloperoxidase content and lysozyme activity values were recorded in rainbow trout fed 0.1% and 0.5% of caper leaf extract for 4 weeks [19]. It is worth mentioning that time and dosage are two important factors which control the efficiency of plant immunostimulants. For example, Christybapita, Divyagnaneswari [24] noticed a significant increase in common tilapia fed with diets containing different concentrations of false daisy leaf for 1 week, while feeding for 2 or 3 weeks didn't showed any significant increase.

Serum protein is an important parameter in humeral immune system in fish. Its composition plays a vital role in keeping fish healthy. Moreover, the most important role played by acute phase proteins is in limiting the spread of infectious agents through repairing tissue damage and killing micro-organisms [25,26]. Present results also demonstrated enhancement in total protein value in all groups that received different concentration of hala extract groups as compared to the control (especially with the dose of 2%). This is in agreement with Dügenci, Arda [27] who reported increases in serum protein levels in rainbow trout fed with 0.1% and 1% of ginger (*Zingiber officinale*), nettle (*Urtica dioica*) and mistletoe (*Viscum album*). Several studies reported an increase of serum protein levels in fish species after using dietary supplement with plants as immunostimulants [6,7,28,29]. Moreover, they suggested that elevation in fish total protein were probably a result of enhancement of the non-specific immune response.

Antiproteases or protease inhibitors are active molecules in the non-specific immune system that inhibit the action of proteases either by binding to their active sites or by 'trapping' the protease to prevent protein hydrolysis [30] and thus limiting the growth of invading bacteria in fish [31]. In the present study, the highest dose of hala extract recorded the highest significant enhancement in serum antiproteases compared to the control. In agreement with our result several studies recorded enhancement in fish species after administration of diets supplemented with medicinal plants [6,32,33]. It is worth emphasizing that the increase in fish immune response depend mainly on dose and time of administration. Moreover, the response also depends on fish species. For example, 0.5% dose of garlic reported the highest antiprotease activity in rainbow trout after feeding for 2 weeks [33]. Although, the highest antiprotease in Asian seabass fed for 2 weeks was reported at 1.5% dose of garlic [34].

Various humoral molecules involved in non-specific immune response have a power to protect the fish from invading microbes [35]. Serum bactericidal activity is a lysin mechanism known for the killing and clearing of pathogenic organisms in fish [31]. In our study, *Y. ruckeri* was used as a model to examine the activity of hala extract to kill the bacterial infection. The strength of immune molecules in fish serum to kill *Y. ruckeri* can detected by the lowest number of bacterial colonies grown on media. Fish groups fed with hala extract revealed higher bactericidal activity compared to control, especially in the group fed with the highest dose (2%). Similarly, using the highest dose of black cumin seed (3%) as food supplement in rainbow trout diet caused higher bactericidal against *Aeromonas hydrophila*, [21]. Also, rohu (*Labeo rohita*) recorded an enhancement in serum bactericidal activity against *A. hydrophila* after feeding for 2 weeks with doses of prickly chaff-flower seed and the activity was elevated with higher concentration of seeds [35].

Challenge with target pathogen is one of the most valuable tests to evaluate the efficiency of immunostimulant to resist microbes. The resistance level of fish can be recognized from the survival percent after a bacterial infection [36]. The results revealed that dietary supplement with hala extract relatively increased the resistance of rainbow trout against *Y. ruckeri*, where the highest doses (2% and 1% respectively) recorded the highest resistance. In agreement with our study using stinging nettle (*Urtica dioica*) as food supplement of rainbow trout diet increased the resistance to *Y. ruckeri*, especially with the highest dose [37]. However, some immunostimulant can enhance the resistance of fish against some bacteria but failed to resist the other. For example an improvement in survival percent of rainbow trout fed with probiotic for 2 weeks was recorded following challenge with *Aeromonas salmonicida*, *Vibrio ordalii*, and *Y. ruckeri*, but not so with *V. anguillarum* [38]. Previous studies showed reduction in mortality against bacterial infections in fish after using plant immunostimulant [3,39,40].

TNF-α, is one of pro-inflammatory cytokines, that mainly produced by activated monocytes/macrophages and regulate the expression of many cytokines [41]. Many studies revealed that using plant immunostimulants in fish can induce pro-inflammatory responses. For example, using 0.1% of caper as supplement in rainbow trout diet caused up-regulation in the expression of *TNF- α* of head kidney [42]. Also, an up-regulation in *TNF-α* expression was observed in common carp treated with *Rehmannia glutinosa* in spleen, head kidney and gut [43]. Moreover, common carp fed with different concentrations of guava leaf powder showed an increase in *TNF-α* expression in the head-kidney, hepatopancreas, and intestine [44]. Similarly our study demonstrated up-regulation of *TNF- α* expression of head kidney in fish groups fed with hala extract compared to control. Increasing in the *TNF-α* levels could be attributed to the activity of the compounds in hala extract like flavonoids and antioxidant [12].

IL-8 is another pro-inflammatory cytokine, that produced in response to many stimulation factors like cytokines, LPS and viruses [45]. It plays an important role in attract T-lymphocytes and neutrophils to sites of inflammation [46]. Similar to *TNF- α* expression, result demonstrated an increase in *IL-8* in rainbow trout head kidney in groups received 0.5% and 1% doses of hala extract. Although, only 0.5% dose recorded significant difference with the control. In agreement with this study, *IL-8* expression increased in rainbow trout head kidney after fed diets supplemented with 1% and 2% of stinging nettle [47] and 0.1% of caper [42]. Interestingly, *IL-8* expression in rainbow trout spleen was unaffected by the green tea supplementation, while in head kidney the expression showed significant increase especially in fish fed with 500 mg kg^{-1} of green tea [48]. Similarly, gilthead seabream fed dietary supplement with 10% fenugreek showed enhancement in IL-8 expression of head kidney after 4 weeks [6].

CD4 is an important co-receptor has been reported in teleosts, expressed on T- helper cell, monocytes and macrophages [49]. *CD4* is binding to major histocompatibility complex (MHC) class I molecules on the antigen-presenting cells, stabilizing the interaction between the T cell receptor complex (TCR) and the MHC [50]. The results demonstrated an increase in *CD4* expression in head kidney in group fed with hala extract compared to control group. Similar observations have been in other fish species after adminstration immunostimulant. For example; Atlantic salmon (*Salmo salar*) injected with lipopolysaccharide (LPS) and β-glucan as immunostimulant, showed an enhancement in the expression of *CD4* in head kidney [51]. Also, an increase in *CD4* expression of posterior intestine of European sea bass (*Dicentrarchus labrax*) has been reported after feeding diets contain low level of synbiotic additive (mannan oligosaccharides) [52]. The slightly higher

expression values of *CD4* of fish group fed with hala extract may indicate an early adaptive immune response.

Lysozyme gene is a bactericidal enzyme that mainly present in lymphoid tissue like head kidney and thymus as well as serum, mucus, gills [53]. There is two types of lysozyme (types I and II), have been identified in the kidney of rainbow trout. Particular, lysozyme type II (*LYZ2*) showed a potential antibacterial activity against four gram-negative bacteria. Such finding supports the role which lysozyme plays in non-specific immune defence in fish [54]. Our study reported the highest level of *LYZ2* expression in head kidney of fish group fed with the lowest dose of hala extract (0.5%), this is agreement with the result obtained from analysis serum lysozyme level for the same group. Similar observation have been reported in common carp fed for 8 weeks with diet supplements with date palm fruit ($200\,ml\,kg^{-1}$) where showed a remarkable increase in the expression level of lysozyme gene in head kidney (*LYZ2*) and serum lysozyme compared to control [55].

The kidney is plays important functions in fish, not only in immune system for production the leucocytes but also as osmoregulatory function [56]. Thus any malfunction in this organ in really preferable. Wilms' tumour suppressor *WT1* is a tumour suppressor gene, any mutation can led to Wilms' tumour, a pediatric kidney cancer [57]. *WT1* is a modulatory gene involved in cell growth and development of the urogenital system development (kidney and gonad) [58]. *WT1* have been identified in fish species [59–61]. The suppression of *WT1* led to appear the edema in zebrafish which suggest that it is involved in pronephros development [62]. *WT1a* and *WT1b* are two types have been reported from *WT1* in zebrafish. Inactivation of wt1a leads to the absence of glomeruli while targeting of *wt1b* resulted in the formation of renal cysts [60]. The result showed an increase in *WT1a* expression in head kidney of fish group treatment with hala extract, especially in the group fed with 2% which recorded the highest value. This could be contributed to the activity of ROS in plant extract compounds to activate the tumour suppressor gene and suggest the role of this plant to work as anti- tumour agent in kidney.

In conclusion, our results demonstrated that dietary supplement with hala extract to rainbow trout for two weeks stimulates the non-specific immune response and increase its resistance toward *Y. ruckeri* infection. The current results suggest that the hala extract, specially the highest dosage (2%) could be considered as a good fish food supplements to enhance the immune system and resist and/or control pathogenic bacterial, in addition to its potential effect to resist the tumour formulation. Future investigations could focus on the effects of long feeding time on the immune status and general health status of fish.

References

[1] M.G. Bondad-Reantaso, R.P. Subasinghe, J.R. Arthur, K. Ogawa, S. Chinabut, R. Adlard, et al., Disease and health management in Asian aquaculture, Vet. Parasitol. 132 (2005) 249–272.

[2] C.P. Alexander, C. John Wesly Kirubakaran, R.D. Michael, Water soluble fraction of *Tinospora cordifolia* leaves enhanced the non-specific immune mechanisms and disease resistance in *Oreochromis mossambicus*, Fish Shellfish Immunol. 29 (2010) 765–772.

[3] E. Awad, A. Awaad, Role of medicinal plants on growth performance and immune status in fish, Fish Shellfish Immunol. 67 (2017) 40–54.

[4] C.-C. Wu, C.-H. Liu, Y.-P. Chang, S.-L. Hsieh, Effects of hot-water extract of *Toona sinensis* on immune response and resistance to *Aeromonas hydrophila* in *Oreochromis mossambicus*, Fish Shellfish Immunol. 29 (2010) 258–263.

[5] J. Hudec, M. Burdová, Kobida Lu, L. Komora, V. Macho, G. Kogan, et al., Antioxidant capacity changes and phenolic profile of *Echinacea purpurea*, nettle (*Urtica dioica* L.), and dandelion (*Taraxacum officinale*) after application of polyamine and phenolic biosynthesis regulators, J. Agric. Food Chem. 55 (2007) 5689–5696.

[6] E. Awad, R. Cerezuela, M.Á. Esteban, Effects of fenugreek (*Trigonella foenum graecum*) on gilthead seabream (*Sparus aurata* L.) immune status and growth performance, Fish Shellfish Immunol. 45 (2015) 454–464.

[7] Adel Milad, Mehdi Gholaghaie, Mohammad Binaii, Poria Khanjany, E. Awad, Effect of dietary *Achillea wilhelmsii* extract on growth performance, and immune status of common carp (*Cyprinus carpio*), Res. J. Pharmaceut. Biol. Chem. Sci. 7 (2016) 1037–1046.

[8] W.T. Abbas, E. Awad, Effect of leek (*Allium ampeloprasum* L.) extract on biotransformation enzymes and innate immunity of catfish (*Clarias gariepinus*) exposed to Benzo [a] Pyrene, Res. J. Pharmaceut. Biol. Chem. Sci. 7 (2016) 2211–2224.

[9] L. Englberger, W. Aalbersberg, M.H. Fitzgerald, G.C. Marks, K. Chand, Provitamin A carotenoid content of different cultivars of edible pandanus fruit, J. Food Compos. Anal. 16 (2003) 237–247.

[10] S. Baba, H.T. Chan, M. Kezuka, T. Inoue, E.W.C. Chan, *Artocarpus altilis* and *Pandanus tectorius*: two important fruits of Oceania with medicinal values, Emir. J. Food Agric. (2016) 531–539.

[11] V. Gupta, J. Niazi, J.K. Kehal, P. Bansal, Anti-inflammatory and anti-nociceptive activity of *Pandanus tectorius* Parkinson, Res. J. Pharmacogn. Phytochem. 2 (2010) 193–195.

[12] O. Omodamiro, C. Ikekamma, In vitro study of antioxidant and anticoagulant activities of ethanol extract of *Pandanus tectorius* leaves, International Blood Research Reviews 5 (2016) 1–11.

[13] R.M. Parry, R.C. Chandan, K.M. Shahani, A rapid and sensitive assay of muramidase, Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine, Royal Society of Medicine, New York, NY, 1965, pp. 384–386.

[14] M.M. Bradford, Foreign patent documents, Biochemistry 72 (1976) 248–254.

[15] S. Lange, B.K. Guđmundsdottir, B. Magnadottir, Humoral immune parameters of cultured Atlantic halibut (*Hippoglossus hippoglossus* L.), Fish Shellfish Immunol. 11 (2001) 523–535.

[16] M.J. Quade, J.A. Roth, A rapid, direct assay to measure degranulation of bovine neutrophil primary granules, Vet. Immunol. Immunopathol. 58 (1997) 239–248.

[17] Y. Kajita, M. Sakai, S. Atsuta, M. Kobayashi, The immunomodulatory effects of levamisole on rainbow trout, *Oncorhynchus mykiss*, Fish Pathol. 25 (1990) 93–98.

[18] B. Austin, D.A. Austin, Bacterial Fish Pathogens: Disease of Farmed and Wild Fish, fourth ed., Springer, Dordrecht Chichester, 2007 Published in association with Praxis Pub..

[19] J. Kumari, P.K. Sahoo, Dietary levamisole modulates the immune response and disease resistance of Asian catfish *Clarias batrachus* (Linnaeus), Aquacult. Res. 37 (2006) 500–509.

[20] S. Bilen, M. Bulut, A.M. Bilen, Immunostimulant effects of *Cotinus coggyria* on rainbow trout (*Oncorhynchus mykiss*), Fish Shellfish Immunol. 30 (2011) 451–455.

[21] E. Awad, D. Austin, A.R. Lyndon, Effect of black cumin seed oil (*Nigella sativa*) and nettle extract (Quercetin) on enhancement of immunity in rainbow trout, *Oncorhynchus mykiss*(Walbaum), Aquaculture 388 (2013) 193–197.

[22] R. Dalmo, K. Ingebrigtsen, J. Bøgwald, Non-specific defence mechanisms in fish, with particular reference to the reticuloendothelial system (RES), J. Fish. Dis. 20 (1997) 241–273.

[23] R. Johnston Jr., Oxygen metabolism and the microbicidal activity of macrophages, Federation Proceedings, 1978, pp. 2759–2764.

[24] D. Christybapita, M. Divyagnaneswari, R. Dinakaran Michael, Oral administration of *Eclipta alba* leaf aqueous extract enhances the non-specific immune responses and disease resistance of *Oreochromis mossambicus*, Fish Shellfish Immunol. 23 (2007) 840–852.

[25] M.H. Larsen, J.L. Larsen, J.E. Olsen, Chemotaxis of *Vibrio anguillarum* to fish mucus: role of the origin of the fish mucus, the fish species and the serogroup of the pathogen, FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 38 (2001) 77–80.

[26] L. Gerwick, R. Steinhauer, S. Lapatra, T. Sandell, J. Ortuno, N. Hajiseyedjavadi, et al., The acute phase response of rainbow trout (*Oncorhynchus mykiss*) plasma proteins to viral, bacterial and fungal inflammatory agents, Fish Shellfish Immunol. 12 (2002) 229–242.

[27] S.K. Dügenci, N. Arda, A. Candan, Some medicinal plants as immunostimulant for fish, J. Ethnopharmacol. 88 (2003) 99–106.

[28] E. Awad, B. Austin, Use of lupin, Lupinus perennis, mango, *Mangifera indica*, and *stinging nettle, Urtica dioica*, as feed additives to prevent *Aeromonas hydrophila* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum), J. Fish. Dis. 33 (2010) 413–420.

[29] M. Dorucu, S.O. Colak, U. Ispir, B. Altinterim, Y. Celayir, The effect of black cumin seeds (*Nigella sativa*) on the immune response of rainbow trout (*Oncorhynchus mykiss*), Proceedings of the Middle East & North Africa (MENA) Region Animal Wealth Research Conference Future of Animal Wealth, Cairo International Convention Center, Egypt, 16-18 October, 2008, Massive Conferences and Trade Fairs, 2008, pp. 543–550.

[30] M. Laskowski Jr., I. Kato, Protein inhibitors of proteinases, Annu. Rev. Biochem. 49 (1980) 593–626.

[31] A. Ellis, Innate host defense mechanisms of fish against viruses and bacteria, Dev. Comp. Immunol. 25 (2001) 827–839.

[32] E.S. Awad, Studies on Plant Based Dietary Supplements for Control of *Aeromonas Hydrophila* Infections in Rainbow Trout (*Oncorhynchus mykiss* Walbaum), Heriot-Watt University, 2010.

[33] E.J. Nya, B. Austin, Use of garlic, Allium sativum, to control Aeromonas hydrophila infection in rainbow trout, Oncorhynchus mykiss (Walbaum), J. Fish. Dis. 32 (2009) 963–970.

[34] A.D. Talpur, M. Ikhwanuddin, Dietary effects of garlic (*Allium sativum*) on haemato-immunological parameters, survival, growth, and disease resistance against *Vibrio harveyi* infection in Asian sea bass, *Lates calcarifer* (Bloch), Aquaculture 364 (2012) 6–12.

[35] Y. Rao, B. Das, P. Jyotyrmayee, R. Chakrabarti, Effect of Achyranthes aspera on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*, Fish Shellfish Immunol. 20 (2006) 263–273.

[36] D. Wassom, E. Kelly, The role of the major histocompatibility complex in resistance to parasite infections, Crit. Rev. Immunol. 10 (1990) 31–52.

[37] M. Adel, C.M.A. Caipang, M.A. Dawood, Immunological responses and disease

resistance of rainbow trout (*Oncorhynchus mykiss*) juveniles following dietary administration of stinging nettle (*Urtica dioica*), Fish Shellfish Immunol. 71 (2017) 230–238.
[38] P. Robertson, C. O'Dowd, C. Burrells, P. Williams, B. Austin, Use of *Carnobacterium* sp. as a probiotic for Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*, Walbaum), Aquaculture 185 (2000) 235–243.
[39] E. Baba, Ü. Acar, S. Yılmaz, F. Zemheri, S. Ergün, Dietary olive leaf (*Olea europea* L.) extract alters some immune gene expression levels and disease resistance to *Yersinia ruckeri* infection in rainbow trout *Oncorhynchus mykiss*, Fish Shellfish Immunol. 79 (2018) 28–33.
[40] E.J. Nya, B. Austin, Development of immunity in rainbow trout (Oncorhynchus mykiss, Walbaum) to Aeromonas hydrophila after the dietary application of garlic, Fish Shellfish Immunol. 30 (2011) 845–850.
[41] J. Zou, S. Peddie, G. Scapigliati, Y. Zhang, N. Bols, A. Ellis, et al., Functional characterisation of the recombinant tumor necrosis factors in rainbow trout, *Oncorhynchus mykiss*, Dev. Comp. Immunol. 27 (2003) 813–822.
[42] S. Bilen, Y.C. Altunoglu, F. Ulu, G. Biswas, Innate immune and growth promoting responses to caper (*Capparis spinosa*) extract in rainbow trout (*Oncorhynchus mykiss*), Fish Shellfish Immunol. 57 (2016) 206–212.
[43] J.-L. Wang, X-l Meng, R-h Lu, C. Wu, Y.-T. Luo, X. Yan, et al., Effects of *Rehmannia glutinosa* on growth performance, immunological parameters and disease resistance to *Aeromonas hydrophila* in common carp (*Cyprinus carpio* L.), Aquaculture 435 (2015) 293–300.
[44] S.S. Giri, S.S. Sen, C. Chi, H.J. Kim, S. Yun, S.C. Park, et al., Effect of guava leaves on the growth performance and cytokine gene expression of *Labeo rohita* and its susceptibility to *Aeromonas hydrophila* infection, Fish Shellfish Immunol. 46 (2015) 217–224.
[45] M. Seppola, A.N. Larsen, K. Steiro, B. Robertsen, I. Jensen, Characterisation and expression analysis of the interleukin genes, IL-1β, IL-8 and IL-10, in Atlantic cod (*Gadus morhua* L.), Mol. Immunol. 45 (2008) 887–897.
[46] N. Mukaida, A. Harada, K. Matsushima, Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions, Cytokine Growth Factor Rev. 9 (1998) 9–23.
[47] E. Awad, W.J. Mitchell, B. Austin, Effect of dietary supplements on cytokine gene expression in rainbow trout, *Oncorhynchus mykiss* (Walbaum), J. Fish. Dis. 34 (2011) 629–634.
[48] S. Nootash, N. Sheikhzadeh, B. Baradaran, A.K. Oushani, M.R.M. Moghadam, K. Nofouzi, et al., Green tea (*Camellia sinensis*) administration induces expression of immune relevant genes and biochemical parameters in rainbow trout (*Oncorhynchus mykiss*), Fish Shellfish Immunol. 35 (2013) 1916–1923.
[49] H. Suetake, K. Araki, Y. Suzuki, Cloning, expression, and characterization of fugu CD4, the first ectothermic animal CD4, Immunogenetics 56 (2004) 368–374.
[50] L. Moore, T. Somamoto, K. Lie, J. Dijkstra, I. Hordvik, Characterisation of salmon and trout CD8α and CD8β, Mol. Immunol. 42 (2005) 1225–1234.
[51] L.B. Hølvold, Immunostimulants Connecting Innate and Adaptive Immunity in Atlantic Salmon (*Salmo salar*), Universitetet i Tromsø, 2007.
[52] S. Torrecillas, F. Rivero-Ramírez, M. Izquierdo, M. Caballero, A. Makol, P. Suarez-Bregua, et al., Feeding European sea bass (*Dicentrarchus labrax*) juveniles with a functional synbiotic additive (mannan oligosaccharides and *Pediococcus acidilactici*): an effective tool to reduce low fishmeal and fish oil gut health effects? Fish Shellfish Immunol. 81 (2018) 10–20.
[53] B. Magnadóttir, S. Lange, S. Gudmundsdottir, J. Bøgwald, R. Dalmo, Ontogeny of humoral immune parameters in fish, Fish Shellfish Immunol. 19 (2005) 429–439.
[54] B. Grinde, Lysozyme from rainbow trout, *Salmo gairdneri* Richardson, as an antibacterial agent against fish pathogens, J. Fish. Dis. 12 (1989) 95–104.
[55] S.H. Hoseinifar, M. Khalili, R. Rufchaei, M. Raeisi, M. Attar, H. Cordero, et al., Effects of date palm fruit extracts on skin mucosal immunity, immune related genes expression and growth performance of common carp (*Cyprinus carpio*) fry, Fish Shellfish Immunol. 47 (2015) 706–711.
[56] I. Henderson, N. Hazon, K. Hughes, Hormones, ionic regulation and kidney function in fishes, Symposia of the Society for Experimental Biology, 1985, pp. 245–265.
[57] K.M. Call, T. Glaser, C.Y. Ito, A.J. Buckler, J. Pelletier, D.A. Haber, et al., Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus, Cell 60 (1990) 509–520.
[58] J.A. Kreidberg, H. Sariola, J.M. Loring, M. Maeda, J. Pelletier, D. Housman, et al., WT-1 is required for early kidney development, Cell 74 (1993) 679–691.
[59] N. Klüver, A. Herpin, I. Braasch, J. Drieβle, M. Schartl, Regulatory back-up circuit of medaka Wt1 co-orthologs ensures PGC maintenance, Dev. Biol. 325 (2009) 179–188.
[60] B. Perner, C. Englert, F. Bollig, The Wilms tumor genes wt1a and wt1b control different steps during formation of the zebrafish pronephros, Dev. Biol. 309 (2007) 87–96.
[61] D. Jiang, J. Chen, Z. Fan, D. Tan, J. Zhao, H. Shi, et al., CRISPR/Cas9-induced disruption of wt1a and wt1b reveals their different roles in kidney and gonad development in Nile tilapia, Dev. Biol. 428 (2017) 63–73.
[62] H.-J. Hsu, G. Lin, B-c Chung, Parallel early development of zebrafish interrenal glands and pronephros: differential control by wt1 and ff1b, Development 130 (2003) 2107–2116.