Aquacult Int
https://doi.org/10.1007/s10499-018-0274-y

The immune modulatory effect of oregano (*Origanum vulgare* L.) essential oil on *Tilapia zillii* following intraperitoneal infection with *Vibrio anguillarum*

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Received: 18 August 2017 / Accepted: 10 May 2018

Abstract The current study aimed to evaluate the possible effect of Origanum essential oil on innate immune parameters as well as the hematological profiles of *Tilapia zillii* following challenge with *Vibrio anguillarum*. Fifty-four of *Tilapia zillii* weighing 180 ± 10.2 g were randomly distributed into three identical closed recirculating seawater systems. The study included three groups (G1, G2, and G3) repeated in triplicates. Fish of the first two groups were fed on a basal diet without herbs, whereas fish of the last group were fed on a basal diet supplemented with Origanum essential oil at concentration 1 g kg^{-1} for 15 days. Subsequently, fish of G2 and G3 subjected to a peritoneal inflammation by intraperitoneally injecting *V. anguillarum* (5.5×10^5 CFU mL^{-1}), whereas fish of G1 injected with saline and served as control. Fish of all groups were then sampled at 4, 12, and 24 h post injection. No mortalities were observed in both basal and Origanum fed groups. However, some specimens of fish fed basal diet showed dorsal fin erosions, eroded mouth, and detached skin. Although the kinetics of RBCs, WBCs, Hb, and differential leukocyte values remained unchanged in fish fed different diets at the beginning of the trial, significant increases in those values were observed in fish fed Origanum essential oil at 12 and 24 h post injection. Similarly, an augmentation of plasma proteases, antiproteases, and lysozyme activities were recorded in fish fed Origanum essential oil at the same particular sampling points. A significant enhancement in plasma bactericidal capacity was only recorded in fish fed Origanum essential oil at 12 and 24 h post challenge compared to those fed basal diet. In conclusion, Origanum essential oil had a pronounced influence on the innate immunity and increased the fish resistance to *V. anguillarum*. These data gave insight into the potential use of Origanum in prophylactic strategies against threatening pathogens.

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Published online: 29 May 2018

Keywords Origanum oil · Pathogenicity · Innate immune parameters · Bactericidal activity · *Vibrio anguillarum*

Introduction

Aquaculture is considered one of the most important animal food-producing sectors that can globally supply world population with protein sources and cover the gap of food shortage due to over population. This increasing fish demand has to be supported by a concomitant increase in aquaculture production due to fisheries stagnation in the last years (FAO 2014, p. 223). Recently, farming and husbandry of *Tilapia zillii* were extensively used because of their high protein content, large size, rapid growth, and palatability. Tilapia are suited to many low innovating farms because of their ability to utilize natural aquatic foods, reproduce under captivity, and tolerate wide ranges of adverse environmental conditions (Fagbenro 2001, p. 26). Aquatic diseases are contributed one of the most threatening problems facing fish industries particularly, during fish rearing and husbandry. Vibriosis is considered one of the most prominent pathogens frequently affecting a broad range of fish species (Lee et al. 2002; Alcaide 2003, p. 184). Vibrionaceae constitutes 60% of the heterotrophic bacteria population that widely distributed in the coastal seawaters and/or brackish ones. The fish immune system is an exceptionally advanced framework that has the capacity to outfit living beings with the ability to oppose pathogenic microorganisms described by two pathways, innate and acquired immunity. The innate immunity contributes a significant number of humoral and cellular factors that assume an important part as the first line of defense against invading pathogens (Ellis 1999, p. 223). The real inflammatory process is rapid, destructive, and self-limiting (Barton 2008, p. 413). It was proposed that feed additives could modulate several aspects of the fish immune system (Li et al. 2007). Using of plant extracts as immunostimulants has been bloomed in most of aquaculture sectors during the last decade (Galina et al. 2009; Vaseeharan and Thaya 2014). Many studies revealed significant increases in fish immunological parameters as well as hematological parameters following oral administration of plant extracts (Harikrishnan et al. 2012; Wu et al. 2013). Moreover, Ran et al. (2016) mentioned that supplementation with essential oil product containing equal levels of thymol and carvacrol enhanced phagocytosis activity of head kidney macrophages and elevated the plasma lysozyme activity of tilapia compared with the control group. Therefore, adding plant extract in basal diets has a thematic concept in fish-producing sectors. *Origanum vulgare* is an aromatic plant belonging to the Lamiaceae family. It contributes more than 20 components. Carvacrol and thymol represent the main ingredients of the total oil (Adam et al. 1998). Both components have antimicrobial (Sivropoulou et al. 1996; Lambert et al. 2001), antimycotic (Kocić-Tanackov et al. 2012), insecticidal (Karpouhtsis et al. 1998), and antioxidant (Botsoglou et al. 2002) properties. So dietary supplementation may consider the best strategy to improve the fish immune status and minimize the cost of useless treatment (Grimble 2009, p. 12). In fact, the hematological parameters are considered fundamental indicators for fish health and reflect the physiological state of fish (Davison et al. 1993). Therefore, the current study aimed to evaluate the possible effects of dietary supplementation with Origanum essential oil on *T. zillii* hematological as well as humoral innate parameters following intraperitoneal injection with a subclinical dose of *Vibrio anguillarum*.

Materials and methods

Bacterial growth and inocula preparation

Vibrio anguillarum strain used in the current study was previously isolated from infected *Tilapia zillii* at Lake Temsah during the summer outbreak 2016. The pathogenicity of this strain was experimentally evaluated against the same fish species in a previous study (Eissa et al. 2017), and presented higher morbidity and mortality rates up to 80%. Bacteria were routinely cultured at 25 °C in thiosulfate citrate bile salt sucrose agar (TCBS) (Lab M, UK) supplemented with NaCl to a final concentration of 3% (*w*/*v*) and kept frozen at 20 °C in tryptic soy broth (TSB) (Lab M, UK) supplemented with 12% (*v*/*v*) glycerol until used. The morphology, culture, and biochemical characteristics of the isolate were performed according to the criteria of *Bergey's Manual of Determinative Bacteriology* (Baumann and Furniss 1994), and confirmed using commercial API-20E strips following the manufacture procedures. Molecular identification of bacterial isolate was performed using two sets of specific primers (rpoN-ang5′F, rpoN-ang3′R), representing two variable regions on *Vibrio anguillarum rpoN* gene as described by Gonzalez et al. (2003). For bacterial recovery and inocula preparation, it was performed according to the method described by Avci et al. (2012) with minor modifications. Briefly, stocked bacteria were cultured for 48 h at 25 °C on TCBS supplemented with NaCl to a final concentration of 3% and then inoculated into TSB for additional 12 h under the same temperature with continuous shaking (120 rpm). Exponentially growing bacteria were harvested by centrifugation at 4000×*g* for 30 min, re-suspended in sterile physiological saline (0.9% NaCl solution), and adjusted to the final concentrations of 5.5×10^5, 5.5×10^6, and 5.5×10^7 CFU mL^{-1}. Bacterial counts were performed manually using a Helber count chamber and confirmed by plate count technique.

Diet preparation

The practical basal diet (contains 30% protein, 14.4% carbohydrate, and 13.48% ash) was formulated from fish meal and plenty of plant feedstuffs as protein sources, while the treated diet was prepared by adding 1 g kg^{-1} of Origanum essential oil which contributes 50% carvacrol and 1% thymol as active ingredients (Dostofarm GmbH, Germany). The dose of Origanum essential oil was selected according to several preliminary trials that indicate the significant effectiveness of that dose compared to other 0.5 and 0.75 g kg^{-1} (data are not presented). All dietary ingredients were thoroughly mixed and passed through CBM machine to give pellets with 2-mm diameter. The composition and proximate analyses of experimental diets were presented in (Table. 1).

Experimental setup

Acclimation period

A total of 200 apparently healthy adult *Tilapia zillii* weighing 180 ± 10.2 g were imported from Lake Temsah, Ismailia, Egypt, and declared according to the Egyptian community law, in a good health and disease free**.** To verify the actual health status, the fish were quarantined and left acclimated for 2 weeks in a closed recirculating unit supplied with sand-filtered, UV-treated, aerated seawater at 24 ± 2 °C. The unit contributes two fiberglass tanks of 1000 L

Table 1 Composition and proximate analysis of the experimental diets (g kg^{-1})

Feed ingredients	Experimental diets	
	Basal diet	Origanum essential oil diet
Fish meal (64.5% CP)	350	350
Soy bean meal (40.3% CP)	175	175
Yellow corn	187	187
Wheat flour	80	80
Fish and corn oil[a]	25	25
Wheat bran	150	150
Bone meal	20	20
Vitamin and mineral mix[b]	10	10
Ascorbic acid	3	3
Total	1000	1000
Origanum essential oil	–	1
Proximate analysis %		
Protein	30.51	30.64
Moisture	5.46	6.13
Fat	14.40	14.76
Ash	13.48	12.98

[a] Fish oil and corn oil were added as constant ratio (1:1)/kg

[b] Vitamin and mineral mixture for each 1 kg of mixture contains the following: Vitamin D_3, 0.8 million IU; A, 4.8 million IU; E, 4 g; K, 0.8 g; B_1, 0.4 g; riboflavin, 1.6 g; B_6, 0.6 g, B_{12}, 4 mg; pantothenic acid, 4 g; nicotinic acid, 8 g; folic acid, 0.4 g; biotin, 20 mg; Mn, 22 g; Zn, 22 g; Fe, 12 g; Cu, 4 g; I, 0.4 g; selenium, 0.4 g; and Co, 4.8 mg

Estimated based on values of the diet ingredients (NRC, 1993)

capacity and 100 fish-stocking density. All fish were inspected daily for any abnormalities. Dissolved oxygen was maintained at 5 mg L^{-1}, water temperature was 24 ± 1 °C, pH was 7.4–8.8, salinity averaged was 33 ± 1‰, and 12-h light/12-h dark photoperiod was adopted. Ammonia and nitrite levels in the water were measured once a week using commercial kits and never exceeded 0.02 and 0.3 mg L^{-1}, respectively. Apparently, healthy fish, as indicated by their activity and external appearance, were used during the trials.

Pathogenicity trial

This trial was performed to detect the sub-lethal dose of *V. anguillarum* to be used later in the feeding and challenge trials. For that, 72 fish were randomly assigned into two identical closed recirculating systems; each contributes six tanks with 70 × 60 × 30 cm dimensions. Each tank was filled with 100 L of aerated filtered seawater at salinity 33 ± 1‰, and contributes six fish. Fish of the first system were intraperitoneally injected with *V. anguillarum* at three different concentrations of 5.5×10^5, 5.5×10^6, and 5.5×10^7 CFU mL^{-1}, whereas fish of the other system were inoculated with a sterile saline solution instead of bacteria and served as negative control. All treatments were performed in duplicates. The mortality of the experimentally infected fish was assessed for 2 weeks post challenge. Dead and moribund fish were frequently collected for bacteriological examination. Any survivors at the end of the experiment were sacrificed and examined for *V. anguillarum*. For bacterial recovery, loopful samples from lesions, liver, kidneys, and spleen were inoculated directly into tryptic soy agar (TSA) supplemented with different concentrations of sodium chloride (1.5–8%). After that, the inoculum streaked on specific medium (TCBS) and left incubated for 24 h at 30 ± 1 °C. The

recovered colonies were picked up and examined microscopically and biochemically (Baumann and Furniss 1994), whereas the molecular characterization was performed using specific sets of primers according to Gonzalez et al. (2003).

Feeding and challenge trials

The feeding trial was performed in triplicates for 15 days to assess the effects of short-term dietary supplementation with Origanum essential oil on *Tilapia zillii* immune status following challenge with *V. anguillarum*. For that, 54 fish were equally distributed into three groups (G1, G2, and G3); each contributes three tanks. The total volume of each tank is 100 L and contributes six fish. Fish of the first two groups (G1 and G2) were fed on a basal diet of 30% protein without herbs, whereas fish of the third group (G3) were fed on an experimentally modified diet (basal diet in addition to Origanum essential oil 1 g kg^{-1}). Fish of all groups were fed twice daily to apparent visual satiety at the rate of 2–3% of total body weight. At the end of the feeding trial, all fish were anesthetized by immersion in a mixture of tricaine methanesulfonate (MS222) 50 mg L^{-1} and sodium bicarbonate 25 mg L^{-1}. Subsequently, fish of first group (G1) were inoculated intraperitoneally with normal physiological saline (0.9% NaCl) and served as control, while fish of remaining groups (G2 and G3) were inoculated with 0.1 mL of *V. anguillarum* at concentration 5.5×10^5 mL^{-1}. The dose was selected based on the pathogenicity trial since no mortality was obtained. Afterwards, stimulated specimens (two fish per tank, two technical replicates) were then sampled for blood and plasma collection at 4, 12, and 24 h post challenge.

Hematological procedures

Blood collection was performed from the caudal vein using heparinized syringes. An aliquot of homogenized blood was used for hematological profiles including total white (WBCs) and red blood (RBCs) cell counting and hemoglobin (Hb g dl^{-1}; Sigma-Aldrich assay kit, ref. MAK115). The remaining blood was centrifuged at 10,000×*g* for 10 min at 4 °C, and the obtained plasma was stored at − 20 °C until used. Afterword, blood smears were performed immediately, air dried, and stained with Wright's stain (Hemacolor; Merck) after fixation with formole-ethanol (10 of 37% formaldehyde in absolute ethanol). To facilitate differentiation of neutrophils, a peroxidase activity was conducted as described by Afonso et al. (1998). The slides were microscopically examined using × 1000 magnification power. At least 200 leucocytes were counted and classified as lymphocytes, monocytes, and neutrophils. The relative percentage and absolute value ($\times 10^4$ mL^{-1}) of each cell type were calculated.

Innate humoral parameters

Lysozyme activity was assessed based on the turbidimetric method elucidated by Swain et al. (2007) with some modifications. Briefly, 15 μL of plasma samples of different treatments were seeded in flat-bottomed 96-well plates in duplicate (technical replicates). To each well, 180 μL of freeze-dried *Micrococcus lysodeikticus* (0.5 mg mL^{-1}) previously suspended in 0.05 M sodium phosphate buffer with pH 6.2 was added as lysozyme substrate. One hundred eighty microliters of 0.04 M sodium phosphate buffer-free *Micrococcus lysodeikticus* was added and served as sample blank. Two wells with 200 μL of 0.04 M sodium phosphate buffer were used as a standard blank. The measurement was carried out at 35 °C and 450 nm after 20 min of incubation in a Synergy HT microplate reader. Lyophilized hen egg-white lysozyme (Sigma)

was serially diluted in sodium phosphate buffer (0.04 M, pH 6.2) and used to develop a standard curve. The amount of lysozyme in the sample was calculated using the formula of the standard curve and expressed as microgram per milliliter.

Total anti-protease activity was based on the ability of plasma to inhibit trypsin activity and was assessed according to Machado et al. (2015). Briefly, 10 μL of plasma samples were incubated with equal amount of standard trypsin solution (5 mg mL^{-1} in $NaHCO_3$, 5 mg mL^{-1}, pH 8.3) for 10 min at 22 °C in polystyrene microtubes. Subsequently, 100 μL of phosphate buffer (NaH_2PO_4, 13.9 mg mL^{-1}, pH 7.0) and 125 μL of azocasein (20 mg mL^{-1} in $NaHCO_3$, 5 mg mL^{-1}, pH 8.3; Sigma) were added, and the mixture was incubated for additional 1 h at the same temperature. Finally, 250 μL of trichloroacetic acid were added to the incubation mixture and incubated for 30 min at 22 °C. The mixture was then centrifuged at 10,000×*g* for 5 min at room temperature, and 100 μL of the supernatant was pipetted to a new 96-well plate previously contained 100 μL of NaOH (40 mg mL^{-1}) per well. The OD was read at 450 nm in a Synergy HT microplate reader. Phosphate buffer in place of plasma and trypsin served as a blank, whereas phosphate buffer in place of plasma refers to the reference sample. The percentage inhibition of trypsin activity compared to the reference sample was calculated. All analyses were performed in duplicates (two technical replicates).

Regarding the protease activity, it was measured according to Ross et al. (2000) using the azocasein hydrolysis assay. Briefly, 100 μL of plasma in duplicates (two technical replicates) were incubated with an equal volume of phosphate buffer (NaH_2PO_4, 13.9 mg mL^{-1}, pH 7.0) containing azocasein (20 mg mL^{-1}, Sigma) for 24 h at 30 °C. After that, the reaction was blocked by adding 10% trichloroacetic acid, and the mixture was vigorously shacked and centrifuged at 10,000×*g* for 10 min. The supernatants were then pipetted to a new 96-well plate in triplicates previously contained 100 μL of NaOH (40 mg mL^{-1}) per well, and the OD was read at 450 nm using a Synergy HT microplate reader. Plasma was either replaced by trypsin (5 mg mL^{-1}, Sigma) which served as positive control (100% of protease activity), or by phosphate buffered, which served as negative control (0% activity). The protease activity was calculated using the following formula: Protease activity (%) = ((Reading Abs−Negative control Abs)/Positive control Abs) ×100.

It was suggested that MTT colorimetric assay could be used for measurement of bacteria growth and viability (Zhang and Liu 2002). The principle is that the yellow-colored MTT was reduced into blue formazan by the act of living bacteria. The intensity of that coloration was directly proportional to the bacterial population. Therefore, the bactericidal assay was carried out using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay as described elsewhere by Machado et al. (2015). *Vibrio anguillarum* was utilized in the bactericidal activity assay. Bacteria were cultured as described above and exponentially growing bacteria were harvested and re-suspended in sterile HBSS and adjusted to 1×10^6 CFU mL^{-1}. A standard plate counting technique using TSA was used to confirm the bacterial concentration of the inoculum. Briefly, 20 μL of plasma samples were placed in U-shaped 96-well plate in duplicate. HBSS was added to some wells instead of plasma and served as positive control. To each well, 20 μL of bacteria were added, and the plates were incubated for 2.5 h at 25 °C. Finally, 25 μL of MTT (1 mg mL^{-1}; Sigma-Aldrich) were then added to each well and left incubated for 10 min at 25 °C to allow formazan formation. Plates were then centrifuged at 2000×*g* for 10 min, and the precipitate was dissolved in 200 μL of dimethyl sulfoxide (Sigma-Aldrich). The absorbance was measured at 560 nm. Bactericidal activity was calculated from analytical triplicates and expressed as a percentage of bacteria surviving in relation to the number of bacteria from positive controls (100%).

Statistical analyses

All results are presented as means ± standard deviation (SD). Data are presented as fold change levels (means ± SD), calculated by dividing each parameter value from fish i.p. injected with *V. anguillarum* by the average value from control fish, i.p. injected with normal physiological saline, minus one. Fold values higher than 0 express an increase. Data were analyzed for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene's test), and when necessary, they were log-transformed before being treated statistically. Data were analyzed by two-way ANOVA followed by Tukey tests and Student *T* tests to ensure the significant variation among the different treatments. All statistical analyses were conducted using the computer package STATISTICA 12 software for Windows. The level of significance used was $p \leq 0.05$ for all tests.

Results

Pathogenicity assays

Fish exposed to 5.5×10^5 CFU mL^{-1} did not show any mortality, whereas those exposed to higher doses of *V. anguillarum* present different degrees of mortalities, depending on bacterial concentration. In fact, 80% of fish infected with the highest dose of *V. anguillarum* died within 6 days following inoculation, while those exposed to 5.5×10^6 CFU mL^{-1} presented 50% of mortality rate. Most of the infected fish displayed scattered hemorrhagic lesions (near the site of injection and at the base of dorsal and pectoral fins), detached skin, and signs of septicemia such as engorged spleen and congested livers (data not shown). The bacteria were successfully isolated from external and internal lesions on TCBS after 48-h incubation period. The colonies appeared pale yellow and exhibited swarming. All isolates were resistant to vibriostat O/129–10 μg and sensitive to vibriostat O/129–150 μg. The Biochemical analysis using API 20E strips showed 89% similarity to *V. anguillarum*. All samples revealed positive results for conventional PCR with amplification bands of 519 bp.

Regarding the feeding trial, the fish adapted rapidly to the experimental diets and consumed all provided diets within 15 min. No mortalities were observed in both basal- and Origanum-fed groups following challenge with 5.5×10^5 CFU $mL^{-1}$of *V. anguillarum*. However, some specimens of fish fed basal diet showed dorsal fin erosions, eroded mouth, and detached skin.

Hematology

In the current study, fold change values of total RBCs, total WBCs, Hb, and peripheral blood leukocytes (neutrophils, monocytes, and lymphocytes) were presented. The kinetics of RBCs values remained unchanged in fish fed different diets at the beginning of the trial, whereas fish fed Origanum essential oil showed significant increases at 12-h (p value = 0.00367) and 24-h (p value = 0.00198) post injection compared to the control group (Fig. 1a). Similarly, an augmentation of Hb values was also recorded in fish fed Origanum essential oil at the same particular sampling times (p value = 0.00015). Moreover, a decrease in blood Hb concentration was also observed in fish fed basal diet from 4 to 24 h post injection (Fig. 1b). The percentage and total concentration of WBCs were increased in fish fed Origanum essential oil compared to the control group throughout the trial (p value = 0.00015) (Fig. 1c). Although no differences

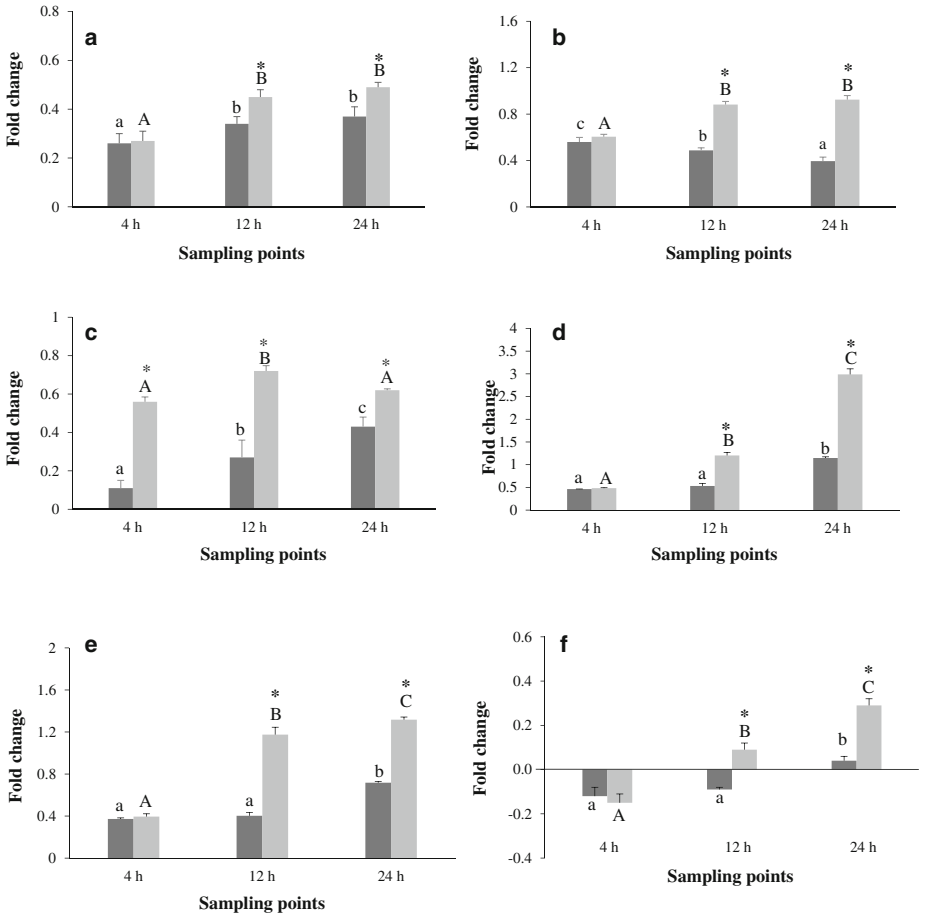

Fig. 1 Red blood cells (RBCs) (**a**), hemoglobin (Hb) (**b**), white blood cells (WBCs) (**c**), peripheral circulating neutrophils (**d**), monocytes (**e**), and lymphocytes (**f**) presented as fold change in *T. zillii* fed basal diet (■) and Origanum essential oil (□) following I/P injection with 10^5 CFU mL^{-1} of *V. anguillarum*. Data are presented as means ± SD ($n = 3$). Different small letters indicate significant changes among the control group, while capital letters stand for differences among Origanum dietary group. Single asterisk indicates significant variations among treated diets for each particular sampling point (two-way ANOVA; $P \leq 0.05$)

in peripheral circulating neutrophil, monocyte, and lymphocyte numbers were observed in fish fed Origanum essential oil relatively to fish fed basal diet at the beginning of the trial, a progressive increase in all values was noticed at 12 and 24 h post challenge (p value = 0.00016) (Fig. 1d–f, respectively).

Innate immune response post challenge

The kinetics of plasma lysozyme activity of both treated and control groups against *V. anguillarum* are presented (Fig. 2a). At the beginning of the trial, no significant variation related to diet composition was observed, whereas significant increases in plasma lysozyme activity were only observed in fish fed Origanum essential oil at 12 and 24 h post challenge (p value = 0.00015). Furthermore, the plasma proteases activity significantly enhanced in fish fed

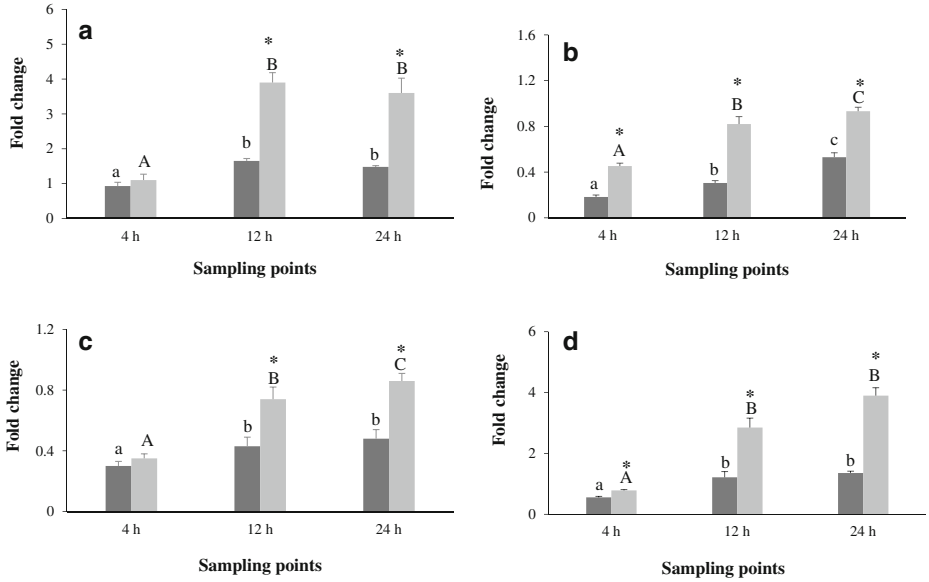

Fig. 2 Lysozyme (**a**), proteases (**b**), antiproteases (**c**), and bactericidal activity (**d**) of plasma presented as fold change in *T. zillii* fed basal diet (■) and Origanum essential oil (□) following I/P injection with 10^5 CFU mL^{-1} of *V. anguillarum*. Data are presented as means ± SD ($n = 3$). Different small letters indicate significant changes among the control group, while capital letters stand for differences among Origanum dietary group. Asterisks indicate significant variations among treatments for each particular sampling point (two-way ANOVA; $P \leq 0.05$)

Origanum essential oil meanwhile the trial (p value = 0.00015) (Fig. 2b). The antiprotease activity in fish fed Origanum essential oil showed similarity at 4 h post challenge while it increased significantly with time compared to those fed a basal diet (p values = 0.00016 and 0.00015 at 12 and 24 h, respectively) (Fig. 2c). Although the plasma bactericidal capacity augmented in time in both dietary treatments, a significant enhancement in bactericidal capacity was only recorded at 12 and 24 h post challenge in fish fed Origanum essential oil compared to those fed a basal diet (p value = 0.00015) (Fig. 2d).

Discussion

Medicinal plant particularly aromatic one has been proven to have a significant role in growth performance, weight gain, survival rate, and expected to have both prophylactic and therapeutic need in poultry-producing sector (Aligiannis et al. 2001; Roofchaee et al. 2013). Although several studies contribute the influence effect of Origanum on muscle protein content, antioxidant activity, feed utilization, and disease resistance of shrimp (Ching 2008), Nile tilapia (Seden et al. 2009), channel catfish (Zheng et al. 2009), common carp, and gilthead seabream (Athanassopoulou et al. 2010), data regarding its immune modulatory effect on *Tilapia zillii* is still not fully elucidated. In general, herbal-based medicines were confirmed to enhance the immune responses and minimize losses from viruses, bacteria, and parasitic infections in either marine or freshwater fishes (Yin et al. 2006; Harikrishnan et al. 2011). Based on this argument, the current study aimed to evaluate the possible effect of Origanum essential oil on *T. zillii* innate immune response, as well as the hematological profiles following experimental infection with *V. anguillarum*.

In the present study, overall hematological parameters, as well as differential leukocytes, were significantly enhanced in fish fed Origanum essential oil compared to those fed a basal diet at the same particular sampling points. Similar augmentation of RBCs, WBCs, thrombocytes, and hemoglobin was recorded in Indian catfish (*Mystus montanus*) following supplementation with four medicinal diets including onion (*Allium cepa*), garlic (*Allium sativum*), ginger (*Zingiber officinale*), and nettle (*Urtica dioica*), which posteriorly resulted in an increase in survival rate following experimental infection with *Aeromonas hydrophila* (Chelladurai et al. 2014). Since, the effects of essential oils are the results of the combined action of their components. Our results are in agreement with those obtained by Pourmoghim et al. (2015), and Yilmaz and Ergün (2015) who did not observe any significant variations in rainbow trout (*Oncorhynchus mykiss*) hematological parameters following supplementation with carvacrol or thymol independently.

It was known that WBCs sustain protection in fish against a wide range of threatening pathogens and several chemical stressors (Harikrishnan et al. 2003). In the current study, the augmented values obtained and get-together data on the profile of leucocytes can reflect on the general immune status of fish. Origanum is well known for its special aroma, and antioxidant and antimicrobial activities (Drăgan et al. 2008). The antimicrobial activity of the essential plant oil was previously elucidated (Ekici et al. 2011; Yildirim et al. 2013). Therefore, the surge increased of WBCs numbers was due to the higher input of Origanum essential oil (1 g kg^{-1}). In the present study, a tenuous lymphopenia was observed during early sampling point, while an increase in circulating lymphocytes was only observed at the end of the trial. Thus, it could be attributed to the migration of lymphocyte toward the site of infection (Lamas et al. 1994). The reduction in lymphocyte numbers has also been reported in fish after bacterial insults (Lamas et al. 1994; Balfry et al. 1997; Garcia et al. 2007; Costas et al. 2013).

Regarding systemic immune responses, plasma proteases, antiproteases as well as circulating monocytes and neutrophils significantly increased in fish fed Origanum essential oil following challenge suggesting an early insight into the dynamics of leucocyte migration. Moreover, peripheral phagocyte numbers, as well as lysozyme activity, increased during infection. In several fish species, the surge of neutrophil migration was predominantly associated with monocyte recruitment (Neumann et al. 2001). The current study proved this assumption since monocytosis was recorded at the same particular sampling points. Cases of blood neutrophilia and monocytosis were also recorded in several fish species fed different medicinal plants (Gopalakannan and Arul 2006; Sahu et al. 2007).

Neutrophils secrete lysozyme, and increases in serum lysozyme activity have been associated with increases in their numbers (Muona and Soivio 1992). Therefore, in the current study, the higher plasma lysozyme activity may be congruent to the observed blood neutrophilia. Similarly, Costas et al. (2013) observed a correlation between the surge increase in Senegalese sole plasma lysozyme activity and blood neutrophilia following *P. damselae subsp. piscicida* infection. Moreover, Balfry et al. (1997) attributed the lack of serum lysozyme activity in *Oreochromis niloticus* following challenge with *Vibrio parahaemolyticus* to the low proliferative number of neutrophils.

Concerning the pathogenicity assay, only some specimens of fish fed basal diet showed dorsal fin erosions, eroded mouth, and detached skin. Plenty amounts of active metabolites have been reported in *Origanum* essential oil, including those with antimicrobial activity: carvacrol, thymol, p-cymene, and γ-terpinene (Karousou and Kokkini 2003), thus reflecting the effluent action of Origanum essential oil on Tilapia immune response, which can be inferred from in vitro analysis of plasma bactericidal activity. Antiproteases were

known to be involved in acute phase reactions (Bayne and Gerwick 2001), including as defense strategy against proteolytic secreting pathogens (Zuo and Woo 1997). Furthermore, lysozyme mucolytic enzyme synthesized by leucocytes was considered a fundamental defense molecule against microbial invasion (Ellis 1999, p. 223.). Volpatti et al. (2013) mentioned that dietary supplementation of carvacrol for 1–4 weeks revealed a higher trend in lysozyme activity in *Dicentrarchus labrax*. Moreover, Diler et al. (2016) observed an increase in lysozyme activity and resistance against *Lactococcus garvieae* in rainbow trout following supplementation with *Origanum onites* essential oil. Therefore, considering the enhanced WBCs response and the role of macrophages and neutrophils on phagocytosis, besides the surge increase in plasma proteases, antiproteases, and lysozyme, it is likely that fish fed Origanum essential oil present increased levels of protection to bacterial insult.

The antimicrobial activity observed in the plasma of fish fed Origanum diet was attributed to carvacrol and thymol; these two active components have the ability to disintegrate the outer membrane of Gram-negative bacteria and thereby increasing the permeability of the cytoplasmic membrane (Helander et al. 1998). The role of carvacrol on the resistance of *Oreochromis niloticus* to *Edwardsiella tarda* and resistance of *D. labrax* to *Vibrio anguillarum* was briefly explained (Rattanachaikunsopond and Phumklachor 2010; Volpatti et al. 2013). Similarly, incorporation of oregano essential oil to channel catfish (*Ictalurus punctatus)* diet presented a significant increase in the hepatosomatic and viscerosomatic index, antioxidant activity, and revealed high survival rate after *A. hydrophila* infection (Zheng et al. 2009). In conclusion, Origanum essential oil at a concentration of 1 g kg^{-1} appears to exert positive effects on *T. zillii* immune status via improving the peripheral leucocyte response and plasma bactericidal capacity. Among acute inflammation, this exerted immunity is translated to an increase of both cellular and humoral responses. The current data gave insight into the potential use of Origanum essential oil in prophylactic strategies against predictable threatening pathogens, hopefully, to cope to the problem of antibiotic resistant. Further detailed immunological and molecular studies are still required to expand the application of these results in aquaculture as a prophylactic measure.

Compliance with ethical standards

Ethical approval All the animals were maintained in accordance with the National and International Institutional Guidelines for the Care and Use of Animals for Scientific purposes.

Competing interests The authors declare that they have no competing interest.

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