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Antibacterial activity of *Oliveria decumbens* against *Streptococcus iniae* in Nile tilapia (*Oreochromis niloticus*) and its effects on serum and mucosal immunity and antioxidant status

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ABSTRACT

The aims of this study were to investigate the antibacterial, immunostimulatory and antioxidant properties of different derivatives of *Oliveria decumbens*, in vitro and in vivo. The GC-MS spectrometry analysis showed γ-terpinene as the most frequent compound in essential oil, whereas carvacrol and thymol were the most common ones in aromatic water. Plant essential oil and hydroethanolic extract showed a positive in vitro bactericidal activity against *Streptococcus iniae* as evaluated by disc diffusion, minimum inhibitory concentration and minimum bactericidal concentration methods. Also, in vivo resistance against *S. iniae* and immune and antioxidant responses of Nile tilapia (*Oreochromis niloticus*) were assayed after 60 days treatment with *O. decumbens* derivatives. Plant hydroethanolic extract and essential oil and their 1:1 combination were added to diet at 0 (negative control), 0.01, 0.1 and 1% (w:w). The plant aromatic water at doses of 0.0312, 0.0625 and 0.1250% were also used as bath treatment. The results showed that aromatic water at lowest dose was more effective than other treatments in increment of fish resistance against *S. iniae* (7.14% mortality in comparison with 50% mortalities in control fish) and modulation of post-challenge respiratory burst activity. The bactericidal activity and biochemical contents of skin mucus did not change significantly among treatments. The levels of superoxide dismutase and catalase antioxidant enzymes activities in spleen tissue were significantly higher in treatments received extract, essential oils and their combination in comparison to other groups, while treatments did not affect peroxidase level. In conclusion, administration of different derivatives of *Oliveria decumbens* showed remarkable antibacterial activity against streptococcosis and enhanced antioxidant status and post-challenge immunity in Nile tilapia.

1. Introduction

The aquaculture industry has contributed to supply animal protein to human with growing trend during the last 50 years [1] and has reached 80 million tons in 2016 [2]. Nevertheless, outbreaks of infectious diseases is a major problem threatening this advancement [3]. To secure the further development of the aquaculture industry and protect the farming species against disease toll, some preventive agents such as vaccine, antibiotics and synthetic immunostimulants have been utilized in this regard but often not with enough prevention from loss against pathogenic diseases. Vaccines have not been developed or effectively acted against all pathogens in fish [4–6]. In addition, many vaccines are not commercially available or affordable to use in aquaculture [7,8]. Alternatively, antibiotics contemplated as another way to manage disease control in aquaculture with some success [7,8]. But the use of antibiotics has raised the emergence of antibiotic-resistant bacteria issue in aquatic organisms with adverse effect on aquatic life and consumers health [7–11]. This has caused strict laws of antibiotic usage in many regions around the world [8]. Consequently, an urgent need for an alternative in disease control with an environmentally friendly and effective agent has been attracted the interest of many researchers. These include investigations on the use of natural immunostimulants especially plant-based active compounds in essential oil or extracts forms [3,8]. Finally, there is an increasing interest in consuming organic and environmentally friendly food. Therefore, the limitation of chemical products in aquaculture and the use of natural treatments could enhance the consumption of aquaculture products.

Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) is a species farmed since the ancient time and has been the most widespread species for aquaculture [12]. Due to short generation time and advanced

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immune system, this species also considered as a proper fish for aquaculture development research [13]. Streptococcosis is one of the major causes to the staggering losses in tilapia and many other aquaculture species. Streptococcosis is usually caused by *Streptococcus iniae* and *S. agalactiae*. Most outbreaks of streptococcosis in tilapia farms occur during the warm seasons that enhanced the pathogenicity of *Streptococcus* species to tilapia [8,9]. Although some vaccines have been recently designed and successfully administrated against streptococcosis in tilapia and some other species [48,49], antibiotic therapy is still widely used to cope with disease in commercial farms.

Some previous studies showed the positive antibacterial effects of herbal products against *S. iniae*. For example, Rattanachaikunsopon and Phumkhachorn [14] studied the bactericidal effects of *Cinnamomum verum*, *Citrus hystrix*, *Cymbopogon citratus* and *Curcuma longa* against *S. iniae,* which *C. verum* was the most potent antibacterial agent. Also, Pirbalouti et al. [15] investigated the growth inhibitory effects of 10 Iranian medicinal plants ethanolic extracts and essential oils against *S. iniae*, and the essential oil of *Satureja bachtiarica* showed maximal antibacterial potency. Harikrishnan et al. [16] also reported that the dietary supplementation of *L. indica* extract enhanced the immunological parameters and increased disease resistance in *Epinephelus bruneus* against *S. iniae* infection.

Oliveria decumbens is an herb from Apiaceae family, native to western and southern Iran [17,18]. Previous studies have elaborated the high in vitro antimicrobial activity of this herb against gram positive and gram negative bacteria [19]. Also the in vivo resistance against *Aeromonas hydrophila* infection was reported in common carp (*Cyprinus carpio*) fed on diet supplemented with *O. decumbens* [20]. Therefore, we consider of interest in studying its bactericidal potential against *S. iniae* and the immunostimulant and antioxidant activities in Nile tilapia.

Essential oil, aromatic water and aqueous, alcoholic and hydro-alcoholic extracts are the most common forms of herbal plants derivatives used in humans and animals [6,18,21]. Both essential oil and aromatic water are obtained by distillation method. During distillation, part of the essential oil components (hydrophilic or polar components) become dissolved in and remain in the distillation water and the product is called aromatic water, which is also known as hydrolate, hydrosol or distillate water [18]. Essential oil and aqueous or hydro-alcoholic extracts of different herbal species have been widely used in aquaculture [4,5,8,10,21], but to the best of our knowledge, there is no report available on the usage of plant aromatic water in aquaculture.

Moreover, for the effective use of immunostimulants, in addition to dosages and method of administration as the main determining factors, the treatment duration and the type of derivative also need to be taken into consideration [21]. Therefore, the objectives of this present study were to investigate the in vitro bactericidal effects of *O. decumbens* and its in vivo potential to enhance the antioxidant enzyme activity, serum and mucosal immunity and resistance against *Streptococcus iniae* in Nile tilapia. Additionally, the most potent derivatives of *O. decumbens*, and its effective dose as well as the optimal supplementation period in fish were investigated.

2. Material and methods

2.1. Plant collection and composition analysis

The aerial parts of *O. decumbens* including stem and flowers were collected from Khonj, Fars province, Iran. The essential oil and aromatic water were extracted by distillation method using a semi-industrial clevenger (NamaGol, Isfahan-Iran). For distillation, 20 g of plant metrials was mixed with 100 ml distilled water. During distillation, steam supplied from a boiller was passed the plant materials and removed the essential oil out. The steam was then cooled using a condenser and the essential oil (the upper phase lighter than water) was separated from aromatic water or hydrolate by using a separation funnel. Finaly, each 20 g plant meterials yielded to 1 g essential oil (equal to 5% of plant materials used) and 75 mL aromatic water. The extracted oils were dehydrated by sodium sulfate and stored in a sealed dark glass at 4 °C until further use. The aromatic waters were placed in dark container and kept in cool and dark place without any further processing. The hydroethanolic extract of plant was prepared by maceration method. Briefly, 20 g of dry plant powder was mixed with 300 ml of water and ethanol 70% (1: 15 v/v) for 72 h and the solvent was separated by rotary evaporation. The hydroethanolic extract was freeze-dryed and kept in 4 °C until use [17,18]. The compositions of essential oil and aromatic water was analyzed by Gas Chromatography - Mass Spectrometry (GC-MS). GC-MS analysis was performed by an Agilent 7890-A GC (Agilent Technologies, CA) coupled with a mass spectrometerdetector (Model 5975C) equipped with a HP-5 MS fused silica capillary column (30 m × 0.25 mm i.d.; film thickness 0.25 μm; J & W Scientific, Folsom). Helium was used as a carrier gas. The ion source and interface temperatures of 230 °C and 280 °C were employed, respectively. An ionization voltage of 70eV was applied. The mass range was set between 45 and 550 amu. The oven temperature programme was the same used for GC-FID.

2.2. In vitro antimicrobial activity of plant derivatives

Disc diffusion method was used to determine the antibacterial activity of *O. decumbens* hydroethanolic extract, essential oil and aromatic water against *S. iniae* as described by Nurtjahyani and Hadra [11]. Briefly, the bacterium at 10^7 concentration was added to the petridish with Muller Hinton (MH) agar medium. Then the steril discs impregnated with 15 mg of essential oil, aromatic water (at their initial concentrations obtained by distillation without any dilution due to their liquid forms) and hydroethanolic extract (after dissolving in the equal volume of water due to its solid form) were placed on the plate. After 24 h incubation at 25 °C, the diameter of the zones of inhibition around each of the discs was taken as measure of the antibacterial activity. The test was carried out in three replicates and all steps were performed in sterile condition. Enrofloxacin was also included in the experiment as the positive control, whereas water (as solvent for extract) were involved as the negative control.

2.3. Minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration of plant essential oil, hydroethanolic extract and aromatic water were determined using continuous dilution in 96 well plate as recommended by Hajlaoui et al. [22]. The microplate wells were prepared by dispending 90 μl of LB media and 10 μl of bacterium with absorbance adjusted to 0.5 McFarland standard turbidity. Then, 100 μl of aromatic water (~ equal to 100 mg) at its initial concentration (see section 2.1) was added to first well and serially diluted (dilution factor 1:1) for other wells. For essential oil, 100 μl of a stock containing 4 mg/mL of essential oil dissolved in 5% DMSO was added to first well and serially diluted in the same way for aromatic water. In case of plant hydroethanolic extract, 100 μl of a stock solution containing 64 mg/mL of freeze-dried extract dissolved in distilled water was added to first well and serially diluted for other wells as described above. The final volume in each well was 200 μl. Positive (essential oil, hydroethanolic extract and aromatic water free medium containing bacterium) and negative (media with continuous dilutions without bacterium) controls were also included in the plate. After 24 h incubation at 25 °C, the well with no absorbance in 600 nm by bacterial growth was considered as MIC. Before MIC measurement, 5 doses of DMSO from 10 to 0.312% were prepared and their inhibitory effects on the bacterium growth were studied. DMSO at doses more than 5% showed significant growth inhibitory effects. Accordingly, for MIC measurement, the DMSO dose at 2.5% was used in first well and diluted serially in consecutive wells.

For MBC measurement, 25 μl of calculated MIC dose or higher concentrations without any bacterial growth were cultured in MH agar for 24 h in 25 °C and the lowest concentration in which no bacterial colony growth observed was considered as MBC [23].

2.4. Diet preparation and in-vivo experimental design

Diet preparation and in-vivo experimental design were carried out as described in Vazirzadeh et al. [24]. Commercial feed (21 Beyza Mill Co, Shiraz, Iran) with 37% crude protein, 10% crude fat, less than 10% moisture and 4000 kcal/kg digestible energy as basal diet was mixed with essential oil or hydroethanolic extract at desired concentration (0.01, 0.1 and 1%). The essential oil and hydroethanolic extract were dissolved in 30 mL sunflower oil and distilled water, respectively, before mixing with feeds. To reduce the water leaching, feed was coated with 3% gelatin solution (50 mL/kg). For feeding trail, a total of 520 Nile tilapia (45 ± 5 g) were purchased from a local fish farm in Bafgh, Yazd province, Iran and were kept in aquariums (ca. 70l). After two weeks of acclimatization, fish were randomly divided into 26 groups each of 20 individuals (13 treatments, in duplicate). Fish were fed at 3% body weight ratio daily. Fish in 9 treatments fed on diet supplemented with *O. decumbens* essential oil, hydroethanolic extract and their 1:1 combination at doses of 0.01, 0.1 and 1%. Three treatments were also received plant aromatic water at doses of 0.0312, 0.0625 and 0.1250% in bath treatment. By the time the study was carried out there were no study available on the application of plant aromatic water in aquaculture, so we calculated the amount of aromatic water used based on the essential oil: aromatic water ration produced in distillation process (see section 2.1). Hence, aromatic water was added to each tank based on the mentioned ratio and in accordance with total weight of fish biomass in each aquarium and daily feeding ratio. Since aromatic water was not dryable due to its aqueous form, the desired abovementioned volume in each treatment was added to each aquarium in bath treatment. A treatment of fish fed on a basal diet without any plant derivatives, but coated with same concentration of gelatin solution was considered as control.

2.5. In vivo bacterial challenge with Streptococcus iniae

Before starting the challenge experiment, to have a bacterium at exponential growth phase, the *Streptococcus iniae* (provided by the School of Veterinary, Shiraz University) was cultured in MH plate and after 24 h, 5 fish of same species but other than those used in treatments were injected at 10^7 and 10^9 concentrations, intraperitoneally. After 3 days, bacterium samples were taken from the kidney of 3 dead fish and cultivated in brain heart (BH) plate at 30 °C for 24 h [24]. Then the bacterium was collected and the concentration was measured spectrophotometrically and by serial dilution of cultured bacterium. Finally, for the challenge test, at the end of trial (day 60) fish in each treatment were injected with 1.45×10^9 bacteria in 0.1 saline solution, intraperitoneally. The mortality of fish was monitored for 14 days (until day 74 experiment) and post infection survival was calculated using the following formula [24]:

Relative percentage survival (RPS%) = [Number of surviving fish after challenge/Number of fish injected with bacteria] × 100.

2.6. Blood sampling

To study the effects of bacteria challenging on biochemical and immunological parameters, blood samples were collected from six tilapia individuals from each aquarium 14 days after challenge (at day 74 of the experiment). Fish were bled from caudal vein using non-heparinized 2-mL syringes. Samples were centrifuged at 12000 rpm for 20 min at 4 °C and the sera were kept at −20 °C for biochemical and immunological analysis.

2.7. Mucus and tissue sample collection

To study the effects of different treatments on the mucosal immunity and antioxidant status of fish, at days 20, 40 and 60, five fish were sampled from each aquarium (10 fish from each treatment) for mucus collection. Mucus samples were collected according to Hoseinifar et al. [17] using polyethylene bags containing 50 mM NaCl. Mucus samples were centrifuged at 1500 rpm for 10 min and the supernatant were preserved in −20 °C for further analysis. From the 10 fish sampled in each treatment for mucus collection at each sampling time, six fish were killed after deep anesthetization by clove powder (200 mg/l), spleen samples were taken and after immediate fixation in liquid nitrogen kept at −20 °C for antioxidant assays. The resting four fish were returned to aquarium alive.

2.8. Mucus and serum bactericidal activity

The mucus and serum bactericidal activity was measured according to Vasudeva Rao et al. [25] with minor modification for mucus. Ten μl of a serially diluted (1:10) suspension of *S. iniae* and PBS with the absorbance of 0.5 at 546 nm was mixed with 100 μl of mucus or serum and incubated at 25 °C for 1 h. After incubation, 100 μl of this solution was cultured on agar nitrite plate and the number of colonies was counted after 24 h. The bactericidal activity of mucus or serum was reported as the average colony of each plate divided into mean colony numbers of the control group.

2.9. Serum and mucus biochemical and immunological parameters

The amounts of serum triglyceride (TG), cholesterol (Chol), alkaline phosphatase (Alp), alanine aminotransferase (SGOT), arginine aminotransferase (SGPT), total protein (Tpr), albumin (alb) and globulin (Glb) were measured in fish challenged with *S. iniae* using commercial kits (Pars Azmoon, Iran) following the manufacturer protocols.

The respiratory burst activity was also measured using NBT according to colorimetric method as recommended by Siwicki [26]. Besides, the levels of alkaline phosphatase (Alp) and total protein (Tpr) of mucus were measured using commercial kits (Pars Azmoon, Iran) as described by Hoseinifar et al. [27].

2.10. Antioxidant enzymes assay

For antioxidant enzymes assays, 1 g of spleen sample was homogenized in 4 mL of potassium phosphate buffer (50 mM, pH = 7) containing EDTA and polyvinylpyrrolidone (PVP). The mixture was centrifuged at 10000 rpm for 10 min at 4 °C and supernatant stored at −20 °C for subsequent analysis. Superoxide dismutase (SOD) activity was measured according to the reduced absorption rate of Nitro-blue-tetrazolium (NBT) under the presence of SOD according to Beauchamp and Fridovich [28].

Catalase (CAT) activity was measured on 240 nm absorbance change caused by peroxide hydrogen in 1 min based on the method described by Zengi and Yilmaz [29].

Peroxidase activity was also estimated according to Chance and Maehly [30] based on the guaiacol oxidation by the enzyme and development of orange color.

2.11. Statistical analysis

Prior to statistical analysis, the normality of the data was checked by Shapiro-Wilk test. Two- way analysis of variance (ANOVA) using GLIMIX procedure in SAS software was applied to distinguish the main effects of treatment and time and their interaction. The used covariance structure was compound symmetric. The corrected least square means (LSD) of treatments were compared by Tukey post hoc test to determine any significant difference ($P < 0.05$) among treatments and in

Table 1
Active compositions of *O. documbens* essential oil and aromatic water using GC-MS.

	Compound	RI	% in the Sample
Essential oil			
	α-Thujene	373	0.281
	α-PINEN	406	0.151
	β-Thujene	590	0.187
	Sabinene	609	1.901
	β-Myrcene	673	0.457
	α-Phellandrene	752	0.015
	δ -3-Carene	785	0.035
	α-Terpinolene	822	0.405
	p-Cymene	873	12.721
	Limonene	896	5.5
	γ-Terpinene	1093	25.866
	Terpinolene	1276	0.045
	β-ocimene	1349	0.074
	Unknown	1842	0.051
	Terpinene-4-ol	1914	0.153
	α -Terpineol	2018	0.041
	Thymol	2778	20.316
	Carvacrol	2828	18.767
	Thymol acetate	2843	0.091
	Unknown	2881	0.31
	Myristicin	4559	9.886
	Elemicin	4814	0.747
Aromatic water			
	n-Decane	999	3.281
	p-Cymene	1024	0.634
	g-Terpinene	1057	0.921
	Linalool	1099	1.383
	Terpinen-4-ol	1176	0.322
	a-Terpineol	1190	0.145
	n-Dodecane	1198	0.684
	Unknown	1221	0.345
	Thymol	1286	37.635
	Carvacrol	1297	52.934
	Unknown	1301	1.229
	Thymol acetate	1354	0.112
	n-Tetradecane	1398	0.282
	Myristicin	1521	0.084

different sampling times.

3. Results

3.1. Herbal compounds

The GC- MS analysis separated 22 active compounds from *O. decumbens* essential oil (Table 1) that most abundant was γ-Terpinene (25.88%) followed by thymol, carvacrol and p-cymene with 20.32%, 18.77% and 12.72% frequency, respectively. Based on the result of GC-MS analysis of aromatic water , totally 14 active compounds were detectable mainly carvacrol (52.94%) and thymol (37.63) as presented in Table 1.

3.2. Antibacterial properties of essential oil, hydroethanolic extract and aromatic water

The results of disc diffusion for bactericidal activity of essential oil, aromatic water and hydroethanolic extract of *O. decumbens* against *S. iniae* are presented in Table 2. Results revealed growth inhibition diameters of 69 ± 1.4 mm in three replicates of the essential oil. Aqueous form of the extract (water to extract 1: 1 ratio) also showed a growth inhibition diameters of 0.8 ± 0.08 mm. Plates containing aromatic water showed no inhibitory halo against *S. iniae.*

3.3. MIC and MBC

The results of MIC and MBC of different derivatives of *O. decumbens* is reported in Table 2. As expected, the MIC an MBC of derivatives were dose-dependent. Essential oil displayed the highest anti-bacterial activity, with an MIC of only 0.5 mg/mL and MBC of 2 mg/mL, whilst aromatic water had the lowest anti-bacterial activity with MIC of 18.75 mg/mL and MBC of 75 mg/mL.

Table 2
In vitro antibacterial activity of essential oil, aromatic water and extracts of *O. decumbens* measured by disc diffusion (zones of growth inhibition, mm), MIC (mg/ml) and MBC (mg/ml) methods against *S. iniae*.

Methods	Essential Oil	Aromatic water	Extract
Disc diffusion (mm ± SD)	69 ± 1.4	0	0.8 ± 0.08
MIC (mg/ml)	0.5	18.75	4
MBC (mg/ml)	2	75	16

No inhibition zone was observed in aromatic water.

3.4. Mucus and serum bactericidal activity

No significant differences were observed in mucus and serum bactericidal activity among different treatments on day 40 of the experiment (P ≥ 0.05) (Fig. 1). It is noteworthy that bactericidal data for days 20 and 60 are not presented here as they were lost during the experiment.

3.5. Bacterial challenge

Relative percentage survival rate of Nile tilapia treated with different derivatives of *O. decumbens* 14 days after challenge with *S. iniae* are shown in Table 3. This highlighted that fish received aromatic water at doses of 0.0312% in bath treatment had the lowest rate of mortalities

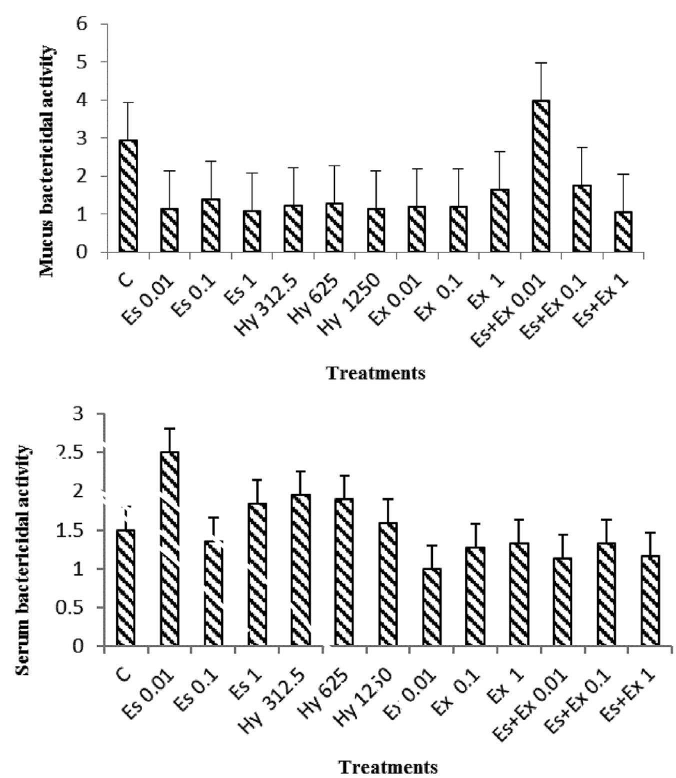

Fig. 1. Mucus and serum bactericidal activity of Nile tilapia treated with different doses of essential oil, extract and hydrolate of *Oliveria decumbens*. Each bar represents mean ± S.E. M. There were no significant difference among treatments (P ≥ 0.05). C: control; Es: essential oil; Hy: hydrolate; Ex: extract; Es + Ex: combination of essential oil and extract. Bacteria is reported as the number of colonies of each samples/colonies of control group.

Table 3
Relative percentage survival (%) of Nile tilapia received different derivatives of *O. decumbens* 14 days after challenge with *S. iniae*.

Treatments	RSP (%)
Control	50.00
Essential oil	
0.01%	57.14
0.1%	21.42
1%	28.57
Extract	
0.01%	64.28
0.1%	35.71
1%	57.14
Extract + Essential oil	
0.01%	35.71
0.1%	64.28
1%	42.85
Aromatic water	
0.0312%	7.14
0.0625%	42.85
0.1250%	64.28

(7.14%) among the treatments compared to control group with 50% mortality and therefore significantly increased the resistance of fish against *S. iniae*. Following that, the group of fish fed on diet containing 0.1% essential oil had the second highest survival rate after the bacterial challenge (21.43% mortality). Some groups such as those receiving aromatic water at doses of 0.062 and 0.125% in bath treatment, as well as those received hydroethanolic extract and combination of extract and essential oil at the dose of 1% in diet, showed higher mortality rates compared to the control group.

3.6. Serum biochemical and immunological parameters after challenge with *S. iniae*

No significant differences were observed among the different treatments for biochemical parameters including TG, Chol, Alp, SGOT, SGPT, Tpr, Alb and Glb after challenging with *S. iniae* (Table 4). But, the level of respiratory burst activity was significantly changed between treatments; the highest level was observed in fish received aromatic water at 0.0312% dose in bath treatment and the lowest level was observed in group received the combined treatment of hydroethanolic extract-essential oil at 0.01% dose in diet (Table 4).

3.7. Mucus parameters

The mucus alkaline phosphatase didn't show a significant difference among sampling times or treatments (Tables 5 and 6). The same result was observed for mucus total protein (Tables 5 and 6). Also the interaction effects of treatment × time were not significant for mucosal parameters (Table 7).

3.8. Antioxidant activity

The main effect of treatment on antioxidant enzymes activities in Nile tilapia treated with *O. decumbens* derivatives are presented in Table 5. Significant differences ($P < 0.05$) were detected in SOD levels of spleen between all experimental groups. The lowest level of SOD activity was observed in aromatic water 0.1250% group, whereas the highest level was observed in extract 0.1 group. The main effects of time on antioxidant enzymes are displayed in Table 6. While no significant differences were observed among sampling times for SOD, but the interaction effect of treatment × time was significant for SOD (Table 8). Accordingly, the lowest value of the enzyme was measured in samples of aromatic water 0.1250% group on day 20 (567.5 U/g) whereas the highest values were recorded in the hydroethanolic extract 0.01%, 1% and 0.1 treatments (1391, 1360 and 1359 U/g, respectively) on day 20.

Regarding the peroxidase enzyme, ANOVA did not identify a significant difference among either of treatments (Table 5) or at sampling times (Table 6). However, the effect of treatment × time interaction was significant. For this enzyme, the combined group of hydroethanolic extract-essential oil 0.1% had the highest value (7687 μM/g) in day 20 and the lowest value were observed in control group at day 60 (2490 μM/g) and in aromatic water 0.0625% group at day 20 (2381 μM/g) as displayed in Table 9.

The main effect of treatment was significant for CAT (Table 5). In this regard, the fish receiving 0.1% essential oil in diet showed the highest level of catalase (2855.5 μM/g), whilst the lowest value for this enzyme (1631.8 μM/g) was recorded in dose 0.01% of essential oil and hydroethanolic extract combination. Nevertheless, the main effect of time and the interaction effect of treatment × time was not significant for CAT (Tables 6 and 10).

4. Discussion

Essential oil and aromatic water compositions analysis showed the presence of γ-Terpinene, Thymol, Carvacrol and p-Cymene as the most abundant compounds in *O. decumbens* in the present study. This was in accordance with previous report of this plant [31]. A study has reported 10 active compounds in the *O. decumbens* essential oil which main compounds with highest frequency were γ-terpinene, myristicin, thymol, ρ-cymene and carvacrol, respectively [32]. Additionally in another research analyzing *O. decumbens*, thymol was the most frequent among other 12 compounds [19]. Therefore, our results on *O. decumbens* compounds were somehow consistent with other available studies. Slight deviance in herbs compositions observed among studies can arise from different geographic variation, growth stage, and harvesting time (season) of the herb [19].

As observed in our GC-MS analysis, herbs are reported to composed of biologically active compounds as a significant source of antimicrobials [19]. The results of the in vitro antimicrobial activity of *O. decumbens* indicated that essential oil had a significant inhibitory effect on *S. iniae* bacterium surpassing the hydroethanolic extract and aromatic water. It has been reported that *O. decumbens* showed higher degree antimicrobial activity against gram-positive than gram-negative bacteria [32]. Recently, the antibacterial activity of *O. decumbens* has been described against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Aspergilus niger, Candida albicans* [19]. Therefore, it is suggested as an alternative to antibiotics for bacterial infection treatment. It is believed that thymol and carvacrol act as an antimicrobial agent by inhibiting the ergosterol biosynthesis and membrane degradation in bacteria [19].

Lack of difference in the serum and mucus bactericidal activity against *S. iniae* in different treatments of Nile tilapia received *O. decumbens* in day 40 was surprising because the plant showed higher antibacterial activity in vitro. It can be inferred that lack of positive bactericidal activity is due to the inappropriate sampling time. Therefore, it was likely to observe a significant bactericidal activity if samples were also measured by days 20 and 60. Contrary to our results, increase in bactericidal activity in rainbow trout has been reported after some herbal treatments [25,33]. Also, increased bactericidal activity in all groups receiving ginger in barramundi (*Lates calcarifer*) [34] and *Allium sativum* in *Labeo rohita* [35] was reported.

The effectiveness of any immunostimulant can be assessed by a bacterial challenge and often used as a final indicator of the health status of the fish after nutritional trials [36]. Streptococcosis has reported as the cause of extensive financial losses in tilapia farming [9]. Treatment with *O. decumbens* derivatives mainly aromatic water markedly decreased the mortality rate of Nile tilapia after challenging with *S. iniae*, of which may be related to antibacterial compounds such as thymol, ρ-cymene and carvacrol which found in herbs. More

Table 4
Effects of different derivatives of *O. decumbens* on the serum biochemical and immunological parameters at 14 days after challenge with *S. iniae* in Nile tilapia.

	Biochemical parameters					Immunological parameters			
Treatments	TG (mg/dl)	Chol (mg/dl)	SGOT(U/L)	SGPT(U/L)	Alp (U/L)	NBT (OD)	Tport (g/dl)	Alb (g/dl)	Glb (g/dl)
Control	117.34 ± 80.06	188.58 ± 25.76	67.50 ± 31.11	2.08 ± 0.63	81.47 ± 16.44	0.84 ± 0.06^{d-h}	3.11 ± 0.30	1.34 ± 0.29	1.75 ± 0.16
Essential oil									
0.01%	95.31 ± 69.42	202.57 ± 22.31	102.38 ± 26.95	0.96 ± 0.77	81.28 ± 14.24	0.83 ± 0.06 $^{d-h}$	3.10 ± 0.26	1.39 ± 0.25	1.71 ± 0.14
0.1%	96.84 ± 97.78	194.41 ± 31.52	140.84 ± 31.11	2.25 ± 0.77	80.97 ± 16.44	0.83 ± 0.05 $^{d-i}$	3.71 ± 0.30	1.78 ± 0.29	1.81 ± 0.16
1%	128.01 ± 80.06	265.89 ± 31.51	101.84 ± 31.11	0.41 ± 0.77	63.37 ± 16.44	0.81 ± 0.06 $^{d-i}$	3.52 ± 0.30	1.71 ± 0.29	1.80 ± 0.16
Extract									
0.01%	134.68 ± 97.81	197.41 ± 31.52	58.01 ± 37.99	1.15 ± 0.77	73.19 ± 20.13	0.79 ± 0.07 $^{d-i}$	3.16 ± 0.37	1.29 ± 0.35	1.86 ± 0.20
0.1%	106.18 ± 80.06	182.25 ± 25.76	62.44 ± 31.11	1.51 ± 0.77	93.63 ± 16.44	0.76 ± 0.06 $^{e-i}$	3.00 ± 0.30	1.38 ± 0.29	1.58 ± 0.16
1%									
Extract + Essential oil	121.34 ± 80.06	209.58 ± 25.76	57.00 ± 31.11	1.63 ± 0.77	90.63 ± 16.44	0.73 ± 0.07 $^{e-j}$	2.98 ± 0.30	1.27 ± 0.29	1.71 ± 0.16
0.01%	102.34 ± 80.06	195.92 ± 26.76	111.00 ± 31.11	0.91 ± 0.77	63.80 ± 16.44	0.66 ± 0.07 $^{e-k}$	3.11 ± 0.30	1.43 ± 0.29	1.69 ± 0.16
0.1%	132.18 ± 97.81	157.41 ± 31.52	160.01 ± 37.99	1.73 ± 0.77	59.69 ± 20.13	0.74 ± 0.06 $^{e-i}$	3.04 ± 0.37	1.58 ± 0.35	1.46 ± 0.20
1%	83.84 ± 80.06	174.92 ± 25.76	80.17 ± 31.11	1.75 ± 0.63	60.30 ± 16.44	0.73 ± 0.06 $^{e-g}$	3.13 ± 0.30	1.37 ± 0.29	1.75 ± 0.16
Aromatic water									
0.0312%	342.51 ± 80.06	242.58 ± 25.76	48.03 ± 37.97	1.15 ± 1.09	91.63 ± 16.44	0.85 ± 0.07 $^{d-h}$	4.07 ± 0.30	2.07 ± 0.29	2.01 ± 0.16
0.0625%	132.93 ± 97.81	188.91 ± 31.52	71.76 ± 37.99	0.57 ± 0.77	75.44 ± 20.13	0.81 ± 0.07 $^{d-i}$	3.21 ± 0.37	1.22 ± 0.35	1.97 ± 0.20
0.1250%	89.01 ± 80.06	193.58 ± 25.76	145.84 ± 31.11	4.22 ± 0.77	68.97 ± 16.44	0.76 ± 0.05 $^{e-i}$	2.95 ± 0.30	1.23 ± 0.29	1.73 ± 0.16
P-Value	0.0560	0.0740	0.6503	0.3582	0.9838	< 0.0001	0.0742	0.9898	< 0.0001

Data are presented as mean ± S.E.M. Means with the different letter within the same column are significantly different ($p \leq 0\ 05$). TG: triglycerides; Chol: cholesterol; SGOT: alanine aminotransferase; SGPT; arginine aminotransferase; Alp: alkaline phosphatase; NBT: respiratory burst activity; Tprot: total protein; Alb: albumin; Glb: globulin.

Table 5
Main effects of treatments on mucus biochemical parameters and antioxidant enzymes of spleen in Nile tilapia received different derivatives of *O. decumbens*.

	Mucus biochemical parameters		Spleen antioxidant enzymes		
Treatments	Alp (U/l)	TPro (g/dl)	SOD (U/g)	POD (μM/g)	CAT (μM/g)
Control	78.30	0.20	803.86^{c-e}	3719.30	2694.67^{a-c}
Essential oil					
0.01%	46.23	0.17	908.75^{c-e}	3589.97	2834.52^{ab}
0.1%	33.62	0.24	780.00^{c-e}	4390.98	2855.50^{a}
1%	88.52	0.21	821.49^{c-e}	4049.12	2836.55^{ab}
Extract					
0.01%	28.65	0.16	1000.75^{a-d}	4132.33	1699.49^{bc}
0.1%	18.98	0.12	1164.15^{a}	3909.77	1639.26^{a-c}
1%	28.73	0.14	1070.97^{a-c}	4882.21	1874.79^{a-c}
Extract + Essential oil					
0.01%	17.15	0.14	1135.71^{ab}	4885.21	1631.81^{c}
0.1%	36.83	0.16	1263.51^{ab}	6832.42	13691.52^{c}
1%	27.57	0.26	1010.57^{-d}	4801.00	1790.19^{a-c}
Aromatic water					
0.0312%	48.95	0.25	777.04^{c-e}	4795.99	2406.09^{a-c}
0.0625%	65.42	0.26	720.25^{de}	4071.18	2389.17^{a-c}
0.1250%	22.85	0.16	638.82^{e}	4511.28	2536.97^{a-c}
P-Value	0.2142	0.6122	< 0.0001	0.3984	0.0005
Pooled S.E.M	0.11	0.05	67.33	458.35	238.32

Data are presented as corrected least square mean ± pooled S.E.M. LS-means with the different letter within the same column are significantly different (p ≤ 0 05). Alp: alkaline phosphatase; Tpro: total protein; SOD: superoxide dismutase; POD: Peroxidase; CAT: catalase.

Table 6
Main effects of sampling times on mucus biochemical parameters and antioxidant enzymes of spleen in Nile tilapia received different derivatives of *O. decumbens*.

	Mucus biochemical parameters		Spleen antioxidant enzymes		
Days	((g/dl TPro	Alp (U/L)	SOD (U/g)	(m/gμ) POD	(m/gμ) CAT
20	32.38	0.17	998.50	4358.8	2053.89
40	26.52	0.19	964.55	4473.4	2123.00
60	57.24	0.21	944.75	4578.14	2182.74
P-Value	0.1789	0.6289	0.2261	0.5846	0.4693
Pooled S.E.M	0.05	0.08	44.63	233.58	108.20

Data are presented as corrected least square mean ± pooled S.E.M. No significant differences were observed among LS-means (p ≥ 0 05). Tpro: total protein; Alp: alkaline phosphatase; SOD: superoxide dismutase; POD: Peroxidase; CAT: catalase.

mortality in higher doses of the plant based immunostimulants has already been reported [24,37,38] and it is believed that higher doses suppress the immune system of fish [8,24]. In addition, the higher level of respiratory burst activity was detected in aromatic water groups which also showed the highest resistance against *S. iniae*. Similarly, higher significant level of respiratory burst activity was reported in *Labeo rohita* following challenge with *Aeromonas hydrophyla* [39]. Macrophages and neutrophils are the main sites for phagocytic activity against the invading microorganisms. During phagocytosis which is also known as respiratory burst, oxygen free radicals are produced by generating O_2^- and its derivatives such as H_2O_2 and OH. Respiratory burst has been reported to have potent bactericidal activities against fish bacterial pathogens [40]. The humoral health factors did not significantly change among treatments subsequent to challenge with *S. iniae*. This means that *O. decumbens* derivatives were not harmful for fish health and can be used safely in Nile tilapia.

Table 7
Treatment × Time interaction effects of different derivatives of *O. decumbens* on mucus biochemical parameters in Nile tilapia.

	((g/dl TPro			Alp (U/L)		
Treatments/Days	20	40	60	20	40	60
Control	0.15	0.38	0.07	21.75	66.25	31.35
Essential oil						
0.01%	0.19	0.15	0.19	17.50	19.95	101.25
0.1%	0.32	0.27	0.14	42.75	28.35	29.75
1%	0.22	0.20	0.20	95.75	23.80	146.00
Extract						
0.01%	0.11	0.13	0.23	20.50	24.45	41.00
0.1%	0.08	0.09	0.18	18.80	12.15	26.00
1%	0.09	0.12	0.22	30.95	29.75	25.50
Extract + Essential oil						
0.01%	0.18	0.14	0.12	12.35	22.10	17.00
0.1%	0.10	0.17	0.22	20.00	15.75	74.75
1%	0.18	0.07	0.52	26.75	19.95	36.00
Aromatic water						
0.0312%	0.14	0.29	0.04	37.80	20.20	10.55
0.0625%	0.31	0.29	0.20	52.75	41.50	102.00
0.1250%	0.18	0.14	0.44	23.25	20.60	103.00
P-Value	0.1002	0.1002	0.1002	0.1040	0.1040	.01040
Pooled S.E.M	0.20	0.20	0.20	0.20	0.20	0.20

Data are presented as corrected least square mean ± pooled S.E.M. No significant differences were observed among LS-means (p ≥ 0 05).

Table 8
Treatment × Time interaction effects of different derivatives of *O. decumbens* on the superoxide dismutase enzyme level (U/g) in Nile tilapia.

	Days		
Treatments	20	40	60
Control	701.78^{b-e}	1011.12^{a-e}	698.67^{b-e}
Essential oil			
0.01%	664.00^{de}	1069.78^{a-e}	992.45^{a-e}
0.1%	787.56^{a-e}	677.78^{c-e}	874.67^{a-e}
1%	813.78^{a-e}	861.78^{a-e}	788.89^{a-e}
Extract			
0.01%	1391.12^{a}	754.67^{a-e}	856.45^{a-e}
0.1%	1359.56^{a}	811.12^{a-e}	1321.78^{a-c}
1%	1360.90^{a}	710.67^{b-e}	1141.34^{a-e}
Extract + Essential oil			
0.01%	1237.78^{a-d}	1053.34^{a-e}	1116.01^{a-e}
0.1%	1259.56^{a-d}	–	1268.45^{a-d}
1%	1330.67^{ab}	589.08^{de}	1112.01 ± $^{a-e}$
Aromatic water			
0.0312%	813.78^{a-e}	750.67^{a-e}	766.67^{a-e}
0.0625%	692.45^{b-e}	876. 89^{a-e}	591.45^{de}
0.1250%	567.56^{e}	587.11^{e}	761.78^{a-e}
P-Value	< 0.0001	< 0.0001	< 0.0001
Pooled S.E.M	113.6	113.6	113.6

Data are presented as corrected least square mean ± pooled S.E.M. LS-means with the different letters are significantly different (p ≤ 0 05).

Based on the findings of this study, there was also no difference in the amount of mucus Tpr in different treatments by *O. decumbens* in Nile tilapia. Also, the serum Tpr, Alb and Glb levels did not changed following challenge with *S. iniae*. Similar to the results of this study, Mansouri et al. [41] didn't find any significant difference of serum and mucus protein in rainbow trout received 0.5 and 1% doses of ancient herb *Myrtus communis* L. in their diets. A dose of 15 g/kg of garlic also did not affect the amount of mucus protein in *Rutilus rutilus* in an 8-week period trial [42].

Table 9
Treatment × Time interaction effects of different derivatives of *O. decumbens* on the peroxidase enzyme level (μm/g) in Nile tilapia.

	Days		
Treatments	20	40	60
Control	3924.81^{ab}	4742.86^{ab}	2490.23^{b}
Essential oil			
0.01%	2989.47^{ab}	4595.49^{ab}	3184.96^{ab}
0.1%	3621.05^{ab}	6396.99^{ab}	3154.89^{ab}
1%	3401.50^{ab}	4400.00^{ab}	4345.86^{ab}
Extract			
0.01%	5016.54^{ab}	2649.62^{b}	4730.83^{ab}
0.1%	4421.05^{ab}	3025.56^{ab}	4282.71^{ab}
1%	4953.38^{ab}	3927.82^{ab}	5765.41^{ab}
Extract + Essential oil			
0.01%	4908.27^{ab}	3491.73^{ab}	6255.64^{ab}
0.1%	7687.22^{a}	–	5978.95^{ab}
1%	4878.20^{ab}	2658.65^{b}	6866.17^{ab}
Aromatic water			
0.0312%	3786.47^{ab}	6180.45^{ab}	4421.05^{ab}
0.0625%	2381.95^{b}	5269.17^{ab}	4562.41^{ab}
0.1250%	4694.74^{ab}	5362.41^{ab}	3476.69^{ab}
P-Value	0.0022	0.0022	0.0022
Pooled S.E.M	846.74	864.74	864.74

Data are presented as corrected least square mean ± pooled S.E.M. LS-means with the different letters are significantly different ($p \leq 0\ 05$).

Table 10
Treatment × Time interaction effects of different derivatives of *O. decumbens* on the catalase enzyme level (μm/g) in Nile tilapia.

	Days		
Treatments	20	40	60
Control	2310.66	2790.36	2978.68
Essential oil			
0.01%	2326.90	3013.20	3163.45
0.1%	3167.51	2775.63	2623.35
1%	3108.63	2883.25	2517.77
Extract			
0.01%	1384.77	1906.60	1807.11
0.1%	1604.06	2213.20	1100.51
1%	7549.24	1993.91	2081.22
Extract + Essential oil			
0.01%	1500.51	1973.60	1421.32
0.1%	1545.18	–	1193.91
1%	1334.01	2268.02	1768.53
Aromatic water			
0.0312%	1935.03	2284.26	2998.98
0.0625%	2024.37	2596.95	2546.19
0.1250%	2909.64	2522.34	2174.62
P-Value	0.7731	0.7731	0.7731
Pooled S.E.M	409.03	409.03	409.03

Data are presented as corrected least square mean ± S.E.M. No significant differences were observed among LS-means ($p \geq 0\ 05$).

Antioxidant systems including low-molecular weight compounds (e.g. ascorbic and uric acid, tocopherols and etc.) and antioxidant enzymes such as SOD, CAT and POD are developed in organisms in order to protect against oxidative stress and converting excess active oxygen radicals such as superoxide anion (O_2-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) into safe oxygen species and protect the cells from free oxygen radical's damages [43–46]. Based on the findings of this present research, treatments of hydroethanolic extract and/or essential oils of *O. decumbens* showed higher levels of antioxidant enzymes activity. Similar result has been observed in a study used *Debaryomyces hansenii* as a dietary supplement in juvenile leopard grouper (*Mycteroperca rosacea*) [47]. Also, the highest levels of antioxidant enzymes were observed by day 20 of present experiment among other sampling times. Same time-dependent changes in antioxidant enzymes following immunostimulants administration in fish were reported previously. For example, SOD and CAT levels in the liver of gilthead seabream (*Sparus aurata*) were significantly higher in the third week following fenugreek (*Trigonella foenum graecum*) treatment than those in the control group, while in the second week they didn't show a significant difference from the control group [43]. A decrease in the value of antioxidant enzymes in higher doses of aromatic water (0.0625 and 0.1250%) was probably due to the immune system suppression by the high concentrations of aromatic water as already reported for higher doses of plant-based immunostimulants [46].

Putting it all together, the results of this study showed that while aromatic water had the lowest antibacterial activity but displayed better in vivo performance against *S. iniae*. Also, despite essential oil and extract displayed higher antioxidant and a strong in vitro bactericidal activity they were not as successful as reducing mortality and when concentrations of 1% were used, they even produced higher mortality. The inconsistent results of in vitro and in vivo studies, maybe suggest that in vitro tests are clearly not a good proxy to identify what plant elements might have a greater potential in the treatment of fish, since higher bioactive plants/extracts can entail higher toxicities. The contradictory results following application of different derivatives of same plants, is probably due to the influence of different extraction methods on the compositions and percentages of bioactive compounds in obtained products as observed in this present study [18,24,33]. Although most of the reported active compounds were same in both essential oil and aromatic water of *O. decumbens*, the relative percentage of each specific compounds varied widely between two derivatives. For instance, carvacrol was the first abundant compound with 52.93% frequency in aromatic water, but it was the third abundant compound (with 18.87% frequency) in essential oil. Moreover, not all compounds reported in essential oil were observed in aromatic water. Therefore, it may be concluded that the performance difference of two derivatives (essential oil and aromatic water) was related to differences in the type and percentages of active compounds. Given the different nature and compositions of plant products, it seems that the best standard way to compare their immunostimulatory and bactericidal potency, in vitro or in vivo, is to use calibrated products with specified amount of known bioactive ingredients. So, given that too many herbal products have been tested in aquaculture so far [3,6,21], it is recommended that future studies focus on the comparison of different products such as extracts, essential oils, and aromatic water with a specific dose of active ingredients.

5. Conclusion

In conclusion, our results showed a remarkable in-vitro and in-vivo antibacterial effect of *O. decumbens* against *S. iniae* in Nile tilapia. While plant essential oil showed the strongest in vitro bactericidal activity, the aromatic water resulted in the highest in vivo resistance against S. *iniae*. Due to successful administration of aromatic water as bath treatment in the present study, we recommend it as a promising and easy way to cope with bacterial disease in aquaculture, especially when oral administration of drugs or immunostimulants is difficult and costly due to body size and feeding behavior of aquatics. The information generated in our study would be useful for developing environmentally friendly strategies in improving resistance to streptococcosis disease.

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Availability of data and materials

The datasets analyzed in the current study are available from the corresponding author on reasonable request.

Authors' contributions

AV conceived and designed the experiment. SJ and AV prepared the diets, performed the trial and collected the experiments data. SJ and AV carried out all immunological and other required analyses.AV, AF and SJ analyzed and interpret the data. AV and AF wrote the first draft of the manuscript. All authors critically reviewed the manuscript for intellectual content and gave final approval for the version to be published.

Conflicts of interest

The authors declare that they have no competing interests.

Ethics approval and consent to participate

The experiments and fish handling were conducted based on the Institutional Animal Care and Ethics Committee of the Shiraz University regulations with minimal suffering of experimental animals.

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Appendix A. Supplementary data

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