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An *In vitro* and *in vivo* study on antimicrobial activity of *Origanum vulgare* extract and its nano form against *Streptococcus iniae* in rainbow trout (*Oncorhynchus mykiss*)

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Abstract:

This study was performed in order to determine the antibacterial activity of oregano, *Origanum vulgare* extract (OVE) and its nano extract (NOVE) on *Streptococcus iniae* in rainbow trout *in vitro* and *in vivo*. Extraction was done under standard percolation method. Then OVE and NOVE were screened to identify their antimicrobial activity against *Streptococcus iniae* by using disk diffusion assay and challenge methods. Also, the MIC and MBC values for OVE and NOVE were determined by micro dilution method. Furthermore, the antimicrobial effect of OVE and NOVE were examined by feeding rainbow trout fingerlings, with *O. vulgare* extract and its nano extract and florfenicol as a positive control. Then, all fish were injected intraperitoneal with *S. iniae* suspension in 3×10^8 CFU ml^{-1} concentration. Mortality rate was investigated for next 14 days after injection; then the spleen of fish samples removed to determine bacteria total count (SBT). *In vitro* results showed that OVE and NOVE had antimicrobial activity which was higher in NOVE. The inhibition zone of NOVE (26.066 mm) was significantly greater than OVE (21.7mm) (p<0.05). Also, the MIC and MBC values with high efficacy against *S. iniae* in NOVE treatment group were 0.25 and 0.5 mg\mL respectively, which was lower than OVE treatment values (p<0.05). *In vivo* results showed that the mortality rate and SBT was significantly lower (p<0.05) in the fish fed with NOVE than OVE and the control group (p<0.05). These results indicated that the *Origanum vulgare* extract and its nano extract supplement are satisfactory as a prophylactic measure against rainbow trout streptococcosis and also for fish health improvement.

Key words: Oregano, Bacterial challenge, *Streptococcus iniae*, Rainbow trout

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Introduction

Global population increase and food demand have been motivating factors leading to additional expansion of intensive fish production. This steady production has led to a massive emergence of diseases. Streptococcosis is one of the diseases that affect numerous fish species like rainbow trout, causing yearly mortality in fish biomass in trout farms in Iran (Soltani *et al*., 2005).

Streptococcus iniae is one of the bacterial species that can cause Streptococcosis infection in rainbow trout. Generally, it is characterized as a Gram-positive coccus that is catalase negative, oxidase negative, non-spore forming, non-motile and typically arranged in short chains (Duremdez *et al*., 2004; Johri *et al*., 2006). *Streptococcus iniae* is one of the most common rainbow trout bacterial diseases and has been isolated from some trout farms in different parts of Iran. The amount of antibiotics used in treatment of fish diseases is increased because of intensive fish production increase. Many studies documented faulty and indiscriminate use of antibiotics in aquaculture leading to increase in antibiotic resistance in various pathogens of fish (Schmidt *et al*., 2000; Agersoa *et al*., 2007); presence of residual antibiotics in seafood and fish products is increased as well (Samanidou and Evaggelopoulou, 2007). Furthermore, bacterial antibiotic resistance is increasing in fish and final consumer which is human (Cabello, 2004).

Herbs are extensively utilized in human and veterinary medicine. Currently, herbs play a considerable role in aquaculture. Several studies reported herbal extracts such as garlic and thyme, as potential antimicrobials against various fish pathogens (Prikhodko *et al*., 1999; Nya and Austin, 2009). One herbal antibiotic is Oregano (Özcalp *et al*., 2010).

Oregano extract or essential oil is a herbal antibiotic based on research studies (Horosova *et al*., 2006). Therefore, in the present study, hydro-alcoholic extracts of oregano in simple and nano forms were examined to determine the potential of their antimicrobial properties as alternative prophylactics against *S. iniea* infection in rainbow trout. Accordingly, we studied the effects of *O. vulgare* extract during *in vitro* and *in vivo* experiments. Based on recent studies the nano form of herbal extracts has greater effects than simple form of the same extract (Khalili *et al*., 2015). In this study, nano *Origanum vulgare* extract was used in order to increase anti-microbial effects of *Origanum vulgare* extract. The aim of this *in vitro* and *in vivo* study was to evaluate antibacterial activity of simple and nano forms of Oregano hydro-alcoholic extract on *S. iniae* in rainbow trout.

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Materials and methods

Preparation of Origanum vulgare extract and its nano form

The aerial parts of oregano (*Origanum vulgare* L.) were collected from Mazandaran - Iran in summer 2014 and plant species were identified and confirmed by a botanist. Oregao was shade-dried for 10 days till weight constancy was achieved. Afterward, the sample was powdered in an electric blender. Oregano extract was prepared according to the standard method of percolation. To do this, chopped dried plant leaves were percolated in 80% ethanol for 72 hours. Then, the slurry was filtered with Whattman No. 1 filter paper and centrifuged for 5min at 5000 rpm. The filtrate was obtained from ethanol using a rotary device, the excess solvent was separated from the extract. For *in vivo* experiment, the extract was dried in 60°C and its ingredients were analyzed using standard gas chromatography method (GC). Table 1 shows the ingredients in the Oregano extract.

Table 1: *Origanum vulgare* extract composition, analyzed by gas chromatography (GC).

Percentage	Name of ingredient
55.6	Carvacrol
1.1	Thymol
12.6	p-Cymene
3.6	-Terpineneγ
3.21	Terpinolene

Nano extract was prepared under the method explained by Hamidi (2015), in which the extract first of all dissolved in acetone and then was coated with an FDA approved polymer. Based on the dissolution test, that was run in this step, about 99% of oregano extract ingredients transformed to nano particles. The particle size and its electrical bar was analyzed using a Zeta sizing device. Figs. 1 and 2 show the size and potential count of the NE particles. Nano extract manufacturing process was conducted in Nano Pharmaceutics Lab of Zanjan Medicinal University.

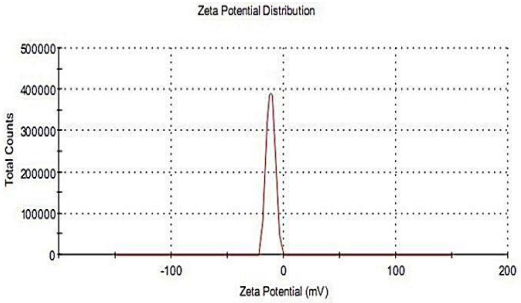


Figure 1: Size dispersion of the particles of nano *Origanum vulgare* extract, analyzed by Zeta sizer.

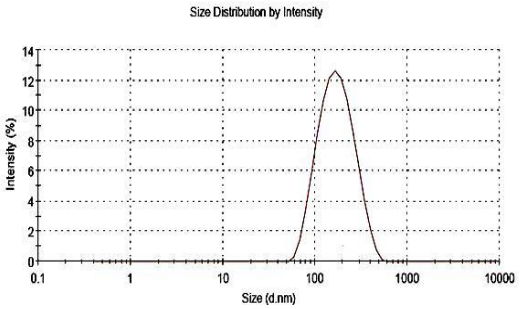


Figure 2: Potential count of NOVE particles, analyzed by Zeta sizer.

Determination of antibacterial activity

In vitro antimicrobial activity was tested using broth microdilution technique (Jorgensen and Turnidge, 2003) and pathogenic strains of *S. iniae* procured from Caspian Sea Ecology Research Center (Mazandaran, Sari). Also, florfenicol was used as control.

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The minimal inhibitory concentration (MIC) was determined using broth microdilution methods. For MIC determination, the inoculum was prepared using 4-6 h of broth culture of bacteria, and adjusted to a turbidity equivalent to a 0.5 McFarland standard, and diluted in nutrient broth media to make a concentration of 1.5×10^8 cfu ml^{-1} for bacteria. Serial dilutions of OE, NE and florfenicol were prepared (each with three replicates) in nutrient broth in 90 test tubes, starting from a stock solution of compounds (1.0 mg ml^{-1} DMSO). An equal volume of bacterial inoculum was added to each tube in all series of tubes except the last tube of each series. The last tube of each series of tubes contains only nutrient broth and used for negative control in spectrophotometry. The inoculated test tubes were then incubated at 25°C for 48 h, and the growth was recorded using a spectrophotometre. The MIC values were defined as the lowest concentration of compounds whose absorbance were comparable with the negative control tubes (without inoculums).

In order to maintain MBC in all tubes without turbidity, tubes were cultured in nutrient agar plates and incubated at 25°C for 48 hours. The smallest concentration which in its culture there was no colony, was used as MBC. Antibacterial activity of OE and NE was studied using standard disk diffusion assay method in MBC concentrations (Burt and Reinders, 2003). This experiment was accomplished using Nutrient agar media.

Fish preparation

All experiments were performed at the experimental challenge lab, Coldwater Fishes Research Center, Tonekabon, Mazandaran, Iran. Four hundreds and eighty rainbow trout fingerlings weighting 15±2 g were obtained from a commercial rainbow trout farm. The fish were maintained in a 400 L polyethylene tank, the water quality and temperature were monitored daily and kept within the acceptable range for rainbow trout. The fish for 2 weeks prior to the experiment were fed commercial feed once daily at a ratio of 1% of their body weight and kept under observation. During the observation period, the fish were randomLy sampled and their kidneys and livers aseptically streaked on nutrient agar (NA) to determine whether the fish were free of bacterial infection. Experiments were conducted in 12 polyethene tanks. Fish were randomLy allotted in 4 experimental groups with three replicates, including 1) negative control (diet without any drug), 2) Oregano extract treatment group (diet with 1% oregano extract), 3) Nano extract treatment group (diet with 1% nano extract) and 4) positive control treatment group (diet with 10 mg/kg of fish weight florfenicol).

Preparation of fish diet

The commercial fish diet used in this experiment was rainbow trout FFT2 produced by Faradaneh Co. The

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experimental drugs were added to the commercial diet using standard oil coating method described by Piper *et al*. (1982).

Experiment trials

To study the *in vivo* effect of OE and NE on *S. iniae* infection, after 14 days of feeding diets all fishes of each group were anaesthetized to reduce stress and were injected with 0.1 ml of bacterial suspension at the dose of McFarland tube number 1(3×10^8 CFU ml^{-1}). Accumulative mortality was recorded until 14 days after the injection. Feeding was continued with a normal diet for 14 days after the infection; and at the end of the experiment samples from the spleen of experimental fishes were taken under sterile conditions based on method explained by Barquero-Calvo *et al*. (2013). All the spleen sampleswere subject to totalbacterial count according to the standard method explained by Barquero-Calvo *et al*. (2013).

Statistical analysis

Data of experimental group were expressed as mean±SD and were compared with each other using one way ANOVA. Significant differences between experimental groups were expressed at a significance level of $p<0.05$.

Results

Oregano extract and its nano extract displayed antibacterial activity against *S. iniae*. The determined minimum inhibitory concentration and minimum bactericidal concentration for extract and nano extract and florfenicol is shown in Table 1. Also Figs. 3 and 4 show the results of disk diffusion analysis for extract, nano extract and florfenicol. Both MIC and MBC of the extract were 1 mg ml^{-1}, but MIC of the nano extract was 0.25 mg $ml^{-1}$and its MBC was 0.5 mg ml^{-1}. Also, disk diffusion assay data showed that antibacterial activity of oregano nano extract was significantly greater than that of the Oregano extract, however, its disk diameter was smaller than florfenicol (Table 2).

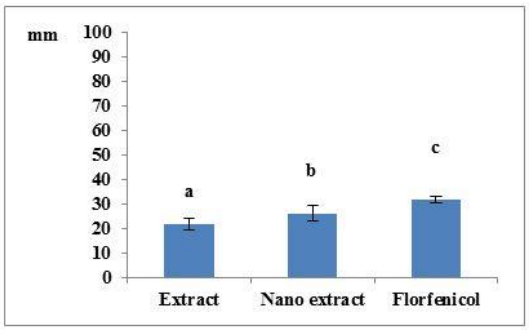


Figure 3: Disk diffusion assay results for OE, NE and florfenicol analyzed in nutrient agar media, (mm disk) (NA).

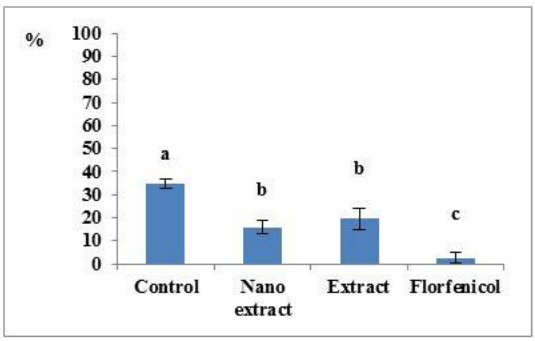


Figure 4: Total mortality in different treatments after the bacterial challenge with *S. iniae* by Peritoneum injection method.

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Table 2: MIC and MBC results for the *Origanum vulgar* extract, its nano extract and florfenicol analyzed by broth microdilution method in nutrient broth (NB).

Florfenicol	Extract	Nano extract	Concentration
-	MIC MBC	-	1mg ml^{-1}
-	-	MBC	0.5 mg ml^{-1}
-	-	MIC	0.25 mg ml^{-1}
-	-	-	0.125 mg ml^{-1}
-	-	-	0.062 mg ml^{-1}
-	-	-	0.031 mg ml^{-1}
-	-	-	0.015 mg ml^{-1}
-	-	-	0.007 mg ml^{-1}
MIC MBC	-	-	0.0035 mg ml^{-1}

In vitro experiment shown that the mortality rate in the extract and nano extract treatment groups was significantly less than that of the control, but mortality in the extract and nano extract treatment groups were more than that of florfenicol (p<0.05). Bacterial total count results in the spleen also showed that colonies counted in the extract and nano extract were significantly less than the those of the control group (p<0.05). Results of bacterial total count is shown in Table 3.

Table 3: Spleen bacterial total count (CFU g^{-1} spleen), analyzed by the method described by Barquero-Calvo *et al*., (2013).

Treatment	Spleen total count in concentration 10^{-2}
Control	$>300^{a}$
Nano *O. vulgare* extract	17 ± 1.5^{b}
O. vulgare extract	110 ± 33^{c}
Florfenicol	14 ± 3.1^{b}

Discussion

The use of herbal ingredients is widely expected to become an alternative therapy in aquaculture as a prophylactic to control fish diseases. *In vitro* and especially *in vivo* studies concerning antimicrobial properties of herbal extracts against bacteria with fish culture importance are still limited. In this study, the antibacterial activity of *Origanum vulgare* extract was examined *in vitro* and *in vivo* in the simple and nano forms of *S. iniae*. Oregano used in this study, is inexpensive and easy to obtain from Iranian market. This plant is reported to have antimicrobial properties (Özkalp *et al*., 2010). Our study determined that the OE and NE had the ability to inhibit the growth of *S. iniae* and provide antimicrobial activity. Also, this *in vitro* and *in vivo* study showed that NE had greater antibacterial activity than OE.

Essential oil of Oregano is known to have antibacterial activity against different bacteria (Özkalp *et al*., 2010). The principal nutraceutical constituents of oregano essential oil are carvacrol and thymol (Sivropoulou *et al*., 1996). Also, it is shown that oregano extract has antibacterial activity against *E. coli* and Lactobacilli (Horosova *et al*., 2006). Other studies showed that antibacterial activity of Oregano was because of Phthalate compounds like Thymol and Carvacrol which are known as detergents in the extract or essential oil (Wedge and Camper, 2000).

In this study, the MIC and MBC were 1 mg ml^{-1} in *Origanum vulgare*

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extract and 0.8 and 0.25 mg ml^{-1} in its nano extract, respectively. On the other hand Gupta *et al*. (2009) found that 62.5 mg ml^{-1} of Cinnamon extract can inhibit Bacillus sp. and *Staphylococcus aureus* on Mueller Hinton agar plates. In another study, MIC of garlic was 2.5 mg ml^{-1} (Thanikachalama *et al*., 2010). In experiments conducted on some of the bacterial strains, the MIC of Oregano extract was 0.016-0.25 mg ml^{-1} (Özcalp *et al*., 2010); while in our study MIC of Oregano extract was 1 mg ml^{-1}. The differences in results may be because of different bacterial strains, but the different value between the results found by Özcalp *et al*. (2010) and ours is acceptable.

The MIC and MBC of NE in this study were significantly smaller than those of OE. Other studies showed that antibacterial activity of nano drugs were significantly greater than those of simple forms. Khalili *et al*. (2015) showed that MIC and MBC of thyme's (*Thymus vulgaris*) essential oil nanocapsule against *A. flavus* were significantly smaller than that of thyme essential oil. Also, other studies showed that nano drugs were more effective than simple forms. Results of disk diffusion analyses also supported these foundlings. Disk diameter in disk diffusion assay for NE was greater than that of OE. This finding showed that antibacterial activity of NE in MBC concentration was greater than OE disk diameter. This result support the findings of *in vivo* experiment.The present study showed that mortality rate of the fish fed with OE and NE was significantly less than that of the control. Similar results showed that feeding catfish (*Clarias gariepinus*) fingerlings with garlic (*Allium sativum* L.) peel reduced mortality after challenging them with *A. hydrophila* (Thanikachalama *et al*., 2010). Tilapia fed with *Cinnamomum verum* extract were observed to have greater survival rates than the control fish, following a challenge with *S. agalactia* (Alsaid *et al*., 2010). Furthermore, bacterial total count in spleen results showed depletion in OE and NE treatment groups compared with the control group. Also, bacterial total count in NE was smaller than that of OE treatment.

Results of a study on tilapia challenged with *Francisella asiatica* showed that 3 days treatment with florfenicol decreased the spleen total count (Soto *et al*., 2010). Some medicinal herbs demonstrated to control fish pathogenic bacteria such as *Euphorbia humifusa, Leucaena glauca, Eclipta alba* and garlic (Sataporn, 2004; Aboud, 2010). These plants are known as herbal antibiotics. Oregano extract in this study showed antibacterial activity and it could be considered as a herbal antibiotic. In the present study, there was no difference in spleen bacterial total count between the NE and florfenicol treatments. So it can be concluded that antibacterial activity of NE was near that of the florfenicol (Table 2).

In conclusion, the results of this study showed that Oregano hydro-

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alcoholic extract and nano extract could be used as an alternative therapy and as an additive to fish food in order to prevent the studied disease (streptococcosis). Administering feed supplemented with OE and NE as a prophylactic may possibly avoid and/or decrease fish mortality. This phenomenon was because of presence of phthalate compositions like thymol and carvacrol in the extract and nano extract. Also, the antibacterial activity of the nano extract was greater than that of the extract. The mechanism of action of nano extract could be difficult to speculate and further studies are required to understand the mechanism and toxicology of Oregano nano extract.

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