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The antifungal effects of *Allium sativum* and *Artemisia sieberi* extracts on hatching rate and survival of rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) larvae

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Abstract

This study was conducted to evaluate the antifungal effects of garlic (*Allium sativum*) and wormwood (*Artemisia sieberi*) ethanolic extracts on survival of rainbow trout (*Oncorhynchus mykiss*) eggs during the incubation and larval stages. The eggs were exposed, in triplicate, to three different doses of garlic (50, 100, 200 ppm) and wormwood (25, 50, 100 ppm) for 30 min under static condition. Malachite green (2 ppm) was also applied as positive control, while none pharmacological intervention was set as negative treatment. The results illustrated no significant difference in eyeing eggs, green eggs and larval stage mortality rate among the treatments ($p<0.05$). the fish eggs exposed to 2 ppm malachite green, 200 ppm garlic extract, and 100 ppm wormwood had the highest survival rate over the eyeing and hatching stages. Similar results were also obtained in larval stage with the highest survival rate in larvae exposed to 2 ppm malachite green (94.3±0.1 %) followed by those exposed to highest garlic (93.1±0.3 %) and wormwood extracts (93.1±0.4 %), respectively. The lowest survival rate was also obtained in eggs and larvae exposed to non-pharmacological intervention. Although the lowest mortality was found in malachite green treatment, findings of the present study illustrated that highest doses of garlic (200 ppm) and wormwood (100 ppm) extracts could be proposed at the highest concentration as a fungicide during the incubation of rainbow trout eggs and larvae culture due to their noncarcinogenic properties compared to the malachite green

Keywords: garlic, wormwood, incubation, larval survival, rainbow trout.

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Introduction

Cold-water fish farming is a section of aquaculture developing in most part of Iran, although it is encountered to some implementation challenges like high mortality during the eggs incubation in hatchery centers often resulted from the fungal disease (Sharif-Rohani *et al*., 2006). Saprolegniasis or cotton wool disease is one of the most harmful cutaneous infection by a variety of water molds mostly in the genus *Saprolegnia* that causes a series of problem during the egg incubation of some species like rainbow trout, *Oncorhynchus mykiss* Walbaum 1792 (Bruno and Wood, 1994). different chemical disinfectants like malachite green, copper sulfate, potassium permanganate, sodium chloride and formalin could be used for treatment of infected fish eggs to saprolegniasis (Hardin, 2001). Due to the genetic diversity in pathogens population, resistant strains formation, and widespread side effects, there is a great interest for replacement of chemical drugs with herbal extracts in sustainable aquaculture activities.

Some plants have active compounds with antimicrobial, immune stimulating, and nutritional properties whose applications is broadly developing in aquaculture industry (Reverter *et al.,* 2014; Syahidah *et al.,* 2015). Due to the easy availability, low price, and high biocompatibility, the plant-derived extracts are of promising sources of bioactive molecules for replacement with the chemical drugs (Bulfon *et al.,* 2015).

Garlic (*Allium sativum*) is one of the most important medicinal plants with a significant amounts of sulfide compounds such as allicin, ajoene, and bioflavonoids showing great antiviral, antifungal, and antibacterial properties (Santhosha *et al.,* 2013). However, Garlic has some other beneficial effects such as fat reduction, liver protection, cholesterol decrement, and clotting time improvement (Sahu *et al*., 2007). Squires *et al.* (2011) reported the highest mortality of *Schistosoma mansoni* and *Echinostoma caproni* after 20 h exposure to 2 mg mL^{-1} ethanolic extract of garlic, while this time was 16- 23 h for *Fasciola hepatica*.

Wormwood (*Artemisia sieberi)* is another herbal plant widely grown as a predominant species in arid and semi-arid regions of Iran that is well used for its antiseptic, antitoxic, antiparasitic, and antimicrobial properties in Iranian traditional medicine (Azadbakht *et al*., 2003; Kazemi and Akhavani, 2009; Mahboubi and Farzin, 2009; Aliabadi *et al*., 2010). Different studies have been recently assessed the effects of *Artemisia annua* extract as an environmentally friendly substitute of chemical compounds against monogenean parasites (Ekanem and Brisibe, 2010; Albert and Ebiamadon, 2010; Elango and Rahuman, 2011).

The present study was conducted to evaluate the effects of *Allium sativum*

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and *Artemisia sieberi* extracts on survival rate of the rainbow trout during the eyeing, hatching and larval stages.

Materials and methods

The present study has been done in a local rainbow trout farm, Aligoudarz, Lorestan province, Iran. Thirty 4-year rainbow trout broodstocks (5 males and 25 females) were randomly selected and anesthetized with 120 mg L^{-1} clove powder to obtain their body weight and length using a digital balance before obtaining their eggs and sperms. The eggs were fertilized in dry method and randomly distributed into 24 separate trout incubator trays with 350 g eggs per tray.

Ethanolic plant extracts (85% purity) were obtained from dry herbs of *Artemisia sieberi* and *Allium sativum* based on the procedure described by Khan *et al*. (2017) and maintained in dark container at 4 ˚C before being used for bioassay.

To evaluate the antifungal properties of herbal extracts against *Saprolegnia*, 36-h post harvested green fertilized eggs of rainbow trout were daily exposed to different doses of garlic (50, 100, 200 ppm) and wormwood (25, 50, 100 ppm). Malachite green (2 ppm) was also applied as positive control, while none pharmacological intervention was set as negative treatment (Soltani *et al*., 2016). The treatments were administered for 30 min by turning off the water flow and dosing the holding water. Aeration was done during treatments to keep the dissolved oxygen concentrations and uniform the extracts dose. All treatments were carried out in triplicates until eggs began to hatch.

During the experimental period, water was supplied from a deep well with the following characteristics: The average temperature 13.1±0.2 ºC, dissolved oxygen 8.0±1.0 mg L^{-1}, carbon dioxide 6.3±0.5 mg L^{-1}, ammonia nitrogen less than 0.01 mg L^{-1}, nitrite nitrogen less than 0.1 mg L^{-1}, pH 7.8±0.3, and carbonate hardness 182.0±0.1 mg L^{-1}. The water flows on eggs and larvae with velocity of 0.8 and 5.0 L min^{-1}, respectively (Soltani *et al*., 2013).

A day after incubation, the dead white eggs were daily siphoned form the trays, while the fertilized eggs were remained in the trays to be counted until the hatch stage. Finally, the survival percentage was assessed during the eyeing and hatch stages based on the following equations (Soltani *et al*., 2009):

Eyeing rate (%) = 100 × number of eyed eggs / total eggs number

Hatching rate (%) = 100 × number of hatched eggs / total eggs number

Survival rate of larvae was also determined after hatching the eggs until the larvae reached to weight of 1 g. Feeding of larvae started after

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absorbing the yolk sac and swimming to the water surface. The larvae with average weight of 0.5 g were fed with commercial food of SFT_2 up to 5% of their body weight for 12 times a day, while larvae with average weight of 1.0 g were fed up to 4.5% of their body weight with SFT_1 and SFT_1. The survival rate of larvae was calculated with the following equation (Nohrman, 1953):

Larval survival rate = 100 × final larvae count / initial larvae count after hatching

Statistical analysis

All statistical analysis was performed by using SPSS 18 software for windows. The data were subjected to normality and homogeneity of variance with Shapiro-Wilk and Kolmogorov-Smirnov tests, respectively. One-way ANOVA was employed to compare the means, while Duncan's new multiple range test was used to determine where the differences occurred. The level of significance was considered at $p<0.05$. The results expressed as mean ± standard error (SE).

Results

The average weight of males and females were 1.0±0.1 and 1.8±0.1 kg, respectively. The survival rate of fish eggs up to the eyeing stage is given in Fig. 1. Eggs in control treatment had the lowest survival rate, while the highest survival rate obtained in eggs treated with malachite green. There was no significant difference in survival rate of eggs exposed to the highest concentrations of *A. sativum* (200 ppm) and *A. sieberi* (100 ppm) extracts ($p>0.05$), although they were significantly lower than that in the eggs treated with malachite green ($p<0.05$).

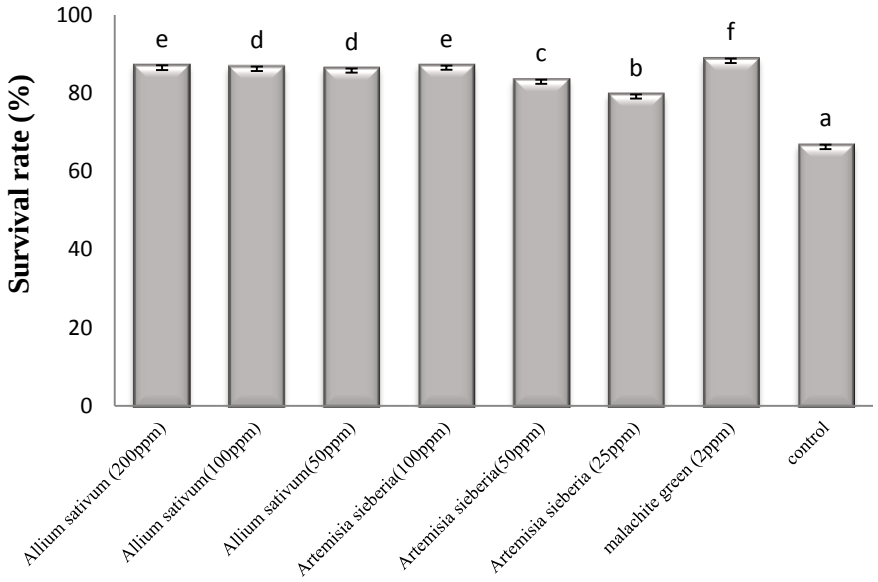

Figure 1: Survival rate of rainbow trout eggs exposed to different herbal extracts from fertilization to eyeing stage. Same letters on each column show no significant difference between the treatments ($p<0.005$).

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Fig. 2 shows the survival rate of rainbow trout eggs exposed to different concentrations of *A. sativum* and *Artemisia sieberi* from eyeing to hatching stage. No significant difference was found between the hatching rate of eggs exposed to 25 and 50 ppm concentrations of *A. sieberi* compared to those exposed to 50 and 100 concentrations of *A. sativum* ($p>0.05$). However, the survival rate of eggs in malachite green treatment was significantly higher than those in the other treatments ($p<0.05$).

Fig. 3 shows the eggs mortality during the incubation period. The lowest mortality was observed in eggs exposed to the malachite green, while the highest rate was found in eggs without pharmacological intervention. The Eggs exposed to 200 ppm of *A. sativum* extract had the lowest mortality among the herbal extract treatments. The eggs exposed to 100 ppm extract of *A. sieberi* had also the lowest mortality rate between the treatments with different concentrations of *A. sieberi* extract.

The results of the survival rate in rainbow trout larval stage up to one g weight exposed to different herbal extracts are shown in Table 1. Based on the present findings, the lowest mortality and the highest survival rate were obtained eggs exposed to 2 ppm malachite green followed by those exposed to 200 ppm *A. sativum* and 100 ppm *A. sieberi* concentrations.

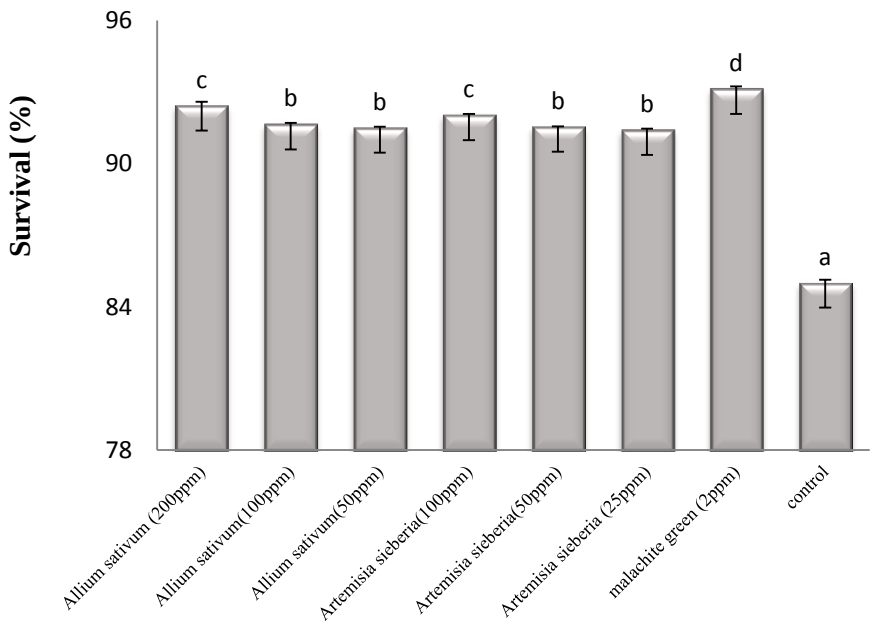

Figure 2: Survival rate of rainbow trout eggs exposed to different herbal extracts from eyeing to hatching stage. Same letters on each column show no significant difference between the treatments ($p<0.005$)

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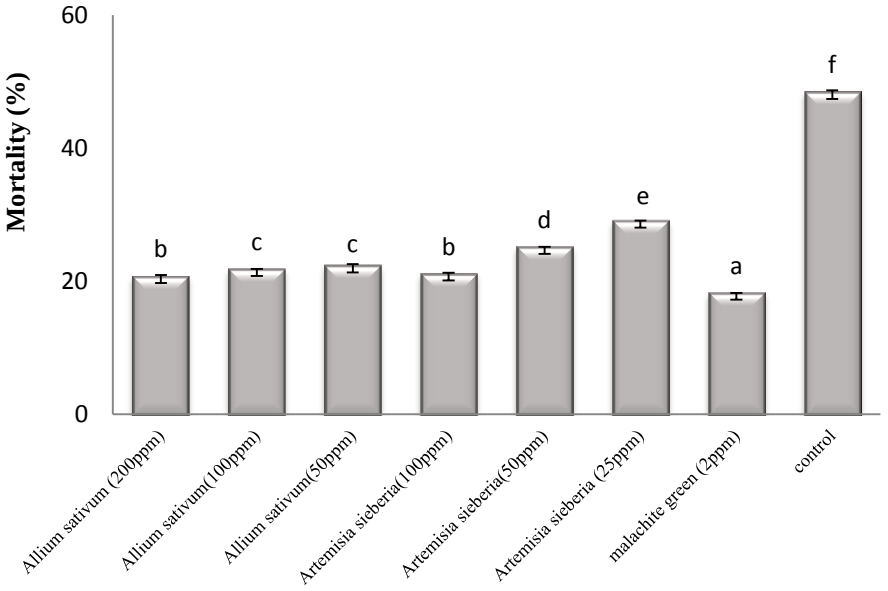

Figure 3: Mortality of rainbow trout eggs exposed to different herbal extracts during the incubation. Same letters on each column show no significant difference between the treatments (*p*<0.005)

Table 1: Survival and mortality rate of rainbow trout larvae exposed to different doses of experimental extracts.

Treatments	Mortality rate (%)	Dead larvae (No.)	Survived rate (%)	Survived larvae (No.)
Allium sativum (200 ppm)	6.9±0.1^{b}	241.3±4.7^{b}	93.1±0.3^{c}	3249.3±8.6^{c}
Allium sativum (100 ppm)	7.7±0.1^{c}	266.0±4.0^{c}	92.3±0.1^{b}	3181.0±8.5^{b}
Allium sativum (50 ppm)	7.7±0.2^{c}	265.5±3.5^{c}	92.3±0.2^{b}	3186.0±34.1^{b}
Artemisia sieberi (100 ppm)	6.9±0.1^{b}	239.0±3.6^{b}	93.1±0.4^{c}	3228.6±6.6^{c}
Artemisia sieberi (50 ppm)	8.2±0.2^{c}	272.3±6.2^{c}	91.8±0.2^{b}	3035.0±10.5^{b}
Artemisia sieberi (25 ppm)	8.5±0.2^{c}	265.6±4.2^{c}	91.5±0.2^{b}	2864.0±5.0^{b}
Malachite green (2 ppm)	5.7±0.2^{a}	207.3±5.1^{a}	94.3±0.1^{d}	3398.0±1.7^{d}
Control	11.8±0.2^{d}	270.3±7.3^{d}	88.2±0.2^{a}	2014.6±9.0^{a}

*Same letters on each column show no significant difference between the means (p<0.005).

Discussion

The low temperature of water and the long time incubation of eggs lead to appropriate conditions for fungal disease in rainbow trout (Ebrahimzadeh Mousavi *et al*., 2010). Malachite green has been usually used as the best chemical substance to preserve the rainbow trout eggs against mold infections. However, Meinertz *et al.* (1995) found detectable residues of malachite green remained in edible tissues of fish exposed to the chemical substances before hatching. Moreover, Andersen *et al.* (2005) found residues of malachite green and its conversion product, leucomalachite green, in market size salmon. Regarding to the health implications concerns in the fish

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consumers and farm workers, the use of malachite green was banned by FDA since 1991. Consequently, considerable efforts have been done to identify natural therapeutic agents as effective as malachite green for fungal treatment of farmed fish species like rainbow trout.

Based on the present research, the rainbow trout eggs exposed to the highest herbal extracts (200 ppm *A. sativum* and 100 ppm *A. sieberi*) had the highest survival rate during the eyeing and hatching stages indicating the effectiveness of garlic and wormwood treatments for fungal treatment of rainbow trout eggs. Comparison of the current findings with dietary supplementation of garlic (50 and 150 mg kg^{-1} diet) to prevent the *Neobenedenia* sp. in barramundi, *Lates calcarifer*, illustrated that longer supplementation could significantly reduce the infection up to 70% (Militz *et al*., 2014). Garlic supplementation to an extruded pellet was found as an effective delivery method for minimizing allicin leakage from the diet (less than 3%). Feeding garlic extract for a period of 30 days significantly reduced infection prevalence and intensity to less than 50% in *L. calcarifer*. The current study in accordance with the previous researches (Lee and Gao, 2012; Gharachorlou and Sadighi Shamami, 2013; Petretto *et al*., 2013; Rashid *et al*., 2013) proven the antifungal activity of *A. sativum* probably due to the synergism between the effective compounds of the garlic extract like α-phellandrene and α-pinene.

The broad antimicrobial and antifungal activities of the herbal extracts against variety of pathogenic fungi and bacteria recommend their application in different pharmaceutical industries. In the present study, larvae exposed to herbal extracts represented higher survival rate compared to those in control group.

Similar findings were also reported with Mousavi *et al*. (2009) when they considered the antifungal activities of a combination of herbal essential oils including *Thymus vulgaris*, *Salvia officinalis*, *Eucalyptus globulus*, and *Mentha piperita* on rainbow trout eggs. Sharif -Rohani *et al*. (2006) also showed that extracts from *Eucalyptus*, *Thymus*, and *Geranium* had positive effects on survival rate of rainbow trout larvae.

According to the current results, survival rate during the incubation to hatching stage was higher in malachite green treatment compared to the other treatments which was in line with other studies about application of herbal extracts for controlling fungal disease (Marking *et al*., 1994; Liu *et al*., 1995; Melendre *et al*., 2006; Mousavi *et al.*, 2009; Khosravi *et al*., 2012; Najafi and Zamini, 2013). However, toxic effects

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of malachite green have been reported on fish and other aquatic animals (Andersen *et al.,* 2005; Culp *et al.,* 2006; Sudova *et al*., 2007). On the other hand, there is no report about the noxious properties of herbal extract on fish and human, which makes them a suitable substituent of chemical agents for preventing the filamentous fungal disease in aquaculture. After the malachite green, the highest dose of garlic and wormwood extracts in the current study had the maximum effects on eggs survived during the incubation and larvae. Given the oncogenesis, tissue bioaccumulation and environmental problems of malachite green, herbal extracts are highly recommended as alternative therapeutic against cotton wool disease in rainbow trout hatcheries (Abdulrahman and Alkhail, 2005).

In conclusion, findings of the present study illustrated that highest doses of garlic (200 ppm) and wormwood (100 ppm) extracts could be proposed at the highest concentration as a fungicide during the incubation of rainbow trout eggs and larvae culture due to their noncarcinogenic properties compared to the malachite green.

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