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The antifungal effects of *Allium sativum* and *Artemisia sieberia* extracts on hatching and survival rates of rainbow trout *Oncorhynchus mykiss* (Walbaum,1972) larvae

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Abstract

This study was conducted to evaluate the antifungal effects of garlic (*Allium sativum*) and wormwood (*Artemisia sieberia*) 80-85% purity ethanolic extracts on rainbow trout (*Oncorhynchus mykiss*) eggs incubation and larvae survival rate. The experimental group divided to three different doses of garlic (50,100,200 ppm), wormwood (25, 50, 100 ppm), green malachite (2 ppm) as positive control group and none pharmacological intervention (negative control group) with three replications. There were significant differences (p<0.05) between treatments in green eggs, eyed eggs from hatched and larval stage mortality rate. The maximum mortality rate in green eggs were shown in negative control group (33.35±014%), 200ppm garlic (13.11±0.09%) and wormwood (13.07±0.07%) respectively. Positive control group (6.9±0.15%), high amount of garlic extract (7.62±0.21%) and 100ppm wormwood extracts (8.01±0.1%) had the lower levels of mortality rate from eyed eggs till hatched respectively .Besides, the third stages (hatching from 1 g) of exam there were significant difference in larval survival rate between treatments (p<0.05). Malachite green had the best level in this time and the maximum levels of garlic and wormwood extracts had same amount of survival rate after control positive group. Base on statistical analysis the lowest point of garlic and wormwood extracts put in same step and no significant differences together. All in all, garlic extract had the best result in each stage than malachite; probably we can replace that as fungicide on (*Oncorhynchus mykiss*) eggs and larvae culture.

Keywords: *Allium sativum*, *Artemisia sieberia*, Egg fertilization, Larvae survive, Rainbow trout

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Introduction

Cold-fresh water fish aquaculture ~~are~~ is developing in most part of Iran, however some obstacles and basic problems in this area like high mortality during incubation of eggs are considerable in hatchery centers (Sharif Rohani *et al*., 2006). One of the most harmful agents in fish farms is fungal diseases (Bruno and Wood, 1994). These kinds of diseases are one of the serious problems during eggs incubation of some species such as rainbow trout. In the treatment of infected fish to Saprolegniasis different chemical disinfectants like green malachite, copper sulfate, potassium permanganate, salt and formalin could be used (Hardin, 2001). Due to genetic diversity in microbial pathogens, resistant strains formation and side effects of used drugs, replacement of them with herbal antimicrobial drugs is of paramount importance which leads to new researches on herbal drugs without serious complications.

Natural products can be an alternative to the above chemicals. Some plants have active compounds with antimicrobial, immuno-stimulating, and nutritional properties and are being used in aquaculture (Reverter *et al.*, 2014; Syahidah *et al.*, 2015). Plant-derived products are a promising sources of bioactive molecules, while being readily available, less costly, and biocompatible (Bulfon *et al.*, 2015). Among these medicinal plants, garlic stands out for having, in its bulb, sulfide compounds such as allicin and ajoene, in addition to bioflavonoids (Lee and Gao, 2012). These compounds have antiviral, antifungal, and antibacterial properties (Santhosha *et al.*, 2013). Garlic has various effects such as fat reduction, anti-hypertension, liver protection, insecticide, cholesterol lowering, increasing the coagulation time and anti-fungal effects (Sahu *et al*., 2007). Wormwood (*Artemisia sieberia)* is another kind of medicinal plant which is considered as dominant community in arid and semi-arid steppes in Iran (Aliabadi *et al*., 2010). *Artemisia prasina* is used in Iranian traditional medicine. It is known as astringent, antiseptic, anti-toxic, anti-parasitic and anti-microbial plant (Azadbakht *et al*., 2003; Kazemi and Akhavani, 2009; Mahboubi and Farzin, 2009). Recently Ekanem and Brisibe (2010); Albert and Ebiamadon (2010); Elango and Rahuman (2011) and Squires *et al*. (2011) try to evaluate the effects of extract of *Artemisia annua* L. against monogenean parasites of *Heterobranchus longifilis* and we take the same idea and try to screen our Egyptian *A.cina on* juvenile *Cyprinus carpio* to find an alternative means for treatment of monogenean diseases of cultured fish using an extract of *A. cina* instead of chemical-based substances that may not be friendly to the environment. In another survey Squires *et al.* (2011) reported that, the mortality was best achieved by ethanolic extracts of Artemisia which, at 2 mg ml^{-1}, killed *S. mansoni* and *E. caproni* in 20 h or less (except for wormwood), *F. hepatica* between 16 and 23 h . Based on this information, objectives of this study are about evaluating the effects of

Allium sativum and *Artemisia sieberia* extracts on growth of fungal isolates from trout farms, determining their minimum inhibitory concentrations in vitro and assessing the most effective one on survival rate of trout eggs.

Materials and methods

The present study has been conducted in Mahichal rainbow trout farm in Aligoudarz, Lorestan province. For this purpose, 5 male and 25 female brood stocks trout (four years old) were selected randomly. The average weight of males and females were 1 and 1.8 kg respectively. Initially, brood stocks were anesthetized with120 mg L^{-1} clove powder and weighed with 0.01 g accuracy before harvesting their eggs and sperms.

Water supply of the culturing and rearing center was from deep well with the following characteristics: The average temperature of 13±0.2 ºC, 8±1 mg L^{-1} O_2, 6.3±0.5 mg L^{-1} CO_2, ammonia less than 0.01 mg L^{-1}, nitrite less than 0.1 mg L^{-1}, pH of 7.8±0.3 and carbonated hardness of 182±0.12 mg L^{-1}.

The water inflows on eggs tray was 0.8 L min^{-1} and it was 5 L min^{-1} for larvae (Soltani *et al*., 2013). Feeding of larvae started with 50% active swimming and their rising to the water surface observation. At first, larvae to 0.5 g were fed with commercial food of SFT_2 (to 5% of body weight), 12 times a day and larvae to 1 g (4.5% of body weight) were fed with SFT_1 and SFT_1. Biometry's of larvae were carried out 5 times in 10 days. Each time 100 larvae were weighed and their average weight calculated with 0.01 accuracy (Soltani *et al.*, 2009). Ethanolic plant extracts used in this study are prepared from *Artemisia sieberia* and *Allium sativum.* They were obtained with routine extraction method from dry herbs (grade purity of 80-85%) and maintained in closed and dark container in refrigerator. In the treatment with *A. sativum* three concentrations have been used (50, 100 and 200 ml L^{-1}) in triplicate.

The treatment operations began with losses of eggs in the first 24 h. This event started 36 h after onset of incubation with 50, 100 and 200 ppm doses and the amount of 12, 24 and 36 ml L^{-1} from garlic extract respectively for 30 min (every other day) as flowing bath until eyed stage. Three concentrations (25, 50 and 100 ml L^{-1}) were applied for treatment with *Artemisia sieberia* in triplicates. The treatment operations began with losses of eggs in the first 24 h.

This event started 36 h after onset of incubation with 25, 50 and 100 ppm doses and the amount of 6, 12 and 24 ml L^{-1} from garlic extract, respectively for 30 min (every other day) as flowing bath until eyed stage. Malachite green treatment was used as positive control like other treatments in triplicates. For treatment part, 2 mg L^{-1} of this compound has been applied for 30 min (Soltani *et al*., 2016). Also an incubator without any medical intervention was allocated to negative control in triplicates. After incubation of fertilized eggs it is better to collect lost eggs after 24 h, but later it should be refused till eyed stage. After this stage lost eggs were counted as well. Ultimately the percentages of hatched eggs were calculated with the following formula

The number of hatched eggs=the number of primary eggs-the number of lost eggs till hatching (Soltani *et al*., 2009).

Percentage of hatching=(the number of hatched eggs×100)/the number of primary eggs

For survival rate determination of hatched eggs into larvae, daily mortalities were recorded (not for more than 1 g in weight), then survival rate of larvae were calculated with the following formula (Ai *et al*., 2004): The number of larvae surviving to 1 g weight=the number of primary larvae after hatching-The number of dead larvae until the end of trial

Survival rate=$(N_t – N_0)×100$

N_t=Number of fish in the beginning of the experiment

N_0=Number of fish at the end of the experiment

At the end of the test Shapiro-wilk test has used for normality of data distribution and homogeneity of variance. One-way ANOVA was employed to analyze the data, and then were compared by Duncan test. The level of significance was considered at $p<0.05$. All statistical analyses were performed by SPSS 18.

Results

Fig. 1 shows the survival rate of eggs to eyed egg stage between treatments. Control group had the highest mortality rate and malachite green had the highest level of survival rate. Furthermore, there is no significant difference between high amount of *A. sativum and A. sieberia* extract ($p>0.05$). There is significant difference between these groups by others same as control and malachite green respectively ($p<0.05$).

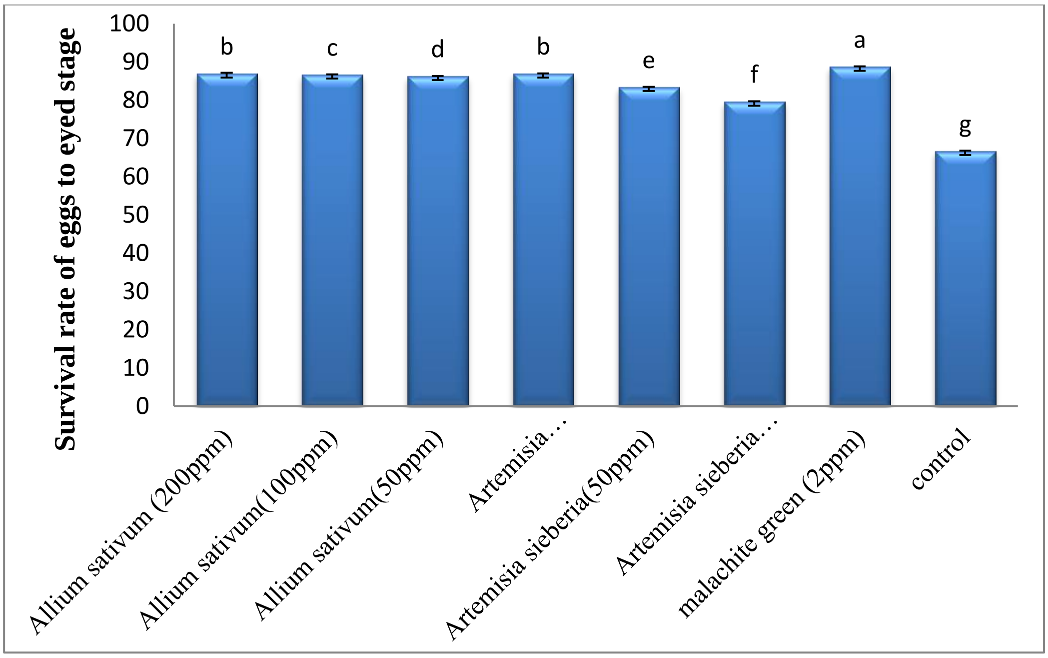

Figure 1: Survival rate of trout eggs to eyed stage in different treatments.
*same and different letters show no significant and significant differences between means respectively.

Fig. 2 presents the survival rate of eggs (eyed stage-hatched) phase. In the second phase observed no significant difference between 25and 50ppm *A. sieberia* concentration by two first treatments of *A. sativum* concentration (p>0.05). The survival rate of eggs in malachite green treatment was higher than other groups (p<0.05).

According to the results, the control group mortality was significantly higher than all treatments (p<0.05), among extracts, the mortality rate in garlic treatments (200 ppm) was significantly lower than that (p<0.05).

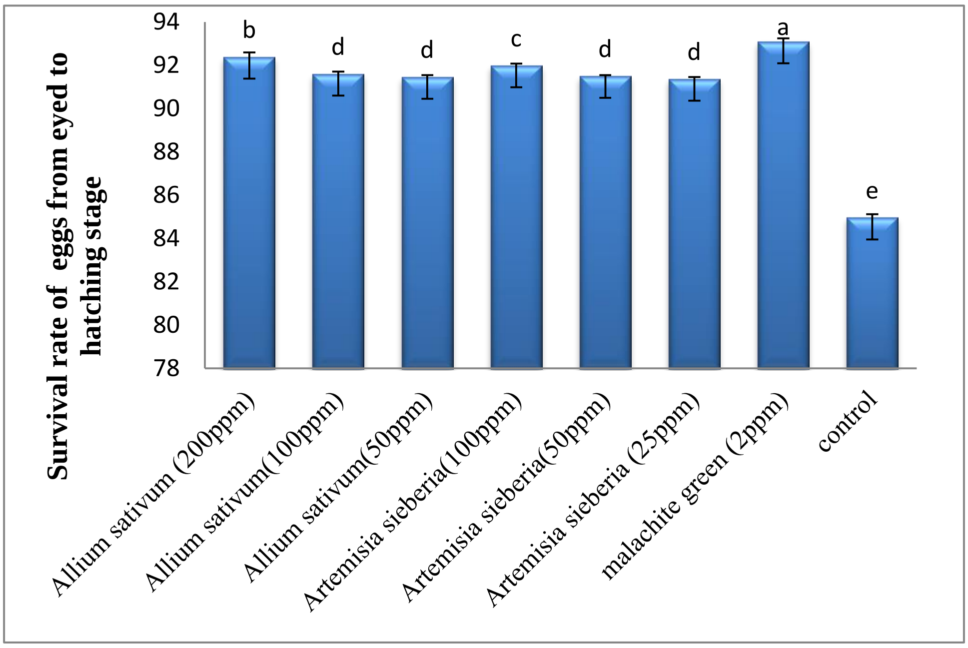

Figure 2: Survival rate of trout eggs from eyed to hatching stage in different treatments.
*same and different letters show no significant and significant differences between means respectively.

Fig. 3 shows the mortality of eggs from initial phase of incubation to eyed stage. The highest mortality rates were observed in control group and those treated with malachite green respectively (p<0.05). The lowest mortality rate was related to the garlic treatment with concentration of 200 ppm (p<0.05).

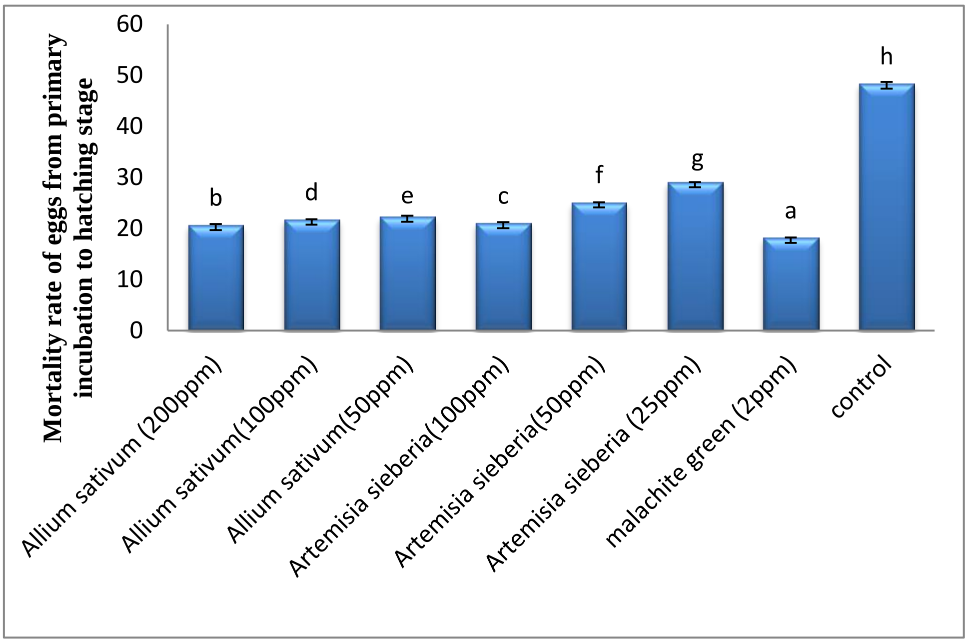

Figure 3: Mortality rate of eggs from primary incubation to hatching stage in different treatments.
*same and different letters show no significant and significant differences between means respectively

The results of the losses and survival of trout larvae from hatching to 1 gr for different treatments are shown in Table 1.

Table 1: Trout larvae number and percentage of survival rate and mortality among different treatments (to 1 g).

Treatment	Mortality rate from hatching time to 1gr (%)	The number of dead larvae	Survived larvae from hatching time to 1g((%)	the number of survived
Allium sativum (200ppm)	6.9±0.06[b]	241±3[b]	93±0.06[b]	3249.3±8.6 [b]
Allium sativum (100ppm)	7.71±0.12[c]	266±4[d]	92.2±0.1[d]	3181±8.5[d]
Allium sativum (50ppm)	7.7±0.17[c]	265.±5.5[d]	92 .71±0.17[d]	3186±34.1[d]
Artemisia sieberia (100ppm)	6.87±0.08[b]	239±3.6[b]	93.1±0.08 [b]	3228.6±6.65[c]
Artemisia sieberia (50ppm)	8.2±0.19[e]	272±6[e]	91.7±0.19[d]	3035±10.5 [e]
Artemisia sieberia (25ppm)	8.47±0.19[d]	265±6[f]	91.5±0.19[d]	2864± 5 [f]
Malachitr green (2ppm)	5.74±0.18[a]	207±31[a]	94.2±0.11[a]	3398± 1.7 [a]
Control (-)	11.82±0.25[f]	270.3±7.3[g]	88.17±0.25[e]	2014.6±9.01[g]

*same and different letters show no significant and significant differences between means respectively.

Based on the results, the lowest loss and highest survival rate were in malachite green, *A. sativum* (200 ppm) and *A. sieberia* (100 ppm) treatments (p<0.05).

Discussion

The low temperature of water and the long time incubation of eggs lead to fungal disease in rainbow trout hatcheries is one of the major casualties in mortality (Ebrahimzadeh Mousavi *et al*., 2010). Malachite green has been used as the best chemical material in controlling of rainbow trout eggs mold infection for many years, however, Meinertz *et al.* (1995) concluded that undetectable residues of malachite green would still remain in fish grown from eggs which had been exposed to the chemical until they reached market size. Moreover, Andersen *et al.* (2005)

found residues of malachite green and of its conversion product leucomalachite green in market size salmon, and because of concerns regarding the consumers and the health implications of the operators at the fish farms, the use of it was banned by FDA since 1991. Therefore, there has been significant effort expended to identify natural therapeutic agents, being effective as malachite green. Base on this survey (200 ppm) *A. sativum* and (100 ppm) *A. sieberia* antifungal extracts properties on trout eggs showed the highest hatching rate which indicates effectiveness of garlic and wormwood treatments. Compared our results with dietary supplementation of garlic (50 and 150 mg kg^{-1} diet) to prevent the *Neobenedenia* sp on farmed barramundi, *Lates calcarifer* from 10 (short-term) and 30 (long term) days, detected that the long term supplementation have significantly reduce the infection, success by up to 70% compared to controls and did not negatively effect on feed but, short term infection success was not influenced and suggesting that a delayed host response must occur to improve resistance to infection by short-term (10 days) supplementation. Incorporation of garlic into a pressure-extruded pellet was found to be an effective method of delivery as only minimal leaching of allicin from the diet occurred (<3% of allicin detected) during the interval of water contact between delivery and consumption, feeding garlic extract to *L. calcarifer* for a period of 30 days significantly reduced infection prevalence (36–50% less) and intensity (50% less) in fish challenged as well. Militz *et al*. (2013a, b) study was in contrast by ours and have been sharply decreasing. In our study, antifungal activity of artemisia is proven and this result is in consistent with other studies such as; Satyal *et al*. (2012), Gharachorlou and Sadighi Shamami (2013), Petretto *et al*. (2013), Rashid *et al*. (2013) were reported due to the synergism between the total components of the extract and mainly attributed to the presence of α-phellandrene and α-pinene as major constituents. The broad spectrum antimicrobial and antifungal activities of the extracts against variety of pathogenic fungi and bacteria, can recommend its incorporation in different pharmaceutical preparations. In the present study, larvae exposed to herbal extracts represented higher survival rate compared to control group. In this regard, similar results were observed in other studies (Sharif rohani *et al*., 2006; Mousavi and Raftos, 2012). For instance, it has been shown that Eucalyptus, Thyme and Geranium extracts had been positive effects on survival rate of trout larvae compared to group without drug interaction (Sharif rohani *et al*., 2006). According to our results, survival rate of eggs from primary incubation to hatching stage was higher in malachite green treatment compared to other groups which is in line with other studies about application of herbal extracts for control of fungal disease process (Marking *et al*., 1994; Liu *et al*., 1995; Melendre *et al*., 2006). Results showed that treatments of rainbow trout eggs with Malachite green (2 mg L^{-1}) greatly improved the hatching success compared to the herbals extract groups. This finding is in agreement with other studies (Sharif-Rohani *et al.*, 2006; Mousavi *et al.*, 2009; Khosravi *et al*., 2012; Najafi and Zamini, 2013) which are reported malachite green at 2 mg L^{-1} treatment gave the best hatching performance in comparison to herbals extract treatments. However, toxicology and teratology effects of malachite green on fishes and other animals have been reported (Andersen *et al.*, 2005; Culp *et al.*, 2006; Sudova *et al*., 2007). However, there is not any report for toxicity effects of the herbal extract and essential oil on fish and human up to present. Therefore, the essential matters in herbal extracts can be a potential substitute for controlling filamentous fungi unlike chemical agents in aquaculture. Hence to this point our results stated that the higher dose of garlic and wormwood extract had significant effects on eggs survived in hatching time after the malachite green. Attention to cancerogenesis, bioaccumulation and environmental pollution of green these med-plants extracts can be replaced for malachite green as an antifungal disease prevention, same as ours Abdulrahman and Alkhail (2005) stated that garlic extract compared with other herbs extracts against the fungal disease. In this regards, It should be noted that garlic and wormwood extracts had no harmful effects on environment, aquatic organisms and human, thus it could be considered as suitable alternative in controlling fungal disease for trout green eggs until hatching stage. Due to the strong impact of antifungal activity of both extracts in the current study by decrease mold infection rate and increase hatch rates in hatcheries and may represent alternative therapeutic treatments in rainbow trout aquaculture and hatchery sectors.

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