Effects of antifungal activity of Daenensis thyme (*Thymus daenensis*)and Mentha (*Mentha longifolia*)essential oils on rainbow trout(*Oncorhynchus mykiss*) eggs hatchability

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Abstract

In this study, rainbow trout (*Oncorhynchus mykiss*) eggs were treated with effective doses of Daenensis thyme**,** *Thymus daenensis* (20, 10 and 5 mgL^{-1}) and Mentha, *Mentha longifolia* (10, 5 and 2.5 mgL^{-1}), during incubation period (20 days) until hatching for 30 minutes per every other day as constant flow bath treatment method. The mortality level in normal control (without any treatment) was significantly higher than other groups ($p<0.05$). Level of mortality in egg treated with Daenensis thyme at 20 mgL^{-1}(12.01±0.357%) was significantly lower than both normal control (33.35±0.889%) and Mentha groups until the eyed-egg stage ($p<0.05$). No significant different was seen between Mentha at 10 and 5 mgL^{-1}mortality untileyed-egg stage($p>0.05$).

The highest hatching rate (78.36±0.340%)was recorded in Daenensis thyme at 20 mgL^{-1}concentrations compared to other groups except Malachite green ($p<0.05$). Eggs treated with Daenensis thyme and Mentha essence showed greater mean percentage of survival and hatching rates compared to the normal control ($p<0.05$). However, essential oil derived from Daenensis thyme at 20 mgL^{-1} probably has the potential to be used as health control of rainbow trout eggs against fungal contamination.

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Introduction

One of the main types of fungal diseases in farmed salmonid fish is saprolegniasis and causes considerable economic loss in the fish farming, especially hatchery sector (Bruno & Wood 1999; Pottinger & Day 1999).The occurrence and severity of sprolegniasis depend on the water sources, water

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temperature, organic load and length of contact time (Ahmadi, Hajimoradloo, Ghorbani, Chitsaz & Soleimani 2012). Malachite green has been used extensively by the aquaculture industry as an effective fungicide throughout the world for many years in the past (Pottinger & Day 1999). However, Malachite green is considered carcinogenic, mutagenic, teratogenic and harmful bioaccumulation chemical material (Meyer & Jorgenson 1983; Meinertz, Stehly, Gingerich &Allen 1995; Culp, Mellick, Trotter, Greenlees, Kodell & Beland 2006; Sudova 2007; Carral, Gonzalez, Celada, Saez-Royuela, Melendre, Gonzalez& Garcıa 2009). The loss of this extremely effective substance in the fish farming industry has driven investigators to look for a non-hazardous material and environmentally friendly which is as effective as malachite green such as medical plants. *Thymus* species are well known as medicinal plants because of having biological and pharmacological properties (Stahl-Biskup & Saez 2002). Daenensis thyme, *Thymus daenensis*, is an endemic aromatic medicinal plant to Iran. Essential oil of *T. daenensis* is a rich source of thymol, which gives high antimicrobial and antioxidant activity to this plant (Akbarinia & Mirza 2008). Essential oil from Mentha (*Mentha longifolia*) is generally used in flavors and fragrances (Iscan, Kirimer, Kurkcuoglu, Baser &Demirci 2002) and furthermore the antibacterial properties of the essential oil from the leaves were recorded (Cowan 1999).

Several studies have concluded that plant essential oils have greater antimicrobial activity than chemical components (Davidson & Parish 1989; Gill, Delaquis, Russo&Holley 2002). Moreover, a number of reports have been issued on the effect of antifungal activity of different herbal oils on aquatic eggs such as rainbow trout eggs (Sharif-Rohani, Ebrahimzadeh-Mousavi, Khosravi, Mokhayer, Bahonar, Mirzargar & Mehrabi 2006; Mousavi, Mirzargar, Ebrahimzadeh-Mousavi, Omid Baigi, Khosravi, Bahonar& Ahmadi 2009; Khosravi, Shokri, Sharifrohani, Ebrahimzadeh-Mousavi & Mousavi 2012), narrow-clawed crayfish (Koca& Cevikbas 2014), Persian Sturgeon (Ahmadi et al. 2012), Iberian rock lizard (Moreira & Barata 2005) and Kutum (*Rutilus frisii kutum*) (Najafi & Zamini 2013).

Nowadays, world follow using safe, effective and eco-friendly substances with decreasing of chemical disinfections. For instance, Khosravi et al. (2012) reported that Zataria (*Zataria multiflora*) essential oil (5 and 10 mgL^{-1}) and *Eucalyptus camaldolensis* (25 mgL^{-1}) increased hatching rates on rainbow trout eggs infected with *Saprolegnia parasitica*. Mousavi et al. (2009) indicated inhibitory effects of the combination of essential oils (*Thymus vulgaris*, *Salvia officinalis*, *Eucalyptus globulus* and *Mentha piperita*) on the filamentous fungiand increase hatching rate in comparison with malachite green on rainbow trout eggs on concentration 10 mgL^{-1}. Moreover, there is a report indicating that Zataria essential oil has immunostimulatory effects in common carps (Soltani, Sheikhzadeh, Ebrahimzadeh-Mousavi& Zaegar2010).

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Chemical disinfections are routinely used in Iranian rainbow trout reproduction centers for controlling of the eggs fungal contamination. This study was intended to assess, two different herbal essential oils, includingDaenensis thyme (*T. daenensis*) and Mentha (*M. longifolia*), as alternative options against saprolegniasis on rainbow trout (*Oncorhynchus mykiss*) eggs.

Materials and Methods

This experiment was carried out at the Abzi Exir Kowsar propagation and breeding center (Lorestan Province, Iran). Broodstock rainbow trout were anesthetized with clove powder (120 mgL^{-1}) and eggs from females (1.6±0.5 kg average weight) were stripped into a dry bowl and fertilized with milt from a ripe male (1±0.2 kg average weight). The mean water temperature was maintained within 13±0.5°C, dissolved oxygen concentration, pH and ammonia nitrogen (AN) were calculated approximately 8±1mgL^{-1}, 7.8- 8.1, less than 0.01mgL^{-1} during the experiment, respectively. Temperature, dissolved oxygen and pH were measured using by a portable multi meter (Instrument Corp. 8603, Taiwan).The water flow rate was adjusted at 0.8 liter per minute with aeration provided throughout the incubation and hatching period.

The *T. vulgaris* and *M. longifolia* oils obtained with hydrodistillation methodfor 4 hours using a Clevenger-type apparatus to produce essential oils according to Pirbalouti, Malekpoor, Enteshari, Yousefi, Momtaz& Hamedi (2010). Phenytoin sodium (20 $mgkg^{-1}$) was added to the essential oils before using to dissolve in water (Mousavi et al. 2009; Soltani et al. 2010).

All incubators were completely cleaned and disinfected with formalin, before transferring eggs into incubators. The fertilized eggs (8.4 kg) were then divided to 24 groups, randomly. The treatment trials started 24 hours post-incubation and were continued until hatching stage. Three concentrations of Daenensis thyme (5, 10, 15 mgL^{-1}) and Mentha (2.5, 5, 10 mgL^{-1}) were used in this study against mold contamination. All treatments were applied for 30 minutes per every other day as constant flow treatment method. Malachite green (Merck, Germany) was used as a positive control at concentration of2 mgL^{-1}(Kitancharoen, Yamamoto& Hatai 1998). There was no treatment in normal control group. The experiment was continued for 20 days (from fertilized eggs to hatching stage). During the experiment, no handling and transferring of eggs were performed (Mousavi et al. 2009).

During this period, eggs were monitored everyday and the ones showing mold contamination were counted. At the end of incubation period, hatching rate of eggs was calculated.

All data were subjected to a one-way analysis of variance (ANOVA). Significance of the differences between means was tested using Duncan's multiple range test ($p<0.05$). Each treatment was three replicate and sample volume in each stage of the treatment was 40%.

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Results

Effects of different disinfectant materials on the successful of hatching during different stages are shown in Table 1. Among essential oils treatments, Daenensis thyme at 20 mgL^{-1} (12.01±0.357%) showed the lowest mortality among eggs until eyed-egg stage ($p<0.05$). But the lowest mortality was observed in Malachite green group (11.29±0.639%), which it was significant than the other treatments ($p<0.05$). The lowest mortality of eggs from eyed-egg to hatching stage was belong Malachite green (6.93±0.354%), followed by Daenensis thyme at 20 mgL^{-1} (9.24±0.299%)($p<0.05$).In contrast, the highest mortality was obtained in normal control treatment in all egg stages (Table 1; $p<0.05$).

In surveying of hatching rate, the highest were Malachite green and Daenensis thyme at 20 mgL^{-1} treatments with 81.78±0.827 % and 78.36±0.340 %, respectively. However, the lowest ratio was significantly observed in control (51.61±0.153%) than other treatments (Fig. 1; $p<0.05$).

All the essential oils demonstrated fungi static properties compared to normal control ($p<0.05$).

Table 1 Hatching success of rainbow trout eggs among different treatments based on different concentrations of essential oils and Malachite Green (Mean±SD)

Group	Concentration (mgL^{-1})	Initial eggs (No.)	Total mortality of eggs (No.)	Mortality of eggs until eyed-egg stage (%)	Mortality of eggs from eyed-egg to hatching stage (%)
Daenensis thyme	20	$4406±9^{ab}$	$936±4^{b}$	$12.01±0.357^{b}$	$9.24±0.299^{b}$
	10	$4417±21^{c}$	$1016±7^{bc}$	$12.93±0.447^{bc}$	$10.08±0.547^{bc}$
	5	$4404±30^{a}$	$1032±18^{c}$	$13.08±0.098^{c}$	$10.36±0.376^{bd}$
Mentha	10	$4417±18^{c}$	$1299±2^{cd}$	$20.04±0.144^{d}$	$9.37±0.256^{c}$
	5	$4422±14^{ab}$	$1369±20^{d}$	$20.10±0.286^{d}$	$10.47±0.229^{d}$
	2.5	$4433±18^{d}$	$1391±20^{d}$	$20.77±0.576^{dc}$	$10.62±0.261^{d}$
Malachite green	2	$4408±19^{b}$	$803±34^{a}$	$11.29±0.639^{a}$	$6.93±0.354^{a}$
Normal control	-	$4428±9^{ba}$	$2143±16^{f}$	$33.35±0.889^{f}$	$15.04±0.603^{f}$

Values with differing letters are significantly different ($p<0.05$) from the other values in the same column.

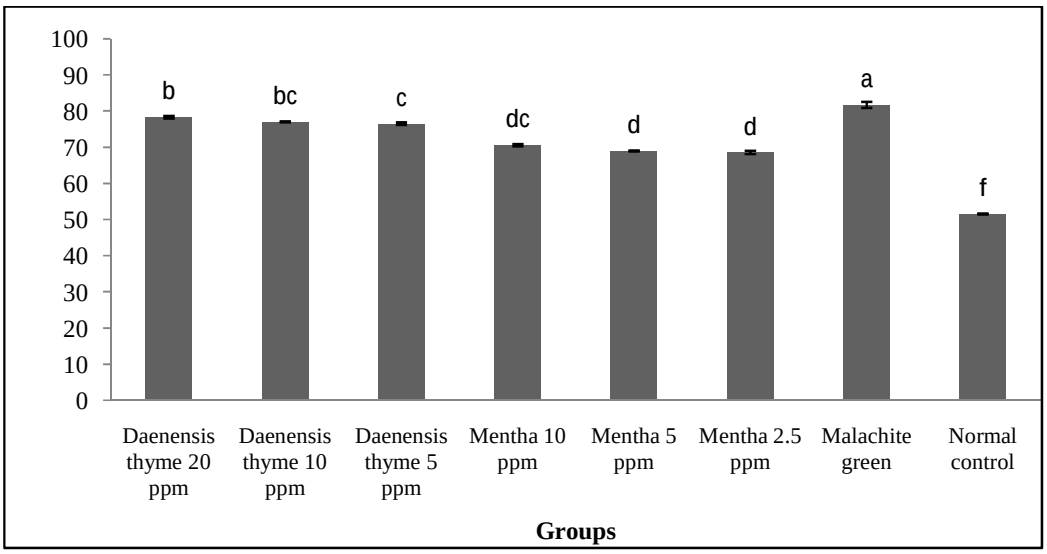

Figure 1 Effects of essential oils on hatching rate (%) of rainbow trout eggs at 20 days (Mean±SD). Different upper case superscripts indicate significant differences ($p<0.05$).

Discussion

Fungal infestations can be controlled chemically with antifungal agents and physically by removing fluffy, cotton-like, and white to grayish dead eggs. Manual removal of dead eggs can be safely performed only after the eyed stage (Jensen & Alderdice1989) and therefore the spreading of fungi before this period must be prevented by other means. In modern large-scale fish hatcheries, chemical control of fungi is the preferred method (Hoskonen, Heikkinen, Eskelinen & Pirhonen 2013). Several investigators concluded that essential oils have greater antimicrobial (Davidson & Parish1989; Gill et al.2002) and aquatic fungicidal (Mori, Hirose, Hanjavanit & Hatai 2002; Sharif-Rohani et al. 2006; Khosravi et al. 2008;Mousavi et al. 2009; Ahmadi et al.2012; Khosravi et al.2012) activities than some chemical components.

Malachite green is 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, Roybal & Turnipseed (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.

Data analysis showed that the best hatching rate among essential oils experiments was recorded in Daenensis thyme at 20 $mgL^{-1}$when

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the rainbow trout eggs were bathed for 30 min. Based on previous report (Moreira & Barata 2005), natural monoterpene phenol like thymol is the main components of Daenensis thyme essential oil and it has been associated with strong antimicrobial attributes (Palaniappan & Holley 2010). The possible mechanisms of phenolic compounds are appeared to bedegradation of the cell wall, damage to cytoplasmic membrane, damage to membrane proteins, leakage of cell contents and depletion of the protein motive force (Burt 2004).

In similar studies, Mousavi et al. (2009) reported that Eucalyptus essential oilin dose of 200 mgL^{-1} is effective in control of Saprolegniasis and resulted in higher hatching rate in rainbow trout eggs.Najafi &Zamini(2013) reported that dosage 100 mgL^{-1} Eucalyptus essential oilcontrolled ***Rutilus frisii kutum*** eggs infected with Saprolegnia. In the other experiment, Eucalyptus essential oil increased hatchability of ***Acipencer persicus*** eggs with concentration of 200 mgL^{-1} (Ahmadi et al. 2012). Bouchard, Patel & Lahey (2000) found that rainbow trout eyed eggs treated with clove oil at 1 g/lit had the lowest rate of fungus infection. Similarly, Khosravi et al. (2012) showed thart ***Zataria multiflora*** at concentration of 25 mgL^{-1}, ***E. camaldolensis*** at concentration of 25 mgL^{-1}, and ***Geranium herbarium*** at concentration of 100mgL^{-1} for 60 min daily were the best treatments to the prevention of fungal attack, the increase of hatching rate and the eyed egg survival rate of rainbow trout egg. However, the recommended doses of herbal essential oils used in the above studies to improve hatchability of fish eggs are significantly higher than the doses obtained from Daenensis thyme in the present study. This result illustrated that antibacterial and antifungal actives of Daenensis thyme may higher than Eucalyptus, clove, Zataria and Geranium essential oils according to its specific components. In contrast, ***Satureja cuneifolia*** essential oil showed highest hatching rates (44.5±0.57%) in infected rainbow trout egg with ***Saprolegnia parasitica*** in lower concentration (5 mgL^{-1}) (Metin, Dıler, Dıdınen, Terzıoglu & Gormez 2013).

According to the results, all essential oils especially Daenensis thyme group improved mortality rate of the eggs until eyed-egg stage compared to normal control. This finding recommended that using of Daenensis thyme essential oil after eyed-egg stage is safe and inhibit spreading of fungi after this period. In contrast, some chemical agents such as hydrogen peroxide treatments during blastopore formation increase rainbow trout egg mortality making the chemical bettersuited to use after the eyed-egg stage (Gaikowski, Rach, Olson, Ramsay & Wolgamood et al. 1998) or the concentration should be decreased during estimated blastopore formation (Barnes & Gaikowski 2003).

The results showed that treatments of rainbow trout eggs with Malachite green (2 mgL^{-1}) greatly improved the hatching success compared to the essential oils groups. This finding is in agreement with other studies (Sharif-Rohaniet al. 2006; Mousavi et al. 2009;Khosravi et al. 2012; Najafi &Zamini

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2013) which are reported Malachite green at 2 mgL^{-1} treatment gave the best hatching performance in comparison to essential oils treatments. However, toxicology and teratology effects of malachite green on fish and other animals have been reported (Andersen et al. 2005; Culp et al. 2006; Sudova 2007). However, there is not any report for toxicity effect of the herbal extract and essential oil on fish and human up to present. Therefore, the essential oils can be a potential substitute for controlling filamentous fungi unlike chemical agents in aquaculture.

The Daenensis thyme essence with 20 mgL^{-1} can decrease mold infection rate and increase hatch rates in hatcheries and may represent alternative therapeutic treatments in rainbow trout aquaculture and hatchery sectors. Essential oils of this study need more trials for evaluating toxicological effects on rainbow trout eggs at high doses.

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اثرضدقارچی اسانس های آویشن دنایی (*Thymus daenensis*) و پونه کوهی (*Mentha longifolia*)در قابلیت تخم گشایی ماهی قزل آلای رنگین کمان

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چکیده:

دراین مطالعه، تخم ماهی قزل آلای رنگین کمان با غلظت های موثر از اسانس های آویشن دنایی (20، 10 و 5 میلی‌لیتر در لیتر) وپونه کوهی (10، 5 و 2/5 میلی‌لیتر در لیتر) درطول دوره انکوباسیون تا زمان تخمگشایی (20 روز) به مدت 30 دقیقه به صورت حمام یکروز در میان با آب جریاندار تحت درمان قرارگرفتند. میزان مرگ و میر (بدون تداخل دارویی) به طورمعنی داری ازتمام تیمارها بیشتر بود ($p<0/05$). میزان مرگ و میر تخم در تیمار درمان شده با اسانس آویشن دنایی با غلظت 20 میلی‌لیتر در لیتر (12/01%) به طور معنی داری از تیمار کنترل (33/35±0/889%) و تیمارهای پونه کوهی تا مرحله چشم زدگی تخم کمتر بود ($p<0/05$). اختلاف معنی داری بین میزان مرگ و میرتیمارهای پونه کوهی با غلظت 10 و 5 میلی‌لیتر در لیتر تا مرحله چشم زدگی مشاهده نشد ($p<0/05$). بیشترین نرخ تخم گشایی در آویشن دنایی با غلظت 20 میلی‌لیتر در لیتر درمقایسه با سایر تیمارها بجز تیمار سبز مالاشیت ثبت شد ($p<0/05$). تخم‌های درمان شده با اسانس‌های آویشن دنایی و پونه کوهی میانگین درصدبقا و تخمگ شایی بالاتری نسبت به تیمار کنترل نشان دادند ($p<0/05$). در حالی‌که، احتمالاً اسانس آویشن دنایی باغلظت 20 میلی لیتر در لیتر پتانسیل استفاده شدن به عنوان یک ماده کنترل کننده سلامت تخم ماهیان قزل آلای رنگین کمان درمقابل آلودگی های قارچی را دارد.

کلمات کلیدی: فعالیت ضدقارچی، آویشن دنایی، اسانس، سبز مالاشیت، پونه کوهی.

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