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Evaluation of Antifungal Activity of New Combined Essential Oils in Comparison with Malachite Green on Hatching Rate in Rainbow Trout (*Oncorhynchus mykiss*) Eggs

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Abstract: The aim of this study was introducing a new herbal constitution for malachite green in hatcheries. In this study, antifungal activities of a new Combination of Essential Oils (CEO) from the herbs; *Thymus vulgaris*, *Salvia officinalis*, *Eucalyptus globulus* and *Mentha piperita* in *Oncorhynchus mykiss* eggs and its effects on hatching rate in comparison with malachite green (a specific treatment for the control of saprolegniasis) were studied. After fertilization, eggs were transferred to incubators and then treated with five concentration of the combined essential oils with concentrations: 10, 50, 100, 150 and 200 ppm, respectively and malachite green (1 ppm) using constant flow treatment method for incubation period. One incubator was as control without any treatment The water quality factors were controlled over the study period. In the end of hatching rate, the mold infection and hatching rate were calculated. The hatching percentage in five treatment groups of CEO were 69.99% at 10 ppm, 63.61% at 50 ppm, 62.1% at 100 ppm, 60.53% at 150 ppm, 54.63% at 200 ppm, 60%. 83 at 1 ppm of malachite green group and 53.48% in control group, respectively. The results revealed significant antifungal effects of the combined essential oils in comparison with malachite green on fish eggs so that it could decrease mold infection and increase hatching rate on concentration 10 ppm ($p<0.05$). This indicated that this combination of essential oils may be a promising antifungal agent in aquaculture.

Key words: Mold infection, combined essential oils, malachite green, salmonid hatcheries

INTRODUCTION

Unfertilized and dead fish eggs are susceptible to fungal infection particularly from the family Saprolegniaceae. During egg incubation, these fungi produce mycelia which grow and spread from the nonviable to the healthy eggs suffocating them and causing mortality (Lilley and Inglis, 1997; Hussein and Hatai, 2002; Forneris *et al*., 2003).

Periodical removal of dead eggs is effective to control fungal growth. However, this practice requires a great human effort and handling could damage healthy eggs (Carral *et al*., 2004). Therefore, using agents and drugs with antifungal activities must be considered.

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Until recently, malachite green was the most frequently used disinfectant in aquaculture and especially in salmonid hatcheries and it was considered practically irreplaceable (Post, 1987; Lilley and Inglis, 1997). Malachite green is a basic dye, readily soluble in water. Fungicidal effects of malachite green have been known since the mid-1930s. Malachite green became even more important when its effectiveness against water fungi (*Saprolegnia* sp.) was demonstrated (Olah and Farkas, 1978; Sudova *et al*., 2007). Malachite green was the most effective fungicide used for many years in aquaculture. However, based on toxicologic and teratogenic effects of malachite green, using this chemical agent in aquaculture has been banned in USA since 1991, in Italy since 1994, in EU since 1997 and in Iran since 2003 (Summerfelt *et al*., 1997; Forneris *et al*., 2003; Stammati *et al*., 2005; Rohani *et al*., 2006; Olesen *et al*., 2007; Sudova *et al*., 2007). However, this chemical agent as fungicide is used in fish hatcheries, aquariua and ornamental fish in Iran.

Scientifics compared antifungal activities of some chemicals but all them had limitation in aquaculture due to their potential toxicity on fish, environment and human. (Kitancharoen *et al*., 1997; Barnes *et al*., 1998).

Since 1990s, the European Union has put into effect the regulations concerning aquaculture that limit the use of products that have a great impact on the environment. The latest European Community directives limit the use of chemotherapeuticals (Forneris *et al*., 2003). In the past decade interest on the topic of antimicrobial plant extracts has been suggested. Various spices and herb extracts have been used for the purpose of food preservation and appetizer promotion as well as medicinal purposes (Mishra and Dubey, 1994; Cowan, 1999). In particular, extracts from many oriental plants and herbs such as cinnamon, clove, garlic, sage, oregano, thyme, rosemary, mint and vanilla have been known to possess antimicrobial effects (Smith *et al*., 1998; Tassou *et al*., 2000; Nilsen and Rios, 2000; Valero and Salmeroj, 2003; Pinto *et al*., 2007).

Furthermore, some studies on antibacterial and antifungal activities of essential oils and herbal extracts on aquatic animal have been reported by Gonzales *et al*. (1989), Adam *et al*. (1998), Marino (2001), Rouhani *et al*. (2006) and Bajpai *et al*. (2007). However, only a few reports have studied combinations of these products for their synergistic antimicrobial activities (Lee *et al*., 2007).

In this study, antifungal effects of a new combination of essential oil in comparison with malachite green were evaluated for controlling mold infections and hatching rate of rainbow trout (*Oncorhynchus mykiss*) eggs.

MATERIALS AND METHODS

This study was conducted through a project during the period from December 2006 to September 2007 and included the following parts:

Combined Essential Oils

The Combined Essential Oils (CEO) used in this study were extracted from the herbs, *Thymus vulgaris* (thyme), *Salvia officinalis* (common sage), *Eucalyptus globulus* (blue gum eucalyptus) and *Mentha piperita* (peppermint). The herbs were collected from an experimental field in the Zardband region located in the North Eastern of Tehran, Iran. The CEO was composed of 60% herbal essences with 40% solubilizing and stabilizing agents in water. Air-dried leaves and stems of the herbs (90 g from each herb) were subjected to hydro distillation for 4 h using a Clevenger-type apparatus to produce essential oils according to the method recommended by the European Pharmacopoeia (Schulz *et al*., 2004). The final combination of essential oils was prepared using an emulsifier. It was composed of 30% *Salvia officinalis*, 30% *Thymus vulgaris*, 20% *Mentha piperita* and 20% *Eucalyptus globulus* extracts. The CEO was dried over anhydrous sodium sulfate and stored in a sealed vial at low temperature (5-10°C) before analysis.

The composition of the CEO was determined by gas chromatography (GC) and by GC coupled with mass spectrometry (MS). GC analysis was performed using a Shimadzu GC-9A gas chromatograph equipped with a DB-5 fused silica column (30 m $\times$ 0.25 mm, film thickness

0.25 μ). Oven temperature was held at 40°C for 5 min and then increased to 250 °C at a rate of 3°C min^{-1}. Injector and detector (FID) temperatures were 260°C. Helium was used as a carrier gas with a linear velocity of 32 cm sec^{-1}. Percentages of different components were calculated by electronic integration of FID peak areas without the use of response factor correction.

GC-MS analyses were carried out on a Varian 3400 GC-MS system equipped with a DB-5 fused silica column (30 m × 0.25 mm i.d.). The oven temperature ranged from 40 to 240°C increasing at a rate of 4°C. The transfer line temperature was 260°C. The carrier gas was helium with a linear velocity of 31.5 cm sec^{-1} and a split ratio of 1/60. Ionization energy was 70 eV (scan time 1 sec and mass range 40-300 amu).

The different components within the CEO were identified by comparisons of their mass spectra with those of a database of known spectra (Stenhagen *et al*., 1974) or with authenticated reference compounds. Identities were confirmed by comparison of their retention indices either with those of authenticated compounds or with data published in the literature (Stenhagen *et al*., 1974).

Malachite Green

Malachite green (oxalate salt) was procured from merck company branch in Tehran, Iran. This chemical is used in microbiology lab for staining and as an antifungal agent in fish hatcheries.

Study of Hatching Rate

The current study was carried out at the Shahid Bahonar Salmonid Hatchery located in Kelardasht city in the north of Iran. The water source of incubator was from river and spring water with the following characteristics over this study: mean temperature 10±1°C, total hardness as $CaCO_3$ 190±10 mg L^{-1}, pH 7-7.5, dissolved oxygen (DO) 8-9 mg L^{-1}. Temperature was measured using a temperature meter and dissolved oxygen, TDS and pH were measured using biochemical kits (Karazma Biochemical Company). The water flow rate was monitored at 15 L min^{-1}. The water quality parameters were monitored daily during the experiment.

All incubators were completely cleaned and treated with different treatments for 1 h, before transferring eggs into California incubators.

During the study 3 kg of rainbow trout eggs (approximately 42000 eggs) were collected from 20 female (average age: 3.5 years, average weight: 1.7 kg) and 30 mL sperm from four male rainbow trout (average age 2.5: years, average weight: 1 kg). After fertilization with dry method, the fertilized eggs were divided to 7 groups and were transferred to the incubators.

Each treatment was replicated. All trials had the same conditions and supplied from the same source water. Treatment groups were CEO with 10, 50, 100, 150 and 200 ppm (Rohani *et al*., 2006), malachite green as positive control group with 1 ppm and one negative control group without any treatment.

Treatments were administered daily for 1 h as constant flow treatment method (Noga, 1996) and lasted for 22 days, from eggs to eyed eggs. During this period no handling and transferring of eggs were performed.

When the eggs were eyed, they were checked every day and the ones showing mold infection were counted, removed and recorded up to hatching period.

At the end of incubation period, final mortality and infected eggs counted and mortality rate was recorded. Also, number of larvae were estimated by volumetrically sampling and counting the number of larvae per sample and then extrapolating to the total volume. This experiment was lasted 46 days (from green egg to starting of hatching period).

Data Analysis

The data obtained from our study were analyzed with the student t-test and variance analysis and Kruskal-wallis to compare differences between tests and controls. Mean, standard deviation and some statistical indexes calculated and p-values lower than 0.05 ($p<0.05$) were considered to reflect significant differences between treatments.

RESULTS AND DISCUSSION

Forty six different components were detected. The main components were: 1,8-cineol (21.37%), thymol (13.86%), camphor (7.92%), α-thujone (7.71%), menthon (6.8%) and menthol (6.2%) (Table 1). The results of this study showed that the combined essential oils inhibited mold infection on rainbow trout eggs (Fig. 1). These results indicated significant antifungal activity of malachite green

Table 1: Composition determined by GC-MS analysis of the essential oils from *Salvia officinalis*, *Thymus vulgaris*, *Eucalyptus globulus* and *Mentha* piperita that were used to formulate the combined essential oil (CEO) tested in this study. The estimated composition of the CEO is also shown

Compound	*S. officinalis*	*T. vulgaris*	*E. globulus*	*M. piperita*	CEO
	(%)				
Salvene	0.3	-	-	-	0.09
Tricyclene	0.2	1.2	-	-	0.42
α-pinene	3.7	-	2.18	0.4	1.626
Camphene	6.6	0.6	-	-	2.1
Verbenene	1.6	-	-	-	0.48
Sabinene	0.2	1.8	-	0.6	0.72
β-pinene	0.4	0.1	0.69	0.6	0.408
Myrcene	0.9	2.4	0.69	-	1.128
α-Terpinene	0.2	2.1	1.66	-	1.022
ρ-cymene	0.2	17.6	1.27	-	5.594
Limonen	2.4	1.2	1.45	4	2.17
1,8 –cineole	9.6	0.3	88	4	21.37
γ-Terpinolene	0.1	14.8	-	-	4.47
Terpinolene	0.2	-	0.25	-	0.11
βThujone	6.4	-	-	-	1.92
α-Thujone	24.7	1	-	-	7.71
Camphor	26.4	-	-	-	7.92
Isopulegol	0.2	-	-	-	0.06
Pinocamphone	1.1	-	-	-	0.33
Borneol	4.2	1.1	-	-	1.59
Terpinene-	4-010.7	1.7	0.52	1	1.024
α-Terpineol	0.5	-	1.59	-	0.468
Myrtanol	1	-	-	-	0.03
Thymol	0.2	46	-	-	13.86
Bornyl acetate	1.3	-	-	-	0.39
Eugenol	0.1	-	-	-	0.03
α-Humulene	0.9	-	-	-	0.27
Caryophyllen oxide	0.2	-	-	-	0.06
Guaiol	3.3	-	-	-	0.99
α-Phellandrene	-	0.1	0.54	-	0.138
Trans sabinene	-	1	-	-	0.3
Linalool	-	2.3	0.16	-	0.722
Menthyl thymol	-	0.7	-	-	0.21
Menthyl carvacrol	-	0.4	-	-	0.12
Carvacrol	-	2.5	-	-	0.75
Cisβ-ocimene	-	-	0.18	-	0.036
Trans pinocarveol	-	-	0.33	-	0.066
α-Terpinyl acetate	-	-	0.49	-	0.098
Menthone	-	-	-	34	6.8
Menthofuran	-	-	-	1.8	0.36
Isomenthone	-	-	-	6.15	1.23
Menthol	-	-	-	31	6.2
Carvone	-	-	-	0.4	0.08
Menthyl acetate	-	-	-	3	0.6
β-Cariyophyllene	-	-	-	2	0.4
Germacrene	-	-	-	1.3	0.26
Other minor Component	-	-	-	-	3.27

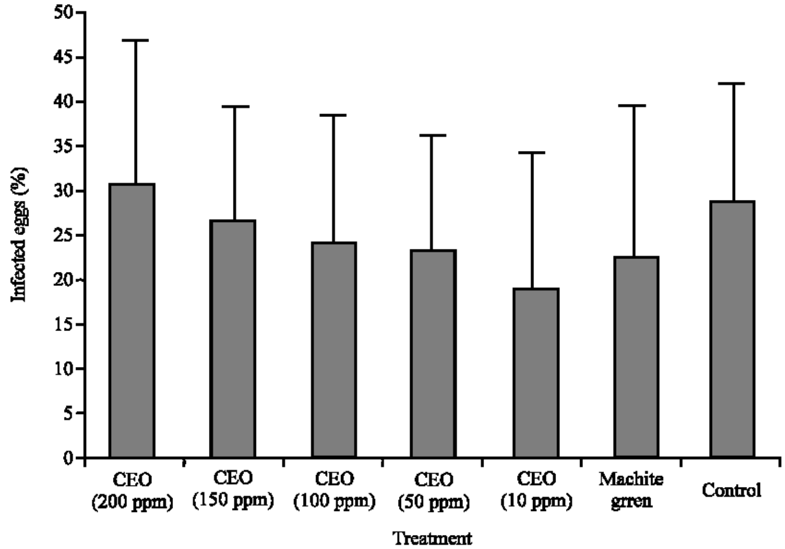

Fig. 1: Effects of the Combined Essential Oils (CEO) on per cent of mold infection with different concentrations of the CEO in comparison with malachite green

and 3 groups of CEO (10, 50, 100 ppm) in comparison with control group without any treatment ($p<0.05$). Also, between different concentrations, the CEO at concentration of 10 ppm was the most effective treatment. Furthermore, antifungal activity of CEO group (10 ppm) was significantly higher than malachite green group (1 ppm) ($p<0.05$) and it was evident of the better activity against water mold infection in hatcheries than malachite green.

The results of the hatching rate obtained at the end of incubation period showed that the CEO increases hatching rate of incubated eggs. The results shown in Fig. 2 indicated that the CEO at concentrations of 10, 50, 100 and 150 ppm and malachite green at 1 ppm concentration increased hatching percentage in comparison with negative control group without any treatment. Also, activities of the CEO at concentrations of 10, 50 and 100 ppm on hatching rate were better than malachite green and between the treatment groups, the maximum hatching rate was obtained from the CEO at concentration of 10 ppm and the minimum hatching rate was related to the CEO at concentration of 200 ppm.

In this study, antifungal activities of the combined essential oils on rainbow trout (*Oncorhynchus mykiss*) eggs and its effects on hatching rate were determined.

The most important components of the CEO used in the this study were 1, 8-cineol, thymol, camphor, α-thujone, menthon and menthol. These components showed antifungal activities against some fungal strains in other studies (Adam *et al*., 1998; Cowan, 1999; Marino *et al*., 2001; Iscan *et al*., 2002; Burt, 2004; Pina-Vaz *et al*., 2004). Their mechanisms of action have been determined and are attributed with disturbance of cell membranes, disrupting the proton motive force, electron flow, active transport and resulting in coagulation of intracellular contents (Denyer and Hugo, 1991; Burt, 2004). Some researchers supposed that combinations of essential oils have greater antifungal activity than their individual components due to their synergistic effects (Davidson and Parish, 1989; Cowan, 1999; Pina-Vaz *et al*., 2004; Duarte *et al*., 2005; Lee *et al*., 2007).

Based on the results of mold infection rates, the CEO could decrease and inhibit fungal growth on *Oncorhynchus mykiss* eggs in hatchery during incubation period. These results are in agreement with earlier reports on antifungal activities of essential oils (Bajpai *et al*., 2007; Braga *et al*., 2007).

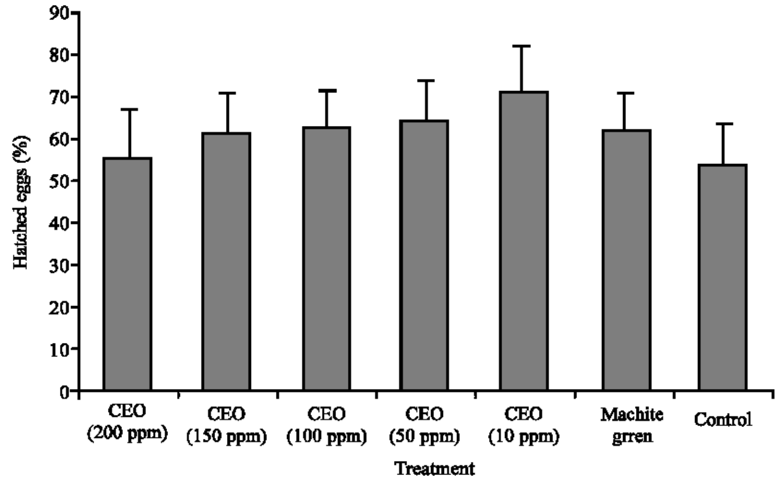

Fig. 2: Effects of the Combined Essential Oils (CEO) on hatching percentage with different concentrations of the CEO in comparison with malachite green and control

Rouhani *et al*. (2006) reported that some essential oils can use in aquaculture as an alternative therapeutic for malachite green in salmonid hatcheries, but they were not supposed the better antifungal effects. Nevertheless, our results indicated that the CEO decreased mold infection on (1989), Gill *et al*. (2002) and Lee *et al*. (2007), higher antifungal activity of the CEO could be in relation to synergistic effects of this combination.

Furthermore, the results indicated that the CEO increases hatching success rate in low concentrations which are in agreement with another research done on hatching success rate (Rohani *et al*., 2006), but they did not find more hatching rates in essential oils groups in comparison *Oncorhynchus mykiss* eggs more than malachite green. According to Davidson and Parish with malachite green. Nevertheless, results obtained here , represented that the CEO at concentrations of 10, 50 and 100 ppm had more hatching rate than malachite green and the most positive effects on hatching rate between all treatment groups was related to 10 ppm concentration. Also, minimum hatching rate was seen with concentration 200 ppm of the CEO. Decreasing of hatching rate in 200 ppm was suggested some toxic components of the CEO. According to Lima *et al*. (2004) and Pinto *et al*. (2007) studies, some components of essential oils have cytotoxic effects on high concentrations. These reports are in agreement with our results which suggested that low level of hatching rate may be in related to Thujone component in this combination (Burt, 2004; Pinto *et al*., 2007).

The CEO can provide an effective alternative to synthetic prophylactic or therapeutic treatments. Further analysis can prove the CEO to be more cost effective, less harmful to aquaculture species and less damaging to the environment than existing pharmaceuticals.

This study provides a first step in identifying appropriate combinations of essential oils for use in fish farm and hatcheries.

CONCLUSION

The combined essential oils with low concentrations can decrease mold infection rate and increase hatching percent in hatcheries through the synergistic effects. Some studies have concluded that combinations of essential oils have greater antimicrobial activity than their individual components (Davidson and Parish, 1989; Gill *et al*., 2002). These combinations of compounds may provide an alternative for chemical therapeutics in aquaculture. Accordingly, the combined essential oils examined in this study have revealed to affect mold infection on *Oncorhynchus mykiss* eggs and increase larvae yield. Finally, the combined essential oils need more studies for decreasing toxicological effects on eggs at high doses. In future, such formulations may represent alternative therapeutic treatments in aquaculture and hatcheries.

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