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Research Article:

Antifungal effects of alcoholic extract of *Thymus vulgaris* on Siberian sturgeon (*Acipenser baerii*) eggs compared with malachite green effects

Shahrani M.[1]; Azari Takami G.[1*]; Sharif Rohani M.[2]; Motallebi A.[3]; Yazdani Sadati M.[4]

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Abstract

The economic losses due to fungal contamination in development of sturgeon and the side effects of malachite green in the environment are very important. Therefore, it is essential to investigate and introduce suitable natural substances instead of malachite green. The aim of this study was to compare antifungal effect of *Thymus vulgaris* with malachite green on fungi isolated from Siberian sturgeon eggs obtained from International Sturgeon Research Institute. The samples were inoculated in culture media (SDA) and Glucose-Peptone Agar at 25°C for 5-7 days and then isolated fungi were identified. The antifungal effect of *T. vulgaris* extract was evaluated by disc diffusion method and MIC and MFC of fungi was measured. In this study, *Saprolegnia* spp., *Fusarium* spp., *Rhodotorula* spp., *Penicillium* spp., *Trichoderma* spp., *Chrysosporium* spp. and sterile hyphae were isolated and identified from fertilized eggs of *A. baerii*. According to the results, *T. vulgaris* extract had a significant effect on all isolated fungal species. The most effective sample was observed on *Saprolegnia* and the least effective ones were *Fusarium* and *Trichoderma.* The MIC and MFC of alcoholic extract for *Saprolegnia* spp., *Fusarium* spp., *Rhodotorula* spp., *Penicillium* spp., *Trichoderma* spp., *Chrysosporium* spp., sterile hyphae were ≥0.75, 3, 1.5, 1.5, 0.75, 1.5, ≤0.75 mg/ml, and ≥1.5, ≥3, 3, 3, 1.5, 3 and 1.5 mg/ml, respectively. Due to high frequency of *Saprolegnia* spp. in *A. baerii* eggs, and the significant effect of alcoholic extract of *T. vulgaris*, it could be used as a good alternative for malachite green to reduce the frequency of fungal contamination and its economic losses during reproductive seasons of *A. baerii*.

Keywords: *Acipenser baerii*, *Thymus vulgaris*, Antifungal effects, Malachite green

1-Health and Aquatic Diseases Department, Science and Research Branch, Islamic Azad University, Tehran, Iran

2-Iranian Fisheries Science Research Institute (IFSRI), Agricultural Research Education and Extension Organization (AREEO), Tehran, Iran

3-Science and Research Branch, Islamic Azad University, Tehran, Iran

4-Caspian Sea International Sturgeon Research Institute, Agricultural Research Education and Extension Organization (AREEO), Rasht, Iran

*Corresponding author's Email: takami85@hotmail.com

Introduction

Siberian sturgeon (*Acipenser baerii*) is one of the most prominent members of the sturgeon's family. Despite its import in Iran, it can be mentioned by the following advantages: easy and suitable adaptation to breeding conditions, resistance to environmental changes, rapid growth, and adaptation to a wide range of foods, ability to grow in favorable conditions, early maturity and fast caviar producing. In recent years, rapid development of fish farming as well as problems caused by fungal contamination led to attention to the important role of fungi in fish life (Kouhpaye *et al.,* 2007). Aquatic fungi considered as one of the most important harmful factors in aquatic health, which are important after viral and bacterial diseases. These diseases are considered to be serious problems in incubation period of many species, including eggs of sturgeons (Ghiasi, 2008). Evidence from recent past suggested that about half of the sturgeon eggs in breeding centers may be out of production due to fungal diseases (Willoughby, 1994; West, 2006; Azari Takami, 2009).

Aquatic fungal diseases were isolated and reported from wild and cultured species worldwide (Alderman, 1982). Several studies have been done on fungal contamination in fish eggs, surface and internal organs of different fish including, fungal infection of *Rutilus frisii*, larvae, fry and skin of *Acipenser persicus*, fungal contamination of *Rainbow trout* eggs and fungal contamination of Cyprinidae in Iran (Hussein and Hatai, 2002; Jalilpour *et al.,* 2006). The presence of these pathogens in water resources and some conditions such as high density, manipulation, and reproductive stress levels, have led them to the occurrence of fungal infections in fish and their eggs (Firouzbakhsh *et al.*, 2009).

Different types of *T. vulgaris* belong to the *Lamiaceae*, grow in west and north of Iran. This plant has a variety of uses in traditional medicine and food industry. Therapeutic parts of this plant include its top branches and dried leaves (Akbari, 2007; Bababaalian Amiri, *et al*., 2020 a,b).

Antifungal effects of *T. vulgaris* have been proven in animals and laboratory animals (Akbari, 2007; Soković *et al.*, 2008). Effective antimicrobial compounds in *T. vulgaris* contain flavonoids such as epinephrine, luteolin and volatile oils containing carvacrol and thymol (Soković *et al.*, 2008). In Iran, other studies have also been carried out on the effects of some medicinal plants as an alternative to malachite green and formalin for control and prevention of fungal infection in fish farms in the stage of incubation (Adel *et al.*, 2015; Keykha *et al.*, 2015; Shafiei *et al.*, 2016).

One of the most important causes of egg mortality in sturgeon and bony fish hatcheries is fungal contamination, especially *Saprolegnia* spp., which has led to huge economic losses (about 10 million pounds annually) in the world aquaculture industry (Hosseini Fard, 2007; Ghiasi, 2008). Many species of *Saprolegnia* have been considered as opportunistic and secondary invasive

agents, due to stressful and environmental conditions such as poor water quality and exchange, low dissolved oxygen, high ammonia, high density, high level of organic matter, changes of temperature and sexual maturation or spawning cause mortality in fish and their eggs (Ghiasi, 2008).

The aim of this study was to compare antifungal effect of *Thymus vulgaris* with malachite green on fungi isolated from Siberian sturgeon eggs obtained from International Sturgeon Research Institute.

Materials and methods

Isolation of fungal agents from fertilized eggs of Siberian sturgeon

During this research, which took place in 2017 in the International Sturgeon Research Institute, Siberian sturgeon eggs were selected from 2 Siberian sturgeons (mean weight 7±1 kg) and transferred to 15 Californian Incubators after fertilization with sperm. At the beginning of the reproduction season of Siberian sturgeon in April, 250 fungal infected eggs with sterilized forceps were randomly taken from incubators in three stages (First, middle and end of incubation) and transferred to sterile tubes containing sterile tap water.

In this work the treatment groups, include 0 ppm (control), 1ppm (Malachite Green), 500, 750, 1000 and 1250 ppm (*T. vulgaris* extract) with 3 replications (Keykha *et al.*, 2015).

Isolation and purification of fungi

Tubes containing fungal infected eggs were transferred to the laboratory Petri dishes after sampling and washed 3 times with sterile tap water and cultured to the Sabouraud Dextrose Agar (SDA) medium and the specific culture medium of *Saprolegnia* fungi (Glucose-Python Agar). In order to prevent growth of bacterial agents in the culture media, two types of Gentamicin and chloramphenicol were applied at a dose of 250 mg/L. The culture mediums were incubated at 25°C for 5 to 7 days and growth pattern of fungi was studied daily (Sharif Rohani *et al.*, 2006)**.**

Identification of isolated fungi species

After growth of fungal colonies for microscopic examination, a part of colonies were placed on a microscope slide containing lactophenol cotton blue. Identification of fungi was performed using identification keys (Beakes *et al.*, 1994; Willoughby 1994; Shahbazian *et al.*, 2010).*T. vulgaris* extract prepared from Barij Essence medicine company (Kashan, Iran) from top branches and dried leaves. It was performed by maceration method and was kept in the refrigerator at 4°C and prepared according to the instructions (Salaby and Razin, 1992).

Malachite green used in this study was provided by Merck Company in Germany.

Investigation on the effect of T. vulgaris extract and malachite green on isolated fungi

In order to investigate the result of antifungal effect of alcoholic extract of *T. vulgaris* and malachite green, fungal suspension was prepared from each of the pure cultures of isolated fungal agents. The prepared fungal suspensions were standardized by 0.5 McFarland standards (1×10^6 fungal cells per milliliter).

Investigation of antifungal effect of T. vulgaris extract with disc diffusion method

After preparation of fungal suspensions, a sterile swab smeared with fungal suspension and inoculated at the surface of Muller Hinton Agar culture medium. Then the plates were located in a suitable place for 3 to 5 minutes to absorb their excess liquid and penetrate into the agar. Blanks discs were smeared with 500, 750, 1000 and 1250 ppm of *T. vulgaris* extract. Malachite green disc (1 ppm) was also prepared individually (Keykha *et al.*, 2015). In this study, discs that smeared with sterile tap water were used as negative control. The culture medium was incubated for 96 hours at 25°C for 24 hours, the growth rate and diameter of the inhibitory growth zone of fungus were measured and recorded by standard ruler.

Determine Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

MIC was determined using microdilution method. In this way, serial dilutions of alcoholic extract of *T. vulgaris* (0 as control, 2, 4, 8, 16 and 32 mg per ml) were added to Sabouraud Dextrose Broth medium and inoculated 0.1 milliliter of $1 \times 10^6 \frac{CFU}{mL}$ fungal suspension. Culture mediums were incubated for 48 hours at 25°C. After that, tubes were compared in terms of turbidity due to the growth of fungi with the control tube and the lowest concentration of *T. vulgaris* that inhibited growth of fungi and/or absence of turbidity in the tube was considered as MIC. For determining MFC, the dilution of MIC and higher concentration transferred with sampler at 10µL to SDA culture medium for 48 hours at 25°C and the lowest concentration of extract that completely inhibited growth of fungi without turbidity (transparent), was considered as MFC (Mousavi *et al.*, 2012).

Statistical analysis of data

In this study, three replications were considered for each treatment. After counting and recording fungal infected eggs, data were analyzed statistically. Analyses of data related to the results of the research was conducted with one-way ANOVA, Duncan's multiple range test and t-test at a probability level of ($p \leq 0.05$) using SPSS software (version 20).

Results

In this study fungal species *Saprolegnia* spp. (38%), *Fusarium* spp. (0.7%), *Rhodotorula* spp. (8.6%), *Penicillium* spp. (11.3%), *Trichoderma* spp. (10.6%), *Chrysosporium* spp. (10%), and sterile hyphae (14.6%) were isolated and identified from fertilized eggs of Siberian sturgeon (Fig. 1).

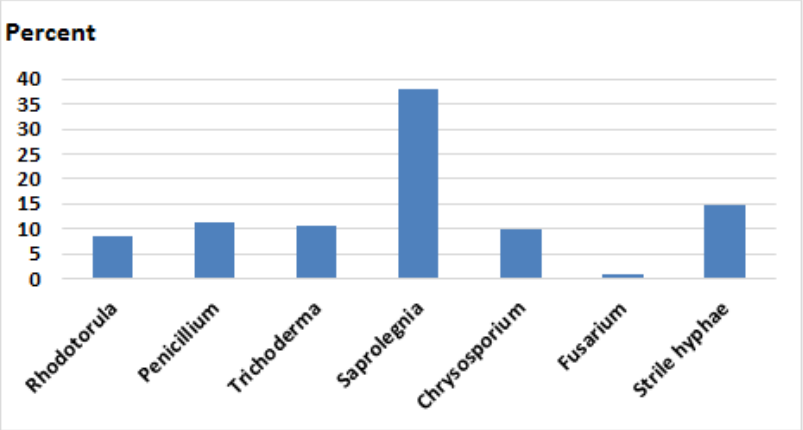

Figure 1: Percentage of isolated fungi from fertilized eggs of Siberian sturgeon.

Results of evaluating percentage of fungal infected eggs in each treatment are presented in Table 1. Regarding the results obtained in all fungal species, the type of treatment had a significant effect on the number of fungi in fertilized eggs of Siberian sturgeon ($p<0.05$). The results of this study showed that the concentration of 1250 ppm of *T. vulgaris* extract and 1ppm malachite green had similar effect in control of the fungal species with no significant difference between them. On the other hand, there was no significant difference between treatments of 500 and 750 ppm of *T. vulgaris* extract and control treatment in most of the studied fungal species ($p\geq0.05$). Considering the amount of fecundity in analysis of variance (Table 2), the most affected treatment was *Saprolegnia* spp. and least affect were *Fusarium* spp. and *Trichoderma* spp. Comparison of average number of each fungus in different treatments was done by Duncan's multiple range test. The results of the test (Fig. 2) showed higher performance of 1000 and 1250 ppm of *T. vulgaris* extracts and malachite green.

The results of MIC and MFC of alcoholic extract of *T. vulgaris* on fungal infected eggs of Siberian sturgeon are shown in Table 3.

Table 1: Average number of fungal infected eggs of Siberian sturgeon in each treatment.

Fungus	Control	1 ppm malachite green	500 ppm *Thymus vulgaris*	750 *Thymus vulgaris*	1000 ppm *Thymus vulgaris*	1250 ppm *Thymus vulgaris*
Rhodotorula	2.00 ± 1.00^{a}	0.00 ± 0.00^{b}	1.333 ± 0.577^{a}	1.00 ± 0.577^{ab}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
Penicillium	3.00 ± 1.00^{a}	0.00 ± 0.00^{c}	1.666 ± 1.154^{ab}	1.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
Trichoderma	1.666 ± 0.577^{a}	0.333 ± 0.157^{c}	1.333 ± 0.577^{ab}	1.00 ± 0.00^{ab}	0.667 ± 0.577^{b}	0.333 ± 0.157^{c}
Saprolegnia	12.66 ± 3.511^{a}	0.667 ± 0.577^{c}	3.333 ± 1.527^{b}	2.33 ± 2.309^{bc}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
Chrysosporium	3.333 ± 1.527^{a}	0.00 ± 0.00^{c}	1.00 ± 0.577^{b}	0.666 ± 0.577^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
Fusarium	1.666 ± 0.577^{a}	0.00 ± 0.00^{b}	1.00 ± 0.577^{ab}	0.666 ± 0.577^{ab}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
Sterile hyphae	4.00 ± 1.732^{a}	0.333 ± 0.157^{c}	1.00 ± 0.577^{b}	1.00 ± 1.00^{b}	0.666 ± 0.577^{b}	0.333 ± 0.157^{c}

*Dissimilar alphabets in fungi indicate significant difference among the averages.

Table 2: The effect of treatment type on the number of isolated fungi.

Fungus	p-value	F	MS	df	SS
Rhodotorula	0.007	5.629	2.189	5	10.944
Penicillium	0.000	11.457	4.456	5	22.278
Trichoderma	0.046	3.200	0.889	5	4.444
Saprolegnia	0.000	20.764	70.367	5	351.833
Chrysosporium	0.001	8.236	5.033	5	25.167
Fusarium	0.046	3.200	1.422	5	7.111
Sterile hyphae	0.066	5.822	5.822	5	29.111

F=fecundity, MS=mean of squares, df=degree of freedom, SS=sum of squares.

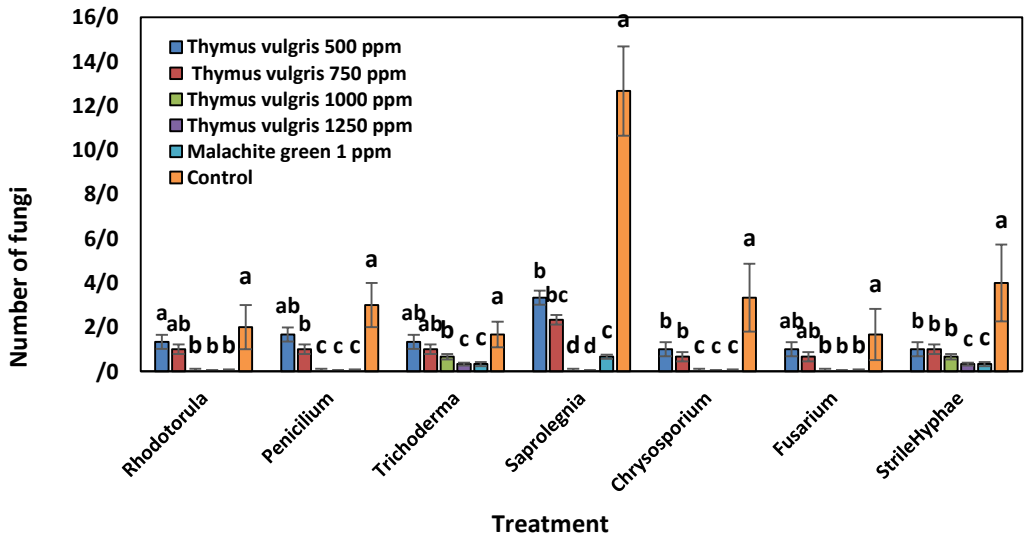

Figure 2: Comparison of average number of fungus in different studied treatments.

Table 3: MIC and MFC of alcoholic extract of *T. vulgaris* on fungal infected eggs.

Studied fungus	MIC $\left(\frac{mg}{mL}\right)$	MFC $\left(\frac{mg}{mL}\right)$
Rhodotorula	1.5	3
Penicillium	1.5	3
Trichoderma	≤ 0.75	1.5
Saprolegnia	≥ 0.75	≥ 1.5
Chrysosporium	1.5	3
Fusarium	3	≥ 3
Sterile hyphae	≤ 0.75	1.5

Discussion

Fungal contamination is one of the causes of damage in aquaculture industry in Iran and as a determinant of health status of fish farms (Azari Takami, 2009). One of effective factors in occurrence of mortality in sturgeon and other bony fishes hatchery system is contamination of fertilized eggs with fungi, especially *Saprolegnia*, which has caused grate losses in aquaculture industry (Shahbazian *et al.*, 2010). High prevalence of treatment-resistant infections due to increased resistance to antibiotics and problems with the use of chemical drugs, including their side effects and their high costs, has increased the importance of using medicinal plants. Malachite green leads to carcinogenesis and reduce fertility and environmental problems when it comes to the natural cycle (Sharif Rohani *et al.*, 2006; Hosseini Fard, 2007).

In this research, which is conducted on Siberian sturgeon in spring, *Saprolegnia* fungi had the highest frequency and *Fusarium* had the lowest frequency. This issue reflects the fact that *Saprolegnia* fungi are very important in fungal infected Siberian sturgeon eggs and despite changing environmental factors and management conditions of farms there is a wide range of *Saprolegnia* contamination compared to other fungal species. Ghiasi (2008) identified *Saprelignia parasitica* from infected eggs of sturgeon and *Rutilus frisii* in hatcheries of Mazandaran Province with morphological and molecular methods. This fungus was also isolated and identified by Czeczuga *et al.* (1995) from eggs of several sturgeon species. The results of the current study and previous studies indicate that *Saprolegnia* species are dominant pathogens in sturgeon eggs in hatchery system and the main cause of mortality in fishes (Czeczuga *et al.*, 1995). Considering the important role of stressor agents in occurrence of Saprolegniasis, reducing stress conditions by improving environmental factors and management conditions, such as proper water quality and exchange of water, reducing the amount of organic matter, avoiding high density of fish, unnecessarily manipulating and using suitable disinfectants in hatcheries can be useful in controlling and reducing economic loss due to Saprolegniasis (Ghiasi, 2008). The results of this study showed that 1,000 and 1250 ppm of *T. vulgaris* extract had the same effect as malachite green in

controlling species of studied fungi with no significant difference among them ($p \geq 0.05$). Regarding this point, due to high frequency of *Saprolegnia spp.* in Siberian sturgeon (*Acipenser baerii*) eggs, *T. vulgaris* extract can be a good alternative to reduce frequency of *Saprolegnia* and as a consequence significant damage caused by it. One of the main components of *T. vulgaris* is thymol and carvacrol, which can be attributed to antifungal property of this plant. Carvacrol is a kind of iso-thymol, which by inhibiting the activity of ATPase enzyme increases non-specific permeability of cell membrane of microorganisms and as a result, increases their sensitivity to plants and antimicrobial compounds (Najib-Zadeh *et al.*, 2011). According to Sharifian *et al*. (2009) antifungal property of carvacrol in other plants, such as *Satureja khuzestanica hortensis* and *Myrtus communis* has been proven. In 2017 Pazira identified anti-fungal activity of *T. vulgaris* on a number of fungi *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, and *Saprolegnia* isolated from *Cyprinus carpio* skin. Based on the results, the highest inhibitory effect of *T. vulgaris* showed was on *Aspergillus flavus* and lowest inhibitory effect was on *Saprolegnia* (Pazira, 2017). Moussavi and Raftos (2012) examined antifungal effects and toxicity of essential oil of a combination of *T. vulgaris*, *Salvia officinalis*, *Eucalyptus globules*, *Mentha piperita* on rainbow trout compared with malachite green, which showed that the combination of the four essential oils could be used as an antifungal agent in hatcheries and fish farms, which confirms antifungal effect of *T. vulgaris*. Centeno *et al.* (2010) investigated antifungal effect of *Rosmarinus officinalis* and *T. vulgaris* extract in *Aspergillus flavus* and *Aspergillus ochraceus*, which showed that both of the extracts at low concentration could have a significant antifungal effect on these two species, which is consistent with the present study. According to the results of this study, all of the studied fungal species were affected by alcoholic extract of *T. vulgaris*, which indicates a wide range of antifungal effects of alcoholic extract of *T. vulgaris*.

Viuda-Martos *et al.* (2007) showed that *T. vulgaris* essential oil reduced the growth of mycelium of *Aspergillus* in amounts of 2 to 8 mL. Moghtader (2012) investigated the antifungal effect of *T. vulgaris* essential oil with thymol in *Aspergillus niger*. The results showed that the essential oil of *T. vulgaris* was more effective than streptomycin sulfate (72%) and gentamicin (8 mg/mL) was stronger than synthetic thymol 10% dilution. Akbari (2007) investigated antifungal effects of *T. vulgaris* and *Origanum vulgare L.* on clinical isolates of *Candida albicans* resistant to fluconazole which the results of this study showed that the extract of both plants were able to inhibit growth of the isolates. Among the extracts, *T. vulgaris* had the highest antifungal effect (0/49-125 mg/mL) and then *Origanum vulgare L.* essential oil,

aqueous extract of *T. vulgaris* and *Origanum vulgare L.* were in next category. A study on antifungal activity of amphotericin B with *T. vulgaris* essential oil in *Candida albicans* showed that the essential oil of *T. vulgaris* has an antifungal activity similar to amphotericin B and can also be used to treat fungal diseases (Giordani *et al.*, 2004). The difference in MIC and MFC values in different studies can be attributed to differences in microorganisms, composition of the extracts in concentration of each extract, in culture medium and the viscosity of the extract and environmental factors (Adel *et al.*, 2015). Regarding records of use of medicinal plants in fish farms for controlling and prevention of fungal diseases during incubation, which is one of the most important factors in reproduction and sturgeon breeding industry, based on the results of this study and antifungal effect of *T. vulgaris* extract at concentration of 1000 and 1250 ppm compared with malachite green in order to reduce side effects in aquaculture and human consumption, it is prioritized to control fungal contamination in Siberian sturgeon eggs by *T. vulgaris*. Therefore, it is recommended that in the future, the purified compounds in *T. vulgaris* extract, histological study and effective antifungal agents should be investigated in order to reduce Saprolegniasis in hatchery systems. To reduce the costs extraction and preparation of *T. vulgaris* and easy access to this plant compared with malachite green, it can be concluded that *T. vulgaris* extract is a good replacement for malachite green.

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