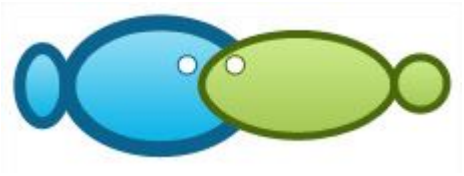

# *In vitro* test of inhibition effect of *Lemna minuta*, *Chlorella vulgaris* and *Spirulina* sp. extracts on *Saprolegnia parasitica*

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**Abstract**. One of the most important oomycete pathogens in fish is *Saprolegnia parasitica*. Its development in freshwater pools leads to major problems in freshwater hatcheries. The aim of the present study was to test extracts from *Spirulina* sp., *Chlorella vulgaris* and *Lemna minuta* against fish pathogen *S. parasitica.* The ethanol, aqueous and methanol extract of these three aquatic plants were prepared. From the rainbow trout fish farm in Tvarditsa, Bulgaria we obtained eggs with signs of saprolegniasis. From the infected eggs, the mycelia of the pathogenic fungal microorganism were separated and cultivated. The methanol extracts from the three tested species did not show activity against the fish pathogen *S. parasitica*. For ethanol extracts of the plants we found a very good inhibitory effect on the studied pathogen. *C. vulgaris* had the strongest inhibitory effect of the three tested plant extracts.

**Key Words**: antifungal, plant extract, *Saprolegnia*.

**Introduction**. *Saprolegnia* species cause diseases on amphibians, crustaceans and fish (Jiang et al 2013). The species belonging to the genus *Saprolegnia* (class Oomycetes) are considered as opportunistic facultative parasites to be the causative agents of the saprolegniasis (Shin et al 2017). One of the most important oomycete pathogens in fish is *Saprolegnia parasitica* (Van West 2006). Initially, it penetrates the epidermal tissues and it usually starts as a cotton wool like shape, white to dark gray or brownish growth over the head region or dorsal fin of fish and then it covers the whole body surface (Willoughby 1994; Hashemi et al 2012). The development of *Saprolegnia* sp. in freshwater pools leads to major problems in freshwater hatcheries (Rach et al 2005). Saprolegniasis is strongly connected with environmental stress, and it can be secondary invader to viral, bacterial and parasitic infectious agents to the fish (Shin et al 2017). Continuous growth of production in the aquaculture sector has led to more pressure on hatcheries to supply an increasing amount of trout (*Oncorhynchus mykiss*) (Caruana et al 2012). As a result, hatchers are increasing loading the eggs to meet the required production. For this reason, there is an increase in infections (Rach et al 2005). Therefore it is very important to control *Saprolegnia* outbreaks during incubation of eggs because the infection can lead to loss of all eggs or to reduce their survivability (Barnes et al 1998). In the past malachite green has been used to control these infections (Alderman 1985). Subsequently, the compound was forbidden, because is considered carcinogenic, mutagenic, teratogenic, and undesirable effects on animal health have been identified (Stammati et al 2005). This required the discovery and application of safe and appropriate alternatives. Chemical products, bacterial isolates, and UV irradiation have been researched on to find the replacement or alternative strategies for banned malachite green (Ali et al 2014; Heikkinen et al 2016). Researches were conducted with hydrogen peroxide ($H_2O_2$), formalin (37% formaldehyde v / v), sodium chloride (NaCl), copper sulfate but there is a risk for human health and the amount entering the environment. For example, small amounts of formaldehyde may also transfer into rain,

fog, and clouds (Schreier et al 1996; Arndt et al 2001; Caruana et al 2012). This requires the search for compounds of natural origin to provide a more environmentally friendly alternative. In the scientific literature there is insufficient information for use of plant extract to control fungal growth in freshwater fish (Rai et al 2002; Pirbalouti et al 2009; Singh et al 2013). The aim of the present study was to test extracts from *Spirulina* sp., *Chlorella vulgaris* and *Lemna minuta* against fish pathogen *Saprolegnia parasitica.*

## Material and Method

***Plant material***. *L. minuta* were collected from the town of Banya – Plovdiv region in a small (about 30 $m^2$) warm swamp formed by a hot mineral water flowing out from "Bancheto" bath (42°32'226''N 24°50'213''E) in April 2018. *C. vulgaris* (SKU: 100-CVC00-50) was supplied from the Algae depot – USA (www.algaedepot.com). Algae cultivation was initiated in a laboratory bioreactor with BBM medium (http://www.ccap.ac.uk/media/ documents/BB_000.pdf). The dry weight of algal biomass was used to prepare extract. *Spirulina* powder bio (ZOYABG@) was purchased from a pharmacy.

***Extract preparation***. The plant extracts were prepared by soaking of 5 g of dry, macerated plant material in 20 mL 100% ethanol for 24 h. Then the material was filtered through a 20 µm mesh before being placed in a rotary evaporator to reduce the volume to 5 mL. Each test compound was diluted by 1:10. The same procedure was used to prepare methanol plant extracts. Water extract of aquatic plants were made according to Dellavalle et al (2011). The plants extracted in water solution at proportion 1:10. The received homogeny solutions were filtered and centrifuged at 7000 rpm for 30 minutes. Afterwards the extracts were filtered with sterile syringe filters with the size 0.2 µm (Minisart, Sartorius Stedim Biotech GmbH, Germany). The extract samples were stored in universal bottles and refrigerated at 4°C prior to use. Control variants are methanol and ethanol, respectively. The experiments were performed in triplicate.

***Strain tested***. Eggs with signs of *Saprolegnia* were provided by the rainbow trout farm in Tvarditsa, Bulgaria. From the infected eggs, the mycelia of the pathogenic fungal microorganism were separated. It was crushed in a mortar with distilled water. The resulting solution was incubated with hemp seeds (Butty et al 1989). After 3-4 days, cotton-seed colonies were stored on the seeds. The columns were washed three or four times with distilled water reused to infect new hemp seeds at a temperature of 14-16°C for 48-72 hours. Subsequently, small pieces of fungal pathogen were washed with distilled water and transferred to pre-prepared glucose-peptone agar. This process was repeated 4-5 times until pure culture was obtained. Finally, the pathogenic oomycete was used to infect three Petri dishes with glucose-peptone agar. The culture was incubated at 20°C and was harvested every 3 days. On the basis of morphology, texture, color (presence of pigments), colony size and species indicators, it was established that the pathogenic microorganism refers to the genus *Saprolegnia*, species *S. parasitica* (Seymour 1970; Buller 2014).

***Microbiological medium***. Glucose-peptone agar was prepared as follows: 12 g of agar was mixed with 1 g D - (+) - glucose, 1 g soybean peptone, 0.128 g magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 0.014 g potassium dihydrogen orthophosphate ($KH_2PO_4$) in 1 L of distilled water, and the resulting medium was placed in an autoclave. After removal from the autoclave the following micronutrients were added: 29.3 mg $L^{-1}$ calcium chloride dihydrate ($CaCl_2.2H_2O$), 2.4 mg $L^{-1}$ iron (III) chloride hexahydrate ($FeCl_3.6H_2O$), 1.8 mg $L^{-1}$ manganese ($CuSO_4.5H_2O$) and 0.4 mg $L^{-1}$ zinc sulphate heptahydrate ($ZnSO_4.7H_2O$), followed by the addition of 0.25 g $L^{-1}$ of penicillin and steptomycin for inhibiting bacterial growth. Then the medium was spilled in Petri dishes.

***Antifungal test***. Pre-prepared media were plated in Petri dishes containing glucose-peptone agar at a 1:10 medium extract concentration. The residue of the pathogenic

oomycete from the *Saprolegnia* 0.7 mm was cut from a 3-day culture and placed in the center of the Petri dish. The culture was then incubated at 24±2°C. Each test was conducted in three replicates. The antimycotic effect was determined by measuring the radial growth of the pathogen and calculated by the following formula:

$$\text{Pathogenic mycelium growth (\%)} = \frac{\text{mycelium growth (experimental variant)}}{\text{mycelium growth (control variant)}} \times 100$$

Data analyses were conducted by using descriptive statistics, one-way Analysis of Variance ANOVA (MS Office, 2010).

**Results and Discussion**. The methanol extracts from the three tested species did not show activity against the oomycetes from *Saprolegnia*. Aqueous extracts of the studied species suppressed the development of the pathogen. The growth of mycelia of the fungal microorganism is suppressed by 31.8% when treated with *L. minuta* water extract as the differences were statistically proven ($p < 0.01$) (Figure 1). The growth of oomycete *S. parasitica* is suppressed by 31.5% when treated with *C. vulgaris* aqueous extract as the differences were statistically proven ($p < 0.001$). *Spirulina* had the strongest inhibitory effect of the three tested plant extracts. It suppresses the growth of fungus colonies by 34.7%, and the differences were statistically proven ($p < 0.001$).

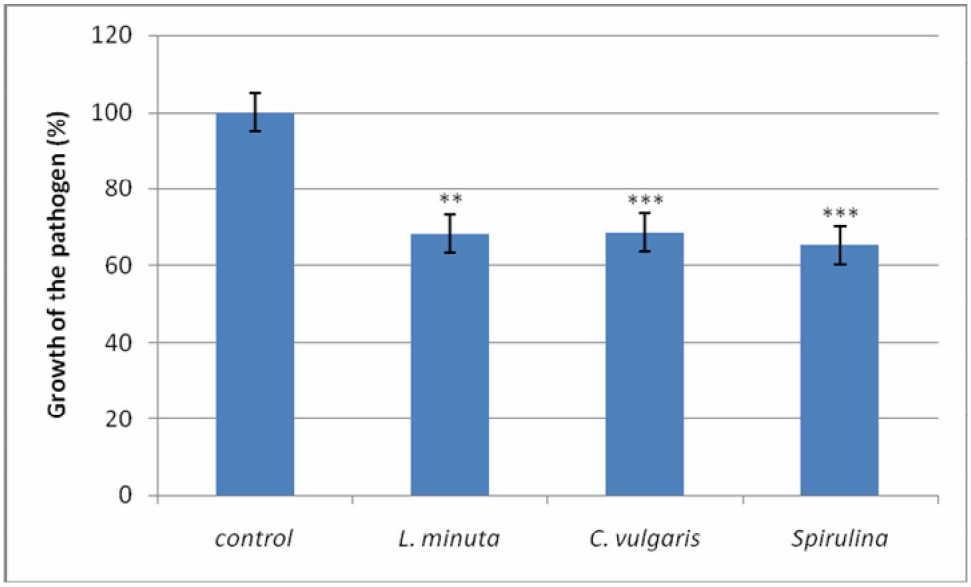


Figure1. Growth of the *S. parasitica* by treatment with aqueous extract of *L. minuta, C. vulgaris, Spirulina* sp. and control variant (**$p < 0.01$, ***$p < 0.001$).

For ethanol extracts of the plants we found a very good inhibitory effect on the studied pathogen.

The growth of oomycete *S. parasitica* is suppressed by 37.5% when treated with *L. minuta* ethanol extract as the differences were statistically proven ($p < 0.001$) (Figure 2). Colony diameter of *S. parasitica* after treatment of plant extracts compared to the control is presented in Figure 3. In literary review, we did not find studies of *Lemna* extracts on *S. parasitica*. Due to the fact that the ethanol extract of *L. minuta* shows a suppressive effect on this pathogen, it is necessary to conduct additional tests with different concentrations and extracts.

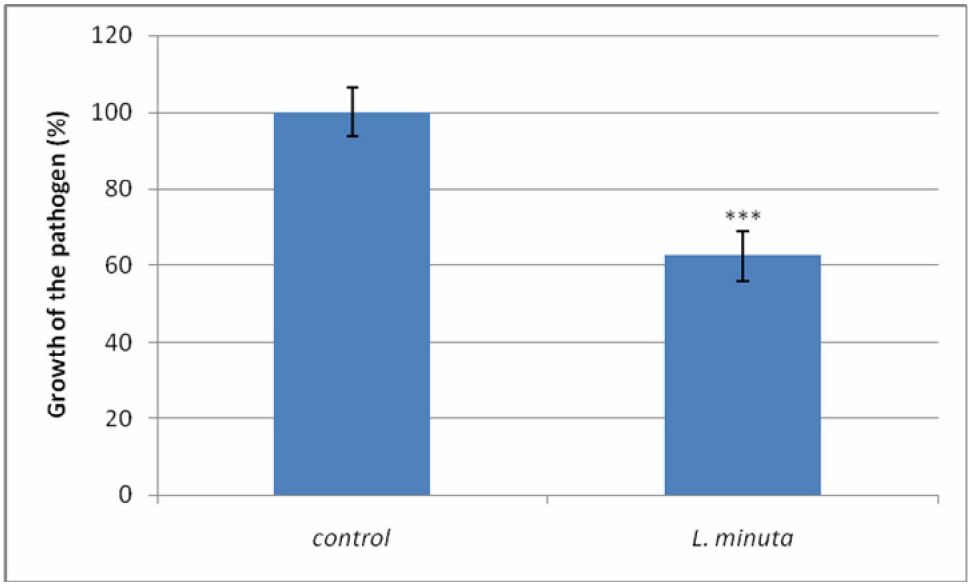

Figure 2. Growth of the *S. parasitica* by treatment with ethanol extract of *L. minuta* and control variant (***p < 0.001).

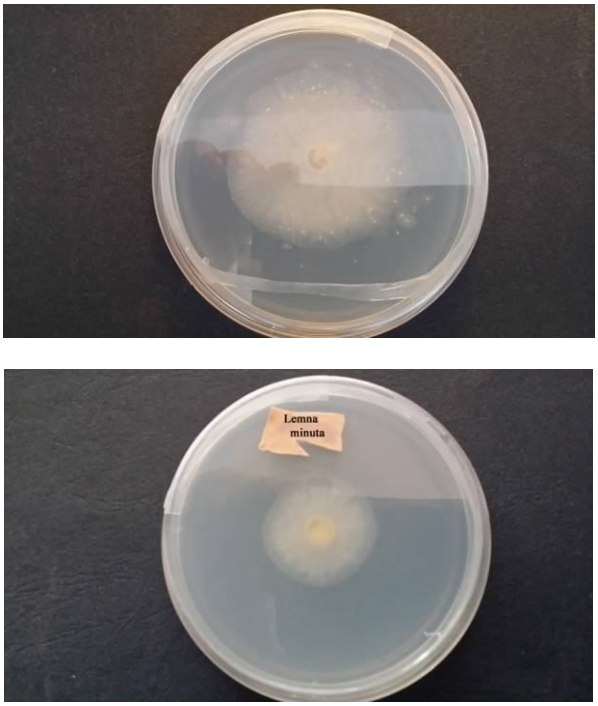

Figure 3. Effect of *L. minuta* ethanol extract on *S. parasitica* growth, versus control variant.

Singh et al (2013) evaluated the antifungal activity of aqueous extracts of terrestrial plants *Brassica nigra*, *Andrographis paniculata, Targetes erectus* and *Pinus roxberghaii* against *S. parasitica*. Their results revealed that all the tested extracts possess high degree of antifungal activities against the test pathogen. Due to the fact that the algae contain many active biological components in their cells, it is assumed that their extraction and impact will inhibit fungal pathogens development. Therefore, we conducted experiments on the effect of extracts of green and blue-green algae on *S. parasitica*. The strongest inhibitory effect of the three tested plants extract had *C. vulgaris*. It suppresses the growth of fungus colonies by 45%, and the differences were statistically proven ($p < 0.001$) (Figure 4). Colony diameter of *S. parasitica* after treatment of plant extracts decreases almost by half compared to the control (Figure 5). Cortés et al (2014) established that red algae *Ceramium rubrum* contains metabolites that possess antifungal activities against these fish pathogens.

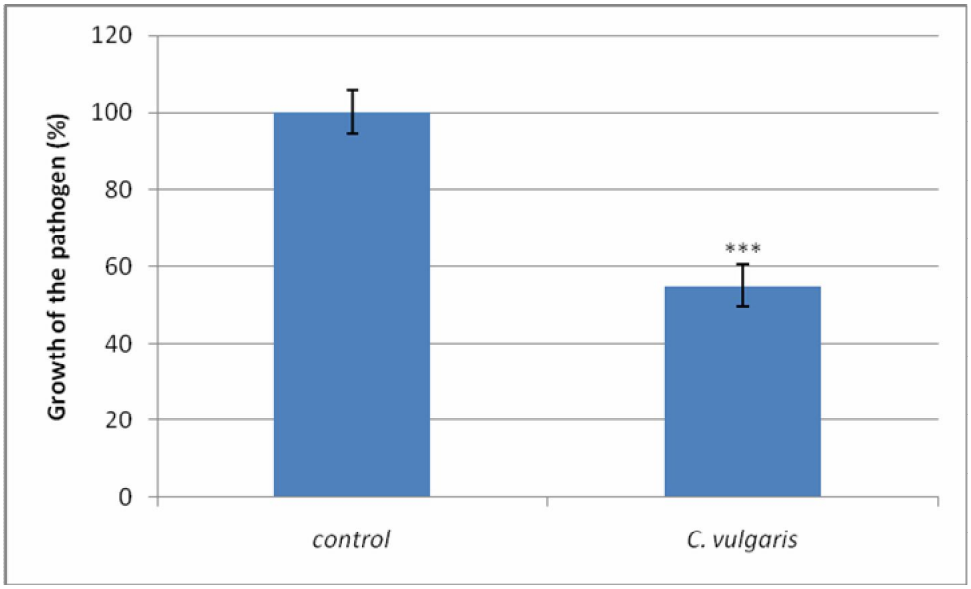


Figure 4. Growth of the *S. parasitica* by treatment with ethanol extract of *C. vulgaris* and control variant (***p < 0.001).

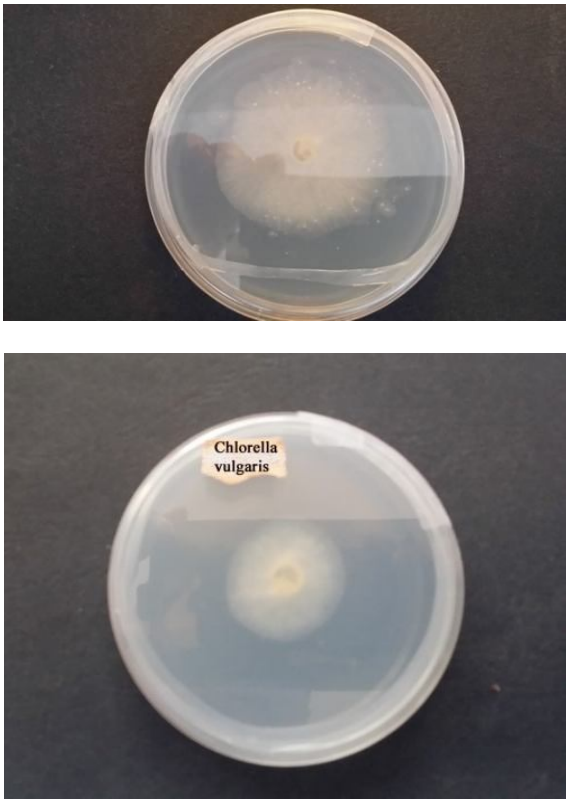


Figure 5. Effect of *C. vulgaris* ethanol extract on *S. parasitica* growth, versus control variant.

A good inhibitory effect also showed the ethanol extract of *Spirulina* on the growth of the fish pathogen. It suppresses the growth of fungus colonies by 40%, and the differences were statistically proven ($p < 0.001$) (Figure 6, Figure 7).

The present research is the first study that has reported the antifungal activity of these three aquatic plants (*L. minuta, C. vulgaris* and *Spirulina* sp.) against *S. parasitica* In our *in vitro* studies it was found that ethanol plant extracts contain fungicidal active substances and can be used against the fish pathogen *S. parasitica*. The results not only reflect the antifungal activity of ethanol extracts of the three tested plants against *S. parasitica* but also have a prospect, especially for the local fish farmers who cannot afford the high cost of chemicals and synthetic fungicides. Further *in vivo* studies are needed,

which could reduce the severe losses due to saprolegniosis in eggs and larvae of cold water fish during breeding and rearing.

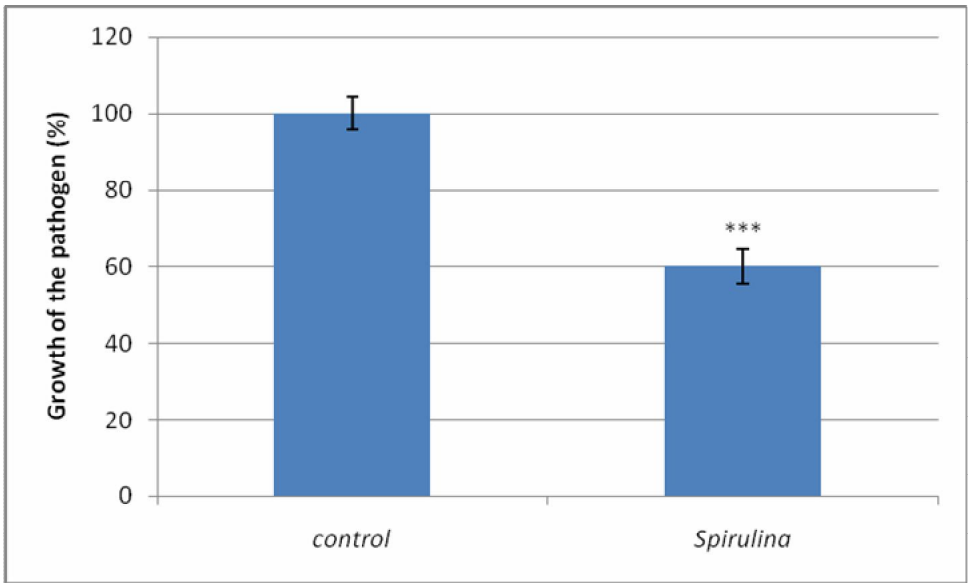


Figure 6. Growth of the *S. parasitica* by treatment with ethanol extract of *Spirulina* sp. and control variant (***p < 0.001).

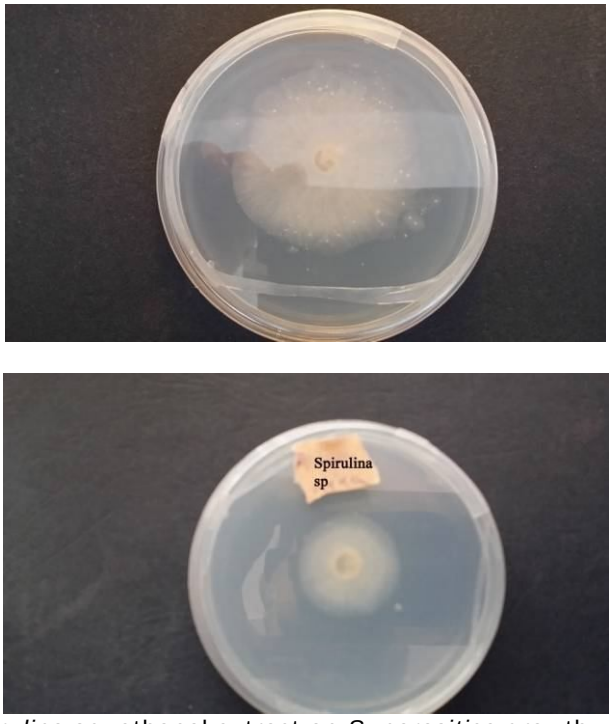


Figure 7. Effect of *Spirulina* sp. ethanol extract on *S. parasitica* growth, versus control variant.

**Conclusions**. For the first time an *in vitro* test of the inhibition effect of *Lemna minuta* on the fish pathogen *Saprolegnia parasitica* was conducted. The methanol extracts from the three tested species *Lemna minuta*, *Chlorella vulgaris*, *Spirulina* sp. did not show activity against the fish pathogen *S. parasitica*. For ethanol extracts of the plants we found a very good inhibitory effect on the studied pathogen. Of the three extracts studied, the strongest inhibitory effect was observed in *C. vulgaris*.

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