In vitro antibacterial effect of *Lemna minuta*, *Chlorella vulgaris* and *Spirulina* sp. extracts against fish pathogen *Aeromonas hydrophila*

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Abstract. The aim of the present study was to test extracts from *Spirulina* sp., *Chlorella vulgaris* and *Lemna minuta* against fish pathogen *Aeromonas hydrophila.* The water extract of these three aquatic plants were prepared. The plants were 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. The bacterial strain of *Aeromonas hydrophila* (ATCC 7965) was used in current research. The bacterial activities of plant extract against *A. hydrophila* were tested with disk diffusion method. The aqueous extracts of all three test plants showed a good inhibitory effect against the fish pathogen. From the in vitro test conducted it was observed that the aqueous extract of *C. vulgaris* showed the highest zone of inhibition against *A. hydrophila* (12 mm).
Key Words: antibacterial, fish pathogen, microalgae, *Lemna*.

Introduction. The species of genus *Aeromonas* are widely distributed in different types of waters - river, marine, drinking, waste. These pathogens are isolated from hydrobionts, meat, milk, vegetables (Havelaar et al 1992). They cause gastroenteritis in humans. *Aeromonas hydrophila* strains are hemolytic, cytotoxic and enterotoxigenic (Orozova et al 2008). It can also contaminate drinking water at deficiencies in the treatment facilities or at an insufficient level of purification. *A. hydrophila* is considered opportunistic pathogens and generally found in the gastrointestinal tract of fish (Yavuzcan-Yildiz et al 2005). When environmental conditions change, such as rapid temperature changes, water quality degradation, stress, overcrowding, high levels of nitrates and carbon dioxide, the bacteria begin to cause disease (Lakshmanaperumalsamy et al 2005). *A. hydrophila* is found in various freshwater and marine fish species. It is isolated from the heart, skin, liver, kidneys and gills of sick fish (Adanir & Turutoglu 2007). Upon penetration into the body of the fish, the pathogen spread by blood to the organs. It produces enterotoxin, which causes cell death and eventual tissue destruction (Al Laham & Al Fadel 2014). These pathogens cause hemorrhagic septicemia, mortality and deterioration in product quality (Hettiarachchi & Cheong 1994). According to different studies, the fish is most obsessed with microorganisms of the genus *Aeromonas* (Janda & Abbott 1998; Sharma & Kumar 2011). *A. hydrophila* is resistant to low temperatures, which is important in storing food. Therefore, it is necessary to find safe and effective factors that have antimicrobial effect and suppressing the growth of this pathogen. In recent years, various plant extracts have been successfully used to treat different pathogens in aquaculture (Bansemir et al 2006; Turker et al 2009). The aim of the present study was to test extracts from *Spirulina* sp., *Chlorella vulgaris* and *Lemna minuta* against fish pathogen *A. hydrophila.*

Material and Method. *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 May 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.

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 strain of fish pathogen *Aeromonas hydrophila* ATCC 7965 was used in tests. The suspension of bacteria *A. hydrophila* with concentration 1.2 x 10^6 CFU mL^{-1} was spread on petri dishes contained Mueller-Hinton agar. Disc diffusion method for determination of inhibition of fish pathogen *A. hydrophila* from extracts of different aquatic plants was used. Prior in vitro tests sterile discs (Himedia) with 6 mm diameter were impregnated with different plant extracts and were placed with sterile pincers on sterile media. For 48 hours at 28°C the petri dishes were incubated. Antibacterial activity of extracts was determined by measuring the diameter of inhibition zones. As a control variant the disc impregnated with distilled water was used.

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

Results. In the literature review, we did not find a study of *L. minuta* extract against fish pathogen *A. hydrophila*. Our in vitro tests with aqueous extract of *L. minuta* show good inhibition effect against this pathogen, and the diameter of its inhibition zone was 11 mm and the differences was statistically proven ($p < 0.05$) (Figure 1). This is due to the fact that aquatic plants contain many biologically active metabolites. Das et al (2012) established that ethanol extract of another duckweed *Spirodela polyrrhiza* showed promising antibacterial activities against *A. hydrophila* (10-20 mm zone of inhibition). Our study has shown that the *L. minuta* aqueous extract has an inhibitory effect against a fish pathogen and it is useful to explore other species of the genus with different extracts.

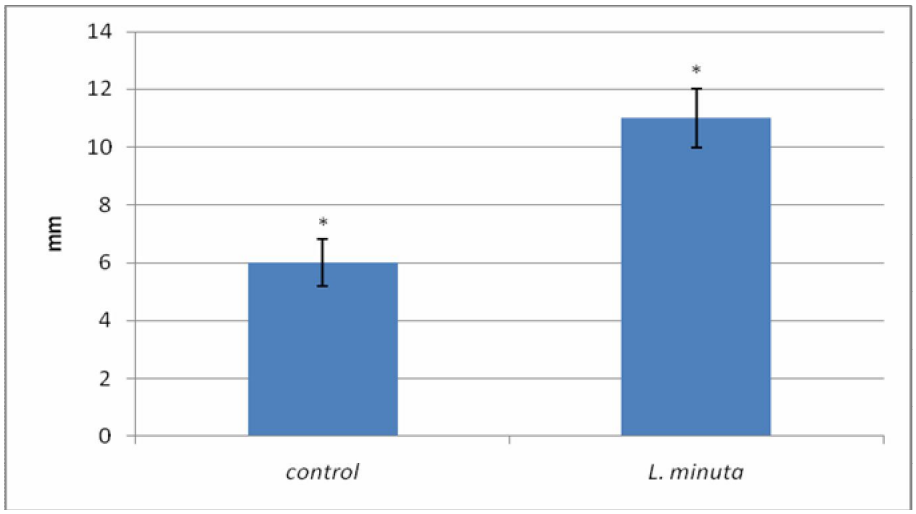

Figure 1. Diameter of inhibition zone in control and *L. minuta* extract ($p < 0.05$).

Algae live in ever-changing and extreme environmental conditions. Therefore they need to quickly and efficiently adapt and produce secondarily biologically active metabolites in response to this (Rodríguez-Meizoso et al 2010). The diseases have increased in recent years due to heavy infections and the pathogenic bacteria becoming resistant to drugs because of the indiscriminate use of antibiotics (Sirakov et al 2019). It is necessary to develop new alternatives to antibiotics due to decreased efficiency and resistance of

pathogen to them (Velichkova et al 2018). The use of natural products in disease control is recommended. They can be used against various types of pathogenic organisms. Mudimu et al (2014) analyzed 97 microalgae cultures and established that the highest bioactive effect of microalgae was inhibition of 70% against the gram-positive bacteria. The second highest bioactive effect was inhibition of 60% against the gram-negative bacteria. *Chlorella* sp. had antibacterial activity against bacteria and fungi (Chowdhury et al 2015). For antibacterial activities of *C. vulgaris* against various strains of fish and shellfish pathogens reported some authors (Pradhan et al 2004; Shabanzad et al 2017). These results are in agreement with that of our current experiment. Das & Pradhan (2010) found that aqueous extract of *C. vulgaris* showed strong antibacterial activity from 11 to 15 mm against *A. hydrophila*. In our study aqueous extract from this green microalga was with the greatest diameter of inhibition zone (12 mm) compared to other aquatic plants against the same fish pathogens (Figure 2).

Shabanzad et al (2017) found the diameter of inhibition zone of *C. vulgaris* hexane extract against *A. hydrophila* was 9.6-11 mm. This is with 8.3% lower inhibitory effect compared to the *Chlorella* aqueous extract in our study.

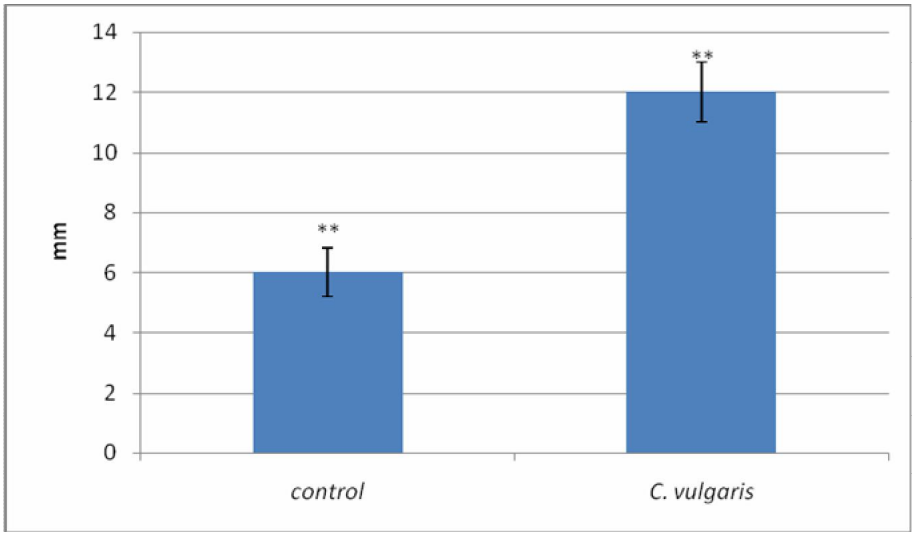

Figure 2. Diameter of inhibition zone in control and *C. vulgaris* extract ($p < 0.01$).

Mian et al (2003) reported antibacterial activities from freshwater Cyanobacteria. The extracts from *Spirulina* have been tested and used as alternatives to antibiotics to enhance the survival and immune competence of cultured aquatic animals (Devi et al 2016). Das & Pradhan (2010) found that aqueous extract of *Spirulina platensis* showed strong antibacterial activity from 8 to 10 mm against *A. hydrophila*. The aqueous extracts of *Spirulina* in this study showed 11 mm inhibition effect against the same pathogen and the differences was statistically proven ($p < 0.01$) (Figure 3). According to Das & Pradhan (2010) some algal extracts have better antibacterial activity than some antibiotics. The contained bioactive compounds in aquatic plants could become good agents for disease control in aquaculture.

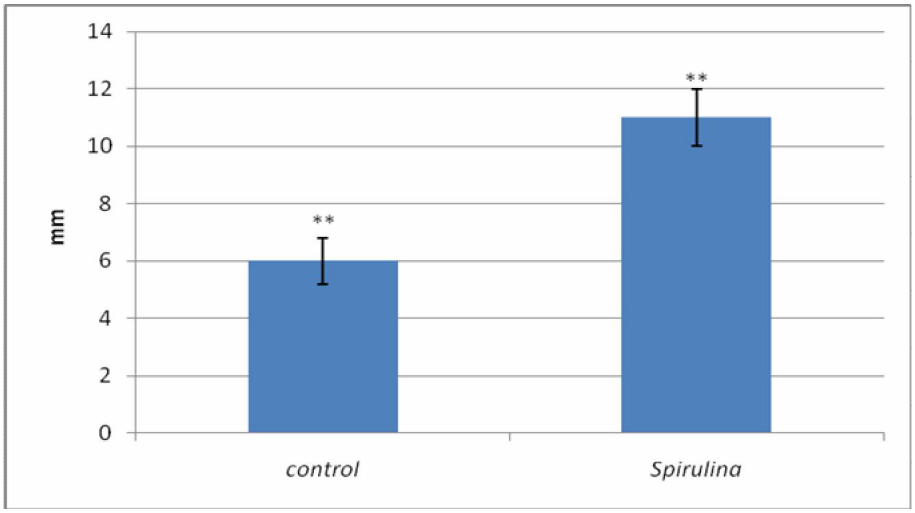

Figure 3. Diameter of inhibition zone in control and *Spirulina* extract ($p < 0.01$).

Conclusions. The aqueous extracts of *Spirulina* sp., *Chlorella vulgaris* and *Lemna minuta* showed a good inhibitory effect against the fish pathogen. The impact of the *L. minuta* extract against *A. hydrophila* was tested for the first time. *L. minuta* extract had an 11 mm diameter of inhibition zone against this fish pathogen. From the *in vitro* test conducted it was observed that the aqueous extract of *C. vulgaris* showed the highest zone of inhibition against *A. hydrophila* (12 mm).

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