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*Original paper*

# *In vitro* test of inhibition effect of extracts from three seaweed species distributed at Black sea on different pathogens potentially dangerous for aquaponics

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## Abstract

Aquaponics is innovative recirculation system where hydrobionts are cultivated together with the plants. The possibilities for control of pathogens in these systems are highly restricted. One possible strategy for inhibition of pathogenic microorganisms in aquaponics is the usage of seaweeds extracts. The study connected with the investigation of inhibition effect of seaweeds from Bulgarian Black sea cost on different pathogens is rare. The aim of current study was to test In vitro the inhibition effect of three seaweed species (Ulva rigida, Cladophora vagabunda, and Ceramium rubrum) distributed at Black sea in front of Bulgarian cost on different pathogens (bacteria and fungi) which are potentially harmful to hydrobionts, plants and consumer of aquaponics products. The ethanol and methanol extracts from investigated seaweed species were prepared. They were tested with agar well diffusion method against the following fish, food borne and plant pathogens: Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, Salmonella typhimurium, Candida albicans, Penicillium verrucosum var. verrucosum, Fusarium graminearum, Fusarium moniliforme and Aspergillus ochraceus. The current study showed that the following extracts from seaweeds distributed in front of Bulgarian Black sea cost possess high inhibition effect (size of inhibition zone higher than 10 mm) against potentially pathogenic microorganisms in aquaponics: ethanol extract of C. vagabunda against B. cereus and A. ochraceus, methanol extract of C. vagabunda against C. albicans, ethanol extract of C. rubrum against E.coli, B. cereus and C. albicans.

**Keywords** : Aquaponics, in vitro tests, inhibition effect, seaweeds, pathogens

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# Introduction

Earth's population will continue to grow up to 9 billion by 2050 (UN, [1]). In order to meet the needs of the continuously growing population, it is necessary to develop technologies that allow a significant proportion of food products to be produced directly in the cities. Thus will reducing the pollution of the environment and lowering transportation costs. One such technology, which allows the cultivation of hydrobionts and plants to be integrated into the urban environment, is aquaponics (GRABER & al. [2]).

Along with the many advantages that this technology possess, one of its disadvantages is that the pathways for reducing the pathogenic microorganisms in it are restricted as the use of pesticides can lead to the toxic effects in the cultivated hydrobionts from one side and on the other hand, the use of the therapeutics on the hydrobionts can lead to their accumulation in plants (RAKOCY & al. [3]).

In aquaculture a large number of microorganisms are referred as a fish pathogens, or potentially dangerous for the humans i.e. S. aureus (SHAH & al. [4]), E. coli (NEWELL & al. [5]; SHIN & al.[6]), B. cereus (KOLANJINATHAN & al. [7]), S. typhimurium SHIN & al.[6]), P. aeruginosa (CHOUDHURY & [8]), C. albicans (PEREIRA & [9]). Therefore, their control in aquaponic systems is of the great importance for the aquaponics hobbyist as well as for the commercial aquaponics farms.

From the other side, the higher humidity in aquaponics greenhouses contributes to the development of pathogenic fungi (from genus Aspergillus, Fusarium, and Penicillium) which are potentially harmful to the plants and could decrease their productivity or deteriorate its commercial realization.

One method proposed by SIRAKOV & al. [10] to solve this disadvantage is the isolation of beneficial bacteria and their use in the control of pathogenic microorganisms associated with hydrobionts and plants. Another possibility is the use of synbiotics in the aquaponic systems, which can improve the immune system of the fish and the metabolism in the cultivated plants (SIRAKOV & al. [11]).

Different approach to diseases control in aquaponic systems is the use of extracts from different plants and it still remains not investigated. Numerous studies have shown the possibility of using extracts of different plants to control pathogens in hydrobionts, plants or for consumers of agriculture production. Studies investigating the possible double effect of the application of extracts from different plants on hydrobionts and plants pathogens as well as on potentially harmful for the consumer of aquaponic products microorganisms are rare.

One of the possible groups of plants that could be used in this battlefield against pathogenic microorganisms is algae. The effect of their use is strongly dependent of intraspecific variability in the production of second metabolites related to seasonal variation and location of their yield (LIMA-FILHO & al. [12]; TÜNEY & al. [13]; PÉREZ et al. [14]). Studies investigating the antimicrobial potential of seaweed from the Black Sea, in front of the Bulgarian coast on various pathogens, are very limited (KAMENARSKA & al. [15]). The antibacterial effect of the following seaweeds species collected from Bulgarian Black sea cost was tested: Bangia fuscopurpurea, Haliptilon virgatum, Corallina elongata, Gelidium spinosum, Callithamnion granulatum, Ceramium diaphanum var. elegans, Chondrophycus papillosus, Laurencia coronopus, Polysiphonia denudate, Polysiphonia denudate f. fragilis, Stilophora tenella, Punctaria latifolia, Punctaria plantaginea, Colpomenia peregrina, Scytosiphon lomentaria, Zanardinia prototypus and Cystoseira crinite (KAMENARSKA & al.[15]) .

The aim of current study was to test in vitro the inhibition effect of three seaweed species (Ulva rigidaC. Agardh, Cladophora vagabunda (L.) C. Hoek,and Ceramium rubrum (Huds.) C. Agardh) distributed at Black sea in front of Bulgarian cost on different pathogens (bacteria and fungi) which are potentially harmful to hydrobionts, plants and consumers of aquaponics products.

# Materials and Methods

*1. Preparation of Seaweed for extraction*

Three seaweeds species (Ulva rigida C. Agardh, Cladophora vagabunda (L.) C. Hoek, and Ceramium rubrum (Huds. C. Agardh) were collected from coastal areas of Black sea, south from Burgas (Tzarevo municipality, Lozenets village, Bulgaria)

(42°12'32.1"N 27°48'40.0"E). The seaweeds were determined by DIMITROVA-KONAKLIEVA [16]. They were prepared for extraction according to SUJATHA & al. [17] with slight modification. The collected seaweeds were transported to laboratory prior washing them with sea water. The remaining animal castings, sand particles attached debris were removed by soaking them in tap water, followed by rinsing with sterile water. Afterward, the excess water removed by blotting them on filter papers and dried on shade in an air-conditioned room for one week at 20°C and cut into small pieces. They were dried in an oven at 37ºC and powder of them was prepared with the help of electric grinder.

*2. Extraction procedure*

The process of extraction was made by soaking algae's powder in 100% ethanol solvent (1:10 w/v) for 24 hours. The received materials were concentrated in a rotary evaporator to reduce the volume. The received extracts were filtered through a syringe filter with 0.2 µm pore size and collected in plastic containers. The containers were placed at refrigerator until antimicrobial tests to be started. The same procedure was used for the preparation of methanol extracts of seaweeds. The concentration of the methanol used for the extraction procedure was 100%.

*3. Tested pathogens*

In the current study the antibacterial activity of seaweed's extracts against the following potentially harmful for fish and humans pathogens were tested: referent bacterial strains-Staphylococcus aureus ATCC25923, Escherichia coli ATCC25922, Pseudomonas aeruginosa ATCC 27853 and clinical isolates - Bacillus cereus, Salmonella typhimurium, and Candida albicans. The strains were kept at refrigerator at -20°C. Prior to their use in inhibition tests, they were recovered at tryptic soy blood agar (Himedia, India).

The plant's mould strains examined were Penicillium verrucosum var. verrucosum 2003 NRRL F-143, Fusarium graminearum 2294 IMI 155426, Fusarium moniliforme 394 Strain FN-9 and Aspergillus ochraceus 2002 IM-BAS.

*4. Test of antimicrobial activity of seaweed's extracts against pathogens*

*Antimicrobial tests with fish and foodborne pathogens*

The extracts (methanol and ethanol) were tested for antimicrobial activity by the diffusion method in wells (agar well method) (GHOSH & al. [18]) with some modifications consistent with the type of strains tested: instead of brain heart infusion agar was used Mueller Hinton agar (Himedia, India). The plates were incubated for 24 hours under aerobic conditions instead of microaerophilic. Briefly, from 24 h bacterial and yeast colonies of trypticase soy blood agar, inoculums were prepared in physiological saline corresponding to 0.5 of the McFarland standard ($1.5x10^8$cfu.mL$^{-1}$). The wells were formed with a sterile 6 mm diameter well probe at the distance of 2 cm from the center after pre-application of the inoculum with a sterile cotton swab. The wells were filled with 100 µl of the extracts. Positive control with gentamicin at a concentration of 12.5 µg.mL$^{-1}$ and negative with the respective solvents was performed. The wells were incubated at 37°C for 24 hours under aerobic conditions. Antimicrobial activity was assessed in the presence of a growth suppression zone of ≥ 8.0 mm (MOHAMMADI-SICHANI & al. [19]).Tests were performed three times to determine the reproducibility of the results.

*5. Antimicrobial tests with plant pathogens*

The methanolic and ethanolic plant extracts were screened for antifungal activity by agar well diffusion method (PEREZ & al. [20]). The 72 hours old fungal cultures were grown on potato glucose agar (glucose 20.0 g, potatoes 200.0 g, yeast extract 2.0 g, agar 20.0 g, pH 5.6). 20 mL of potato glucose agar was poured in every Petri dish. After solidification, 0.1 mL inoculum of the fungal strains (1-2 x $10^4$ CFU.mL$^{-1}$) was introduced on the surface of the agar plate and the wells were made by using sterile cork borer of size 6.0mm. 0.1ml of methanolic and ethanolic plant extracts were introduced in the wells. An incubation period of 3-7 days at $23^0$ C was maintained for observation of antifungal activity of plant extracts. The antifungal activity was evaluated by measuring zones of inhibition of fungal growth surrounding the plant extracts in the wells. The zones of inhibition were

measured in mm and the experiment was carried out in triplicates. Antifungal activity was assumed in the presence of aninhibition zone ≥ 8.0 mm. The complete antifungal analysis was carried out under strict aseptic conditions. Methanol and ethanol were used as a negative solvent control.

*6. Data analysis*

The antimicrobial tests were conducted in a completely randomized design with three repetitions, for each treatment. The received data were analyzed by ANOVA single factor at a significance level of $P<0.05$. The analyses were made by using the SPSS program.

## Results and Discussions

*1. Antimicrobial tests with fish and foodborne pathogens*

The current In vitro test showed advantageously low and moderate inhibition effect of tested seaweeds extracts (U. rigida, Cl. vagabunda, and C. rubrum) against fish and foodborne pathogens (Table 1). The ethanol and methanol extracts of U. rigida showed lower than 10 mm inhibition zone in antagonistic tests against tested pathogens (Table 1). They showed the highest inhibition effect against fish pathogen S. aureus and it was higher with 60% than the average size of inhibition zone found out for the control variant. The ethanol and methanol extracts of Cl. vagabunda showed the highest inhibition effect against B. cereus and C. albicans and the inhibition zones were higher respectively with 71.6% and 103% compared with the sizes of inhibition zone found for the control variants. The sizes of inhibition zone of ethanol extract of C. rubrumin In vitro tests with pathogens E.coli, B. cereus and C. albicans was higher respectively with 68.1%, 66.6%, and 84.3% compared with the size of inhibition zone in control variant where only ethanol's inhibition effect was tested.

The positive control showed the higher inhibition effect of Gentamicin against tested fish and foodborne pathogens compared with the tested seaweeds extracts except for S. typhimurium where the ethanol extract of Cl. vagabunda showed the highest inhibition effect (8.6±0.4) (Table1).

**Table 1.** The diameter of inhibition zone of antimicrobial screening of algae extracts determined by theagar diffusion method

| Test algae extract | *S. aureus* | *E. coli* | *B. cereus* | *S.typhimurium* | *P. aeruginosa* | *C.albicans* |
|---|---|---|---|---|---|---|
| ***Ulva rigida* EtOH** | 9.6±1.15* | 9.3±0.5* | 8.3±0.5* | 8±0.5* | 8±0.5* | 9±0.5* |
| ***Ulva rigida* MeOH** | 9.6±1.15* | 8±0.2* | 8±0.5* | 7±0.7$^{ns}$ | 8.06±0.15* | 9.1±0.1* |
| ***Cladophora vagabunda*EtOH** | 9.6±0.5* | 9.3±0.5* | 10.3±0.5* | 8.6±0.4* | 8.3±0.5* | 9.3±1.15* |
| ***Cladophora vagabunda* MeOH** | 9.5±0.5* | 8.1±0.1* | 7.9±0.45* | 7.4±0.5* | 7.1±0.1* | 12.2±0.8* |
| ***Ceramium rubrum* EtOH** | 9.3±0.5* | 10.1±0.4* | 10±0.3* | 7.9±0.2* | 8.2±0.2* | 11.06±0.2* |
| ***Ceramium rubrum* MeOH** | 7.9±0.4* | 7.7±0.3 | 6.2±0.2$^{ns}$ | 6.1±0.1$^{ns}$ | 8.1±0.1* | 9.03±0.3* |
| ***EtOH*** | - | - | - | - | - | - |
| ***MeOH*** | - | - | - | - | - | - |
| ***Gentamicin*** | 21.1±0,4* | 17.3±0,2* | 23.0±0,4* | - | 15±0,2* | |

Diameter of inhibition zone (mm) against plant's mould (Staphylococcus aureus ATCC25923, Escherichia coli ATCC25922, Bacillus cereus (clinical isolate), Salmonella Typhimurium (clinical isolate), Pseudomonas aeruginosa ATCC 27853) and fungal strain Candida albicans (clinical isolate), EtOH=Ethanol, MeOH=Methanol; diameter of inhibition zone equal to 6 mm =absence of inhibition effect (-), diameter of inhibition zone in range from 6 mm to 8.5 mm=low inhibition effect, diameter of inhibition effect in range from 8.6 mm to 10mm=moderate inhibition effect; diameter of inhibition zone equal or higher than 10 mm =higher inhibition effect;
Asterisk (*) denotes a significant different at P<0.05, ns Difference not statistically significant;

*Antimicrobial tests with plant pathogens*

The inhibition effect of tested seaweeds extracts against plant's pathogenic fungi was lower than this found out for fish and foodborne pathogens (Table 2). The highest inhibition effect was found out for ethanol extract of Cladophora vagabunda against Aspergillus ochraceus and the inhibition zone was higher with 68.3% compared with the size of inhibition zone in the control variant. The moderate inhibition effect was also observed in tests conducted with the ethanol extracts of Cladophora vagabunda and C.rubrum respectively against Aspergillus ochraceus and Fusarium moniliforme (Table 2).

**Table 2.** Inhibition of mycelial growth of different plant pathogens affected by different herbal extracts

| **Test algae extract** | ***Aspergillus ochraceus*** | ***Fusarium moniliforme*** | ***Fusarium graminearum*** | ***Penicillium verrucosum*** |
|---|---|---|---|---|
| ***Ulva rigida* EtOH** | - | - | - | 7,1±0.1* |
| ***Ulva rigida* MeOH** | - | - | 7±0.3$^{ns}$ | - |
| ***Cladophora vagabunda* EtOH** | 10.1±0.1* | 7.5±0.5$^{ns}$ | - | - |
| ***Cladophora vagabunda*MeOH** | 8.5±0.3* | 9±0.4* | 8±0.2* | 7±0.1* |
| ***Ceramium rubrum* EtOH** | 9±0.1* | - | - | - |
| ***Ceramium rubrum* MeOH** | 8±0.2* | - | - | - |
| ***EtOH*** | - | - | - | - |
| ***MeOH*** | - | - | - | - |

Diameter of inhibition zone (mm) against plant's fungi strains (Penicillium verrucosum var. verrucosum 2003 NRRL F-143, Fusarium graminearum 2294 IMI 155426, Fusarium moniliforme 394 Strain FN-9 and Aspergillus ochraceus 2002 IM-BAS.), EtOH=Ethanol, MeOH=Methanol; diameter of inhibition zone equal to 6 mm =absence of inhibition effect (-), diameter of inhibition zone in range from 6 mm to 8.5 mm=low inhibition effect, diameter of inhibition effect in range from 8.6 mm to 10mm=moderate inhibition effect; diameter of inhibition zone equal or higher than 10 mm =higher inhibition effect; Asterisk (*) denotes a significant different at P<0.05, ns Difference not statistically signifcant;

Our study showed the low antagonistic activity of methanol and ethanol extracts of Ulva rigida against tested pathogens and received results were similar to the results received from TÜNEY & al. [13] where the same seaweed species from Aegean sea were tested for the antimicrobial activity. The current tests were conducted with a dry matter of seaweeds which is distinguished with lower antagonistic effect against pathogens compared to the higher antimicrobial activity found out in a fresh matter of seaweeds possible due to the lower quantity of volatile compounds or fatty acids in the samples according to TÜNEY & al. [13]. We did not find differences in antimicrobial activity in an extract of Ulva rigida when different solvents were used (methanol and ethanol). TÜNEY & al. [13] found strong inhibition effect

against different pathogens when diethyl ether was used as a solvent resulted probably in different content or quantity of active substances which were extracted during the extraction process.

The current test with Cladophora vagabunda showed high antagonistic effect against bacterial pathogen B. cereus and pathogenic fungus C. albicans and A. ochraceusand moderate antagonistic effect against pathogenic micro-organisms S. aureus, E. coli, S. typhimurium and Fusarium moniliforme. Our study is in confirmation of research made by HORINCAR & al. [21] which determined the antagonistic effect of C. vagabunda collected from the Romanian Black sea against S. enteritidis, B. subtilis, and E.coli. The same authors explored the volatile composition and fatty acids content and found that the main volatile components presented in C. vagabunda are hexanal (11.2%), octane (9.8%), nonanal (6.7%), octanal (6.7%), 2,5,5-trimethyl-2-hexene (4.7%), 3-hexen-2-one(4%), and o-cymene (3.6%) and fatty acid composition was composed generally of palmitic and arachidonic acids. HORINCAR & al. [21] found out that MUFAs and PUFAs content in C. vagabunda was 42%.

Ethanol extract of Ceramium rubrum showed a high antagonistic effect against E.coli, B. cereus and C. albicans during conducted from us In vitro tests. The study made by CORTÉS & al. [22] showed that the antimicrobial ability of C. rubrum primarily results from the availability of the lipophilic extract. Other study showed that the type of solvent has a primary meaning for antibacterial activity of C. rubrum because a different type of solvent has different antimicrobial capabilities and it was demonstrated that that methanol extract from C. rubrum seaweed had a higher inhibitory effect than nonpolar n-hexane extract (DUBBER & al. [23]). In our study, the ethanol's extract showed a higher antagonistic effect in C. rubrum.

Another factor influenced antimicrobial activity in seaweeds is the geographical location (HELLIO & al. [24]). The microbiological investigation of antagonistic activity of seaweeds in front of Bulgarian cost are limited (KAMENARSKA & al. [15]) and present commercial interest.

The antagonistic effect of seaweeds against pathogenic microorganisms is promoted mainly from the content of two group compounds- volatile compounds and fatty acids. Inhibition effect of volatile compounds is connected with the significant changes in membrane permeability in cells of pathogens (TROMBETTA & al. [25]). It is already known that polyunsaturated fatty acids could kill pathogenic microorganisms by disrupting their cell membrane, leading to cell lysis (LE et al. [26]). These characteristics made from seaweeds possible object for biological treatment of pathogens in such multifunctional system as the aquaponics where the fish and plants are presenting.

## Conclusions

The current study showed that the following extracts from seaweeds distributed in front of Bulgarian Black sea cost possess high inhibition effect against potentially pathogenic microorganisms in aquaponics: ethanol extract of C. vagabunda against B. cereus and A. ochraceus, the methanol extract of C. vagabunda against C. albicans, the ethanol extract of C. rubrum against E.coli, B. cereus, and C. albicans. The future study connected with different doses and the ways of application of seaweeds extracts possess antimicrobial activity should be tested in vivo in the aquaponics.

## Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

## Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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