Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Food Science and Technology
Open Access

Antibacterial Activity of *Ulva Lactuca* Against Important Aquaculture Bacterial Strains

Sofia PAPPOU*, Michail Angelos VALSAMIDIS, John BATJAKAS, Vasileios BAKOPOULOS

Department of Marine sciences, University of Aegean, University Hill, Lesvos Island, 81100 Mytilene, Greece
** Corresponding author: S. Pappou e-mail: mard18005@marine.aegean.gr*

SHORT COMMUNICATION

Abstract

Efforts are made to produce functional aquaculture diets capable of promoting fish growth and health while being sustainable at social, economic and environmental levels. One of the emerging threats in aquaculture has become the antibiotic resistance phenomena due to antimicrobial drugs. Therefore, functional feed additives of marine origin have been introduced globally as an alternative to fish antibiotics. Towards the selection of natural and sustainable resources of bioactive compounds, seaweed has been proven to be a source of valuable substances showing antibacterial activity. Aim of this study is to evaluate the inhibition of bacterial growth caused by an ethanolic extract of the macroalgae *Ulva lactuca* against important aquaculture bacterial strains. *Vibrio anguillarum* O1, *Photobacterium damselae* sub. *piscicida* and *Tenacibaculum maritimum,* were incubated for 48 hours in sterile Brain Heart Infusion Broth and tested for resistance to the extract using broth cultures. The algal extract successfully inhibited the growth of all strains. The optimum inhibition was achieved for VAO1 and PHDP by the undiluted and 1:1 diluted extract, while for TMAR, by the 1:3 dilution as well. The antibacterial activity that the extract provided is considered to be the result of the containing bioactive compounds of the algal strain, such as polysaccharides, carotenoids and phenolic compounds.

Keywords: algal extracts; antibacterial activity; aquaculture; fishfeed additives

Received: 15 September 2023
Accepted: 10 November 2023
Published: 15 November 2023

DOI:
10.15835/buasvmcn-fst:2023.0027

INTRODUCTION

Optimization of aquafeeds is a subject of particular interest for the aquaculture sector, and efforts have been made to produce novel functional diets capable of promoting both fish growth and health while being sustainable at social, economic and environmental levels (Ferreira et al., 2021). Infectious diseases are recognized to be one of the most frequent and devastating problems in aquaculture. Antibiotics represent an important tool to prevent infectious disease outbreaks and to reduce economic losses associated with illness, but the expansion of fish farming has induced an increasing trend in the consumption of antibiotics around Europe (Lagana et al., 2011). Therefore, one of the most serious emerging threats in aquaculture has become the antibiotic resistance phenomena triggered by the selective pressure on bacterial populations played by antimicrobial drugs. While most research focusing on antibiotic resistance concerns bacteria of clinical relevance, only a few studies have addressed environmental isolates of bacterial species such as *Vibrio* spp. and *Photobacterium damselae* subsp. *piscicida*, recognized as the etiological agents of the most widespread and devastating diseases in Mediterranean aquaculture such as vibriosis and pasteurellosis (Lagana et al., 2011). Towards the selection of natural and sustainable resources of compounds exhibiting antimicrobial activities, research has been focused on organisms of marine origin (Afzal et al., 2022). Seaweed has been proven to be a potential source of

antibacterial compounds towards both Gram-negative or Gram-positive pathogenic bacteria. Extracts of seaweed species belonging to Chlorophyta, Phaeophyta and Rhodophyta have been reported to exhibit a broad spectrum of both antibacterial and antifungal activities (Natrah et al., 2015). The content of bioactive compounds derived from marine algae that are responsible for the aforementioned antimicrobial activity, have been ascribed to a variety of metabolites, such as polysaccharides, polyunsaturated fatty acids, phlorotannins and other phenolic compounds, as well as carotenoids (Rizzo et al, 2017; Afzal et al., 2022; Silva et al., 2020).

One of the most important factors that cause variations in the level of antibacterial activity is the method of the extraction and the location of the bioactive compounds within the cell. A variety of solvents have been tested regarding the antibacterial activity of the algae extracts, such as methanol, ethanol, water, chlorophorm, dichloromethane, hexane, e.t.c. (Natrah et al., 2015; Silva et al., 2020). The different solvents, depending on their polarity, along with different extraction methods, provide a different efficiency in extracting the bioactive compounds that are responsible for the antibacterial effects (Rizzo et al., 2017). For example, in the study of Rizzo et al., 2017, water extracts showed broader and higher inhibitory activity than ethanol extracts against the tested pathogens. In the *in vitro* study of Ferreira et al., 2021, algae extracts were prepared in distilled water and at a low concentration, to better simulate the expected effects of algae in aquafeeds without prior physical or chemical processing, and revealed considerably high antimicrobial activities for a variety of algae species.

Although aqueous extracts have shown high antibacterial properties, which are attributed mostly to the extracted polysaccharides, many of other natural antimicrobial compounds are not water-soluble. For that reason, in the current study, the selected extract was produced using a mixture of ethanol and water 70:30 v/v as a solvent, in order to be evaluated according to its antibacterial activity. The extraction conditions were the result of previous research and process optimization, so as to maintain the optimum antioxidant activity through the extraction of both phenolic compounds and carotenoids' content of *Ulva lactuca* (Pappou et al., 2022).

Macroalgae of the *Ulva* species have been previously shown to provide antimicrobial properties against a wide range of pathogenic strains such as *Edwardsiella tarda, Escherichia coli, Pseudomonas fluorescens, Salmonella typhi,* and *Staphylococcus aureus,* while using solvent systems of methanol, ethanol and chloroform (Rebecca, 2012; Avila-Romero et al., 2023). In addition, water-soluble polysaccharides of the *Ulva* species have been tested with promising results against important bacterial pathogens such as *Photobacterium damselae* subsp. *damselae*, and *Vibrio anguillarum* amongst many others (*A. salmonicida* subsp. *Salmonicida, A. hydrophila, V. cholerae* and *V. harveyi*), by Rizzo et al., 2017. Within that study, it was suggested that the results were attributed to the polysaccharides' ability to disrupt the bacterial cell wall and membrane, leading to the dissolution of the protein and the leakage of essential molecules, resulting in cell death and therefore provide antimicrobial action.

Vibrio anguillarum and *Photobacterium damselae* subsp. *piscicida* represent important bacterial pathogens for various marine fish species and in particular the European sea bass, causing severe economic losses in aquaculture production. *V. anguillarum* is the causative agent of vibriosis, often described as an opportunistic infection, occurring either as lethal and acute haemorrhagic septicaemia or as subacute/chronic disease. *Photobacterium damselae* is the aetiological agent of pasteurellosis orphotobacteriosis, a deadly septicaemic disease evolving in chronic formation of granulomas in internal organs due to bacterial accumulation (Mosca et al., 2014). Photobacteriosis also causes serious economic losses in various wild and farmed fish species, while susceptibility to this disease has been reported in newly hatched sea bream larvae causing mortalities of 50-100% (Hanif et al., 2004). Meanwhile, bacteria of the genus *Tenacibaculum* cause tenacibaculosis, which includes lesions on the body, necrosis, frayed fin, tail rot, eroded mouth, and sometimes necrosis on the gills and eyes. The disease can lead to mortality and can leave afflicted species susceptible to secondary infections from the open lesions (Ferreira et al., 2021). The disease is currently widely spread in Europe and poses a threat to the culture of a wide range of marine fish species.

In the recent study of Ferreira et al., 2021, two micro- (*Nannochloropsis oceanica* and *Chlorella vulgaris*) and two macroalgae (*Gracilaria gracilis* and *Ulva rigida*), were tested for their antimicrobial activity against a variety of farmed fish pathogenic bacteria (i.e. *Vibrio anguillarum, V. harveyi, V. parahaemolyticus, Aeromonas hydrophila, Yersinia ruckeri, Edwardsiella tarda*, PHDP and *Tenacibaculum maritimum*). The single algae, as well as their blends displayed bactericidal and bacteriostatic activities against most of the tested pathogenic bacteria, with the most promising results being observed against *T. maritimum*, producing 40–45% of activity (Ferreira et al., 2021).

Within the present study, the three bacterial strains of *Vibrio anguillarum* O1 (VAO1), *Photobacterium damselae* subsp. *piscicida* (PHDP) and *Tenacibaculum maritimum* (TMAR) were used in order to evaluate the inhibition activity of a certain ethanolic extract of high antioxidant activity derived from the green macroalgae *U. lactuca*.

MATERIALS AND METHODS

Algal Extract

The *U. lactuca* extract was obtained according to previous research and process optimization (Pappou et al., 2022), using the classic extraction method with maceration for 24h at 25°C. Ethanol/water 70:30 v/v was used as a solvent, at 1:10 w/v biomass to solvent ratio. The supernatant was then concentrated in a rotary evaporator for

the removal of ethanol resulting in the final extract concentration of 50mg/ml. The subsequent aqueous extract was sterilized using Whatman filters (FP 30/0.45μm). The filtrated extract was diluted in sterile conditions using 2% NaCl at 1:7, 1:3, and 1:1 v/v, according to Table 1.

Bacterial cultures

Three aquaculture pathogen bacterial strains, namely *Vibrio anguillarum* O1 (VAO1), *Photobacterium damselae* sub. *piscicida* (PHDP) and *Tenacibaculum maritimum* (TMAR), were obtained from the Laboratory of Ichthyology, Aquaculture and Fish Diseases of the Aegean University and was incubated for 48 hours in sterile Brain Heart Infusion Broth (BHIB) prepared according to PanReac-AppliChem.

In vitro inhibition study

All the bacterial strains were tested for resistance or sensitivity to the different dilutions of the algae extract using broth cultures. Broth micro- or macro-dilution is one of the most basic anti- microbial susceptibility testing methods (Balouiri et al., 2016). Each dilution of the algae extract was added to sterile BHIB at a 1:4 v/v ratio, in order to create five groups of samples according to the level of extract addition, namely 100%, 50%, 25%, 12.5% and 0% as a positive control (Table 1). 50μl of each bacterial culture were then added to each sample in triplicates and incubated for 5 days at room temperature (25°C). During the incubation period the optical density (OD) of each sample was measured at 610nm every 24hours using a HITACHI U-2900 spectrophotometer. Results were finally analyzed using IBM SPSS statistics.

Table 1. Dilution levels of the *U. lactuca* ethanolic extract used that was used in the *in vitro* inhibition study

Dilution Groups	Broth (ml)	*U. lactuca* extract (ml)	2% NaCl (ml)	Dry Ulva extract (mg)	% Extract in the bacterial culture
100%	4.00	1.000	0.000	50.0	20.0
50%	4.00	0.500	0.500	25.0	10.0
25%	4.00	0.250	0.750	12.5	5.0
12.5%	4.00	0.125	0.875	6.25	2.5
Control+	4.00	0.000	1.000	0.00	0.0

RESULTS AND DISCUSSIONS

Effect of the different *U. lactuca* extract dilutions on the bacterial growth

Figure 1 presents the inhibition activity of the *U. lactuca* extract, in four different dilution levels and their comparison to the control culture, against the bacterial strain *V. anguillarum* O1. The measurements of optical density (OD610nm) are plotted for all the five dilution groups of Table 1 against the five days' time of incubation. It is observed that the two dilution levels of 50 and 100% tend to group at the lowest levels of bacterial growth throughout the whole experimental period. Bacterial cultures of the 25 and 12.5% extract inclusion groups, tend to follow the growth pattern of the control culture in slightly smaller numbers of OD610nm, but considerably higher than the 50 and 100%.

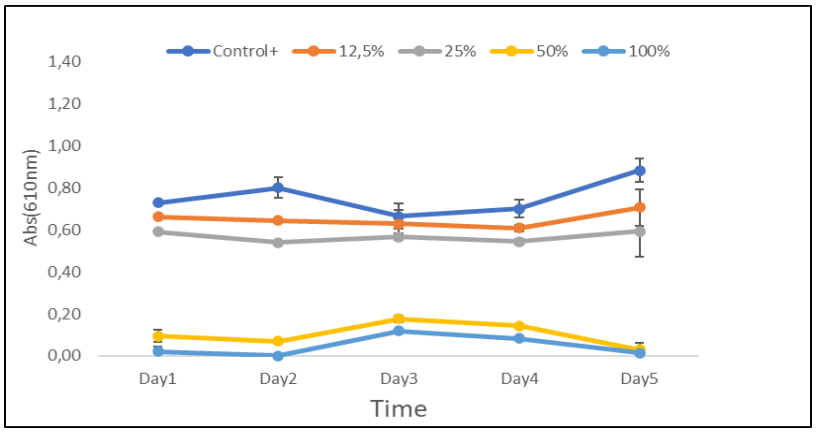

Figure 1. Optical Density (OD610nm) of *V. anguillarum* O1 cultures in the presence of different dilution levels of the ethanolic extract of *U. lactuca* for five days of bacterial growth.

Results of one-way Anova revealed that there was a statistically significant difference in VAO1 growth between at least two groups of the extract dilutions (F (4, 20) = 174.1666, p = <0.00001). Tukey's HSD Test for multiple comparisons found that the mean value of the bacterial growth was significantly different between the Control and 12.5% extract (p = 0.0468), the Control and 25% extract (p = 0.0002), the Control and 50% extract (p = 0.0000), the Control and 100% extract (p = 0.0000), the 12.5% and 50% extract (p = 0.0000), the 12.5% and 100% extract (p = 0.0000), the 25% and 50% extract (p = 0.0000), and finally the 25% and 100% extract (p = 0.0000) as described in Table 2. On the other hand, there was no statistically significant differences between the 12.5% and 25% dilutions (p=0.1571), as well as the 50% and 100% (p=0.5157).

Table 2. Tukey's HSD Test for multiple comparisons for the inhibition study of VAO1 between the different levels of *U. lactuca* extract's dilutions.

VAO1 Tukey's HSD Test	Control	12.5%	25%	50%	100%
Control		*	*	*	*
12.5%			-	*	*
25%				*	*
50%					-
100%					

*Statistical significance <0.05

The results of the inhibition activity against the bacterial strain *P. damselae* sub. *piscicida* are presented in Figure 2. It is observed that the two dilution levels of 50 and 100% tend to group also at the lowest levels of bacterial growth throughout the whole experimental period. On the contrary compared to inhibition activity of VAO1, the 25 and 12.5% dilutions, despite grouping with the control culture in higher numbers of bacterial growth, they each follow different patterns throughout the five days of incubation. The control population seems to keep a steady bacterial growth until Day3, and increase exponentially after that. Culture 12.5% starts increasing after Day2 but reaches lower OD values on Day5 compared to the control. Finally, the culture containing the 25% concentration of extract, shows an increase in bacterial growth on Day2, but exhibits a decrease on OD values for the rest of the experimental period until Day5.

Results of one-way Anova revealed that there was a statistically significant difference in PHDP growth between at least two groups of the extract dilutions (F (4, 20) = 20.02174, p = <0.00001). As described in Table 3, Tukey's HSD Test for multiple comparisons found that the mean values of the bacterial growth were significantly different between the Control and 50% extract (p = 0.00003), the Control and 100% extract (p = 0.00004), the 12.5% and 50% extract (p = 0.00004), the 12.5% and 100% extract (p = 0.00006), the 25% and 50% extract (p = 0.00254), and finally the 25% and 100% extract (p = 0.00346). No statistically significant differences were reported between the Control and 12.5% (p=0.99), the Control and 25% (p=0.27), the 12.5% and 25% (p=0.38), and the 50% and 100% dilutions (p=0.99).

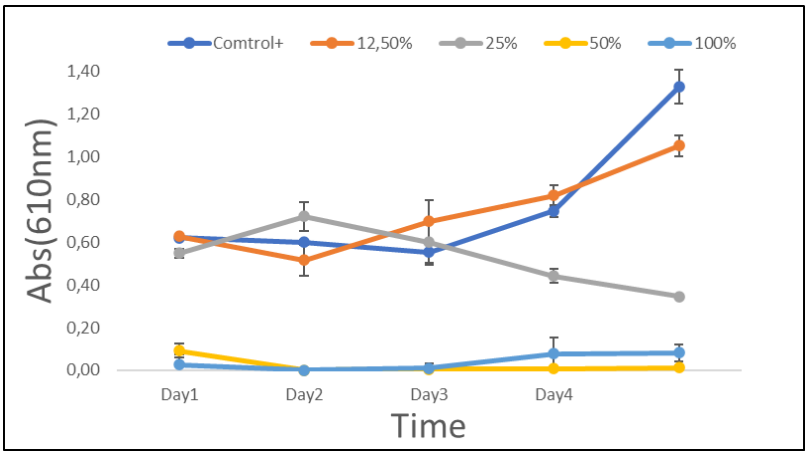

Figure 2. Optical Density (OD610nm) of *P. damselae* sub. *piscicida* cultures in the presence of different dilution levels of the ethanolic extract of *U. lactuca* for five days of bacterial growth.

Table 3. Tukey's HSD Test for multiple comparisons for inhibition study of PHDP between the different levels of *U. lactuca* extract's dilutions.

PHDP Tukey's HSD Test	Control	12.5%	25%	50%	100%
Control		-	-	*	*
12.5%			-	*	*
25%				*	*
50%					-
100%					

*Statistical significance <0.05

The results of inhibition activity regarding the *U. lactuca* extract against the bacterial strain *T. maritimum* are presented in Figure 3. It is observed that the two dilution levels of 50 and 100% of the extract tend to group at the lowest levels of bacterial growth throughout the whole experimental period as it was observed for VAO1 and PHDP, but in this case, the 25% follows also the same trend. The culture containing 12.5% of the extract, also exhibits the lowest OD as 25, 50 and 100% cultures for the first 24 hours, but begins to show bacterial growth exponentially after Day2. The control population reveals a steady growth of TMAR until reaching on day5 almost the same amounts of OD as the 12.5% culture.

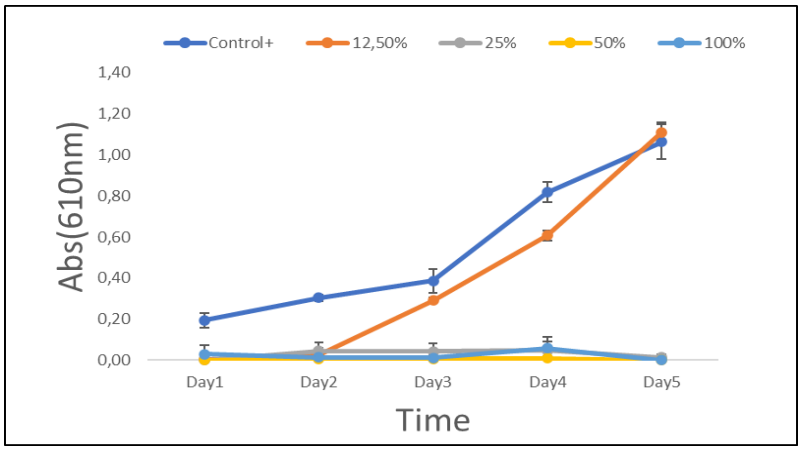

Figure 3. Optical Density (OD610nm) of *T. maritimum* cultures in the presence of different dilution levels of the ethanolic extract of *U. lactuca* for five days of bacterial growth.

Results of one-way Anova revealed that there was a statistically significant difference in PHDP growth between at least two groups of the extract dilutions (F (4, 20) = 4.7522, p = <0.0074). Tukey's HSD Test for multiple comparisons found that the mean values of the bacterial growth were significantly different between the Control and 25% extract (p = 0.0378), the Control and 50% extract (p = 0.0286), and the Control and 100% extract (p = 0.0350) as shown in Table 3.6. There were no statistically significant differences observed between groups of the Control and 12.5% (p=0.91), the 12.5% and 25% (p=0.19), the 12.5% and 50% (p=0.15), the 12.5% and 100% (p=0.18), the 25% and 50% (p=0.99), the 25% and 100% (p=0.00), and finally the 50% and 100% (p=0.99).

Table 4. Tukey's HSD Test for multiple comparisons for inhibition study of TMAR between the different levels of *U. lactuca* extract's dilutions.

TMAR Tukey's HSD Test	Control	12.5%	25%	50%	100%
Control		-	*	*	*
12.5%			-	-	-
25%				-	-
50%					-
100%					

*Statistical significance <0.05

Comparison between the inhibition activity of the three bacterial strains

The comparison of the bacterial growth of the three different strains is shown in Figure 4, specifically for the cultures containing the highest concentrations of *U. lactuca* in order to further investigate the inhibition activity of VAO1, PHDP and TMAR. The bacterial cultures of PHDP and TMAR in the presence of the undiluted *U. lactuca* extract (100%), follows the same pattern of growth during the first four days, while on Day5, PHDP reveals a slightly higher OD610nm. Although VAO1 does not exceed the bacterial growth of the other two strains throughout the incubation period, it reaches a peak of bacterial growth in the middle of the incubation period, on Day3.

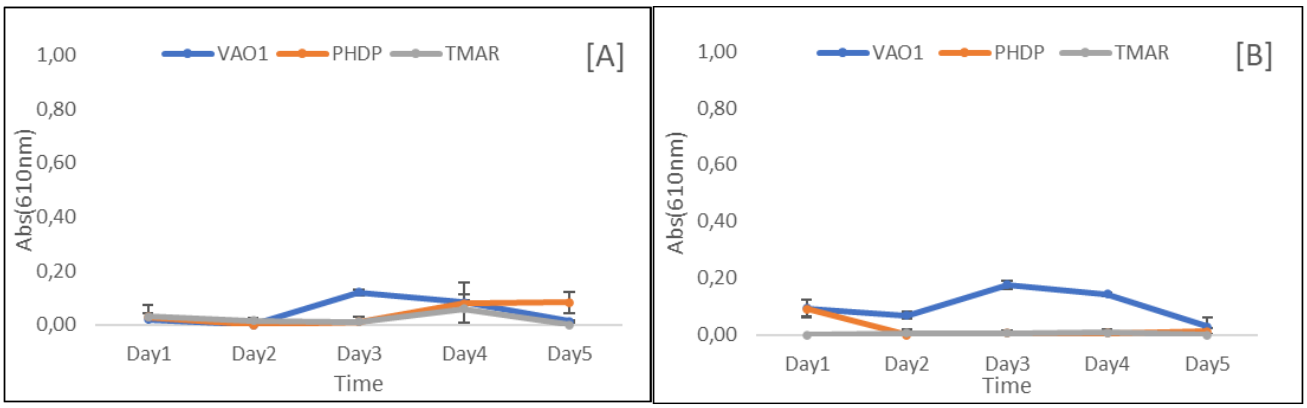

Figure 4. Optical Density (OD610nm) of *V. anguillarum* O1, *P. damselae* sub. *piscicida* and *T. maritimum* cultures in the presence of *U. lactuca* 100% [A] and 50% [B] extract, for five days of incubation.

Supporting the previous observations, as shown in Table 5, T-tests between the three bacterial strains showed no statistically significant differences between their growth in the presence of the 100% extract. Therefore, inhibition of VAO1, PHDP and TMAR growth was equally successful against the undiluted *U. lactuca* extract.

Table 5. P-values of the t-tests between the three bacterial strains in the *in vitro* study of the 100% dilution level of *U. lactuca*.

[100%] T-test P-values	VAO1	PHDP	TMAR
VAO1		0.42	0.19
PHDP	-		0.20
TMAR	-	-	

The bacterial cultures of PHDP and TMAR in the presence of the 50% *U. lactuca* extract, also follow the same pattern of growth apart from the first day of incubation where PHDP exhibits a slightly higher OD610nm. VAO1 has higher bacterial growth than the other two strains throughout the whole incubation period. As it was observed for the 100% extract, VAO1 exhibits its highest OD on Day 3. For Days 4 and 5 there is a decline in bacterial growth as well. On the contrary to the 100% extract, T-tests showed statistically significant differences in the performance of the 50% extract on the bacterial growth. More specifically, the performance of VAO1 differs significantly from both PHDP and TMAR (Table 6), supporting the aforementioned observations.

Table 6. P-values of the t-tests between the three bacterial strains in the *in vitro* study of the 50% dilution level of *U. lactuca*.

[50%] T-test P-values	VAO1	PHDP	TMAR
VAO1		***0.02**	***0.003**
PHDP	-		0.16
TMAR	-	-	

*Statistical significance

During the first 24 hours of incubation, the 100% *U. lactuca* extract was able to inhibit the growth of all three aquaculture bacterial strains equally, while for the 50% extract, the bacterial strain TMAR exhibited the lowest

growth. Overall, the *U. lactuca* extract successfully inhibited the growth of VAO1, PHDP and TMAR throughout the 5 days of incubation.

The compounds which are suggested to be responsible for the potent antibacterial activity of the algae extracts belong to a broad range of structural and functional classes, e.g., polyphenols, alkaloids, terpenes, polysaccharides, fatty acids, sterols, lactones, proteins and peptides. The *U. lactuca* ethanolic extract which was used in the particular *in vitro* study was evaluated regarding its content in phenolic compounds, carotenoids and antioxidant activity within previous study by Pappou et al., 2022. It was found to contain high amounts of chlorophylls, a wide variety of carotenoids namely all-trans-neoxanthin; 9-cis-neoxanthin; all-trans-violaxanthin; all-trans-astaxanthin; all-trans-lutein; 9-cis astaxanthin; 9-cis-lutein and phenolic compounds that were identified to be gallic acid, caffeic acid, catechin, and rutin.

Carotenoids along with chlorophylls are pigments present in algae, whose functions vary from being the central component for photosynthesis to conferring a photoprotective nature and characteristic colors to algae (Bhowmick et al., 2020). Both chlorophylls a and b have been found to inhibit the growth of different species of Streptococci, Staphylococci, and Lactobacilli, while major carotenoids, such as beta carotene and astaxanthin, have also shown considerable antibacterial activity (Bhowmick et al., 2020). In addition, according to Bhowmick et al., 2020, algal-derived polyphenols, apart from their antioxidant properties, display other potential features such as antibacterial and antifungal. Although the mechanisms of action of seaweed metabolites have not been clearly elucidated, according to earlier research, some phlorotannin antibacterial effect may be connected to their ability to integrate with microbial proteins, such as cell membranes and enzymes, and cause cell disintegration by inhibiting the oxidative phosphorylation pathway in microorganisms (Lomartire et al., 2023).

Last but not least, Lomartire et al., 2023, suggested that the cell wall, cytoplasmic membranes, and DNA may be the primary targets of polysaccharides, providing in such a way, antibacterial and antioxidant activities. In this case we know for a fact that the certain macroalgae strain that was used, reached a level of up to 50% polysaccharides of dry biomass (Pappou et al., 2022). Finally, the extraction solvent ethanol / water 70:30, was chosen not only because of the successful extraction of carotenoids and phenolic compounds, but also due to the considerable ability of water to extract polysaccharides from algae.

CONCLUSIONS

The main goal of the present study was to evaluate the inhibition activity of an ethanolic extract of the green macroalgae *U. lactuca* against bacterial strains of *Vibrio anguillarum O1 (VAO1), Photobacterium damselae subsp. piscicida (PHDP)* and *Tenacibaculum maritimum (TMAR)*. The results of the *in vitro* study showed inhibition of growth for all the important aquaculture bacteriab that were tested, although factors such as the algae cultivation conditions as well as the extraction techniques can strongly influence the antibacterial activity of the algal extracts. Therefore, these results signify the need of extraction techniques' optimization towards the optimum exploitation of algae (Silva et al., 2020). Finally, although there is evidence that macroalgae extracts are a source of antimicrobial compounds (Bhowmick et al., 2020), significant further research is required in order to understand the mechanisms by which the macroalgal substances affect the bacterial cells.

Author Contributions: B.B., S.P. Conceived and designed the analysis; S.P. Collected the data; J.B., V.B. Supervised; S.P. Performed the analysis; S.P. Wrote the paper; V.B., J.B. Revised the paper.

Funding Source: This project has received funding from the European Union's Horizon Europe Framework Programme (HORIZON) under the Marie Skłodowska-Curie grant agreement No 101086261.

Acknowledgments

The implementation of the doctoral thesis was co-financed by Greece and the European Union (European Social Fund-ESF) through the Operational Programme «Human Resources Development, Education and Lifelong Learning» in the context of the Act "Enhancing Human Resources Research Potential by undertaking a Doctoral Research" Sub-action 2: IKY Scholarship Programme for PhD candidates in the Greek Universities.

Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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