Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(3): 3329 – 3339 (2025)
www.ejabf.journals.ekb.eg

Anti-Microbial Activities of Sea Lettuce, *Ulva rigida,* Extracts Against Pathogenic Microorganisms

Gamze Turan[1*], Selin Aktar Kiremitci[2], İsmail Öztürk[3], Mesude İsar[1], Nihal Kutlu Çalışkan[4], Edis Koru[1], Gözde Elgin Cebe[5], Pınar Akdoğan Şirin[6], Caner Şirin[6], Ulviye Karacalar[1], and Semra Cirik[1]

[1]Ege University, Fisheries Faculty, Aquaculture Department, Bornova, İzmir, 35100 Türkiye
[2]İstinye University, Pharmacy Faculty, Pharmaceutical Botany Department, Zeytinburnu, İstanbul, 34010 Türkiye
[3]Katip Çelebi University, Pharmacy Faculty, Pharmaceutical Microbiology Department, Çiğli, İzmir, 35620 Türkiye
[4]Republic of Türkiye, Minister of Agriculture and Forestry, Central Fisheries Research Institute, Yomra, Trabzon, 61250 Türkiye
[5]Ege University, Pharmacy Faculty, Pharmaceutical Botany Department, Bornova, 35100 Türkiye.
[6]Ordu University, Fatsa Marine Sciences Faculty, Fisheries Technology Engineering Department, Fatsa, Ordu, 52400 Türkiye

Corresponding author: gamze.turan@ege.edu.tr

ARTICLE INFO

Article History:
Received: April 22, 2025
Accepted: June 27, 2025
Online: June 29, 2025

Keywords:
Ulva cultivation,
Ulva extraction,
Pathogenic bacteria,
Anti-bacterial activity,
Bioactive compounds

ABSTRACT

The aim of this study was to determine the antibacterial properties of the green macroalgae *Ulva rigida*, which was both genetically and morphologically identified and cultivated under laboratory conditions. The study focused on its effects on Gram-positive microorganisms, including *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, and *Bacillus subtilis* ATCC 6633, as well as Gram-negative microorganisms, such as *Escherichia coli* ATCC 25922, *Salmonella enterica* ATCC 13311, and *Pseudomonas aeruginosa* ATCC 27853. Through the use of disc diffusion and micro-dilution methods, the antibacterial properties of *Ulva* were scrutinized in regards to both Gram-positive/negative bacteria. Except hexane extracts of *Ulva*, the other four solvent (acetone, diethyl ether, ethanol, and methanol) extracts of *Ulva* showed antimicrobial activities on all bacteria types.

INTRODUCTION

The progression of antibiotic resistance in pathogenic bacteria has prompted researchers to investigate novel antimicrobial agents derived from diverse freshwater and marine environments, including microalgae and macroalgae (or seaweed) **(Kamimoto, 1956; Haefner, 2003; Smit, 2004; Montalvao *et al.*, 2016; Pereira, 2018; Vizetto-Duarte *et al.*, 2019)**. To cope with physical and chemical alterations in their environment, algae have developed improved resistance and adaptive mechanisms that enable them to produce a diverse array of compounds via various metabolic pathways **(Montalvao *et al.*, 2016)**. When the compounds in the algae responsible for the

antimicrobial activity were analyzed, they were found to be algal fluorotannins, bromoditerpenes, halogenated furanones, lectins and sesquiterpenes. These unique molecules are found within alkaloids, carbohydrates, flavonoids, lipids, phenolic compounds, proteins, saponins, and tannins **(İsar *et al.*, 2021)**. Bioactive sesquiterpenes of *Ecklonia kurome*, a brown alga, are defined as having antifungal and antimicrobial effects against *Bacillus megaterium* **(Kuniyoshi *et al.*, 2005)**. The crude extracts of *Ecklonia kurome* contain a wide variety of phlorotannins that were tested against multidrug-resistant pathogens **(Schultz*, et al.*, 1992; Nagayama *et al.*, 2002; Gopal *et al.*, 2008)**.

Two bromoditerpenes were isolated from *Sphaerococcus coronofifolius*, a species of red macroalgae that showed antibacterial and antimararial activities **(Etahiri *et al.*, 2001)**. Diethyl ether extracts of seaweeds collected from the shores of Urla (Türkiye) showed antibacterial and antifungal effects, as well being effective against *Candida* sp., *Enterococcus faecalis, Streptococcus aureus, S. epidermidis, Pseudomonas aeruginosa,* and *Escherichia coli* **(Özdemir *et al*., 2006; Tüney *et al.*, 2006)**.

While isoobtusol has some activity against *Klebsiella pneumoniae* and *Salmonella* spp. **(Vairappan, 2003)**, elatol extract isolated from the red alga *Laurencia majusculee* has antimicrobial activity against *Staphylococcus epidermidis, Klebsiella pneumoniae,* and *Salmonella* spp. Halogenated furanones isolated from *Delisea pulchrahas* possess broad-spectrum antibiotic activity **(Zang *et al.*, 2009)**.

A red macroalga, *Hypnea musciformis*, isolated from the southeastern and southwestern coasts of India had antibacterial properties, while a species of green macroalgae, *Ulva fascia* showed a broad spectrum antibacterial activity by inhibiting *Bacillus cereus, Escherichia coli, Bacillus subtilis, Aeromonas hydrophila, Vibrio fischeri,* and *V. harveyiat* **(Selvin & Lipton, 2004)**.

Methanol extracts of macroalgae isolated from the Gulf of Mannar, India, showed different activities on different pathogenic organisms **(Manikandan *et al.*, 2011)**. For example, a Brown alga, *Padina tetrastromatica*, is effective against resistant bacteria such as *Stocheospermum marginatum*, the source of urinary tract infections. The red alga *Grateloupia lithophila* shows higher activity against both multi-drug-resistant and non-resistant bacteria compared to *Caulerpa* sp., *Gracilaria corticata*, and *Valoniopsis pachynema*, which exhibit lower activity **(Manikandan *et al.*, 2011)**.

The methanol extract of 26 species of red macroalgae isolated from the Moroccan coast showed their effectiveness against *Entrococcus faecalis* (ATCC-29212), *E. faecalis* (ATCC-29213), *Escherichia coli, Klebsiella,* and *Pneumonia* **(Pratt *et al.*, 2010)**. *Hypnea musciformis*, a red algae, exhibits different functions among other species of algae as it has an inhibition zone of 10 to 35mm **(Pratt *et al.*, 2010)**.

Thirty-two species of algae from Karachi (Pakistan) were found to be effective against bacteria **(Muhammad & Shameel, 2004)**. For example, *Codium shameelii* and *Iyengaria stellata* were effective on Gram-positive bacteria while *Colpomenia sinuosa*

was effective on Gram-negative, *Sargassum ilicifolium* was effective on Gram-negative bacteria, *Cystoseira indica* was effective on Gram-positive/negative bacteria, a red alga *Botryocladia leptopodahas* was effective on both positive/negative bacteria, and *Champia compressa* was effective on only Gram-negative bacteria **(Muhammad & Shameel, 2004)**.

Crude extracts of *Sargassum cinereum*, a brown macroalga, were found to be effective against pathogenic bacteria species, and it was concluded that this species has great potential to be used in the medical field **(Divya *et al.*, 2011)**.

The aim of this study was to determine the antimicrobial effects of green macroalgae *Ulva rigida* naturally distributed in İzmir Bay (Aegean coast of Türkiye) on both Gram-positive/negative bacteria. To achieve this goal, *Ulva* extracts were prepared using five different solvents (ethanol, methanol, acetone, diethyl ether, and hexane). The antimicrobial effects on pathogenic microorganisms were then determined using the disc diffusion and microdilution methods.

MATERIALS AND METHODS

Supply of *Ulva* samples

During this study, green seaweed *Ulva rigida* samples were obtained from the Macroalgae Cultures in the Algae Culture Laboratories at Ege University, Fisheries Faculty, in Bornova and Urla Research Center where they were cultivated in Guillard's F/2 culture medium **(Guillard & Ryther, 1962; Guillard, 1975)**. *Ulva rigida* cultures were originally obtained from natural stocks located at Bostanlı Harbor (İzmir Bay, Türkiye), and their identification was previously carried out based on various morpho-anatomical characteristics using the methodologies outlined by **Taşkın *et al.* (2008)**, **İsmail and Mohamed (2017)** and **Taşkın *et al.* (2019)**. The species name followed the taxonomy provided by **Guiry and Guiry (2024)**. Moreover, the identification was confirmed via the AlgaeBase website and rbcL gene amplification **(Thompson *et al.*, 1994; Hall, 1999)**. For this experiment, *Ulva rigida* cultures were maintained in 1L glass jars enriched with Guillard's F/2 medium (10 mL L^{-1}), under continuous aeration and constant light conditions.

Preparation of *Ulva* extracts

The *Ulva rigida* samples were subjected to drying at 35°C and subsequently transferred to a deep freezer (-20°C) until extraction using 5 different solvents. The preparation of algae extracts involved the utilization of organic solvents, namely methanol (95%), hexane (95%), diethyl ether (99%), ethanol (96%), and acetone (99%). Each 5g dry *Ulva* sample required the addition of 25ml of the respective organic solvent, with 3 replicates performed for each extraction **(Sastry *et al.*, 1994; Zheng *et al.*, 2001)**. The ultrasonic extraction method was employed for this process. Upon completion of the

extraction procedure, the *Ulva* extracts were dried in a rotary evaporator. A portion of the *Ulva* extracts was utilized to assess the antimicrobial effects of *Ulva* on the bacteria.

Bacteria used in the study

Antimicrobial activities of *Ulva* extracts were examined on bacterial strains of *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Salmonella enterica* ATCC 1331, and *Pseudomonas aeruginosa* ATCC 27853. The bacterial strains were preserved in brain-heart infusion broth containing 10% glycerol at a temperature of -80°C. The strains chosen for the study were retrieved from the stock medium in cryotubes, subsequently plated onto Mueller-Hinton Agar (MHA), and obtained following a 24-hour incubation period at 35°C **(Bauer *et al.*, 1966)**.

Antimicrobial activity studies

In order to invastigate the antimicrobial activities of *Ulva* on the bacterial strains, disc diffusion test and microdilution method were applied according to the Clinical and Laboratory Standards Institute (CLSI) and EUCAST guidelines **(Bauer *et al.*, 1966, Kahlmeter *et al*., 2006; Wikler *et al*., 2006)**.

Disk diffusion test

The disc diffusion method was applied accourding to the Clinical and Laboratory Standards Institute (CLSI) and EUCAST guidelines **(Bauer *et al.*, 1966; Kahlmeter *et al*., 2006; Wikler *et al*., 2006)**. As a control, Gentamicin antibiotic discs were utilized.

Microdilution method

The microdilution method was applied according to the Clinical and Laboratory Standards Institute (CLSI) and EUCAST guidelines **(Bauer *et al.*, 1966; Kahlmeter *et al*., 2006; Wikler *et al*., 2006)**. As a control antibiotic, Ciprofloxacin was utilized in this study.

Statistics

All experiments on antimicrobial tests were conducted in triplicate *Ulva* extracts. The average and standard deviation from the three experiments were calculated.

RESULTS

Disk diffusion test results

The results of the disc diffusion test affirmed that the extracts derived from *Ulva* had an effect on Gram-positive bacteria, showcasing their antibacterial properties (Table 1). Except hexane extracts of *Ulva*, the other four solvent extracts of *Ulva* showed antimicrobial effect: for example, Acetone extracts of *U. rigida* showed an anti-*Bacillus subtilis* activity with 7.33mm zone diameter. Ethanol extracts showed an anti-*Bacillus subtilis* activity with 7mm zone diameter and methanol extracts of *U. rigida* showed anti-

Staphylococcus aureus with 7mm zone diameter and anti-*Bacillus subtilis* activities with 8 mm zone diameter. Diethyl Ether extracts showed anti- *Staphylococcus aureus* (with 7 mm zone diameter) and anti-*Bacillus subtilis* (with 7.66mm zone diameter) and anti-*Salmonella enterica* (with 9.66mm zone diameter) effects (Table 1).

Table 1. The bacteria's susceptibility to *Ulva rigida* extracts (100 µg.disc^{-1}), prepared using various organic solvents, and measured by determining the diameter of the inhibition zones (mm)

	Gram-positive microorganisms			Gram-negative microorganisms		
Organic solvent	*Staphylococcus aureus*	*Entrococcus faecalis*	*Bacillus subtilis*	*Escherichia coli*	*Salmonella enterica*	*Pseudomonas aeruginosa*
Acetone	-*	-	7.33±0.0	-	-	-
Diethyl ether	7±0.0	-	7.66±0.0	-	9.66±0.0	-
Ethanol	-	-	7±0.0	-	-	-
Hexane	-	-	-	-	-	-
Methanol	7±0.0	-	8±0.0	-	-	-

*: *(-):* No detection of inhibition zone diameter.

Microdilution method results

Based on the research findings, it was discovered that the microdilution test results demonstrated the effectiveness of extracts derived from *Ulva rigida* on both Gram-positive/-negative bacteria (Table 2). The five solvent extracts of *Ulva* showed antimicrobial effect: for example, acetone extracts of *U. rigida* showed anti-*Staphylococcus aureus* (with 512 µg.ml^{-1} MIC) and anti- *Entrococcus faecalis* (with 512µg.ml^{-1} MIC) effects (Table 2). Ethanol extracts showed anti-*Staphylococcus aureus* (with 1024µg.ml^{-1}MIC), anti- *Entrococcus faecalis* (with 512 µg.ml^{-1} MIC), anti- *Bacillus subtilis* (with 1024µg.ml^{-1}MIC), anti-*Escherichia coli* (with 512µg.ml^{-1} MIC), anti-*Salmonella enterica* (with 512µg.ml^{-1} MIC), and anti-*Pseudomonas aeruginosa* (with 512µg.ml^{-1} MIC) effects (Table 2). Methanol extracts of *U. rigida* showed anti-*Entrococcus faecalis* (with 2048µg.ml^{-1} MIC), and anti-*Bacillus subtilis* (with 2048µg.ml^{-1}MIC) effects. Moreover, diethyl ether extracts of *U. rigida* showed anti-*Staphylococcus aureus* (with 256µg.ml^{-1}MIC), anti- *Entrococcus faecalis* (with 512µg.ml^{-1} MIC) and anti-*Bacillus subtilis* (with 1024µg.ml^{-1} MIC), anti-*Escherichia coli* (with 2048µg.ml^{-1} MIC), anti-*Salmonella enterica* (with 1024µg.ml^{-1} MIC), and anti-*Pseudomonas aeruginosa* (with 2048 µg.ml^{-1} MIC) effects (Table 2).

Table 2. The MIC values of *Ulva rigida* extracts, prepared using various organic solvents, are measured in µg.ml^{-1}

	Gram-positive microorganisms			Gram-negative microorganisms		
Organic Solvent	*Staphylococcus aureus*	*Entrococcus faecalis*	*Bacillus subtilis*	*Escherichia coli*	*Salmonella enterica*	*Pseudomonas aeruginosa*
Acetone	512	512	>2048	>2048	>2048	>2048
Diethyl Ether	256	512	1024	2048	1024	2048
Ethanol	1024	512	1024	512	512	512
Hexane	>2048	>2048	>2048	>2048	>2048	>2048
Methanol	>2048	2048	2048	>2048	>2048	>2048

DISCUSSION

As part of this study, natural extracts of the green macroalga *Ulva rigida*, collected from the Aegean Sea coast of Türkiye, were tested for their antibacterial effects using five different organic solvents: acetone, diethyl ether, ethanol, hexane, and methanol. The *Ulva* extract samples were prepared to assess antibacterial activity through disc diffusion and microdilution methods against bacteria pathogenic to humans and animals, including those affecting fishery products. The Gram-positive bacterial strains tested included *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, and *Bacillus subtilis* ATCC 6633, while the Gram-negative strains included *Escherichia coli* ATCC 25922, *Salmonella enterica* ATCC 13311, and *Pseudomonas aeruginosa* ATCC 27853.

The results of both disc diffusion and microdilution assays confirmed that *Ulva rigida* extracts exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria. Except for the hexane extract, all other solvent extracts demonstrated antimicrobial effects.

The results of the disc diffusion and microdilution assays in this study are consistent with previous research conducted in the Mediterranean Sea region of Türkiye **(Sukatar *et al.*, 2006; Tüney *et al.*, 2006; Taşkın *et al.*, 2011; Gümüş & Ünlüsayın, 2016; Montalvão *et al.*, 2016; Gümüş *et al.*, 2018)**. Similarly, in the study by **Ismail *et al.* (2018)**, biologically directed purification of extracts from *Ulva rigida* demonstrated antibacterial activity, particularly against *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212. **Yücel *et al.* (2023)** investigated the antimicrobial activities of essential oils derived from hexane, dichloromethane, chloroform, and methanol extracts of *U. rigida*, reporting the highest antimicrobial activity against *Pseudomonas aeruginosa* ATCC 9027 in the chloroform and methanol extracts. Additionally, **Djoh *et al.* (2024)** found that *Ulva reticulata* extracts prepared with methanol, ethyl acetate, and chloroform exhibited both antimicrobial and antioxidant activity against *Staphylococcus aureus* Rosenbach, 1884 and *Escherichia coli*. The highest levels of antimicrobial and antioxidant activity in that study were observed in the ethyl acetate extract of *U. reticulata*

CONCLUSION

It has been suggested that new antibiotics may be derived from *Ulva* species to combat bacteria that cause diseases in humans and animals, including fish species. Consequently, future research should focus on identifying the specific bioactive compounds responsible for the antimicrobial effects of *Ulva*, considering variations in cultivation conditions, extraction methods, and *Ulva* species. To support the development of a related industry, standardized protocols must be optimized and aligned with the requirements of relevant stakeholders.

ACKNOWLEDGMENT

Authors thankful to Ege University Scientific Research Projects Programme (BAP-Project number-17-SÜF-010: "Determination of Antimicrobial Effects of Micro-and Macroalgal species against Pathogenic organisms") for providing funding for this study.

AUTHOR'S CONTRIBUTION

Conceptualization, S.C., Ş.G. and G.T.; methodology, S.A.K, İ.Ö and G.T.; software, P.A.Ş.,C.Ş. and M.İ; validation, U.K. and G.T.; moleculer and genetic analysis, N.K.Ç. and G.T.; formal analysis, S.A.K., İ.Ö, and G.T.; investigation, M.İ, P.A.Ş., N.K.Ç., G.E.C., S.C. and G.T.; resources, S.A.K. and G.T.; data curation, P.A.Ş, N.K.Ç. and G.T.; writing—original draft preparation, P.A.Ş., M.İ. and G.T.; writing—review and editing S.C. and G.T.; visualization, P.A.Ş., M.İ., E.K. and G.T.; supervision, G.T. and S.C.; project administration, S.C. and G.T.; funding acquisition, G.T. and S.C. All authors have read and agreed to the published version of the manuscript.

RESEARCH FUNDING

This research was funded by Ege University Scientific Research grant Agreement 17-SÜF-010.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

COMPLIANCE WITH ETHICL STANDARDS

This article does not contain any studies involving animals performed by any of the authors.

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