

RESEARCH ARTICLE

The Antimicrobial Activity of *Enteromorpha sp.* Methanolic Extract and Gelatin Film Solution Against on Some Pathogens

Elif Ayşe Erdoğan Eliuz^{1*} • Nahit Soner Börekçi² • Deniz Ayas²

¹ Department of Food Technology, Technical Sciences Vocational School, Mersin University, Mersin, Turkey

¹ Department of Seafood Processing Technology, Faculty of Fisheries, Mersin University, Mersin, Turkey

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ABSTRACT

Pathogenic microorganisms have been the primary cause of foodborne disease and food poisoning throughout the world for years. The use of natural antimicrobial agents in food coating has been effective in regulating the adverse effects of pathogens in food. Increasing antimicrobial efficacy in these coatings is one of the current issues of the food industry. In the present study, the antimicrobial properties of *Enteromorpha sp.*, which is a marine algae, and gelatin film solution incorporated with *Enteromorpha sp.* methanol extract have been investigated. The contents of *Enteromorpha sp.* methanol extract were determined by Gas chromatography–mass spectrometry (GCMS). The most important components in the extract were methyl palmitoleate, neophytadiene, phytol, methyl linolenate and methyl stearate. The minimum inhibitory concentration (MIC; the lowest concentration of test material which results in 99.9% inhibition of growth) of *Enteromorpha sp.* on *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* were found to be between 10.79 mg/mL and 26.86 mg/mL by spectrophotometric microdilution technique. The antimicrobial effect of gelatin- *Enteromorpha sp.* methanolic extract film solution against the same pathogens was determined by disc diffusion method. The inhibition zone of gelatin- *Enteromorpha sp.* film solution was reported between 0.1 and 5.1 mm against pathogens. After a 24-h incubation, the effectiveness of the film solution was lower (1.3 mm) when compared to the extract on *E. coli* (5.1 mm). As a result, this study clearly showed that *Enteromorpha sp.* could be used as antimicrobial food coating agent, especially, in *E. coli* struggle.

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Introduction

Recently, interest in seaweeds (marine algae) has grown as natural source of pharmaceutical agents. They are rich in polysaccharides,

minerals, proteins and vitamins with recorded antioxidant activity that would increase the human diet's quality as food (Yan et al., 1998). Several studies have demonstrated that seaweeds contain active molecules, protective enzymes, phloroglucinol and phenolics, which

* Corresponding author

E-mail address: eliferdogan81@gmail.com (E.A. Erdoğan Eliuz)

are mostly known as antioxidants (Pavia and Åberg, 1996; Ganesan et al., 2011). Recently, seaweeds are reported to be potentially high bioactive sources that could be useful leads in antimicrobial product development. Their antiviral, antibacterial and antifungal properties are remarkable (Val et al., 2001; Newman et al., 2003). Although marine algae have been traditionally used as important sources of protein, carbohydrate, lipid, vitamin for centuries (Murata and Nakazoe, 2001), currently, their metabolites find out by varieties extraction methods are demonstrated to be unique antimicrobial agents (Lima-Filho et al., 2002; Desbois and Smith, 2015; Kausalya and Rao, 2015).

Food safety and quality in the food industry are major concerns. Food manufacturers are striving to eliminate microorganisms from food products because food surviving microorganisms can lead to food product quality spoilage and cause infection which threatens the public health. Therefore, pathogenic microorganisms are well-considered to be a significant concern for worldwide public health (Nykänen et al., 2000; Srey et al., 2013). In recent years, scientists have focused on edible food coatings containing antimicrobial agents. Synthetic antimicrobial agents can be integrated into edible films and coatings to monitor harmful microorganisms on food surfaces. However, the use chemical preservatives are highly controversial, because they have been shown to cause respiratory or other health problems (Quintavalla and Vicini, 2002). Researches therefore head for alternative strategies to reduce the use of chemical additives in the food industry (Sanchez-Garcia et al., 2010). In this context, the use of natural compounds with antimicrobial properties such as algae which are in rich protein and mineral seems to be an attractive option.

Enteromorpha species, we selected for the study, are cosmopolitan intertidal macroalgae and common edible seaweed species that are abundant in the coastal areas of Asia and Europe around the world (Tan et al., 1999; Callow, 2002). There are hardly any studies on this species spreading in the region of Mersin, Turkey. In the present study, the antimicrobial activity of *Enteromorpha sp.* methanol extract (EME) and gelatin- *Enteromorpha sp.* film solution (EME-F) were firstly researched against *E. coli*, *S. aureus* and *C. albicans* pathogens. Besides, methanolic content were determined as well.

Material and Methods

Plant material and methanolic extraction

Enteromorpha sp. were collected from Viranşehir, Mersin, Turkey in 2019. They were identified and confirmed by comparing them with the specimen located at the Faculty of Fisheries at Mersin University, Turkey. The *Enteromorpha sp.* extraction was performed from approximately 80 g of the dried *Enteromorpha sp.* in 320 mL methanol via shaker incubator for 24 hours at 35°C.

Chemical composition

The qualitative and quantitative composition of *Enteromorpha sp.* analysis was conducted at Giresun University Central Research Laboratories Application and Research Center by GC-MS 7890A-(5975C inert MSD) instrument equipped with an Agilent 19091S-433 column. The chemical composition of *Enteromorpha sp.* methanolic

extract was determined by analyzing GC-MS in the scanning range of $M+=50-550$ m/z. 1 L of the concentrated plant extract was filtered through 0.45 µL syringe filter and injected to GC-MS injection port (250°C) in splitless mode. The extract was eluted using HP5-MS capillary column (30m × 0.25 mm × 0.25 µm) at helium gas (flow rate: 1.75 mL/min) under fixed 21.21 psi of pressure. The study was performed by applying the following sample elution temperature system for a maximum of 70 minutes. The oven temperature was gradually increased after it was kept at 50°C for 2 min. Then, it was increased to 100°C at 5°C min⁻¹ and was held for 5 min. Then, it was increased to 150°C at 5°C min⁻¹ and performed for 8 min. Finally, the temperature was increased to 250°C at 5°C min⁻¹ and it was kept there for 15 min. Characterization of *Enteromorpha sp.* components was based on the library (Wiley and NIST) comparison with the mass spectra of the extract sample (Yabalak, 2018; Sıcak and Eliuz, 2019a).

Antimicrobial activity

The antimicrobial activity of methanol extraction of *Enteromorpha sp.* was researched on several pathogens, namely *E. coli*, *S. aureus* and *C. albicans* using disc diffusion and modified spectrophotometric microdilution technique. Firstly, the inoculums of microorganisms were prepared in 4 mL Tryptic Soy Broth for bacteria, 4 mL Sabouraud Dextrose Broth for yeasts and incubated at 37°C, overnight. After 24 hours, the culture suspensions were adjusted to 0.5 McFarland Standard Turbidity and stored at +4°C until use (Dalynn Biologicals, 2014).

Spectrophotometric microdilution technique

The experiment was performed on 96-well microtiter plates and firstly 50 µL of Mueller Hinton Broth (MHB) medium were added into all wells. Two-fold serial dilutions (50 µL) of EME (286 mg/mL) were made on all x-axis along with ELISA plate. Columns 11 and 12 were used as negative and positive controls. Finally, 5 µL culture of microorganisms was inoculated on all wells except the medium control wells. All of the plates were incubated at 37°C for 24 hours, the growth (turbidity) was measured at 600 nm for bacteria, 415 nm for yeasts. For MIC analysis, the optical density was read both before, T₀ and after 24 hours-incubation, T₂₄. For each plate, MIC was calculated using the following formula: The OD for each replicate at T₀ was subtracted from the OD for each replicate at T₂₄.

$$\text{The Percent Growth} = \frac{OD_{\text{test}}}{OD_{\text{control}}} \times 100$$

$$\text{The Percent Inhibition} = 1 - \frac{OD_{\text{test}}}{OD_{\text{of corresponding control well}}} \times 100$$

for each row of the 96-well plate. We calculated MIC using the R² formula on inhibition curve (Patton et al., 2006; Sıcak and Eliuz, 2019b).

Preparation of coating film solution

The gelatin (Dr. Gusto) and glycerol (Gly) (98% reagent grade) were purchased from Market and Sigma, respectively. The film-forming solution contained 5% (w/w) gelatin, glycerol 3.5% (w/w) and *Enteromorpha sp.* methanolic extract (15%). The pH of the solution was appropriately adjusted to 9-10 with 2 M NaOH. Film solution was

homogenized with a Rotor-Stator homogenizer for 3 min at 23000 rpm in a first step (at about 25°C). The control samples were prepared using the same mentioned-above procedure, except without the addition of the extract. Antimicrobial activities of EME enriched gelatin-based film solutions were determined by disc diffusion method. Antibiotic and EME alone were studied to compare with EME film solution antimicrobial activities. Paper discs (6 mm in diameter) were impregnated on MHA to load 20 µL of ampicillin, EME and EME Film. Then, all samples were incubated at 37°C for 24 hours. The results in the study have recorded the zones of growth inhibition surrounding the disc using digital caliper (Kuppulakshmi et al., 2008). All data on antimicrobial activity were the average of triplicate analyses.

Statistical analysis

All data on antimicrobial activity assay studies were the averages of triplicate analysis. Data were recorded as mean ± SEM (standard error of the mean). Significant differences between means were determined by LSD (SPSS v25; post hoc-one way ANOVA) test and *p* values <0.05 were regarded as significant.

Results and Discussion

Chemical content

The components of the methanolic solution from *Enteromorpha sp.* with their retention time (RT) and area (%) were listed in Table 1. In the present study, methyl palmitoleate (27.78%), neophytadiene (19.36%), phytol (14.98%), methyl linolenate (14.46%), and methyl stearate (5.40%) were the major components in the methanolic extract of *Enteromorpha sp.* followed by other components such as cyclomethicone 7, heptadec-8-ene, linoleic acid, palmitin, 1-vinyl silatrane, 9-octadecenamide.

Antimicrobial activity

The 24-hour incubation of *Enteromorpha sp.* with microorganisms was found to be statistically significant in terms of MIC (*p*<0.05) (Table 2). All microorganisms were found to be sensitive to *Enteromorpha sp.* methanolic extract in broth media. The MICs of

EME were 26.86 mg/mL for *E. coli*, 12.04 mg/mL for *S. aureus* and 10.79 mg/mL for *C. albicans* (*p*<0.05). The antibiotic results were 64 µg/mL, 8 µg/mL and 128 µg/mL for *E. coli*, *S. aureus* and *C. albicans*, respectively.

As shown in Figure 1, EME revealed different inhibition activities towards the three microorganism cells investigated. Inactivation of *E. coli*, *S. aureus* and *C. albicans* by increased doses of the methanolic extract was similar in that they cause an increased in cell death rate. In general, a dose-dependent decrease in the survival of the microorganisms was observed. The applied EME at doses of 4 g/mL led to inhibition maximum 65.71% for *E. coli*, 86.17% for *S. aureus*, and 87.65% for *C. albicans* (*p*<0.05).

Table 2. MICs of EME against tested microbial strains by microdilution method (For positive control: ampicillin (for bacteria) and fluconazole (for yeast) were used as positive control (128 µg/mL))

Microorganism/MIC	EME (mg/mL)	Antibiotics (µg/mL)
<i>E. coli</i> (-)	26.86 ^a ±3.2	64 ^c ±3.9
<i>S. aureus</i> (+)	12.04 ^{ad} ±0.1	8 ^{bd} ±2.8
<i>C. albicans</i>	10.79 ^{ad} ±1.2	128 ^c ±4.8

Note: “±” indicates standard error of the mean. The values shown in different superscript letters are statistically different (ANOVA, *p* < 0.05, LSD test).

The antimicrobial property of gelatin film solution incorporated with *Enteromorpha sp.*

According to the antimicrobial property of gelatin film solution incorporated with *Enteromorpha sp.* methanol extract investigated in our study, EME and EME-F were determined to clear inhibition zone by disc diffusion method. The antimicrobial performance of EME was presented stronger than EME-F on *E. coli* (*p*<0.05). The highest inhibition zone was noted as 5.1 mm for *E. coli* in EME, although the lowest zone was 0.1 mm on *S. aureus* and *C. albicans* in EME and EME-F. The antibiotic results were 4.9 mm, 4.5 mm and 2.4 mm for *E. coli*, *S. aureus* and *C. albicans*, respectively (Table 3).

Table 1. Chemical composition (C) of *Enteromorpha sp.* methanolic extract

<i>Enteromorpha sp.</i>					
RT	C	%	RT	C	%
26.800	cyclomethicone 7	1.01	41.151	linoleic acid	2.19
27.201	2,4-Di-tert-butylphenol	0.76	41.277	methyl linolenate	14.46
32.053	heptadec-8-ene	2.77	41.512	phytol	14.98
33.272	methyl tetradecanoate	0.68	41.752	methyl stearate	5.40
36.099	benzyl (dideuterated) methyl ether	1.04	41.906	1-vinyl Silatrane	0.84
36.505	neophytadiene	19.36	42.049	2-eicosanol	2.32
37.386	methyl palmitoleate	27.78	43.949	palmitin	0.81
40.630	2-Norpinanol, 3,6,6-trimethyl-	0.89	45.837	9-Octadecenamide	1.83
40.968	<i>E,E,E</i> -1,4,8-cyclododecatriene	2.46	Total		96.58

Note: RT is retention time; quantity (%) is more than 0.01.

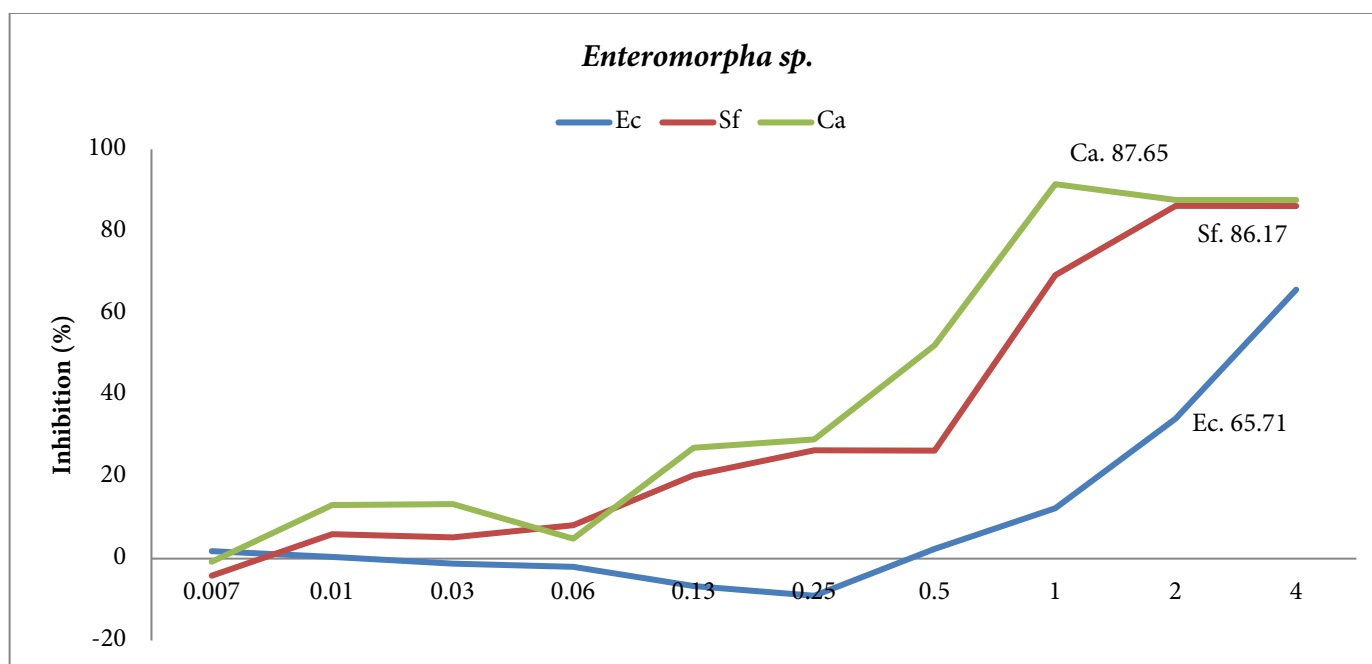


Figure 1. The graph of inhibition (%) – *Enteromorpha sp.* methanolic extract (between 0.007 and 4 g/mL) on *E. coli*, *S. aureus* and *C. albicans*

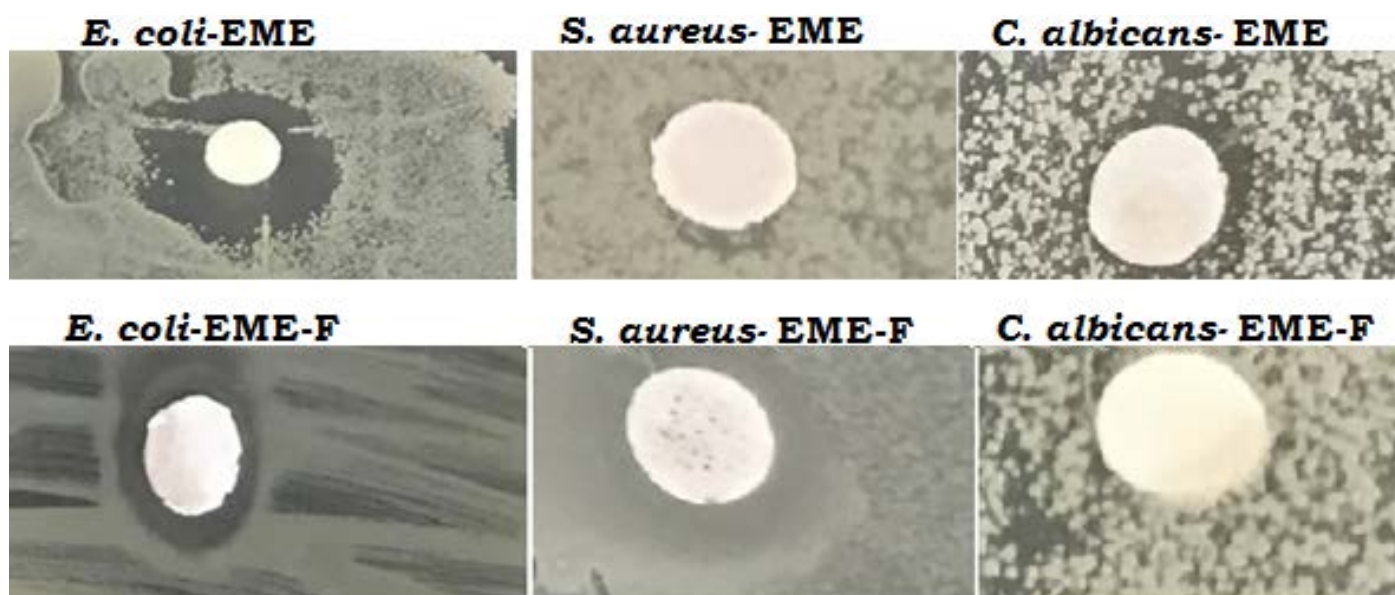


Figure 2. Images of *E. coli*, *S. aureus* and *C. albicans* responses to EME and EME-F extracts

Table 3. The comparing of inhibition zones (mm) of the EME and EME-F against tested microbial strains (For positive control: ampicillin (for bacteria) and fluconazole (for yeast) were used as positive control. IZ: inhibition zone)

Microorganisms		EME	EME Film	Antibiotics
<i>E. coli</i> (-)	IZ	5.1 ^c ±1.6	1.3 ^b ±0.6	4.9 ^c ±0.9
<i>S. aureus</i> (+)	IZ	0.1 ^a ±0.1	0.1 ^a ±0.1	4.5 ^c ±1.2
<i>C. albicans</i>	IZ	0.1 ^a ±0.1	0.1 ^a ±0.1	2.4 ^{bc} ±1.2

The inhibition zones of *Enteromorpha sp.* methanolic extract and gelatin-based film solution of *Enteromorpha sp.* on microorganisms were photographed in Figure 2. In the photograph, both EME and EME-F solution clearly inhibited *E. coli*.

The present study showed that *Enteromorpha sp.* is highly effective against *E. coli* (5.1 mm) compared to *S. aureus* and *C. albicans* (0.1 mm) in disc diffusion method. In contrast, in the liquid culture, the MIC values were close for to all three microorganisms. The highest MIC value was found in *C. albicans* and then *S. aureus* and then *E. coli* between 10.79 and 12.04 and 26.86 mg/mL by microdilution method. The antimicrobial property of the macroalgae is due to its compounds. The inhibition effect on all pathogens of major components such as phytol (Pejin et al., 2014), methyl stearate, methyl nonadecanoate (Oliveira et al., 2013) of *Enteromorpha sp.* was reported previously. The neophytadiene and phytol components, found in *Enteromorpha sp.* abundantly in this study, were also determined in *Dunaliella salina* Microalga by Herrero et al. (2006) and they had antimicrobial potential.

Previous studies showed the different extracts of *Enteromorpha sp.* species are widely effective against the growth of a wide range of pathogens, particularly, *E. coli*, *S. aureus* and *C. albicans*. For instance, Patra et al. (2015) reported that the inhibition zone as between 10.00 mm and 13.33 mm and the MIC value as 12.5 mg/mL against *E. coli*. Senthilkumar et al. (2015) found that methanolic extract of *Enteromorpha flexuosa* exhibited antibacterial activity against both *E. coli* and *S. aureus*. It was found that the aqueous extract of *Enteromorpha sp.* had an inhibition effect at a value of MIC (200 mg/mL) against *E. coli* and *S. aureus* strains (Alghazeer et al., 2013). The antimicrobial activity of *E. intestinalis* methanolic extract was shown in the study of Ibrahim et al. (2015), among the tested microorganisms, *Staphylococcus aureus* (MRSA) were susceptible to the extract and inhibited with the MIC value of above 6.25 mg/mL (Ibrahim et al., 2015).

Pathogenic bacteria have been known throughout the world for years as the prime cause of foodborne disease and food poisoning (Kim et al., 1995). The use of natural antimicrobial agents in food production and food safety has been instrumental in regulating the adverse effects of bacteria in food. The natural antibacterial agents commonly used as preservatives, however, are first of all effective against gram positive foodborne pathogens, whereas they are not very effective on gram negative foodborne pathogens (Trombetta et al., 2005; Bassole and Juliani, 2012; Sfeir et al., 2013; Nazzaro et al., 2013). Based on this topic, the best result of this study is the efficacy of *Enteromorpha sp.* against *E. coli*, a gram negative bacterium. Because, the toxic species of *E. coli*, especially O157: H7, are gram-negative bacteria that can easily multiply in foods such as cheeses, yoghurts, juices, salads, salad dressings, sandwiches, freshly squeezed fruit juices and cause poisoning (Tosun and Gönül, 2003).

Gelatin-based antimicrobial films and coatings used in food products serve a barrier to oxidative and physical stress and extend the shelf life of foods (Cha and Chinnan, 2004; Silva-Weiss et al., 2013). Also, it was reported that the use of films or coatings incorporated with antimicrobial agents were more efficient than adding additives directly to the food. Therefore, they will spread slowly into the environment and will be effective for longer (Ouattar et al., 2000). Especially when we examined *E. coli*, inhibition zone of gelatin solution combined with *Enteromorpha* was less (1.3 mm) than alone *Enteromorpha sp.* (5.1 mm). This shows that the biological agent trapped in the film will retain its effect for a longer time.

Conclusion

In this study, methanolic extract of *Enteromorpha sp.* exhibited inhibition zones on *E. coli* and lower on tested *S. aureus* and *C. albicans*. Conclusively data demonstrates the anti-*E. coli* potential of methanolic extract from *Enteromorpha sp.* It may also be suggested that there is scope for *E. coli* medical therapy. On the basis of this study, further purification of the active compounds and their individual study of antibacterial activity may be suggested. Another important result is that we showed for the first time that the *Enteromorpha* was an antimicrobial coating agent against *E. coli*.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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