DÜSTAD
Dünya Sağlık ve Tabiat Bilimleri Dergisi
JOGHENS
Journal of Global Health & Natural Science
ISSN: 2687-637X Yıl: 2024 Cilt: 7 Sayı: 2

Determination of Antimicrobial Effect of Extracts Produced from Some Freshwater Algae Using Different Methods

Article Type
Research

Received
3 September 2024

Accepted
2 December 2024

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Abstract: Algae are organisms that can be used in agriculture, cosmetics, medicine, industry and many other areas thanks to the many metabolites they contain. There are thousands of species of algae. Some algae species still remain a mystery. In this study, the antimicrobial activity (against *Clostridium perfringens, Enterococcus faecalis, Escherichia coli, E. coli O157:H7, Listeria monocytogenes, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus agalactiae*) of extracts (18 different groups) obtained from three different algal species (*Ulothrix zonata, Fontinalis antipyretica and Spirogyra gracilis*) by two different methods using water, ethanol, methanol and ether were studied by disc diffusion method. According to the results obtained; each algae species, the method of extraction of the extract and the type of solvent used were found to be effective on the antimicrobial properties. In particular the extracts obtained by using the Soxhlet device (Method B) showed more antimicrobial activity against the selected microorganisms, and in this method, it was observed that the antimicrobial activityt was higher in the extracts in which ether was used as solvent.

Keywords: antimicrobial activity, algae, algal extract, *Ulothrix zonata, Fontinalis antipyretica, Spirogyra gracilis*

Bazı Tatlı Su Alglerinden Farklı Yöntemler Kullanılarak Üretilen Ekstraktların Antimikrobiyal Etkisinin Belirlenmesi

Özet: Algler, içerdikleri birçok metabolit sayesinde tarım, kozmetik, tıp, endüstri ve daha birçok alanda kullanılabilen organizmalardır. Alglerin binlerce türü bulunmaktadır. Bazı alg türleri ise hala gizemini korumaktadır. Bu çalışmada; üç farklı alg türünden (*Ulothrix zonata, Fontinalis antipyretica ve Spirogyra gracilis*), iki farklı yöntemle; su, etanol, metanol ve eter kullanılarak elde edilen ekstraktların (18 farklı grup) antimikrobiyal aktiviteleri (*Clostridium perfringens, Enterococcus faecalis, Escherichia coli, E. coli O157:H7, Listeria monocytogenes Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus agalactiae*'ye karşı) disk difüzyon yöntemi ile incelenmiştir. Elde edilen sonuçlara göre; her bir alg türünün, ekstraktın elde edilme yönteminin ve kullanılan çözücü türünün antimikrobiyal özellikler üzerinde etkili olduğu görülmüştür. Özellikle Soxhlet cihazı kullanılarak elde edilen ekstraktların (Yöntem B) seçilen mikroorganizmalar üzerinde daha fazla antimikrobiyal etki gösterdiği ve bu yöntemde çözücü olarak eterin kullanıldığı ekstraktlarda antimikrobiyal etkinin daha yüksek olduğu belirlenmiştir.

Anahtar kelimeler: antimikrobiyal aktivite, alg, alg ekstraktı, *Ulothrix zonata, Fontinalis antipyretica, Spirogyra gracilis*

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INTRODUCTION

Today, resistance to antibiotics is increasing day by day. This problem has prompted the search for new antibiotics. Algae are a promising source of many compounds. There are millions species of algae, many of which have not yet been studied for their antimicrobial properties. Algae are organisms that are primary producers in the ecosystem and are recognised as one of the oldest forms of life, and have been used in many areas, especially in human nutrition, for many years. In addition to meeting the need for oxygen by photosynthesis, algae have been the source of food for many living things. Its use as human food is revealed with the archaeological data made in Chile, which dates back to 14000 years ago (Aktar and Cebe, 2010; Kaba and Caglak, 2006; Muslu and Gökçay, 2020). Algae are becoming increasingly important in terms of being primary producers in the ecosystem, containing very important biological materials in their structures, and the value of the primary and secondary metabolites they form as a result of their biological activities. Appearing as primary metabolites; carbohydrates, proteins, fats and nucleic acids are found in almost many organisms, while secondary metabolites are bioactive compounds formed by the triggering of various factors (water, salinity, pH, disease, stress factors, etc.). These algal metabolites have been proven by many studies that have many beneficial properties such as antibacterial, antifungal, antioxidant, anticancer, antimutagenic and antitumor (Eom et al., 2012; Gökpınar et al., 2013; Kausalya and Rao, 2015; Bhowmick et al., 2020). Antimicrobial properties of algae; It originates from a wide range of structural and functional substances such as polyphenols, alkaloids, terpenes, polysaccharides, fatty acids, sterols, lactones, proteins and peptides thathave significant biological activity. Although some of these metabolites have been well studied and characterized, others have not been fully investigated (Tüney et al., 2006; Aktar and Cebe, 2010; Rengasamy et al., 2014; Bhowmick et al., 2020).

The aim of this study was to determine the antimicrobial activity of 18 different extracts of three freshwater algae(*Ulothrix zonata, Fontinalis antipyretica* and *Spirogyra gracilis*) prepared by different methods and solvents against *Clostridium perfringens* (ATCC 13124), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922) and *E. coli* O157:H7 (ATCC 35150), *Listeria monocytogenes* (RSKK 02028), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Streptococcus agalactiae* (ATCC 13813).

MATERIALS and METHODS

Algae Samples

Algae were collected from the natural spring water in the Palu, Elazıg, Turkey in July – October 2022 and identified by experts in the Biology Department and Fisheries Faculty of Firat University. Using a light microscope, morphological and anatomical structures in the samples were examined. According to the characteristics and identification keys in the taxonomic publications, the samples were identified (Hustedt, 1932; Huber-Pestalozzi, 1968; Prescott, 1982; Etll, 1983; Round and Chapman, 1987; Krammer and Lange-Bertalot, 1991). All moss samples were washed with purified tap water until it was free from all kinds of macro-organisms (insects, flies, etc.) and particles (soil, stones, leaves, etc.) in the algae. It was then passed through distilled water at least three times. The samples were kept at 4±1°C for filtration and then taken to the drying process. To speed up the drying process, the algae samples were cut into small pieces by hand. The drying process was then carried out in a fan oven at 40°C until it reached a constant weight. At the end of the drying process, attention was paid to ensuring that the moisture content in the product was less than 10%. The dried algae were ground and taken into the extraction process.

Extraction Procedure

The extraction methods used to determine the antimicrobial properties of algae vary widely. These include Soxhlet extraction and the use of organic solvents, enzymes, or bacterial fermentation; temperatures, times, pH ranges, and concentrations may vary (Rodriguez-Bernaldo et al., 2010; Savaroglu et al., 2011; Gümüş and Ünlüsayın, 2016; Shannon and Abu-Ghannam, 2016). Considering all these, two main extraction methods used in the studies were modified and used. Each extraction method is represented by a different letter and given below.

A: 5 grams were taken from the dried and powdered sample and extracted with 1/30 (gr/ml) ethanol, methanol and water separately in a water bath at 60°C for half an hour. After this process, each sample was vortexed for 30 minutes and mixed thoroughly. After waiting at room temperature for two days, it was centrifuged at 3500 rpm for 10 minutes and then filtered through filter paper and kept in an oven at 40°C until the solvents were (Farasat et al., 2013; Gümüş and Ünlüsayın, 2016; Durgun, 2018; Keskinkaya et al., 2020). It was dissolved in water, ethanol and methanol to a final concentration of 200 mg/ml.

B: 4 grams of the prepared algae samples were taken and extracted with 80% ethanol, 80% methanol and ether for 8 hours in a Soxhlet device. It was then kept at 40°C until the solvents disappeared. It was dissolved with di methyl sulfa oxide (DMSO) with a final concentration of 200 mg/ml (Demirel, 2006; Savaroglu et al., 2011).

Antimicrobial Testing

For testing the antibacterial activity, *Clostridium perfringens* (ATCC 13124), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922) and *E. coli* O157:H7 (ATCC 35150), *Listeria monocytogenes* (RSKK 02028), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923) and *Streptococcus agalactiae* (ATCC 13813) bacterial strains were selected. They were obtained from the Department of Microbiology, Faculty of Veterinary, Firat University. The work was carried out in the Microbiology Laboratory, Department of Microbiology, Firat University, Faculty of Veterinary. The disc diffusion method was used to determine the antimicrobial effect. Algae extracts were soaked in 50 µL on empty sterile discs with a diameter of 6 mm and left to dry for about 30 minutes at 30°C. Meanwhile; Pre-activated bacterial cultures were adjusted to 0.5 density using the MacFarland device and inoculated with sterile swaps in three different directions using the swabbing technique in petri dishes prepared with Mueller Hinton Agar (MHA) (Oxoid). Then, discs containing algae extracts, positive control discs (Vancomycin 30 µg (V) (Oxoid) and Neomycin 10 µg (N) (Oxoid)) and negative control discs (50 µL impregnated ethanol, methanol and DMSO) were placed on these plates. Prepared petri were incubated at 37°C for 24 hours. After the incubation period was completed, the inhibition zones around the discs were measured using millimetric rulers (NCCLS, 1993; Abedin and Taha, 2008; Prakash et al., 2011; Gümüş and Ünlüsayın, 2016).

RESULTS

The diameters of the inhibition zones recorded in millimeters are given in Table 1. No activity was observed for the negative controls, solvent and blank discs. The results of positive controls (Vancomycin 30 µg (V) (Oxoid) and Neomycin 10 µg (N) are given in Table 2.

Table 1. The diameters of the zones formed by the algae extracts obtained with different solvents and methods against microorganisms (mm).

Method	Alg Species	Types of Solvent	*C. perfringens*	*E. faecalis*	*E. coli*	*E. coli* O157:H7	*L. monocytogenes*	*P. aeruginosa*	*S. aureus*	*S. agalactiae*
Extracts Obtained By Method A	*U. zonata*	Water	_	_	_	5±0.23	_	9±0.23	_	_
		Ethanol	_	_	_	_	_	8±0.2	5±0.3	_
		Methanol	9±0.4	8±0.2	_	6±0.15	_	_	6±0.2	_
	F. ntipyretica	Water	_	_	_	_	_	_	_	_
		Ethanol	5±0.2	_	_	_	_	9.5±0.3	6±0.2	_
		Methanol	_	_	_	_	_	9±0.4	_	_
	S. gracilis	Water	_	_	_	_	_	10±0.3	_	_
		Ethanol	_	_	_	_	_	6±0.2	_	_
		Methanol	_	_	_	_	_	7±0.3	_	_
Extracts Obtained By Method B	*U. zonata*	Ethanol	14±0.41	8±0.41	8±0.4	7±0.3	4±0.2	7±0.4	16±0.31	4±0.15
		Methanol	11±0.32	7±0.32	_	6±0.25	2±0.12	_	12±0.32	2±0.03
		Ether	19±0.54	8±0.4	8±0.35	8±0.2	6±0.5	9±0.2	17±0.34	10±0.12
	F. ntipyretica	Ethanol	13±0.52	8±0.3	6±0.42	7±0.33	3±0.22	7±0.3	7±0.22	6±0.1
		Methanol	12±0.42	7±0.42	_	6±0.2	2±0.12	_	5±0.12	4±0.33
		Ether	17±0.34	8±0.12	8±0.45	8±0.3	7±0.31	10±0.4	15±0.31	10±0.2
	S. gracilis	Ethanol	12±0.5	7±0.6	6±0.23	8±0.12	2±0.33	7±0.2	12±0.33	6±0.05
		Methanol	10±0.32	8±0.51	_	8±0.2	3±0.21	7±0.3	8±0.21	5±0.05
		Ether	15±0.4	9±0.31	8±0.33	8±0.23	6±0.31	7±0.6	15±0.31	8±0.13

Table 2. Positive control disc results (mm).

	Vancomycin 30 μg (V)	Neomycin 10 μg (N)
C. perfringens	27±0.56	22±0.43
E. faecalis	15±0.52	8±0.43
E. coli	11±0.56	10±0.15
E. coli O157:H7	12±0.56	18±0.65
L. monocytogenes	16±0.6	10±0.5
P. aeruginosa	12±0.5	10±0.4
S. aureus	23±0.25	12±0.33
S. agalactiae	20±0.52	18±0.45

DISCUSSION

Colostridium perfringens; It is very important because it can cause food poisoning, which can have both negative effects human health and cause economic losses (Diane et al., 2010; Sert, 2014). In the study, on *C. perfringens*; It is observed that there is zone formation (between 10±0.32 mm and 19±0.54 mm) in the

extracts obtained with method method B (with the Soxhlet device). Zone formation is less in method A. In a study investigating the antimicrobial effects of ethanol extracts of 30 different brown algae species on *C. perfringens* (Lee et al., 2009), it was highlighted that especially *Ishige okamurai*, *Ecklonia stolonifera, Sargassum siliquastrum, Sargassum thunbergii*, *Colpomenia bullosa*, and *Ecklonia cava* algae species are effective. It has been reported that ethanol extract of *Myagropsis myagroides* type algae collected from the coasts of Korea and Japan and evaluated in the Phaeophyta family is effective against *C. perfringens* (Lee et al., 2011). As can be seen when reviewing the relevant literature, the algae species, the method of obtaining the extract and the solvent used were found to be effective on the antimicrobial properties. In fact, in the study; It was observed that the effects of extracts prepared from the same algae species on *C. perfringens* were different.

Against *E. faecalis*, which is very important due to its pathogenic aspect and as an indicator of fecal contamination in food; although the antimicrobial properties of 18 different extracts prepared with different solvents from three different algae species varied (Table 1), it was observed that the highest effect was observed in the extract from *Spirogyra gracilis* using ether (9±0.31 mm). Kolanjinathan and Stella (2009) found that extracts of macroalgae (*Sargassum myricystum, Turbinaria conoides, Hypnea musiformis, Gracilaria edulis* and *Halimedia gracilis*) from the south-eastern coast of India prepared with methanol, ethanol and acetone are potential sources of bioactive compounds by acting on *E. faecalis*. They reported that it should be studied by taking antibiotics.

In the antimicrobial effect analyzes against *E. coli*, it was seen that the extracts obtained by method A showed no effect, the extracts obtained by method B using ether and ethanol with the Soxhelet device were effective, but there was no effect in the groups in which methanol was used as a solvent. According to the results of antimicrobial effect the prepared algae extracts on *E. coli* O157:H7 (Table 1); In the groups obtained with water and methanol from the extracts formed from *U. zonata* by method A; An effect of 5±0.23 mm to 6±0.15 mm was observed. It was found that the extracts obtained by using the Soxhelet device showed an effect on *E. coli* O157:H7 in all solvents used, and this effect varied between 6 mm and 8 mm. Tuney et al. (2007), examined the antimicrobial properties of 98 extracts from 13 different algae species collected from the shores of Izmir in various forms against different groups of microorganisms; it was found that many algae species did not show any effect on *E. coli*, some species were effective, and the most ether group, followed by ethanol, was determined in terms of the solvent used. In fact, in our study; while none of the extracts obtained by method A had any effect on *E. coli*, an effect was observed in the groups obtained by using ether and ethanol in method B. In this respect, the study is in harmony with the relevant literature. In the study investigating the antimicrobial effects of six different algin extracts prepared with methanol on *E. coli* O157:H7; It was reported that the highest effect was obtained *C. barbata* species and zones with 22.33 ± 0.57 mm diameter were formed. The second highest effect was demonstrated by zones measuring 12.33±0.57 mm from the *Cladostephus spongiosus* species. It has been reported that no effect was observed in other algae species (Taşkın et al., 2007).

Antimicrobial property against *L. monocytogenes*, an important pathogen that can cause many problems in food, especially meat and meat products; while none of the extracts obtained by method A were effective, the extracts obtained by method B were found to be effective. Ether was found to be the most effective solvent (zones between 6 mm and 7 mm). In a study investigating alternative antimicrobials against *L. monocytogenes*, the effects of various marine algae were investigated by disc diffusion method. It was suggested that methanolic extracts of *Ecklonia cava* algae showed the highest efficacy and could contribute to the development of an alternative phytotherapeutic agent against Listeria infections (Nshimiyumukiza et al., 2015).

In a study where the antimicrobial properties of *Spirogyra aequinoctialis* algae extracts were determined by disc diffusion method against various microorganisms, it was determined that it is highly effective against *E. coli* O 157:H7, *S. aureus* is the most susceptible, and *L. monocytogenes* is moderately sensitive (Pabuccu and Yücer 2022).

P. aeruginosa; It is a very important microorganism as it can cause many nosocomial infections, shows rapid and increasing resistance to antibiotics, and therefore facesmany difficulties in its treatment (Gündüz et al., 2004; Ciftci et al., 2005; Ince et al., 2014; Oner et al., 2022). In the study; The largest inhibition zones on *P. aeruginosa* were determined in the extract of *F. antipyretica* obtained by using ether (10±0.4), and in the extract of *S. gracilis* algae species using water by method A (10±0.3). The methanol extract of the marine algae of *Gracilaria changii* species against *P. aeruginosa*; It has been suggested that 6.25 mg/ml has an antimicrobial effect by forming an inhibition zone with a diameter of 13 mm and this effect is at a good level (Sasidharan et al., 2010). Methanolic extracts of algae such as *Mikroket tenera, Nitella tenuissima* and *Sphaeroplea annulina* showed significant antibacterial activity against *P. aeruginosa*, respectively; It was reported to form inhibition zones with a diameter of 19.5±0.06 19±0.50 19.5±0.20 mm (Prashantkumar et al., 2006).

S. aureus is a bacterial species that can cause many bacterial food poisoning and infections seen today, has a high pathogenicity, and has become increasingly important with the emergence of many drug-resistant strains (Erol, 2007; Foster, 2017; Taylor and unakal, 2022). In the study, the greatest antimicrobial effect detected against *S. aureus* was observed in the extracts obtained using ether in method B (highest: 17±0.34 mm, lowest: 15±0.31 mm) (Table 1). No effect was observed in any extract obtained from *S. gracilis* by method A. In conclusion; The antimicrobial effect investigated against *S. aureus*; It can be said that it varies according to the type of algae, the method used and the type of solvent. In a study investigating the antimicrobial properties of green algae of the species *Scenedesmus protuberans*, it was found that the antimicrobial effect varies depending on the solvent used. In this situation; It has been reported that as the polarity of the solvents used to extract *S. protuberans* decreases, their antimicrobial effect increases (Demirel, 2006).

S. agalactiae is a very important bacterium in terms of public health, as it can cause a wide variety of infections such as meningitis, sepsis, skin and soft tissue, puerperium and urinary tract (Savcı et al., 2018; Ayhancı et al., 2020). In the study, when the diameters of the zones created against *S. agalactiae* were examined, it was found that while none of the extracts obtained by method A showed antimicrobial activity, inhibition zones were formed in all groups created with the Soxhelet device (lowest 2 mm, highest 10 mm). According to the results of the study investigating the phytochemical composition, antioxidant and antibacterial activity of Philippinesea green algae (*Ulva pertusa*); of *S. aureus, E. coli, Aeromonas hydrophila, A. sobria* and *Vibrio harveyi*, depending on the concentration used. It has been reported to have a strong inhibitory effect on *S. agalactiae* and inhibiting its growth. In addition, it has been stated that *U. pertusa* contains bioactive compounds with good antioxidant capacities and because of these properties, they are promising candidates that can be used in the synthesis of new drugs (Pakingking et al., 2022).

CONCLUSION

As a result; it was observed that each algae species, extraction method and solvent type was effective on the antimicrobial properties. It was observed that the extracts obtained by using the Soxhlet device (Method B) showed more antimicrobial effect on the selected microorganisms, and the effect of ether

was higher in this method. It is believed that algae and such studies are important in the research of new and plant-based drugs as the antibiotic resistance is gradually increasing.

Acknowledgment

This study includes part of the master's thesis. The research was supported by Firat University Scientific Research Projects Coordination Office (FUBAP) as Project number SUF.22.02.

Conflict of interest

There is no conflict of interest among the authors.

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How to cite this article/Bu makaleye atıf için:

Özpolat, E., & Sapmaz, M. (2024). Determination of antimicrobial effect of extracts produced from some freshwater algae using different methods. *JOGHENS-Journal of Global Health & Natural Sciences, 7*(2), 107-116. https://doi.org/10.56728/dustad.1542958