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RESEARCH ARTICLE / ARAŞTIRMA MAKALESİ

Assessment of the Antibacterial Potency of *Usnea* sp. against Foodborne Pathogens

Usnea sp.'nin Antibakteriyel Etkisinin Gıda Kaynaklı Patojenlere Karşı Değerlendirilmesi

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Abstract
The increase in the incidence of foodborne diseases has been demonstrated by epidemiological studies, and the adverse impact on the socio-economic development of countries has been also reported by health authorities. The combat against foodborne pathogens through the use of natural biosources has become the focus of recent research. Lichens produce several secondary metabolites with various biological activities including antibacterial, antifungal, anti-cancer etc. due to competition with other living things in their surrounding environment. In this perspective, we aimed to investigate the antibacterial properties of *Usnea* sp. that collected from Kastamonu, Turkey against five foodborne pathogens in the present study. These tested bacteria included both Gram-positive and Gram-negative ones. Our data demonstrated that the acetone extracts of *Usnea* sp. had antibacterial efficiencies especially against Gram-positive bacteria tested (*Clostridium perfringens*, *Staphylococcus aureus*, and *Bacillus cereus*) at varying percentages. This potential antibacterial activity of *Usnea* sp. suggests that it can be used in the food industry. Since it has already been reported to be used in dishes or ingredients of bread, it gives the idea that it may be used as a food additive (such as a preservative, extending shelf life). However, detailed studies for its toxicity or the dosages that do not be toxic should be done.
Keywords: Foodborne pathogen, lichen, *Usnea*, antibacterial.

Öz
Gıda kaynaklı hastalıkların görülme sıklığındaki artış, epidemiyolojik çalışmalarla ortaya konulmuş ve bu nedenle ülkelerin sosyoekonomik kalkınmalarına olan olumsuz etkisi de sağlık otoriteleri tarafından rapor edilmiştir. Doğal biyokaynakların kullanımı yoluyla gıda kaynaklı patojenlere karşı mücadele, son yıllardaki araştırmaların odak noktası haline gelmiştir. Likenler, çevrelerindeki diğer canlılarla rekabet etmeleri nedeniyle antibakteriyel, antifungal, anti-kanser vb. gibi çeşitli biyolojik aktivitelere sahip birçok ikincil metabolit üretirler. Bu açıdan, bu çalışmada Türkiye, Kastamonu'dan toplanan *Usnea* sp.'nin beş gıda kaynaklı patojene karşı antibakteriyel özelliklerini araştırmayı amaçladık. Bu bakteriler hem Gram-pozitif hem de Gram-negatif olanları içermiştir. Verilerimiz, *Usnea* sp.'nin aseton özütlerinin özellikle test edilen Gram-pozitif bakterilere (*Clostridium perfringens, Staphylococcus aureus,* ve *Bacillus cereus*) karşı değişen oranlarda antibakteriyel etkinliğe sahip olduğunu göstermiştir. *Usnea* sp.'nin bu potansiyel antibakteriyel aktivitesi, gıda endüstrisinde kullanılabileceğini düşündürmektedir. Daha önce yemeklerde veya ekmek malzemelerinde kullanıldığı bildirildiğinden, gıda katkı maddesi (koruyucu, raf ömrünü uzatan gibi) olarak kullanılabileceği fikrini vermektedir. Ancak toksisitesi veya toksik olmayan dozları için detaylı çalışmalar yapılmalıdır.
Anahtar Kelimeler: Gıda kaynaklı patojen, liken, *Usnea*, antibakteriyel.

I. INTRODUCTION

Foodborne diseases are of great importance in terms of public health because they cause many complications and death. The Centers for Disease Control and Prevention (2018) declared that 47.8 million people suffer from foodborne diseases, and amongst them, 128.000 hospitalizations and 3.000 deaths are reported each year in the United States [1]. Considering the epidemiology of foodborne diseases, some reasons for the worldwide increase of foodborne illness are attributed to large-scale production and distribution of food, increase in international food trade, rapid population increase, tourism increase, microbial adaptation, and antimicrobial resistance, etc. [2]. Foodborne diseases are considered as related to destitution in undeveloped countries, the danger for the transport of contaminated food across countries leads to increased cases of foodborne diseases. On the other hand, the exact incidence ratios of food poisoning cases cannot always be monitored and surveillance by public healthcare systems due to unreported cases or outbreaks [3, 4]. Unfortunately, there is not sufficient statistical data even in developing countries. To evaluate the incidence of foodborne illness requires some skill practices in food microbiology, chemistry, epidemiology, etc. Although these competencies can be provided in developing countries, there is some

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lack of monitoring and surveillance systems for foodborne diseases [5]. Also, it is sometimes difficult to distinguish which diarrheal disease is food-related because all gastroenteritis is not food-related every time. Therefore, the precise global incidence of foodborne diseases is being investigated in several studies [4]. Foodborne pathogens such as *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Listeria monocytogenes, Campylobacter jejuni, Clostridium perfringens,* and *Pseudomonas aeruginosa* are responsible from various diseases via contaminated by food or food processing equipment [6]. These bacteria are reported to be associated with acute such as abdominal pain, diarrhea, fever, malaise, sometimes bloody stools, dehydration, exhaustion, headache, and chronic complications such as erythema nodosum, hemolytic uremic syndrome, chronic kidney disease, Guillain-Barré syndrome (GBS), hemolytic-uremic syndrome, meningitis, pancreatitis etc. [7].

The pivotal importance of food safety has been emphasized in nutrition and food security since the primary affected group from these diseases are infants, young children, the elderly, and the sick [8]. These facts prompted the researchers to investigate the ways to combat food-related pathogen microorganisms and to prolong the shelf life of processed food to both protect the consumers' health and also ensure waste management [9]. Although more emphasis is placed on food processing technologies, transport, hygiene rules, and people know more about foodborne pathogenic microorganisms, governments cannot avoid economic losses and medical costs [10]. In addition, concerns about outbreaks associated with foodborne illnesses emphasize the importance of food safety. In order to ensure food safety, the use of alternative natural resources in food processing has come into prominence. In this respect, there are many studies investigating potential antimicrobial agents which have positive effects on shelf life, microbial degradation, oxidation reactions, etc. [11-15]. Natural biosources such as plants, lichens, animals, etc. are known to have unique compounds with properties of medicinal and numerous biological activities [16].

Lichens are symbiotic living organisms that are composed of their algae or cyanobacteria (photobiont) and fungal (mycobiont) partners that show mutualistic beneficial relationships among themselves [17]. They are one of the natural resources with many biological activities. Lichens can survive in various geographic areas, sometimes even in extreme conditions [18]. From ancient times, lichen species have been utilized for food and ethnomedicinal purposes in various countries. In addition, it has been reported that lichens have abundant nutrients (carbohydrate, crude fiber, and ash) that vary according to the species [19]. On the other hand, various pharmacological activities of lichens such as antioxidant, anticancer, antimicrobial, anti-inflammatory, antidiabetic etc. have been reported in the literature. The health benefits of these organisms are attributed to various secondary metabolites [18]. It has been stated that 2.000 of the 20.000 species belonging to various lichens are localized in different regions in Turkey. Different biological activities of these lichen species were investigated in various studies [20, 21]. It has been reported that some species of lichens such as *Cetraria islandica*, *Cladonia rangiferina, Platismatia glauca, Usnea barbata, Usnea longissima*, etc. have been included in some dishes such as salads or in bread as well as incorporated into traditional healing practices such as poultice and tea [19]. In India, Dagad Phool (Stone Flowers) spice mixture containing various *Parmeliaceae* species (especially *Parmotrema* and *Everniastrum* species) also with *Ramalina* and *Usnea* species. These lichens were also reported to be added to curry as a bulking agent and preservative. The taste of *Usnea* sp. (beard lichen) is sweet to bitter with high carbohydrate (83.5-93%), protein (2-7.5%), and fat source (9%). This fructose lichen is distributed all over the world. The utilization of *Usnea* sp. as a bulking agent in soups, stews, and curries has been reported. For medicinal properties such as analgesic, antibacterial, antifungal, anti-inflammatory, anti-pyretic, anti-tumor, and antiviral etc. of this lichen species, dried, tincture or oil forms are used [22]. The utilization of *Usnea* species as decocted tea in Traditional Chinese Medicine (TCM) was reported in the literature. The usage dose was indicated as 60-120 mg usnic acid from 6-9 g of dried lichen per day [23]. This lichen is not very water-soluble; solvents are used to make an extract of *Usnea* sp. [22]. Usnic acid, a secondary metabolite found in *Usnea*, has been reported to be used in pharmaceuticals and cosmetics sectors with trade names such as Omnigran, Barba de la Piedra and etc., for the treatment of ailments or for personal care in different countries [23]. Based on the antibacterial activities of lichens against various bacteria, the potential to be used as a food additive or to provide hygienic conditions should be evaluated in the food industry.

From this point of view, our goal was to evaluate the potential antibacterial efficacies of the acetone extracts of *Usnea* sp. against five foodborne pathogens, *Bacillus cereus*, *Salmonella* sp., *S. aureus*, *E. coli* O157:H7, and *C. perfringens*, in this study. It was thought that the possible antibacterial effect would be meaningful for the food industry.

II. MATERIAL AND METHODS

2.1. Lichen Samples

The lichen samples belonging to *Usnea* sp. were harvested from Kastamonu province, Turkey weighing 20-30 gr. The identification of samples was performed by the classical taxonomic method via microscopic (stereomicroscope and light microscope) examination [24].

Usnea sp.: Turkey, Kastamonu province, Kapaklı Village, 41.24492, 34.18330, G. Cobanoglu.

2.2. The Preparation of Lichen Extracts

The samples were weighed and pulverized by liquid nitrogen in a porcelain mortar following the washing and drying steps. The samples (10 g) were kept in acetone solvent (100 mL) for 24 h, in a dark place and then, filtration was performed through filter paper. Evaporation of acetone was achieved with a rotary evaporator to obtain crude extracts.

2.3. Bacterial strains

In this study, *B. cereus, Salmonella* sp., *S. aureus, E. coli* O157:H7, and *C. perfringens* were tested. These bacterial strains were isolated from food samples by Eurofins Scientific Food Analysis Laboratory.

2.4. Antibacterial Test

B. cereus, Salmonella sp., *S. aureus, E. coli* O157:H7, and *C. perfringens* were grown in Tryptic Soy Agar at 37 °C for 24 h. The antibacterial tests of *Usnea* sp. extracts against test bacteria were performed in Tryptic Soy Broth via 96-well microplates (Greiner Bio-One, CellStar, F-bottom, with lid). The overnight bacterial cultures were adjusted to an optical density (OD) 0.01 at 600 nm in a 96-well plate. The extracts of *Usnea* sp. were applied for 12 dilutions. Control (untreated), blank wells, gentamicin and chloramphenicol as positive controls were also included in the experiments. As stated in the literature, positive controls were chosen because they had good *in vitro* antibacterial activity against the bacteria tested. Experiments were done in triplicate. The absorbance of bacterial growth was measured for each bacterium for twenty-four hours using Cytation 3 multimode microplate reader (Biotek) at intervals of every 20 minutes.

III. RESULTS AND DISCUSSION

The antibacterial potency of the extracts of *Usnea* sp. was evaluated against *B. cereus* and our data showed inhibitory effects of test materials at the concentrations of 240, 120, 60, 30 and 15 µg/mL by the inhibition rates of 98.22±0.037, 96.03±0.015, 97.20±0.016, 90.71± 0.012 and 94.70±0.014%, respectively. There were moderately suppressive effects at the tested concentrations of 7.5 µg/mL and 3.75 µg/mL with the inhibition percentages of 47.53±0.066 and 41.94±0.205, respectively. Other concentrations tested had no antibacterial effect against *B. cereus*. The positive control (gentamicin) was also tested and completely killed this bacterium (Figure 1).

There was no noteworthy antibacterial effect against *Salmonella* sp. except first applied concentration (240 µg/mL). The other tested concentrations were not effective to inhibit bacterial growth of *Salmonella* sp. The positive control (gentamicin) had an inhibitory effect by the percentage of 98.91 (Figure 2).

We observed a remarkably suppressive effect on the bacterial growth of *S. aureus*. The inhibition percentages were recorded as 100, 100, 98.77±0.008, and 97.18± 0.019 for the first four concentrations (240, 120, 60, and 30 µg/mL), respectively. Moderate antibacterial efficacy was detected at a concentration of 15 µg/mL with an inhibition rate of 47.81±0.26%. A very slight inhibition for the growth of *S. aureus* was determined at the concentrations of 7.5, 3.75 and 1.875 µg/mL (26.13±0.072%, 21.36±0.032% and 12.52±0.038%, respectively). For the concentrations of 0.9375 and 0.46875 µg/mL, there was no antibacterial effect against test bacterium. On the other hand, lower concentrations of the extracts of *Usnea* sp. had a stimulating effect on the bacterial growth of *S. aureus*. Chloramphenicol was tested as a positive control and the inhibition rate was calculated as 97.29% (Figure 3).

On the other hand, we did not observe any inhibitory effect against *E. coli* O157:H7. While no antibacterial effect was observed with the inhibition rates remaining at very low percentages such as 1.5% and 3%, even an increase in bacterial growth was observed at some concentrations, albeit at a very low rate. Gentamicin had an inhibitory effect on the bacterial growth by the percentage of 99.52 (Figure 4).

The antibacterial activity of *Usnea* sp. extract against *C. perfringens* was determined from 240 µg/mL to a concentration of 0.23 µg/mL for the eleven dilutions tested. The inhibition percentages were recorded as 46.74±0.053, 48.82±0.057, 47.77±0.023, 38.15±0.076, 35.42±0.183, 34.21±0.06, 52.71±0.028, 44.06±0.03, 42.11±0.013, 43.75±0.024, and 45.02±0.03. At the twelfth concentration, the inhibition ratio was detected as 21.63±0.05%. The antibiotic gentamicin was also tested in the experiments and the inhibition percentage was calculated as 99.95 (Figure 5).

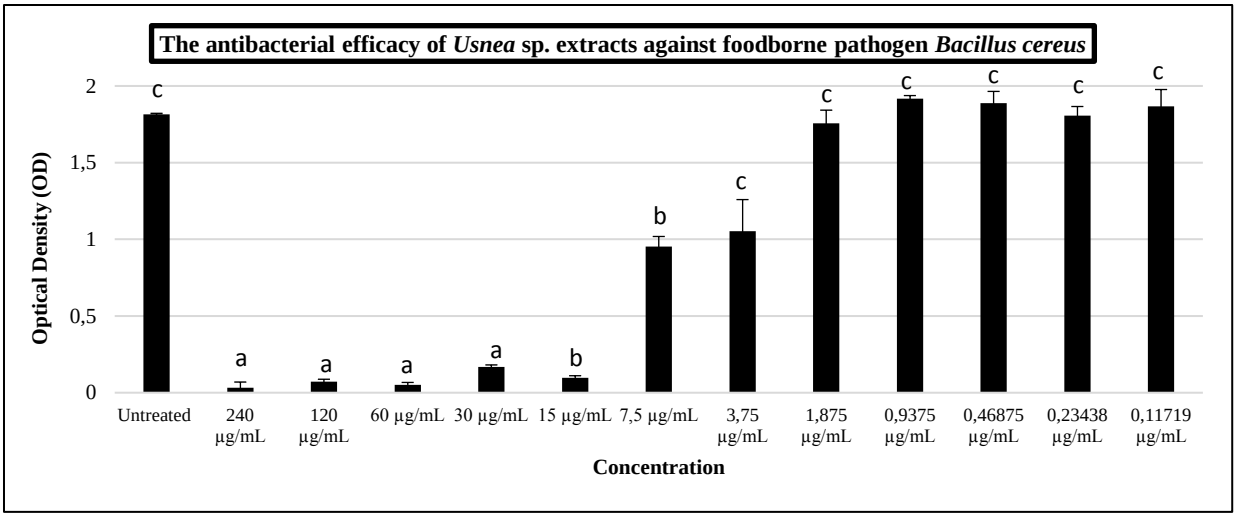

Figure 1. The antibacterial efficacy of extracts of *Usnea* sp. against *B. cereus.*

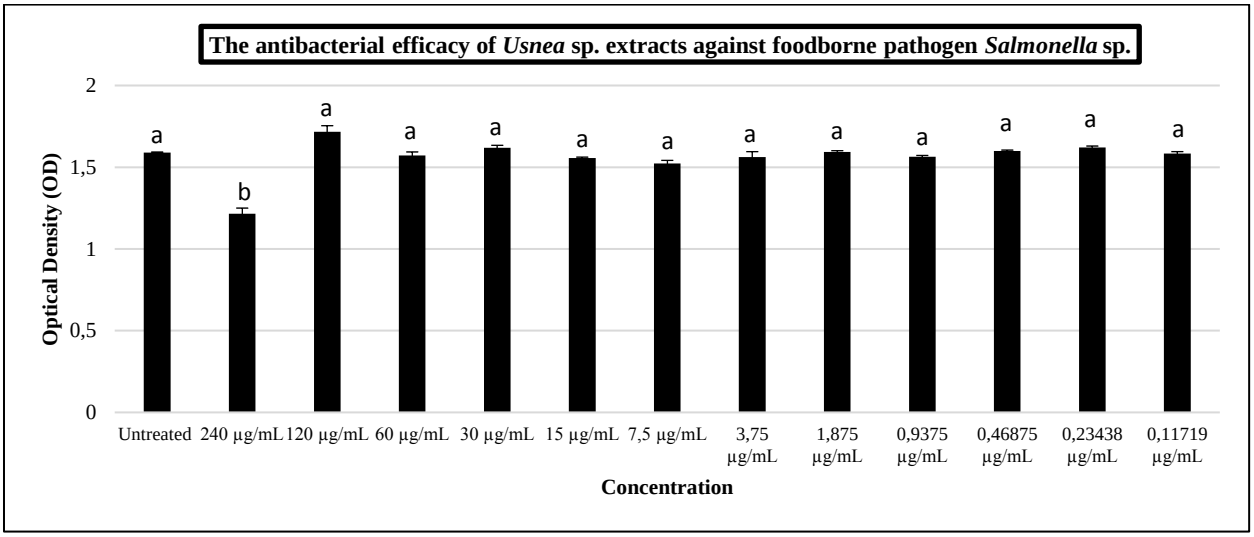

Figure 2. The antibacterial efficacy of extracts of *Usnea* sp. against *Salmonella* sp.

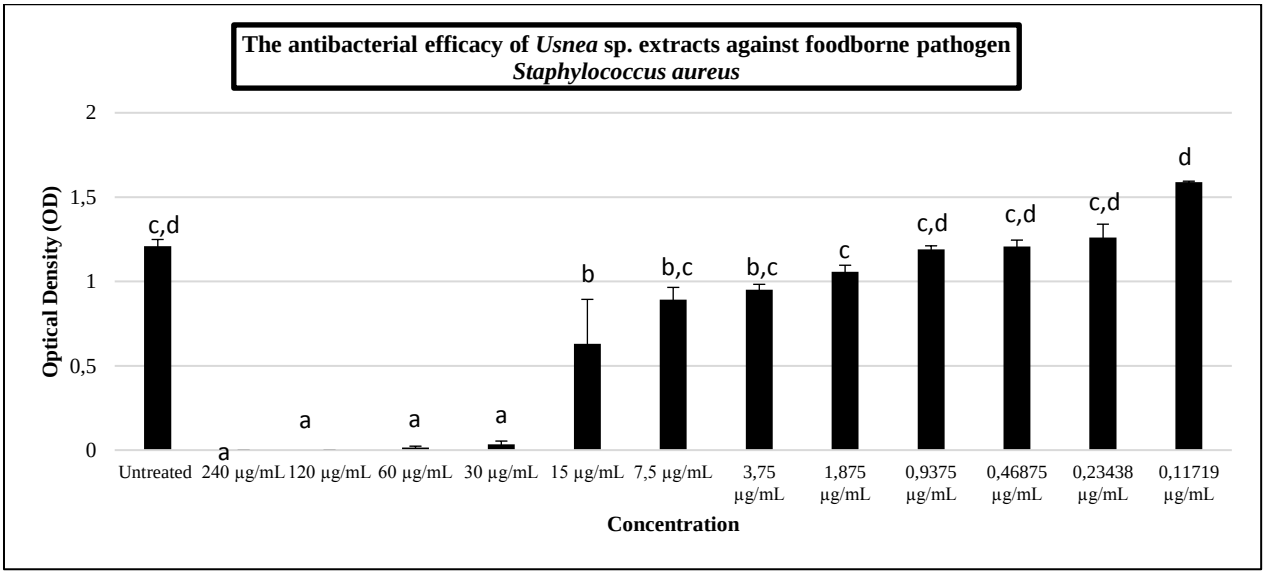

Figure 3. The antibacterial efficacy of extracts of *Usnea* sp. against *S. aureus*.

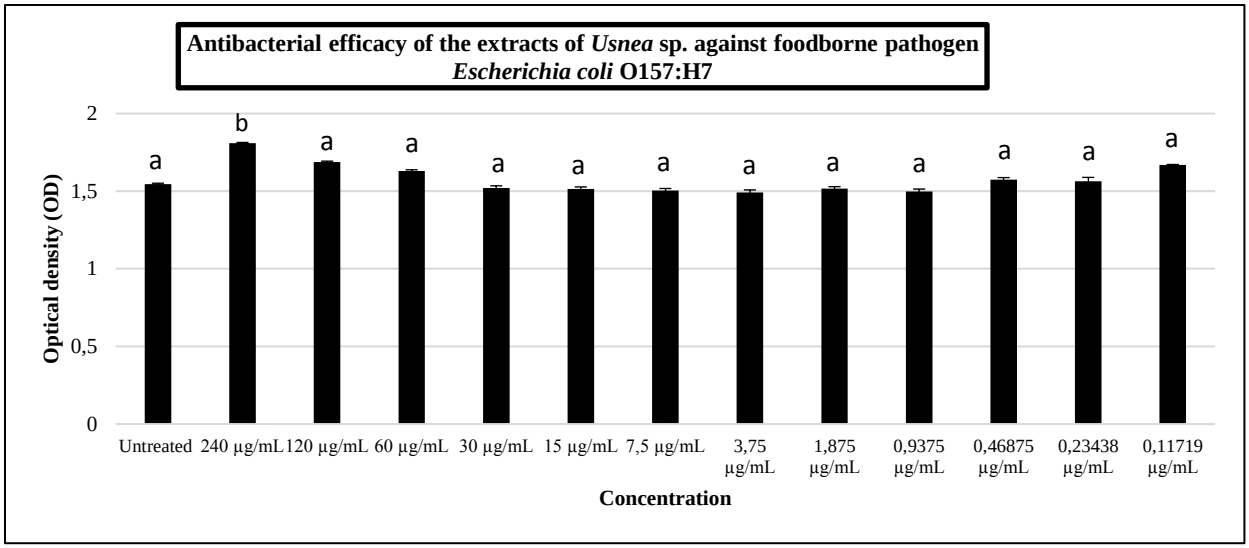

Figure 4. The antibacterial efficacy of extracts of *Usnea* sp. against *E. coli* O157:H7.

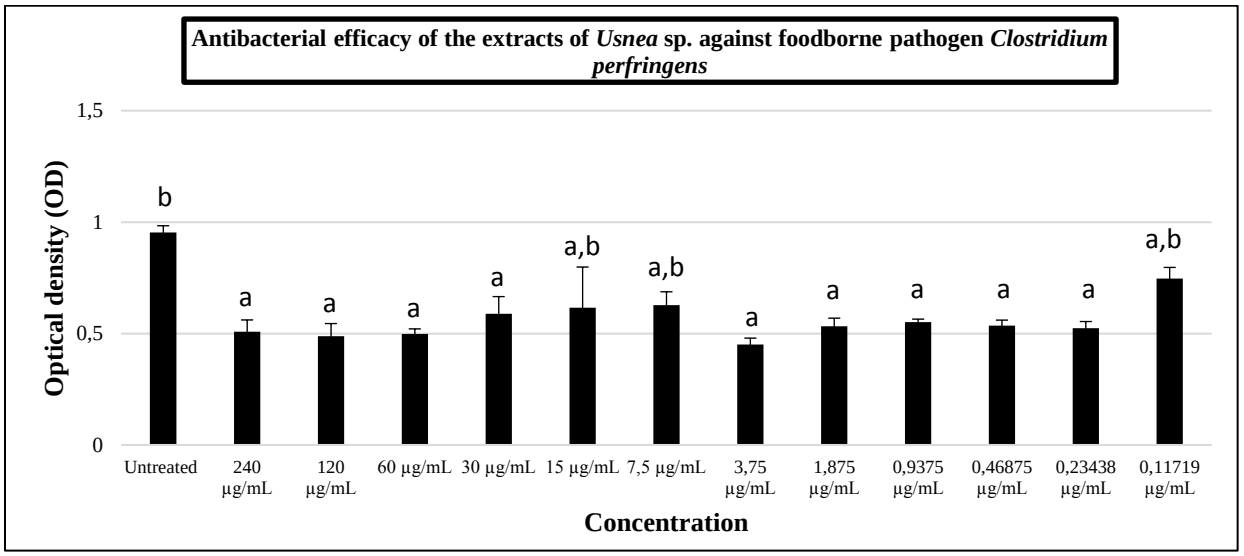

Figure 5. The antibacterial efficacy of extracts of *Usnea* sp. against *C. perfringens*.

In our study, we detected that *Usnea* sp. had inhibitory effects on the bacterial growth of *B. cereus*, *S. aureus*, and *C. perfringens* at varying percentages. Particularly, we selected the five foodborne pathogens, Gram-positive (*B. cereus*, *S. aureus*, and *C. perfringens*) and Gram-negative bacteria (*Salmonella* sp., and *E. coli* O157:H7) to compare the results. We observed the potential antibacterial efficiency against Gram-positive ones. On the other hand, we could not find any activity against Gram-negative bacteria that we tested. In accordance with our data, similar results were also emphasized in the literature. The differences in the cell membranes of these bacterial groups were indicated by the researchers as the reason for the different results between the two groups in studies where antibacterial potencies were tested [25]. Madamombe and Afolayan (2003) studied the antimicrobial efficacy of *U. barbata* against several bacteria and reported less activity against Gram-negative bacteria [26, 27]. Several solvents such as methanol, ethanol, ethyl acetate, hexane, or acetone are used to obtain the secondary metabolites of lichens. In our study, we preferred to test acetone solvent due to its properties to provide wide-ranging extraction of the polar and semipolar constituents. In a study investigating the comparative effects of various solvents conducted by Bharti et al. (2022), less antibacterial activity was reported against Gram-negative bacteria (*E. coli*, *Klebsiella pneumoniae*, and *P. aeruginosa*) by the acetone, methanol, and 70% hydroalcoholic extracts of *U. longissima* [28]. Londoñe-Bailon et al. (2019) evaluated the antibacterial potency of the extracts of *U. antarctica* and *U. aurantiaco-atra* against *S. aureus*, *Vibrio alginolyticus*, and *P. aeruginosa* and they detected antibacterial efficacy against *S. aureus* amongst tested bacteria [29]. Sepahvand et al. (2021) reported the more prominent antimicrobial effect of *Usnea* sp. against some Gram-positive including *S. aureus*, *B. subtilis*, *B. cereus*, and *Mycobacterium tuberculosis* whereas less activity against Gram-

negative bacteria [30]. These results show consistency with our results. For example, the MIC (minimum inhibitory concentration) value for methanol extracts of *U. ghattensis* against *B. subtilis* was reported to be 0.625 mg/mL [31]. Behera et al. (2005) found that the extracts of *U. ghattensis* that obtained via different solvents were effective against three *Bacillus* species, and *S. aureus* at the concentration of 5-10 µg/mL [32]. Similarly, we demonstrated the considerably high antibacterial effect of acetone extracts of *Usnea* sp. at 240 µg/mL concentration with the inhibition percentage of 98.22. Even at lower concentrations including 120, 60, 30 and 15 µg/mL, we observed the continuation of the effect with inhibition rates of 96.03%, 97.20%, 90.71% and 94.70%. We also observed efficacy at concentrations of 7.5 µg/mL and 3.75 µg/mL with inhibition percentages of 47.53 and 41.94, respectively. Furthermore, in accordance with our results, Shrestha (2015) reported no antibacterial effect of *U. hirta*, *U. lapponica*, and *U. strigosa* against *E. coli* [33]. However, the antibacterial potential of *Usnea* sp. against *E. coli* was reported in a study of Manoharachary and Nagaraju (2016) [34]. Bate et al. (2018) reported MIC value of methanol extract of *U. articulata* as 6 mg/mL [35]. These differences may be due to the type of lichen, the solvents used, the dosages applied, the geographical differences of the lichens harvested, the type of bacteria, and the secondary metabolites contained in the lichens. In the literature, it has been indicated that several extracts of *Usnea* sp. which obtained various solvents (*Umbilicaria americana, Usnea articulata, U. baileyi, U. barbata, U. ceratina, U. coralline, U. esperantiana, U. filipendula, U. florida, U. fulvoreagens, U. ghattensis, U. hirta, U. intermedia, U. lapponica, U. longissima, Umbilicaria mammulata, U. pectinate, U. rigida, U. strigosa, U. subflorida, U. subscabrosa, U. undulata*) had antibacterial effects against *S. aureus* [30]. Likewise, we also detected antibacterial potential against *S. aureus* at the concentrations of 240, 120, 60, and 30 µg/mL in our study. Weckesser et al. (2007) reported antibacterial efficacy of *U. barbata* extract against *C. perfringens* ATCC 13124 at low concentrations (MIC, 0.4 µg/mL) [36]. Our results showed moderate activity against foodborne pathogen *C. perfringens*, with the inhibition ratios ranging from 52.71% to 34.21%.

These antibacterial activities were attributed to the secondary metabolites of various lichen species such as depsides, depsidones, dibenzofurans, or xanthones [37]. Phytochemical researches revealed the presence of various many chemicals in different *Usnea* species such as usnic acid, zeorin, ergosterol peroxide, psoromic acid, methyl-β-orcinolcarboxylate, atranorin, barbatic acid, usnic acid, diffractaic acid, usneaceratin A and B, evernic acid, constictic acid, sekikaic acid, squamatic acid, stictic acid, thamnolic acid, norstictic acid, β-orcinol, salazinic acid, protocetraric acid, chloroatranorin, barbatolic acid, lobaric acid, etc. [30, 38, 39]. In particular, usnic acid, a yellowish pigment of *Usnea* sp., has been studied for its potential biological activities. Cansaran et al. (2006) studied six *Usnea* species against various Gram-positive and Gram-negative bacteria. *U. subflorida* was reported to have high antibacterial activities due to its abundant usnic acid content by the researchers [40]. In this study, since we could not detect any efficacy against Gram-negative bacteria, we may consider that potential antibacterial efficiency against Gram-positive bacteria may be due to other aforementioned chemicals. Unfortunately, it would be wrong to draw a definite conclusion from these results. In this respect, further studies have to be performed to analyze which chemicals of *Usnea* species, collected from different geographical regions, are responsible for this efficacy.

IV. CONCLUSION

In the present study, we demonstrated the antibacterial action of *Usnea* sp., collected from Kastamonu-Turkey, against Gram-positive *B. cereus*, *S. aureus*, and *C. perfringens*. Recently, the demands of customers in the food sector are directed towards natural food products. Natural biosources such as extracts or chemicals especially from plants are being investigated for utilization in food science by means of preserving foods (chicken, meat, etc.) or extending the shelf-life of foods. Foodborne diseases have great importance over the world due to the increasing numbers of patients affected by various food pathogens. Controlling these microorganisms means also handling foodborne diseases. Taking into consideration the potential of lichens for antibacterial activities, lichen-based products may be utilized in the food industry for preservation or extending the shelf-life of foods or beverages as food additives or providing hygiene conditions.

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