Antibacterial Properties of Several Lichen Extracts against Two Moderately Halophilic Bacteria from Salted Sheepskins

by

Türkmenoğlu, I.,[1] Toksöz, O.,[1] Berber, D.,[2*] Çağlayan, P.,[3] Birbir, M.[3] and Sesal, N.C.[3]

[1]*Marmara University, Department of Biology, Institute of Pure and Applied Sciences*, Istanbul, Turkey.

[2]*Maltepe University, Fine and Arts Faculty, Gastronomy and Culinary Department*, Istanbul, Turkey.

[3] *Marmara University, Department of Biology, Faculty of Arts and Sciences,* Istanbul, Turkey.

Abstract

It is well known that possible undesirable defects in finished leathers can result from microbial activities on the salted raw hides/skins during storage. The traditionally used salt curing method can control bacterial activities on the raw stock, but it does not seem possible to completely eliminate microbial attacks. Moderately halophilic bacteria can cause serious damage to hides/skins. From this point of view, potential procedures such as applying new antibacterial agents in the leather industry should be considered. Since some lichen species have been indicated for their promising biological efficacies in the literature; most researchers have focused on their potencies in various fields including leather industry from ancient times. In this study, the bacterial growth of proteolytic and lipolytic Gram-negative moderately halophilic bacteria, *Chromohalobacter canadensis* (YN6) and *Halomonas eurihalina* (BL5), from salted sheepskin samples were tested with the extracts of *Usnea* sp., *Plastismatia glauca, Ramalina farinacea, Evernia divaricata, Bryoria capillaris, Hypogymnia tubulosa, Pseudevernia furfuracea* and *Lobaria pulmonaria*. Some of these lichen species were found to be successful to inhibit the bacterial growth of *C. canadensis* (YN6) and *H. eurihalina* (BL5). In conclusion, lichen extracts may be utilized in stacked raw hides/skins in tanneries or warehouses to control moderately halophilic bacteria which causes several defects on leather.

Introduction

Since leather is the most traded product, it has great importance in the world economy.[1] It has been reported that the international trade value of the leather sector is about $80 US billion dollars annually. China, Italy, Korea, India, Russia, and Brazil have a major role in leather production, import, and export worldwide. In particular, footwear takes place at top of the market share.[2] According to The Food and Agriculture Organization (FAO) reports, approximately, seven million tons of hides and one million tons of skins are utilized for the conversion into finished leather every year.[3]

During leather-making processes, every precaution has to be taken to produce the best quality leather. Previous studies have demonstrated that the bacterial population on hides may cause serious defects in the finished product.[4-8] These bacteria can be either halophilic or non-halophilic bacteria depending on different steps in the leather-making process.[9-11] Unfortunately, applied preservation methods cannot prevent these organisms and several problems may be encountered on the finished leather.[6, 7, 12-22] The prevention of these bacterial activities is of high importance, as it means that new problems arise later in the other stages of leather making due to the unhindered bacterial population. To avoid these defective bacterial populations in the storage step of raw hides/skins, the salt curing method is generally preferred in developing and undeveloped countries.[10] However, it seems that this application does not work as expected. The need for alternative applications in the protection of raw hides/skins has arisen due to economic losses experienced in the sector.

As known, commonly encountered defects such as red discolorations, bad odor, holes, and deterioration on raw hides/skins are attributed to the activities of halophilic bacteria.[23] Amongst halophilic bacteria, moderately halophilic ones are known to grow in hypersaline environments due to their ability to produce compatible solutes which help to maintain cytoplasmic homeostasis. Since the optimal conditions for these bacterial population are defined as 3-15% NaCl and pH 5-10, they may easily grow on salted hides/skins.[24] There are several studies focused on these moderately microorganisms in respect to their numbers or their potential defects. The number of moderately and extremely halophilic archaea was reported to be 10^5-10^8 colony-forming unit/gram (CFU/g) from 131 hide samples of 34 different tanneries.[6] In another study, 10^5-10^8 CFU/g of moderately halophilic bacteria and 10^5-10^7 CFU/g of extremely halophilic archaea were determined in four salted sheepskins with discolorations, slimy surface, bad smell, and hair slips.[17] In the same study, a total of 78 moderately halophilic bacterial isolates representing 7 species (*Alkalibacillus halophilus*, *Pseudomonas halophila*, *Acinetobacter johnsonii*, *A. salilacus*, *Salimicrobium salexigens*, *Marinococcus luteus* and *Staphylococcus equorum* subsp. *equorum*), and a total of 101 extremely halophilic archaeal isolates representing 12 species (*Halorubrum tebenquichense*, *H. saccharovorum*, *Halococcus dombrowskii*, *H. qingdaonensis*, *Natrinema pellirubrum*, *H.*

*Corresponding author email: yazi47@hotmail.com

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morrhuae, *H. kocurii*, *H. terrestre*, *H. lipolyticum*, *Halostagnicola larsenii*, *Haloterrigena saccharevitans* and *N. versiforme*) were identified.[17] More detailed studies revealed that new moderately halophilic species, *Thalassobacillus pellis* sp. nov. and *Salimicrobium salexigens* sp. nov., may also be found on salted hide samples.[25,26] Thirteen species of moderately halophilic bacteria (*Salimicrobium luteum, Marinococcus halophilus, Halomonas koreensis, S. album, S. halophilum, H. elongata, H. halmophila, H. eurihalina, H. alimentaria, Oceanobacillus picturae, Thalassobacillus devorans, Chromohalobacter salexigens and Alkalibacillus salilacus*) were reported by researchers from the salt-pack cured hides.[16] Thirty-one moderately halophilic species belonging to the genus of *Staphylococcus, Salimicrobium, Bacillus*, *Salinicoccus, Planococcus, Alkalibacillus*, *Gracilibacillus*, *Oceanobacillus*, *Marinococcus, Halomonas*, *Salinivibrio*, *Chromohalobacter,* and *Idiomarina* and fourteen moderately halophilic bacteria belonging to the genus of Staphylococcus, *Bacillus, Gracilibacillus, Salinicoccus, Halomonas,* and *Chromohalobacter* were isolated and identified from salted sheepskins and salted goatskins, respectively.[18, 27] Due to their proteolytic and lipolytic activities of moderately halophilic bacteria on the salted goat skins,[27] the quality of leather may be affected negatively.

As mentioned earlier, it is of great importance to correctly combat the bacterial population prior to further steps to obtain a valuable product without economic loss. In this view, the bacterial population has to be under control from the beginning of the leather-making processes including the storage of raw hides/skins in warehouses. For this purpose, to control moderately halophilic bacteria with possible degradative and defective effects, different approaches were reported by the researchers such as direct and alternating electric current application,[28-30] bacteriocins,[31] antimicrobial agents such as alkyltrimethylammonium bromide, and a combination of alkyltrimethylammonium bromide, and chlorhexidine digluconate.[19, 22] However, bacteria may develop resistance to commonly used antimicrobial agents. The intrinsic or acquired resistance of bacteria to traditionally utilized antibacterial agents has potential. It has previously been demonstrated that some bactericides cannot be effective against the bacteria with proteolytic and lipolytic activities in soak liquors.[11, 32, 33]

In recent years, natural compounds from plants, microorganisms and lichens have been evaluated as promising alternatives for resistant bacteria.[34] Especially, lichens are reported with their various biological properties. They have been used for medicines, spices, foods, perfumes, and dyes. Studies have revealed that the healing properties of lichens used in the prevention of diseases are due to the acidic secondary metabolites in their structures.[35-37] Approximately 1050 unique secondary metabolites produced by lichens as metabolism products and these metabolites have various features (antimicrobial, antioxidant etc.).[38] Some antibiotic effective compounds are produced by the utilization of secondary metabolites obtained from lichens. Aslan et al. (2006), stated the antibacterial efficacy of methanol extracts of *Evernia divaricata* against *S. aureus, E. faecalis, E. coli* and *P. aeruginosa*.[39] However, it was observed that the antibacterial effects showed differences due to the bacterial species. Çobanoğlu et al. (2010) investigated that the extracts of five lichen species (*Alectoria sarmentosa, Bryoria fuscescens, E. divaricata, Platismatia glauca* and *Ramalina farinacea*), against *P. aeruginosa, E. coli* and *Acinetobacter* and the researchers found effective results depending on the bacteria.[40] In the study conducted by Esimone and Adikwu (1999), *R. farinacea* had antibacterial and antifungal effects.[41]

Lichens that are used for tanning belong to the genus *Cetraria islandica* and *Lobaria pulmonaria*.[42, 43] The efficiency of extracts belonging to *Pseudevernia furfuracea (L.) Zopf.* has been tested in the leather industry.[42] More recently, the potential antimicrobial effects of several lichen extracts against some bacterial species isolated from soak liquor samples have been reported.[44-46]

Considering the problems caused by the halophilic bacterial population in the stages of leather processing, especially in the storage period of raw hides, which affects further steps of the manufacturing, the need for new natural antibacterial agents come into prominence. The presence of the high number of halophilic bacteria in raw hides/skins preservation occurs due to the failure to provide the desired conditions during storage in warehouses and the extra bacterial load that may come from the salts used in salt curing with potential lipolytic and proteolytic activities. Furthermore, there is an adverse environmental impact of salt curing method and the subsequent soaking procedure which results in the discharge of excessive salt. Natural resources can be a solution to minimize the use of salt in raw hide curing, at least these resources can be applied together with salt. Thus, potentially harmful bacteria arising from salt can be kept at a minimum level. It is important to test the antibacterial activity of extracts obtained from natural sources on halophilic bacteria. In the literature, there are few studies on the antimicrobial potency of lichen extracts against moderately halophilic bacteria with proteolytic and lipolytic activities. Researchers isolated some proteolytic and lipolytic Gram-negative moderately halophilic bacteria from salted sheepskin samples.[18] It was stated that the main cause for the presence of bad odor, cream and yellow discolorations, sticky appearance maybe due to these bacterial species.[18] From this point, we tested the antimicrobial properties of the extracts of *Usnea sp.*, *P. glauca, R. farinacea, E. divaricata, Bryoria capillaris, Hypogymina tubulosa, P. furfuracea* and *Lobaria pulmonaria* as natural resources against *Chromohalobacter canadensis* (YN6) and *Halomonas eurihalina* (BL5) which were isolated from salted sheepskin samples in the previous study.[18]

Materials and Methods

Moderately Halophilic Test Bacteria

Moderately halophilic test bacteria (*C. canadensis,* YN6 and *H. eurihalina,* BL5) obtained from the culture collection of the Division of Plant Diseases and Microbiology, Biology Department, Faculty of Arts and Sciences, Marmara University (Turkey) were used in this study. These bacteria were isolated from salted sheepskin samples imported from Greece and Bulgaria and identified with molecular methods in the previous study of Caglayan et al. (2017).[18] While optimal growth conditions for *H. eurihalina* were detected as 10% NaCl, 37°C, and pH 7, *C. canadensis* showed optimum growth at 7.5%-10% NaCl, 30°C-37°C, and pH 7.[18] Complex Agar Medium I (CMI) prepared with 0.5% yeast extract and 2% agar was utilized as the growth medium of test bacteria. The final salt concentration of CMI was 10% with the following composition (SW10, saline water): 0.7% $MgCl_2$, 0.96% $MgSO_4$, 8.1% NaCl, 0.2% KCl, 0.036% $CaCl_2$, 0.0026% NaBr and 0.006% $NaHCO_3$.[48] The colonies of *C. canadensis* (YN6) and *H. eurihalina* (BL5) grown on Complex Agar Medium I were checked for purity and pure colonies were used for the experiments.

Lichen Samples

The samples belonging to *P. glauca, R. farinacea, E. divaricata, B. capillaris, H.tubulosa, Usnea sp., P. furfuracea* and *L. pulmonaria* were collected from Bursa Aladağ region. The classical taxonomic method via microscopic examination was utilized in the identification of lichen samples.

Photos of lichen samples are given below.

Extraction of Lichen Samples

The extraction processes were started by washing and drying the collected lichen samples. These samples were placed in sterile bottles and acetone (ACS, ISO, Reag. Ph Eur) solvent was added to them and kept in a dark environment for 24 hours. After 24 hours, the samples were filtered through filter paper. Acetone was removed by evaporation with the help of a rotary evaporator, and acetone extracts of lichens were obtained and stored for use at +4°C.

Antibacterial Tests

C. canadensis (YN6) and *H. eurihalina* (BL5) were grown in Tryptic Soy Agar supplemented with salt and yeast extract at 37°C for 24 h. Tryptic Soy Broth (including salt and yeast extract) was utilized for the antibacterial tests of acetone extracts of tested lichen samples against test bacteria. Antibacterial efficacy was determined using 96-well microplates (Greiner Bio-One, CellStar, F-bottom, with lid). Firstly, 50 µL of medium was placed into each well in 96-well microplates. Then, 50 µL of the tested lichen extract were added. Two-fold dilution concentrations of the tested lichen extract were made in every subsequent well. After serial dilutions were made, 50 µL of overnight bacterial culture of YN6 and BL5 with an optical density (OD) 600 nm of 0.01 were added to the wells. Therefore, final volume was 100 µL in each well. The acetone extracts of lichen samples were initially added in five dilutions to Tryptic Soy Broth in each well. The acetone extracts were applied at the concentrations of 240,120, 60, 30 and 15 µg/mL (5 dilutions) or 240, 120, 60, 30, 15, 7.5, 3.75, 1.875, 0.9375, and 0.43875 µg/mL (10 dilutions). Depending on the species of lichen or bacteria, some lichen extracts showed efficacy up to 5 dilutions, while others showed efficacy up to 9 dilutions. It was not necessary to decrease the concentration in the groups that did not have an antibacterial effect below 15 µg/mL. Control

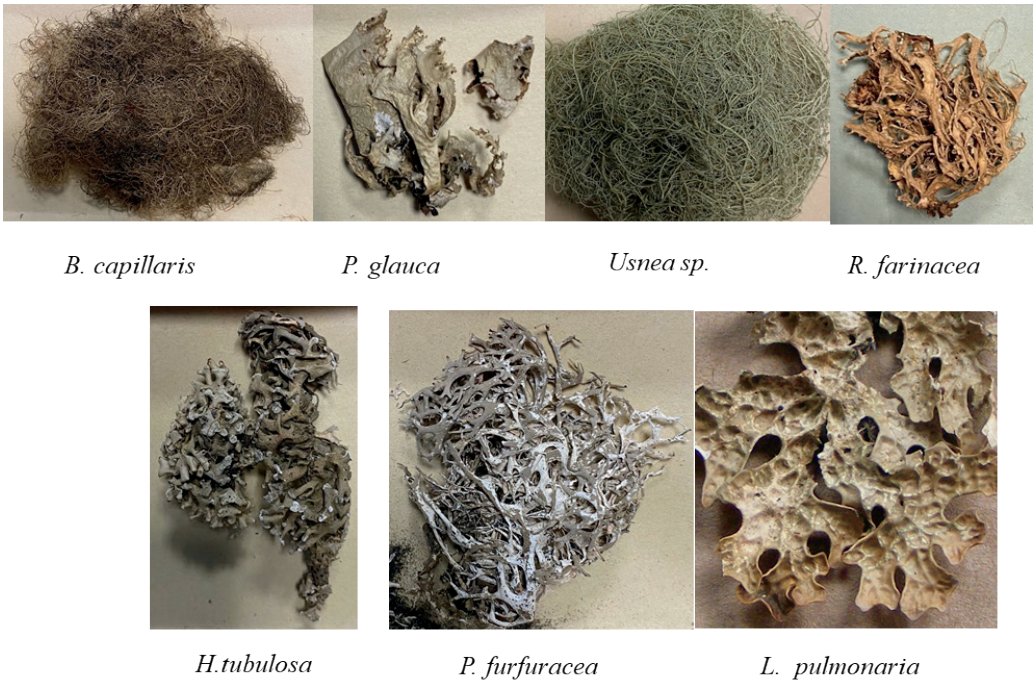

Figure 1. Photos of lichen samples.

(untreated group (medium and bacteria)) and blank (only medium) wells were also included in the experiments. Experiments were done in triplicate. The bacterial growth was evaluated every 20 minutes for 24 h using Cytation 3 multimode microplate reader (Biotek), by measuring the absorbance (OD, 600 nm) The measurement of absorbance provides information on changes in the optical density of the bacterial population. The antibacterial effects of acetone extracts of lichen samples against the test samples were compared with the control ones.[44-47] To test bactericidal or bacteriostatic effects of the tested lichen extracts against the test bacteria, first 10 µL of the test medium were taken from 24 h incubated-96 well microplates which include extracts and bacteria (test groups) and then spread onto the Tryptic Soy Agar medium containing salt and yeast extract. After incubation of the agar medium at 37°C for 24 h, bactericidal or bacteriostatic effects of the tested lichen extracts against the test bacteria were evaluated according to the presence or absence of bacterial colonies on the agar medium.

Statistical Analyses

Statistical analyses were evaluated by SPSS version 16.0 software program with One-way ANOVA (Tukey) to find significant differences between varying concentration groups of extract and untreated groups. A p-value below 0.05 was accepted as significant. The same letters in the figures indicate that there is no significant difference between the concentrations, while different letters indicate a significant difference.

Results and Discussion

As known, salt curing is a traditionally used preservation method to control halophilic and non-halophilic bacteria in different countries. However, these bacteria may cause destructive problems to the finished product. The potential efficacy of lichen extracts against moderately halophilic bacteria isolated from salted skins has not yet been studied in the literature. From this point, the potential antibacterial efficacy of some lichen species against two moderately halophilic bacteria such as *C. canadensis* (YN6) and *H. eurihalina* (BL5), which were isolated and identified from salted sheepskin samples in the previous study[18] has been examined. Therefore, the isolates mentioned above were chosen and the efficiency of the lichen extracts was tested on these microorganisms.

According to the results of the previous study,[18] the isolates, YN6 and BL5 were Gram negative bacteria, and positive for the biochemical activities of catalase, protease, lipase, methyl red degradation, and production NH_3 from peptone. Whereas YN6 was oxidase negative, BL5 was oxidase positive. Both strains were found to be positive for β-galactosidase activity and reduced nitrate to nitrite. Amylase and caseinase were not produced by the strains. BL5 was urease positive and produced H_2S whereas YN6 was indole positive. They were both citrate negative. Both YN6 and BL5 utilized L-arginine, L-glycine, L-alanine, L-tyrosine, and L-proline. In molecular analyses, these strains were assigned to *C. canadensis* (YN6) and *H. eurihalina* (BL5) with similarity percentages of 99.8 and 99.2%.[18] Overall these findings may suggest that *C. canadensis* (YN6) and *H. eurihalina* (BL5) may play an active role in skin biodegradation due to their proteolytic ability and utilization of the amino acids, which are main components of skin structure. As mentioned before, harmful activities of bacteria and skin degradation are unwanted situations for the finished leather as it causes loss of quality.

The need for potential antibacterial agents has been indicated in the literature. In this view, the antibacterial effect of lichen samples against various bacteria was analyzed in most studies. The antibacterial activity of the extracts from *P. furfuracea* lichen against several bacteria and fungi (*Staphylococcus aureus, Bacillus subtilis, B. cereus, Escherichia coli, Klebsiella pneumoniae, Enterococcus faecalis, Pseudomonas aeruginosa, Aspergillus niger, A. fumigatus, A. candidus, A. flavus, Penicillium jensenii, Geotrichum candidum* and *Candida albicans)* growing on the raw skin and chrome-tanned leather was carried out by Türkan, et al. (2013).[42] Furthermore, in recent studies, Berber (2020) and Berber et al. (2020) reported antibacterial activities of some lichen species including *H. physodes, E. divaricata, P. furfuracea, Usnea sp., P. sulcata* and *H. tubulosa* against several bacterial isolates which were obtained from soak liquor samples.[44-47] Berber (2020) demonstrated the potency of the acetone extracts of *H. physodes, E. divaricata, P. furfuracea* and *Usnea sp.* against *B. toyonensis, B. mojavensis, B. subtilis, B. amyloliquefaciens, B. velezensis, B. cereus*, and *B. licheniformis*.[44] More recently, Berber et al. (2020) screened six lichen species for the potential efficiency against *Enterococcus durans*, which was isolated from soak liquor samples.[45] The researchers reported that only the acetone extracts of *P. sulcata*, amongst these lichen extracts, had no antibacterial effect. While other lichen extracts had a considerable antibacterial effect against *E. durans*, *Usnea* sp. in particular had prominent efficacy. In another study, *H. tubulosa* extracts were reported to be more efficient against the aforementioned isolates when compared to the acetone extracts of *P. sulcata*.[46]

In the present study, the acetone extracts of *P. glauca*, *H. tubulosa*, *R. farinacea*, *Usnea sp.*, *E. divaricata*, *L. pulmonaria*, *B. capillaris*, *P. furfuracea* were examined against both test bacteria. Considering that *P. sulcata* extracts had no potential activity on isolates tested from soaked liquor samples in previous studies, *P. sulcata* was not included in this study. On the other hand, to the best of our knowledge, *P. glauca*, *R. farinacea*, *L. pulmonaria* and *B. capillaris* were firstly tested on isolates obtained from salted sheepskins in the leather industry. The acetone extracts were applied at the concentrations of 240-15 µg/mL (5 dilutions) or 240-0.46875 (10 dilutions) µg/mL. Depending on the lichen or bacteria, some lichen extracts showed efficacy up to 5 dilutions, while others showed efficacy up to 9 dilutions. Acetone as a solvent was chosen for the extraction of lichen samples due to its potency of comprehensive extraction of the compounds with various bioactivities. All of the extracts of lichen species were firstly screened

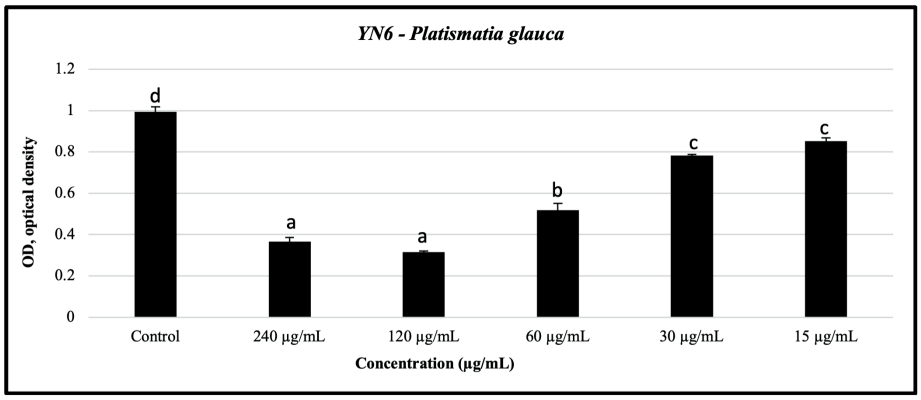

Figure 2. The antibacterial effects of the extracts of *P. glauca* against *C. canadensis* (YN6).

for the possible antibacterial efficacy on both bacteria. According to these preliminary screening studies, the extracts of *L. pulmonaria, B. capillaris* and *P. furfuracea* did not have any antibacterial effect on tested isolates. Otherwise, the extracts of *P. glauca, R. farinacea, Usnea sp., E. divaricata* and *H. tubulosa* were recorded to have various potential antibacterial efficacies against test bacteria.

The acetone extracts of *P. glauca* at concentrations of 240 and 120 µg/mL were shown to have average antibacterial activity against *C. canadensis* (YN6) when compared to the control group with inhibition rates of 63.14 and 68.22%, respectively. On the other hand, the inhibition percentages of *P. glauca* extracts for bacterial growth of YN6 were 47.83, 21.19 and 14.13% at tested concentrations of 60, 30 and 15 µg/mL. In statistical analyses for *P. glauca* extracts against *C. canadensis* (YN6), 240-15 µg/mL groups were found to be statistically significant compared to control groups ($p<0.05$). Significant differences were also detected in 240 µg/mL group when compared to all other treatment groups ($p<0.05$) except 120 µg/mL. In the statistical comparison of 120 µg/mL group, the differences were found to be significant between all groups ($p<0.05$). On the other hand, there were significant differences between 60 µg/mL and 30 µg/mL groups ($p<0.05$). But there was no difference in 30 and 15 µg/mL (Figure 2).

A great inhibition was detected in the bacterial growth of *C. canadensis* (YN6) by the acetone extracts of *H. tubulosa* at the concentration of 240 µg/mL. The inhibition percentages for *H. tubulosa* extracts were 81.12, 57.44, 67.26, 72.13 and 66.73 for the tested concentrations of 240-15 µg/mL, respectively (Figure 2). Statistically significant differences were detected in all treatment groups of *H. tubulosa* against *C. canadensis* (YN6) when compared to control groups ($p<0.05$). Similarly, each treatment group of 240 and 120 µg/mL differed significantly among themselves from the other low concentration groups ($p<0.05$). In 60 µg/mL treatment group, a significant difference has been detected with 30 µg/mL group ($p<0.05$) whereas no difference was observed with 15 µg/mL group. Also, 30 µg/mL group has demonstrated significant difference with 15 µg/mL group ($p<0.05$). This statistical information was included in Figure 3.

The acetone extracts of *R. farinacea* showed average antibacterial effects against YN6 as in *P. glauca* extracts. The concentrations of 240, 120 and 60 µg/mL were more successful according to the other tested ones with the inhibition ratios of 65.37, 71.37 and 54.57%, respectively. No noteworthy antibacterial effect (27.26 and 12.39%) was recorded at the concentrations of 30 and 15 µg/mL (Figure 3). In treatment groups with *R. farinacea* extracts, there were statistically significant differences when compared to control groups ($p<0.05$) except the 15 µg/mL group. The 240 µg/mL treatment group showed no significant difference with 120 and 60 µg/mL but significantly different from 30 and 15 µg/mL groups. In 60 µg/mL treatment group, significant difference has been detected with 30 and 15 µg/mL groups ($p<0.05$). Also, 30 µg/mL group has demonstrated significant difference with 15 µg/mL group ($p<0.05$). This statistical information was included in Figure 4.

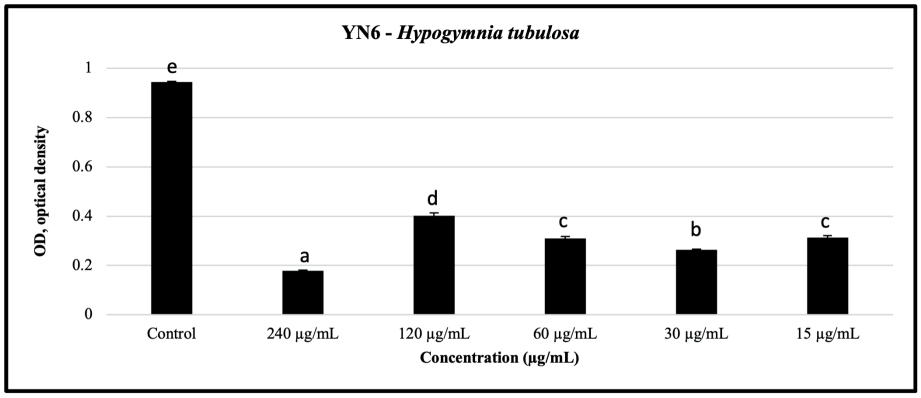

Figure 3. The antibacterial effects of the extracts of *H. tubulosa* against *C. canadensis* (YN6).

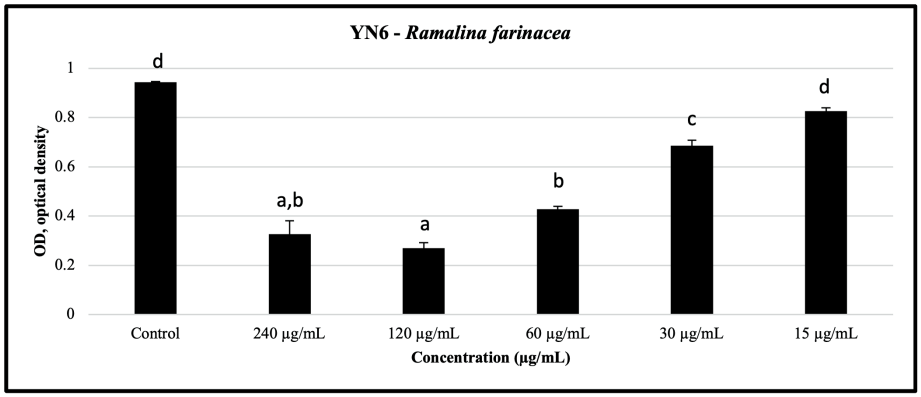

Figure 4. The antibacterial effects of the extracts of *R. farinacea* against *C. canadensis* (YN6).

YN6 isolate was also inhibited by more than 50% by the extracts of *Usnea* sp. at concentrations of 240, 120, and 60 µg/mL. The inhibition ratios were recorded as 89.5, 64.89, 63.58, 44.65, and 37.2% for all concentrations, respectively. Statistically significant differences were observed when all treatment groups were compared with the control group ($p<0.05$). Similarly, a comparison of 240 µg/mL group with the other treatment groups revealed a significant difference ($p<0.05$). At lower concentrations, there was no significant difference between the 120 and 60 µg/mL groups as well as 30 and 15 µg/mL groups (Figure 5).

The antibacterial efficacy was also observed for the extracts of *E. divaricata* against YN6 isolate. At the concentration of 240 µg/mL, 90.6% inhibition was detected on the bacterial growth. Also, antibacterial effects were observed at the concentrations of 120 and 60 µg/mL with inhibition ratios of 62.52% and 59.62%, respectively. The concentrations of 30 µg/mL and 15 µg/mL did not show notable efficiency against YN6 isolate with the inhibition ratio of 38.1 and 14.2, respectively (Figure 6). While no significant difference was found between 120 and 60 µg/mL treatment groups, a significant difference was observed between all other groups ($p<0.05$).

According to our results, the acetone extracts of *H. tubulosa* had considerable efficacy against *H. eurihalina* (BL5) at the concentrations of 240, 120, 60, 30, 15 and 7.5 µg/mL. The growth of BL5 was inhibited by the inhibition ratio of 84.52, 85.5, 90.41, 92.89, 97.12, 99.12%,

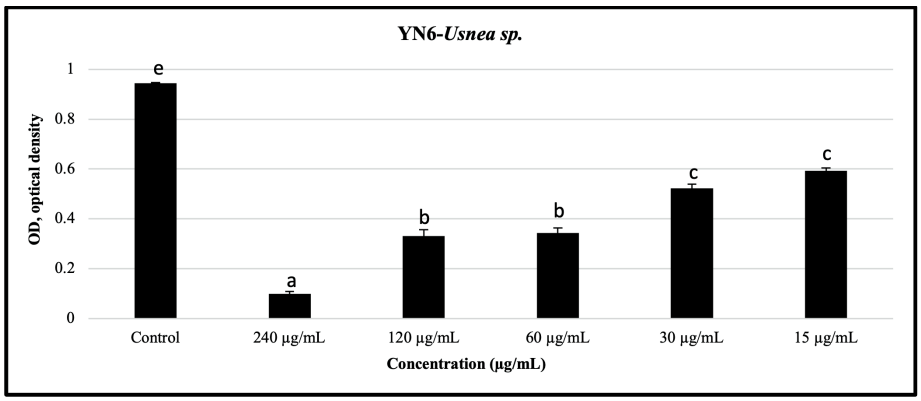

Figure 5. The antibacterial effects of the extracts of *Usnea* sp. against *C. canadensis* (YN6).

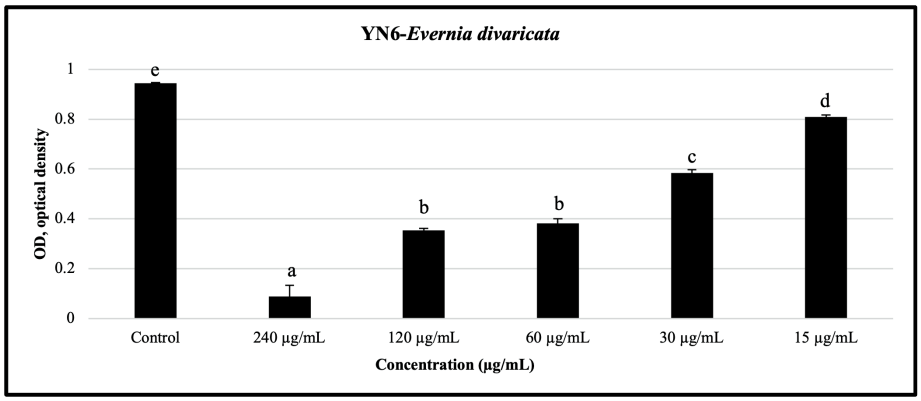

Figure 6. The antibacterial effects of the extracts of *E. divaricata* against *C. canadensis* (YN6).

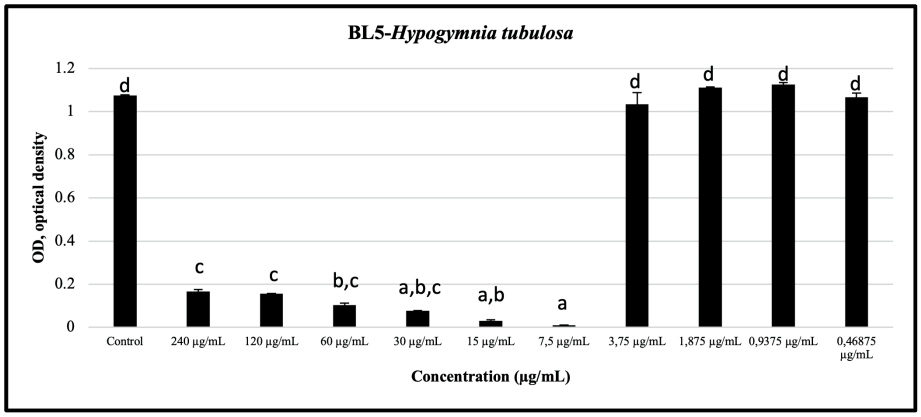

Figure 7. The antibacterial effects of the extracts of *H. tubulosa* against *H. eurihalina* (BL5).

respectively. As seen in Figure 7, the lower concentrations from 7.5 µg/mL (3.75, 1.875, 0.9375, and 0.46875 µg/mL) had no antibacterial efficacy against BL5. There was no significant difference between the 3.75 µg/mL treatment group and the lower concentration groups. In addition, there was no statistically significant difference between the 240 µg/mL treatment group and the 120, 60, 30 µg/mL groups, the 60 µg/mL treatment group and 30 and 15 µg/mL groups, and the 30 µg/mL treatment group and 15 and 7.5 µg/mL groups ($p<0.05$). Apart from these, it was observed that there was a significant difference between all other groups.

The most outstanding results were obtained with *Usnea* sp. extracts against *H. eurihalina* (BL5). As seen in Figure 8, the extracts were successful for the inhibition of BL5 bacterial growth up to 9 dilutions. However, at the last dilution increasing effect was recorded. Compared to control groups, detected inhibition rates were 82.65, 63.26, 87.73, 90.81, 91.72, 91.11, 91.68, 91.21 and 91.13% for 9 dilutions. At the last tested concentration, bacterial growth of BL5 was observed to increase at the percentage of 22.83. In statistical analyses for *Usnea* sp. extracts, all treatment groups, except 0.46875 µg/mL group, showed significant differences when compared to the control group ($p<0.05$). In the 120 µg/mL group, statistically significant differences were observed with the 30, 15, 7,5, 3,75, 1,875, 0,9375 µg/mL groups. There was no significant difference between the 240, 120, and 30 µg/mL groups. The 60 µg/mL group had no significant difference from the 30 and 15 µg/mL groups. Similarly, no significant difference was obtained among the 30 µg/mL group, 15 and 7.5 µg/mL groups.

Our results showed that the extracts of *R. farinacea*, especially at the concentrations of 240-30 µg/mL, had a great suppressive effect on bacterial growth of BL5 with the inhibition percentages of 98.01, 92.20, 98.12, 96.47. On the other hand, lower concentrations from 30 µg/mL, the inhibitory effect was not observed (between 30.84 and 4.31%) (Figure 9). All treatment groups, except the 7.5 µg/mL group, showed significant differences when compared to the control group ($p<0.05$). There was no statistically significant difference between 240, 120, 60, and 30 µg/mL treatment groups. Statistically, significant difference was observed between 60 µg/mL treatment group and the lower concentrations from 15 µg/mL group ($p<0.05$). The 15 µg/mL group showed statistically significant difference with the 7.5, 3.75, 1.875, 0.9375 and 0.46875 µg/mL groups ($p<0.05$). There was also a significant difference between the 7.5 µg/mL group and 3.75-1.875 µg/mL groups ($p<0.05$). On the other hand, there was no significant difference between 3.75 and 1.875, 0.9375 and 0.46875 µg/mL groups. Additionally, no difference was observed for 7.5 µg/mL and 0.9375 and 0.46875 µg/mL groups (Figure 8).

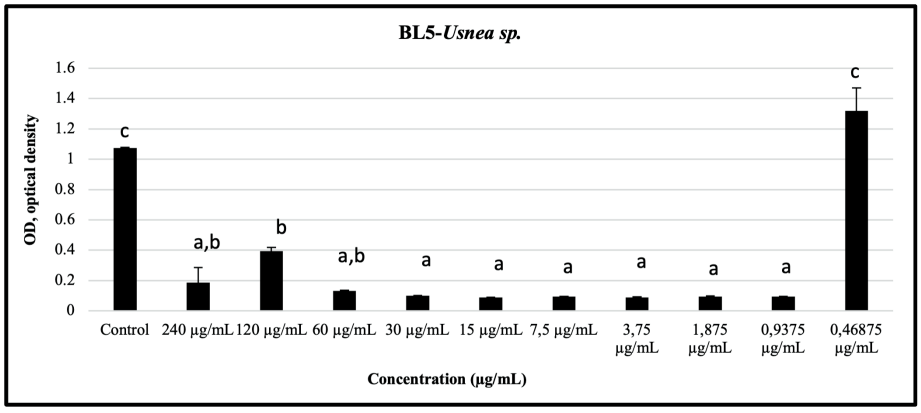

Figure 8. The antibacterial effects of the extracts of *Usnea* sp. against *H. eurihalina* (BL5).

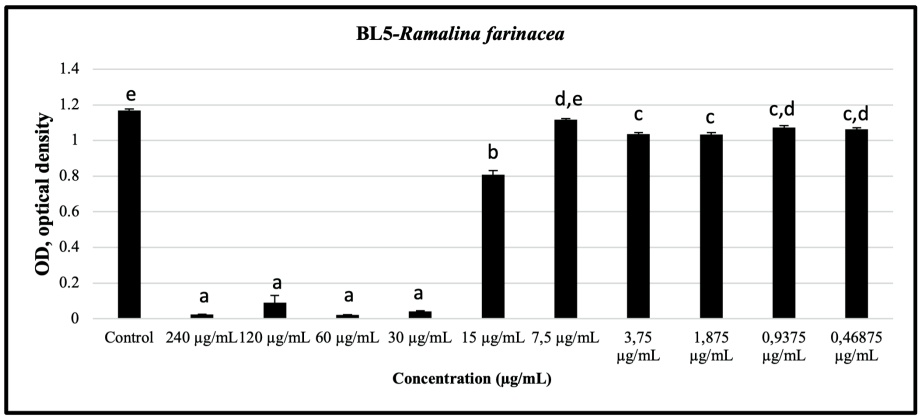

Figure 9. The antibacterial effects of the extracts of *R. farinacea* against *H. eurihalina* (BL5).

At the concentrations of 240, 120, 60, 30 and 15 µg/mL of *E. divaricata* extracts, there was also a notable antibacterial effect against *H. eurihalina* with the inhibition ratios of 91.25, 87.02, 91.04, 98.45, and 98.06%, respectively. 7.5 µg/mL and lower concentrations of *E. divaricata* extracts did not show any efficacy (between 25.70 and 5.89%) against *H. eurihalina*. Data were given in Figure 10. In the comparison of 3.75, 1.875, and 0.9375 treatment groups and the control group, there was no significant difference. On the other hand, other treatment groups showed statistically significant differences when compared to the control group ($p<0.05$). The treatment groups of 3.75, 1.875, 0.9375 and 0.46875 µg/mL have no significant difference among them (Figure 10).

We observed that the lichen extracts had bactericidal activities at the highest concentrations that the potential antibacterial activities were observed, whereas the lower but effective concentrations had bacteriostatic effects against test bacteria.

Overall, these results indicate that lichen extracts have varying effects against tested bacteria. In this respect, lichen extracts being both ecological and effective antibacterial agents against these bacteria may have a potential solution for antibacterial ineffectiveness in the leather industry. Some bacterial strains may cause a quality loss in the final product due to antibacterial resistance that occurred during leather-making processes. Therefore, natural products, especially from plants, have been investigated for their antibacterial potential on the growth of bacteria that may be encountered in the leather industry. In previous studies, antibacterial efficacy for essential oils of *Lavandula officinalis* and *Origanum minutiflorum* was reported. Moreover, myrtle oil (1%) was found to be effective in the soaking process.[48-50] On the other hand, there is no study focused on lichen extracts and/or their compounds against *C. canadensis* (YN6) and *H. eurihalina* (BL5). However, similarly to previous studies, *Usnea* sp. extracts were found to be effective for the suppression of bacterial growth even in very small concentrations.[44-45,47] The extracts of several lichens were known to have more prominent effects against Gram-positive bacteria in the literature. Although test bacteria are Gram-negative in the present study, sufficient antibacterial effects were demonstrated. In this regard, not only Gram-positive but also Gram-negative bacteria may be controlled by the lichen extracts or their compounds with antibacterial properties may be utilized in this sector. As well known, the secondary metabolites of most lichens are responsible for this mentioned antibacterial potential against several bacteria. These active chemical groups of

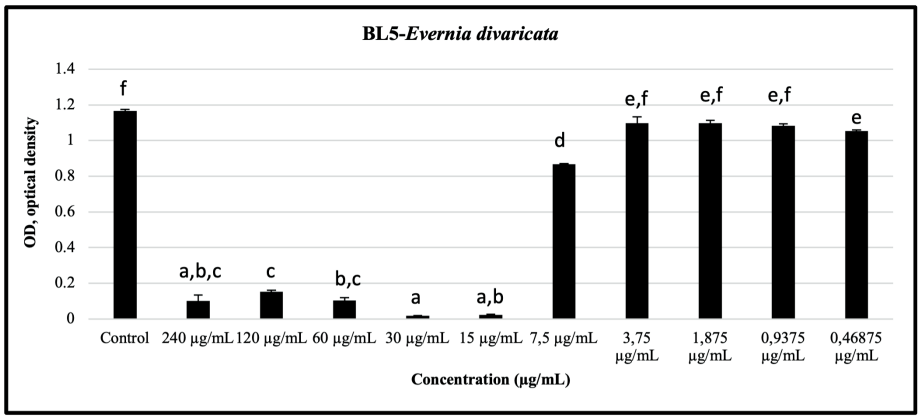

Figure 10. The antibacterial effects of the extracts of *E. divaricata* against *H.eurihalina* (BL5).

lichen - are reported as phenols, terpenes, steroids, anthraquinones, depsides, and depsidones. Some metabolites with antibacterial activities which are mentioned in the literature are usnic acid, lecanoric acid, atranorin, salazinic acid, olivetoric acid, stictic acid fumarprotocetraric acid, protocetraric acid, usnic acid, vulpinic acid, evernic acid, lobaric acid, physodic acid, barbatic acid, divaricatic acid, diffractaic acid, emodin, vulpic acid, psoromic acid, sekikaic acid, caperatic acid, homosekikaic acid etc.[51] For example, usnic acid, diffractaic acid, lecanoric acid, barbatic acid from *Usnea* sp., olivetoric acid, atranorin, chloroatranorin, physodic, oxyphysodic acid, virensic acid, and olivetoric acid acid from *P. furfuracea* and stictic acid, isidiophorin, rhizonaldehyde, rhizonyl alcohol, pulmonarianin, vesuvianic acid, ergosterol peroxide, usnic acid, and diffractaic acid from *Lobaria pulmonaria* (L.) Hoffm. were reported in the literature.[52-55] These results, which vary depending on the bacteria and lichen species, may be due to the differences in secondary metabolites of lichen species. Overall, further detailed studies may clarify which compounds are responsible for these potential antibacterial activities.

Conclusion

In this study, some lichen extracts were found to be potent for the inhibition of bacterial growth of *C. canadensis* (YN6) and *H. eurihalina* (BL5). In this respect, these natural biosources may also be used during storage periods of raw hides in warehouses. Moreover, in recent years, there has been an orientation towards nature in every sector. Lichen extracts are both ecological and non-synthetic. There is a need for further studies to investigate the feasibility of the application for compounds from lichens in raw hides/skins or subsequent processes in leather processes. In this respect, we made a preliminary experiment with acetone extracts of effective lichen species on salted sheepskin samples (data not shown). According to our first data, salted sheepskin samples treated with lichen extracts had considerably inhibitory zones on agar plates. These data suggest that detailed studies have to be performed to show the applicability of these natural extracts/their chemical compounds in the leather industry such as skin preservation.

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