Antibacterial Activities of Lichen Derived Extracts against Different *Bacillus* Species from Soak Liquor Samples

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Abstract

In the leather industry, some bacterial strains may become resistant to antibacterial agents utilized in the soaking process due to long-term use and/or not using in sufficient doses. Alternative approaches or novel agents need to be investigated to overcome antibacterial resistance of bacteria present in the soaking process. These alternative approaches may be from natural resources such as lichens which are known to have various biological activities such as antibacterial, antifungal etc. For this purpose, soak liquor samples from different tanneries were collected and eight isolates from these samples were identified by cultural and molecular techniques. Also, the antibacterial effects of acetone extracts of *Hypogymnia physodes, Evernia divaricata, Pseudevernia furfuracea* and *Usnea sp.* at different concentrations were tested on these isolates. They were all Gram (+), rod shaped, oxidase (+), catalase (+), protease (+). Six isolates had lipase activities. The isolates were assigned to *Bacillus toyonensis, B. mojavensis, B. subtilis, B. amyloliquefaciens, B. velezensis, B. cereus,* and *B. licheniformis* in molecular analyses. The acetone extracts of *Evernia divaricata* totally killed *B. toyonensis*, *B. mojavensis, B. amyloliquefaciens,* and *B. subtilis* at the concentrations of 240, 120, 60 and 30 µg/ml, respectively. These extracts had also significant antibacterial efficacies on *B. cereus, B. velezensis, B. licheniformis* at the concentration of 240 µg/ml. The acetone extracts of *P. furfuracea* had a great inhibitory effect on the growth of most species (80.24-88.65%) only at the concentration of 240 µg/ml. *H. physodes* acetone extracts totally killed *B. amyloliquefaciens* and had considerably high suppressive effect on the growth of other tested bacteria at the concentrations of 120 and 240 µg/ml. *Usnea sp.* acetone extracts had inhibitory effect on *Bacillus* species (86.6-97.9%) even at the 30 µg/ml concentration. In this respect, lichens may provide an alternative approach for the leather industry to overcome bacterial resistance to the antibacterial agents.

Introduction

Microbial growth and degradation of hides/skins due to bacterial activities throughout curing and tanning processes are major problems leading to significant economic losses in the leather industry.[14] High moisture and protein contents of freshly slaughtered hides/skins provide a favorable environment for growth of bacteria and as a result, the metabolic activities of these bacteria cause decomposition of hide/skin substances.[5] Therefore, salt or brine curing methods are traditionally used for preservation of slaughtered hides/skins in many countries. These salted or brine-cured raw hides are stored in warehouses or tanneries until beamhouse processes. This storage period may differ according to warehouses or tanneries. Unless raw hides/skins are preserved adequately, bacterial activities and defects on leather will be inevitable. Moderately or extremely halophilic archaea or salt tolerant-non halophilic bacteria may colonize on raw hides/skins. These various types of bacteria have proteolytic and lipolytic enzymatic activities which may cause red and yellow discolorations of the flesh side of hides/skins, bad odor, hair slip, pin pricks, degradation of hair follicle, holes in grain surface, loose grain, grain peeling, disruption of collagen fibers and uneven dyeing in leathers.[6-15] Since environmental conditions in tanneries are available for microbial growth, bacterial population will continue to increase gradually in the subsequent leather processing stages. Thus, these harmful bacteria have considerably significant effects on leather quality in all of the processing stages.

In beamhouse operations, salted hides/skins are primarily soaked in soaking stage to remove salt, blood, soluble proteins, dirt and manure etc. The duration of soaking process depends on countries, preservation methods and tanneries. In our previous questionnaire study, we determined that the soaking process was generally carried out for 12-18 hours in most tanneries in Turkey.[16] The high bacterial numbers and various bacterial species with degradative properties present in soaking liquor may affect the course of soaking process and leather quality. The duration of the soaking should not be too long and should not be performed at higher temperatures (above 22°C).[3] Since the generation time of bacteria can range from 0.5 to 6 hours, bacteria in soak liquor may reach a significant number within 12-18 hours.[16] Therefore, researchers emphasized that effective bactericides must be used to reduce bacterial damage on leather because of high bacterial numbers in the soaking liquor.[17,18] Due to long-term use of antibacterial agents and not using them in sufficient doses, some bacterial strains may become resistant to these antibacterial agents and for this reason, antibacterial efficacy of utilized bactericides in soaking process should be tested regularly.[18] Antibacterial-resistant strains may transfer their resistance genes by horizontal gene transfer[19,20,21] As a result, bacterial growth can occur even in the

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presence of antibacterial substances in the soaking process.[22-24] In an environment containing different populations of microorganisms each bacterial strain may react distinctly than others. Some species disappear, some change, or sometimes bacterial growth is prevented or some are not affected from antibacterial agents. In the meantime, some bacteria may become dominant and more harmful.[19, 25]

Nevertheless, alternative approaches or novel agents with low toxicity, biodegradable and biocompatible properties need to be investigated since antimicrobial agents may not always have the expected effect. However, some chemicals used in the leather industry are related to occupational diseases (bronchial asthma, liver and renal disorders etc.) and may cause toxic effect on liver.[26] These alternative approaches may be from natural resources. Plant based formulations and lichen derived extracts from *Pseudevernia furfuracea* (L.) Zopf. have been reported in the leather industry.[27-29] Throughout the ages, lichens have been used for various purposes, in particular as dyes, perfumes and remedies in folk medicines. In the leather industry, lichens such as *Cetraria islandica* and *Lobaria pulmonaria* have been used as tanning agent from ancient centuries.[30] Lichens are natural inhabitants of many ecosystems which are stable, ecologically obligate symbiotic organisms between fungus (the mycobiont) and one or more algae or cyanobacterium (photobiont). It is known that lichens synthesize unique secondary metabolites that cannot be synthesized by higher plants. Approximately 1050 lichen substances (aliphatic, cycloaliphatic, aromatic and terpenic compounds) have been reported.[31-32] These metabolites are becoming increasingly important due to the need for new bioactive compounds.[33] Various effects of these secondary metabolites such as antimicrobial, antifungal etc. have been discovered. The potential antibacterial effects of various lichen extracts against several bacteria species were reported in the literature.[34-37]

Taken into consideration of all this knowledge, it can be suggested that lichen species with potential antibacterial properties may be effective solution for antibacterial-resistant bacterial strains in soaking process when compared to industrially utilized antimicrobial agents. For this purpose, soak liquor samples from different tanneries were collected and eight isolates were isolated from these liquor samples. They were identified by cultural and molecular techniques. Also, the antibacterial effects of acetone extracts of lichen species *Hypogymnia physodes, Evernia divaricata, Pseudevernia furfuraceae* and *Usnea sp.* on these isolates.

Experimental

Bacterial Strains

Soak liquor samples were collected from different tanneries in Leather Organized Tannery Region, Tuzla-İstanbul, Turkey. Then, these samples were immediately placed into sterile sample bags and carried on ice during transportation. 20 ml of soak liquor samples were put in a flask containing 180 ml 0.85 % sterile physiological saline solution and placed in a shaking incubator (Edmund Bühler, Germany) for half an hour at 25°C in 100 rpm. Direct and serial dilutions of bacterial suspensions were spread onto the Nutrient agar plates. After incubation at 37°C for 24 h, different cream-white colonies were randomly selected to obtain pure culture and spread onto Luria Bertani (LB) agar plates several times. Morphologically different eight isolates were numbered as Isolate 1 to Isolate 8.

Gram Staining, Oxidase-Catalase Tests and Selective Media

Gram staining was performed using earlier described procedures.[38] For oxidase test, 1-2 drops of 1% dimethyl-p-phenylenediamine hydrochloride solution is placed on the filter paper. The colonies were spread over the filter paper which absorbed dimethyl-p-phenylenediamine hydrochloride. Dark blue-purple color formation within 10 seconds was evaluated as positive oxidase activity. 3% H_2O_2 solution was dropped on the colonies for catalase activity and the formation of gas bubbles was recorded as positive catalase activity.[38] Eosine methylene blue agar (Acumedia Lab, Neogen), Baird Parker RPF (BP) (Acumedia Lab, Neogen) agar, Cetrimide agar (Acumedia Lab, Neogen), Mannitol Salt Agar (MSA) (Acumedia Lab, Neogen) and 5% sheep blood agar were used to confirm the presence of the strains belonging to family *Enterobacteriaceae*, genera of *Staphylococcus*, *Pseudomonas* and *Bacillus*. All experiments were done duplicate.

Protease and Lipase Activity

Protease activities of eight isolates were tested on gelatin agar medium containing 2% gelatin (w/v). After 24 h incubation, the agar plates were flooded with Frazier solution. Clear zones around the colonies evaluated as positive for the protease activity. Lipase activities of the isolates were examined on agar medium containing tripticase pepton (0.8%) and Rhodamin B (0.2 mg/100 ml). Fluorescent orange halos around the colonies were evaluated as positive lipase activity.[39,40]

Molecular Analyses

Genomic DNA of tested isolates were extracted by phenol/chloroform extraction and ethanol precipitation. DNA isolation was confirmed by agarose gel electrophoresis and ethidium bromide staining. All DNA from sample were stored at –20°C until use. The 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR) with the universal bacterial primers 27F (5-AGAGTTTGATCMTGGCTCAG) and 1492R (5-TACCTTG TTACGACTT).[41] Negative control was included in PCR amplifications. PCR amplification was carried out by initial denaturation at 95°C for 4 min, followed by 30 cycles at 95°C for 1 min, 57°C for 1 min, and 73°C for 1 min. the reactions were finished by a final extension at 73°C for 7 min. The PCR products were monitored by agarose gel electrophoresis. These products were purified by *Thermo Scientific*™ GeneJET™ *Gel Extraction Kit.* These purified samples were analyzed by MedSanTek Laboratory Istanbul (Turkey). The 16S rRNA sequence contigs were generated by the software ChromasPro version 2.1.8 (Technelysium Pty. Ltd, Tewantin, Queensland, Australia) Then, consensus sequences were exported in Fasta format for each sample for data analysis. These sequences compared with sequences in the NCBI database using the BLAST search program.

Lichen Samples

Hypogymnia physodes, Evernia divaricata, Pseudevernia furfuraceae and *Usnea sp.* were collected to be 20-30 gr in quantities from fir trees of Kastamonu province in the north-west of Turkey. They were identified through classical taxonomical methods by microscopic examination. Voucher specimens were deposited with the lichen collection of Marmara University Herbarium (MUFE).

Hypogymnia physodes, Evernia divaricata, Pseudevernia furfuraceae and *Usnea sp.:* Turkey, Kastamonu province, Kapaklı Village, 41.24492, 34.18330, G.Çobanoğlu.

Extraction of Lichen Samples

After lichen samples were washed and dried on air, they were weighed and pulverized by liquid nitrogen in porcelain mortar. The lichen samples were taken into sterile bottles, acetone (grade: ACS,ISO,Reag. Ph Eur) was added and then kept in a dark place for 24 hours. Following the evaporation of acetone in a rotary evaporator, crude lichen acetone extracts with extract yields of approximately 2.5% for the lichen species were obtained.

Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) tests were performed in 96-well microplates (Greiner Bio-One, Cell Star, F-bottom, with lid). Tryptic soy agar was added to each well and four-fold serial dilutions of the acetone extracts of *H. physodes, E. divaricata, P. furfuracea* and *Usnea sp.* were made. Final concentration of lichen extract were 240, 120, 60 and 30 µg/ml. Overnight culture of Isolate A was added to obtain a total volume of 100 µl with an OD_{600} of 0.1. The experiments included positive and negative controls. The tests were performed in three replicates. The bacterial growth was evaluated at 20th hour using Cytation 3 multimode microplate reader (Biotek), by measuring absorbance.

Results and Discussion

In this study, eight isolates, which were obtained from soak liquor samples collected from different tanneries in Istanbul Organized Leather Industrial Zone, Turkey, were identified by cultural and molecular techniques. In addition, for the first time, potential antibacterial properties of acetone extracts of *H. physodes, E. divaricata, P. furfuracea* and *Usnea sp.* were evaluated against these isolates.

Previous studies emphasized that bacterial contamination may occur from the beginning of salt curing method due to improper preservation and the number of these bacteria will possibly increase further throughout the soaking process.[42] It has been known that a variety of microorganisms may be present in soak liquor samples. In the present study, eight isolates, which are morphologically different in Nutrient agar, were detected to be Gram positive, rod shaped, oxidase and catalase positive. Whereas the bacterial growth was observed on Mannitol Salt Agar (MSA) and 5% sheep blood agar, no growth was detected on EMB and Cetrimide agar. These results suggested that these isolates may be belonging to the genera of *Bacillus* due to the characteristics of Gram staining, cell morphology and catalase test. As known, *Bacillus* may grow on MSA since this

Table I

Isolate codes, closest relatives, similarities, Gram staining, oxidase and catalase tests, bacterial growth on selective media, protease and lipase activity results of eight isolates collected from soak liquor samples of different tanneries.

Isolate code	Closest relative	Similarity %	Genbank Accession Number	Gram Staining	Oxidase test	Catalase test	EMB agar	BP Agar	Cetrimide Agar	MSA	5% Sheep blood agar	Protease activity	Lipase activity
1	*Bacillus toyonensis*	100	MN428224	+	+	+	–	+¥	–	+Y	α	+	+
2	*Bacillus mojavensis*	99.93	MN120046	+	+	+	–	-/+¥	–	+Y	α	+	+
3	*Bacillus cereus*	100	MN428211	+	+	+	–	+¥	–	+*	β	+	–
4	*Bacillus velezensis*	99.93	MN240443	+	+	+	–	+¥	–	+Y	α	+	+
5	*Bacillus cereus*	100	MN232161	+	+	+	–	+¥	–	+*	β	+	–
6	*Bacillus licheniformis*	98.08	MN368416	+	+	+	–	+¥	–	+Y	–	+	+
7	*Bacillus amyloliquefaciens*	99.72	CP035899	+	+	+	–	+¥	–	+Y	α	+	+
8	*Bacillus subtilis*	100	MN208471	+	+	+	–	+¥	–	+Y	α	+	+

¥Cream colored colonies *Pink–cream colored colonies YYellow colonies + growth – no growth -/+ weak growth

genus is including salt-tolerant members. These suggestions were confirmed by no bacterial colonization on EMB and Cetrimide agar. All isolates had protease activities whereas six isolates had lipase activities. Experimental data of cultural methods were included in Table I.

In molecular analyses, tested eight isolates were identified by comparative partial 16S rRNA gene sequence analysis with the sequences deposited in the GenBank database via BLAST program. It was determined that these isolates have similarities with *Bacillus toyonensis, Bacillus mojavensis, Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus velezensis, Bacillus cereus,* and *Bacillus licheniformis*. The percentage of similarities and GenBank accession numbers belonging to tested isolates were given in Table I.

Before phylogenetic tree construction by using the neighbor-joining method, multiple-alignment was performed in the ClustalW program. Mega X software was used for phylogenetic tree. The evolutionary distances were showed on phylogenetic tree with branch. GenBank accession no. was indicated at each branch.

Recently, Yılmaz and Birbir (2019) isolated *B. mojavensis, B. licheniformis, B. velezensis, B. amyloliquefaciens, B. subtilis, B. atrophaeus, B. paralicheniformis, B. safensis, B. siamensis, B. tequilensis, B. pumilus,* and *B. halotolerans* from curing salt samples. The researchers indicated that curing salt contaminated with *Bacillus* species may lead to dominancy of bacterial species belonging to this genus on hide/skin samples during different stages of leather processing. Similarly, *B. mojavensis, B. licheniformis, B. velezensis, B. amyloliquefaciens,* and *B. subtilis* were also isolated from soak liquor samples in the present study. These results indicate that these *Bacillus* species may come from curing salt samples and may survive during the soaking process despite the antibacterial agent utilization.

In previous studies, the existence of bacteria belonging to the genus *Bacillus* and species of *B. cereus, B. laterosporus, B. liquefaciens, B. megaterium, B. subtilis,* and *B. pumilus* were reported from soak liquor samples[3, 9, 42, 44-46]. In our previous study evaluating the efficacy of antimicrobial agent (the active content, didesyldimethylammonium chloride), *Bacillus mycoides, Bacillus lentus,* and *Bacillus amyloliquefaciens* were isolated despite the applied doubled concentration of antimicrobial agent in soak liquor samples.[18] Unlike these studies, *Bacillus toyonensis* was isolated and identified in this study. Due to high numbers of proteolytic and lipolytic non-halophilic bacteria and the high content of organic matter in the hide/skin, bactericide utilization in sufficient concentration in soaking process sometimes may not be effective to reduce the number of bacteria. The adverse effect of *Bacillus* species on leather quality was evaluated by Rangarajan et al., (2003) and they observed perforations in grain surface due to *Bacillus subtilis, B. megaterium, B. anthracoides, B. pumilus*. They emphasized that antimicrobial agents should be added regularly especially in summer or when hides/skins are soaked for longer periods.[42] In particular, it is difficult to inactivate proteolytic strains which have penetrated into the hide/skin and attached to collagen fibers via high concentrations of bactericides. Therefore, these bacteria will continue to multiply and damage the collagen fibers of the skin.

The antibacterial effect of lichen samples against *Bacillus* genus from various sources was reported in the literature. The acetone and chloroform extracts of *P. furfuracea* (L.) Zopf was also tested

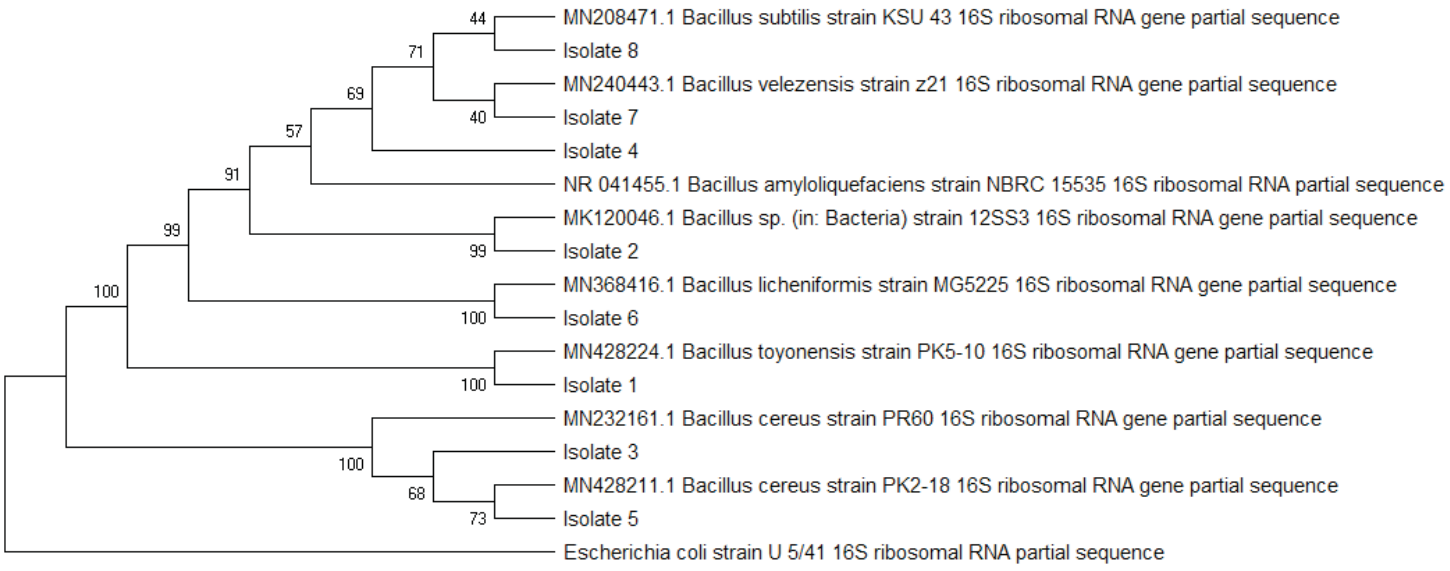

Figure 1. Construction of phylogenetic tree based on 16S rRNA gene sequencing by neighbor joining method (Mega X).
The scale bar represents 0.02 substitutions per nucleotide position.

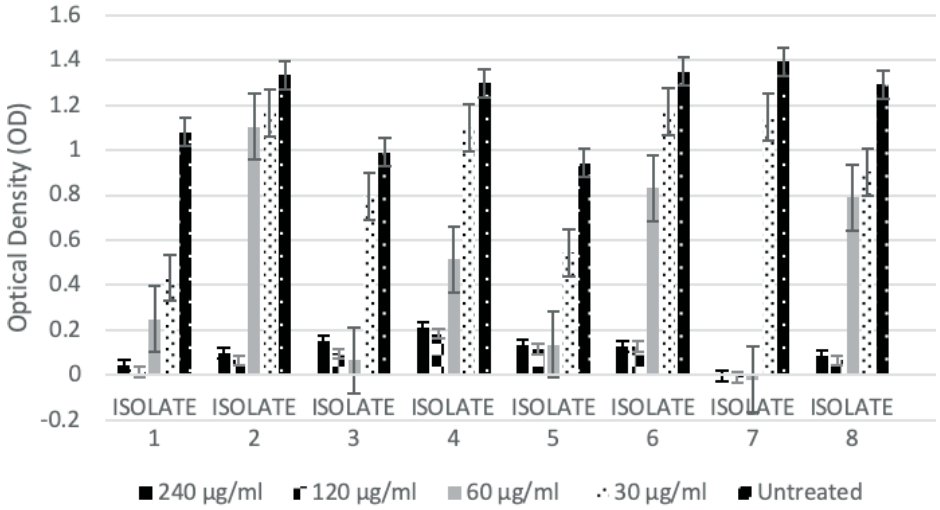

Figure 2. Dose response curves for the antimicrobial activity of thirteen isolates treated with acetone extracts of *Hypogymnia physodes* at the certain concentrations of 240, 120, 60 and 30 µg/ml at 20th hour. Data are shown as optical absorbance over OD 600 nm.

on raw skin and chrome-tanned leather samples against *Bacillus* species.[29] But, there is no study for the evaluation of lichen extracts against isolated bacteria from soak liquor samples. In this respect, the antibacterial effect of lichen species *H. physodes, E. divaricata, P. furfuracea* and *Usnea sp.* against these isolates which were assigned to several *Bacillus* species were evaluated. Acetone was preferred used as a solvent for the extraction of lichen samples in order to provide for wide-ranging extraction of the polar and semipolar constituents that may have potential bioactive properties.

According to results, *H. physodes* acetone extracts totally killed *Bacillus amyloliquefaciens* and had considerably high suppressive effect on the growth of other tested bacteria at the concentrations of 120 and 240 µg/ml. The inhibition percentage for *H. physodes* extracts against *B. toyonensis* (Isolate 1) was recorded as 96.1 and 98.6% at the concentrations of 240, and 120 µg/ml, respectively. Also, the acetone extracts of *H. physodes* had antibacterial effects on *B. mojavensis* (Isolate 2), *B. cereus* (Isolate 3 and 5), *B. velezensis* (Isolate 4), *B. licheniformis* (Isolate 6), and *B. subtilis* (Isolate 8) at the concentrations of 240 and 120 µg/ml. At 60 µg/ml of *H. physodes* extracts, there was also antibacterial effect against *B. toyonensis* (77.1%) and *B. cereus* (93.4%) when compared to the other species. 30 µg/ml of *H. physodes* extracts did not show any efficacy against all *Bacillus* species tested in this study. Data were given in Figure 2. The percentages of bacterial growth inhibition were included in Table II.

The acetone extracts of *E. divaricata* at the concentrations of 240, 120, and 60 µg/ml had significantly antibacterial effects in all tested isolates. *E. divaricata* acetone extracts totally killed *B. toyonensis*, *B. mojavensis, B. amyloliquefaciens, and Bacillus subtilis* at the concentrations of 60, 240, 60 and 240 µg/ml (100%), respectively. Furthermore, these extracts had almost significant antibacterial efficacies on *B. cereus, B. velezensis, B. licheniformis* (96.3-99.4%) at the concentrations of 240 µg/ml. At 30 µg/ml of *E.divaricata* extracts

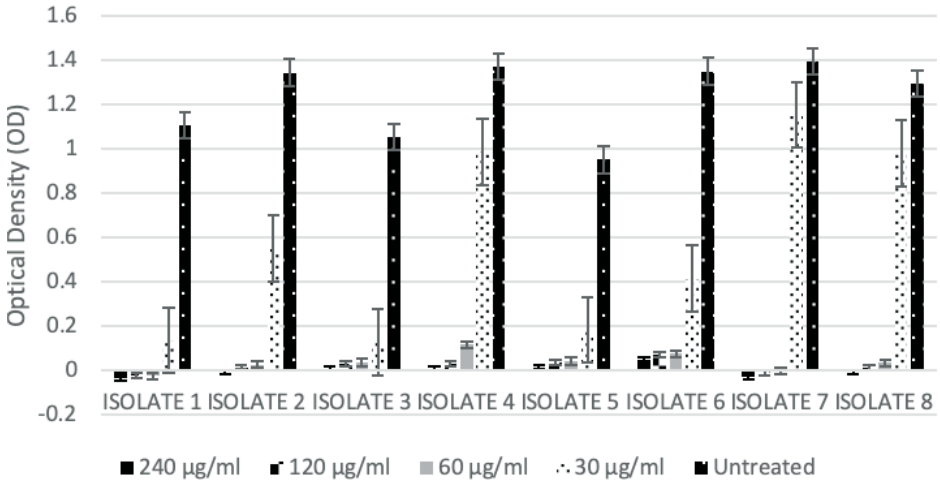

Figure 3. Dose response curves for the antimicrobial activity of thirteen isolates treated with acetone extracts of *Evernia divaricata* at the certain concentrations of 240, 120, 60 and 30 µg/ml at 20th hour. Data are shown as optical absorbance over OD 600 nm.

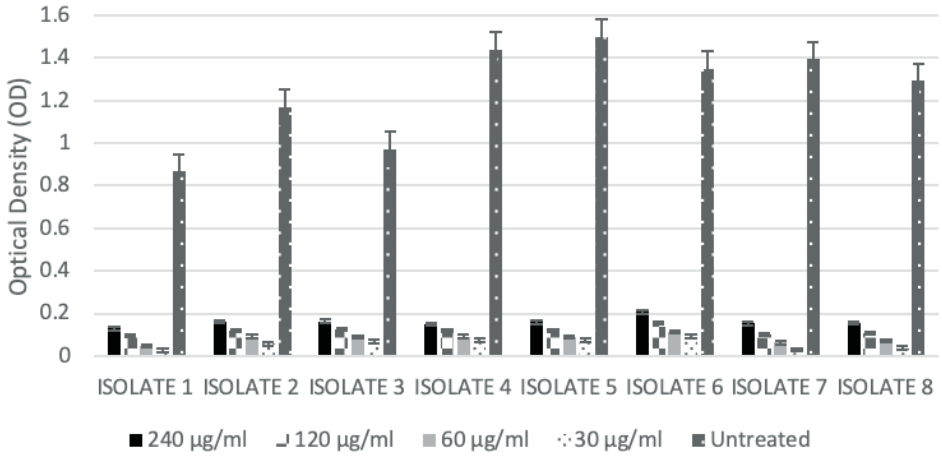

Figure 4. Dose response curves for the antimicrobial activity of thirteen isolates treated with acetone extracts of *Usnea sp.* at the certain concentrations of 240, 120, 60 and 30 µg/ml at 20th hour. Data are shown as optical absorbance over OD 600 nm.

was effective only on *B. toyonensis* (Isolate 1) and *B. cereus* (Isolate 3). The growth of *B. mojavensis* (Isolate 2), *B.licheniformis* (Isolate 6) and *B. cereus* (Isolate 5) were determined to be slightly inhibited by the extracts of *E. divaricata* extracts at 30 µg/ml. Data were given in Figure 3. The percentages of bacterial growth inhibition were included in Table II.

Similarly to the acetone extracts of *E. divaricata*, significant antibacterial effects of *Usnea sp.* in all tested isolates. However, *Usnea sp.* extracts had antibacterial activity even at 30 µg/ml in all isolates whereas *E. divaricata* had significant inhibitory effect against only two species (87.5-87.8%) in that concentration. *Usnea sp.* acetone extracts were all successful in inhibiting bacterial growth of tested bacteria. *Bacillus* species were considerably inhibited or almost killed at a percentage of 84.6-97.9 by the extracts. *Usnea sp.* acetone extracts had inhibitory effect on *Bacillus* species even at the 30 µg/ml concentration with inhibition rate over 90 % except *B.cereus* (86.6%). Data were given in Figure 4. The percentages of bacterial growth inhibition were included in Table II.

P. furfuracea extracts were also tested against isolated *Bacillus* strains. These extracts were not effective at the concentrations of 240 and 120 µg/ml to kill the bacteria but slightly inhibited the growth of them. The acetone extracts of *P. furfuracea* had a great inhibitory effect on the bacterial growth of *B. toyonensis, B. mojavensis*, *B. cereus, B. velezensis, B. licheniformis, B. amyloliquefaciens, Bacillus subtilis* only at the concentration of 240 µg/ml. In two *Bacillus* strains (*B. cereus* and *B. amyloliquefaciens*), 120 µg/ml of *P. furfuracea* extracts had similar antibacterial effect as in 240 µg/ml. Data were given in Figure 5. The percentages of bacterial growth inhibition were included in Table II.

Similarly to these data, the antibacterial effects of tested lichen species in the present study were reported in the literature against

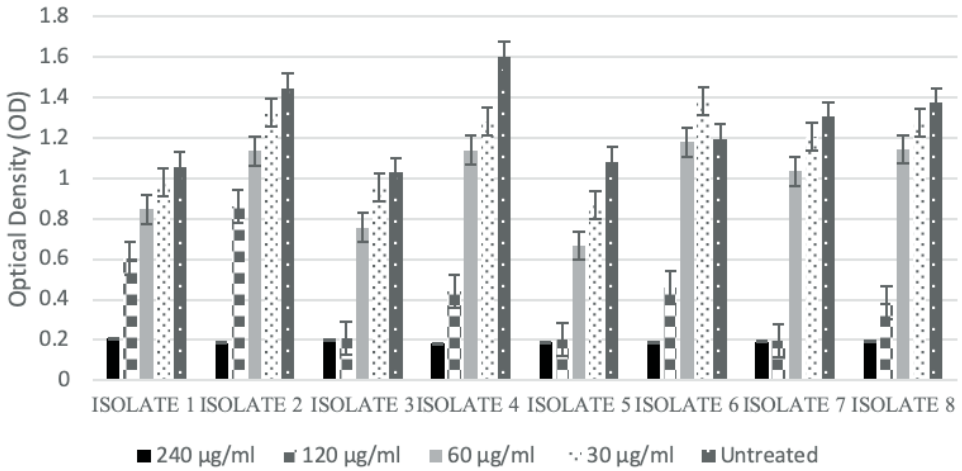

Figure 5. Dose response curves for the antimicrobial activity of thirteen isolates treated with acetone extracts of *Pseudevernia furfuracea* at the certain concentrations of 240, 120, 60 and 30 µg/ml at 20th hour. Data are shown as optical absorbance over OD 600 nm.

Table II

The percentage of bacterial growth inhibition for tested lichen extracts

	ISOLATE 1 ***Bacillus toyonensis***	ISOLATE 2 ***Bacillus mojavensis***	ISOLATE 3 ***Bacillus cereus***	ISOLATE 4 ***Bacillus velezensis***	ISOLATE 5 ***Bacillus cereus***	ISOLATE 6 ***Bacillus licheniformis***	ISOLATE 7 ***Bacillus amyloliquefaciens***	ISOLATE 8 ***Bacillus subtilis***
Pseudevernia furfuracea								
240 µg/ml	80.29	87.05	80.24	88.65	82.49	84.31	85.22	86.05
120 µg/ml	42.70	40.36	79.38	72.44	81.26	61.56	84.83	71.84
60 µg/ml	19.84	21.49	26.38	28.85	38.52	1.37	20.63	16.85
30 µg/ml	6.93	8.41	7.25	19.85	19.85	-15.53	7.60	7.31
Hypogymnia physodes								
240 µg/ml	96.1	92.7	84.5	83.8	86.1	90.3	100	93.4
120 µg/ml	98,6	95,1	90,3	85.9	87.8	90.5	100	94.8
60 µg/ml	77.1	17.2	93.4	60.2	85.7	38.2	100	39.0
30 µg/ml	59.9	12.5	19.8	15.3	42.2	13.2	17.7	30.1
Evernia divaricata								
240 µg/ml	100	100	99.2	99.4	97.8	96.3	100	100
120 µg/ml	100	98.8	97.0	97.8	93.8	94.5	100	98.8
60 µg/ml	100	98.0	96.4	91.3	93.0	94.3	100	97.3
30 µg/ml	87.5	58.9	87.8	28.0	69.6	69.2	17.4	24.2
Usnea sp.								
240 µg/ml	85.1	86.3	83.3	89.7	71.2	84.6	89.0	87.9
120 µg/ml	88.8	89.9	87.0	91.7	78.0	88.7	92.6	91.7
60 µg/ml	94.3	92.1	90.8	93.6	83.7	91.6	95.5	94.5
30 µg/ml	97.1	95.1	92.8	95.0	86.6	93.1	97.9	97.2

various *Bacillus* species. The methanol extracts of *E.divaricata* and *P. furfuracea* collected from Kastamonu province, Turkey were reported to have antibacterial efficacies against *B. megaterium* in a previous study.[47] It has been also reported that *E. divaricata* extracts were successful against *B. subtilis.*[48] *E. divaricata* extracts were also effective against *B.subtilis* in the present study. Rankovic et al. (2009) reported antimicrobial activities of *H. physodes* acetone and methanol extracts against *B. mycoides* and *B. subtilis.*[49] The extracts of several lichens were known to have more prominent effects against Gram positive bacteria in the literature. In this respect, further detailed studies have to be performed against Gram negative bacteria obtained from soak liquor samples.

Conclusion

To our knowledge, there is no study for the evaluation of *H. physodes, E. divaricata, P. furfuracea* and *Usnea sp.* against several *Bacillus* species isolated from different soak liquor samples. The antibacterial effects of *H. physodes, E. divaricata, P. furfuraceae* and *Usnea* sp. acetone extracts against *B. toyonensis, B. mojavensis, B. subtilis, B. amyloliquefaciens, B. velezensis, B. cereus,* and *B. licheniformis* was demonstrated in this study. Since antimicrobial agents may not always have the expected effect, novel agents must be investigated for the reason of bacterial resistance problems against utilized antimicrobial agents in the soaking process. Lichens may be preferred for ecological and toxicological aspects. In this respect, this study may provide an alternative approach to the leather industry to overcome bacterial resistance to the antibacterial agents. Taken together, the extracts of *H. physodes, E. divaricata, P. furfuraceae* and *Usnea* sp. must be investigated in detail for their chemicals and this/these potential chemical(s) may be utilized in the leather industry alone or in combination with antibacterial agents against antibacterial-resistant bacteria.

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