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Antibacterial Potential of Six Lichen Species against *Enterococcus durans* from Leather Industry

Evaluation of acetone extracts obtained from several lichen species as alternative natural antibacterial agents

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Antibacterial resistant bacteria are a significant problem in the hide or skin soaking process due to their destructive properties on finished leather. Lichens may be a solution to overcome this resistance problem. *Enterococcus durans* (99.86%) was isolated from soak liquor samples. For screening of possible antibacterial effects of lichen acetone extracts, six lichen species (*Hypogymnia tubulosa*, *H. physodes*, *Evernia divaricata, Pseudevernia furfuracea, Parmelia sulcata* and *Usnea* sp.) were examined by nine-fold dilution against *E. durans*. *H. tubulosa*, *H. physodes* and *E. divaricata* extracts showed antibacterial effects at the concentrations of 240 µg ml^{-1}, 120 µg ml^{-1} and 60 µg ml^{-1} whereas the extracts of *P. furfuracea* had an antibacterial effect at 240 µg ml^{-1} and 120 µg ml^{-1}. On the other hand, *P. sulcata* had no antibacterial effect. The most successful lichen extract was determined to be *Usnea* sp. at the concentrations of 240 µg ml^{-1}, 120 µg ml^{-1}, 60 µg ml^{-1}, 30 µg ml^{-1} and 15 µg ml^{-1}. In conclusion, lichen extracts seem to have potential antibacterial efficacies against *E. durans.*

1. Introduction

The leather industry produces and exports high-quality products with high added value to the world market. However, several bacterial problems during leather-making processes are reflected in finished products and lead to economic losses. After the flaying process in slaughterhouses, microflora on hide or skin surfaces change due to bacterial contamination originating from faeces, air, dust or the animal skin itself and some bacteria easily colonise (1–4).

The soaking process is the first tannery operation that recovers water loss during raw hide or skin curing applications. There are some criteria to be taken into consideration during the soaking process of raw hides or skins. Especially prolonged soaking provides a convenient milieu for bacterial activity and damage to hides or skins may occur. Due to reduced salt content and high protein and lipid constituents, hides or skins become defenceless against bacterial attacks in the soaking process (5–8). It has been reported that the number of bacterial populations in soak liquors may be up to 10^5 colony forming unit (CFU) ml^{-1} (5). But in a previous study, it was demonstrated that total bacterial numbers were considerably higher than 10^5 CFU ml^{-1} in soak liquor samples (9). The adverse effects of the soaking process on the hide quality originate from

degradative enzymatic properties of bacteria such as protease and lipase activities. These enzymatic activities can irreversibly affect the structure of hide or skin substances that cannot be fixed at the subsequent stages of hide processing (10). High numbers of bacteria with protease and lipase activities cause unwanted defects such as hair-slip, putrefaction, grain peeling, loose grain, holes on the hides or skins and light stains on the suede surface (1, 3, 11–15).

Antibiotics are used in various industries as well as in the treatment of diseases. The World Health Organization declared that antimicrobial resistance in most countries and industrial sectors has increased dramatically (16, 17). The emergence of antibiotic-resistant bacteria due to improperly used antibiotics in humans, animals and agriculture has been reported in the literature (17). In the leather industry, to control bacterial numbers and their degradative properties on hides or skins, various antibacterial agents are utilised during the soaking process of beam house operations. The normal microflora in animals comprises many harmless bacteria but any of them may become resistant to commonly utilised antibacterial agents due to intrinsic or acquired resistance (17, 18). The resistant bacteria may survive despite bactericides and may transfer their resistance properties to others through horizontal gene transfer (5, 9, 18). Bactericides may remain ineffective against proteolytic and lipolytic bacteria in soak liquors because of high organic content in soak liquors (9, 19). The existence of many non-halophilic bacteria was demonstrated in the presence of an antimicrobial agent at twofold increased concentration (0.8 g l^{-1}) (19). This finding emphasises the antibacterial resistance of bacteria in the soaking process. More recently, it was reported that antimicrobial agents used in the soaking process could not control multidrug-resistant *Enterobacteriaceae* from soaked sheepskins and cattle hides treated with an antibacterial agent (20).

Over the past decades, it has been suggested that alternative compounds from natural resources may overcome the antimicrobial resistance of many bacteria. Previously, the potential of lichen derived extracts from *P. furfuracea* (L.) Zopf was reported in the leather industry (21). Lichens are symbiotic organisms between a fungus and one or more algae or cyanobacteria. They synthesise unique secondary metabolites that cannot be synthesised by higher plants (22, 23). Secondary metabolites from numerous lichen extracts have been reported to have biological activities such as antibacterial activity against Gram-positive and Gram-negative bacteria (24–27). It has been reported that approximately 2000 of the 20,000 lichen species in the world are in Turkish lichen mycota. There are many studies evaluating the bioactivities of lichen species in Turkey against different bacterial species (25–27). In the previous study, the acetone extracts of *H. physodes, E. divaricata, P. furfuracea* and *Usnea* sp. at different concentrations were tested on some *Bacillus* species which were isolated from soak liquor samples. These extracts were detected to have potential antibacterial effects (28).

From this point, lichen species may have potential antibacterial efficacies against various antibacterial-resistant bacterial strains in the soaking process which cannot be exterminated by antimicrobial agents. Therefore, the antibacterial effects of acetone extracts of lichen species *H. tubulosa, H. physodes, E. divaricata, P. furfuracea, P. sulcata* and *Usnea* sp. against Isolate 1 (*E. durans*), which has protease and lipase acitivities, was evaluated in the present study.

2. Materials and Methods

2.1 Sample Collection

Three soak liquor samples were collected from Istanbul Leather Organized Industrial Zone, Tuzla, Istanbul, Turkey. These samples were immediately placed into sterile sample bags and carried on ice during transportation. Direct and serial dilutions were spread onto nutrient agar plates. The morphologically different colony was picked up to obtain the pure culture of the isolate and was numbered as Isolate 1.

2.2 Biochemical and Molecular Analyses

Gram staining, catalase, oxidase, lipase and protease activities were examined. Protease activity of Isolate 1 was examined on gelatin agar medium containing 2% gelatin (w/v). The agar plates were flooded with Frazier solution following 24 h incubation. Clear zones around the colonies were evaluated as positive for protease activity. Lipase activity was tested on Tween® 80 agar medium containing 1% (w/v) Tween® 80. After incubation, opaque zones around the colonies were accepted as evidence of lipase activity (29, 30).

Genomic DNA of Isolate 1 which was determined to have protease and lipase activities were extracted by phenol/chloroform extraction and ethanol precipitation. DNA isolation was confirmed by agarose gel electrophoresis. DNA samples 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-TACCTTGTTACGACTT). Negative control was included in PCR amplifications. PCR amplification was carried out by an 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 also monitored by agarose gel electrophoresis. These products were purified by GeneJET™ Gel Extraction Kit (Thermo Scientific™, Thermo Fisher Scientific, USA). These purified samples were analysed by Medsantek Ltd Co, Istanbul, Turkey. The 16S rRNA sequence contigs were generated by the software ChromasPro version 2.1.8 (Technelysium Pty Ltd, Australia). Then, consensus sequences were exported in FASTA format for each sample for data analysis. These sequences were compared with sequences in the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST®) search program.

2.3 Lichen Samples

The lichen samples belonging to *H. tubulosa, H. physodes, E. divaricata, P. furfuracea, P. sulcata* and *Usnea* sp. were collected from fir trees of Kastamonu province in the north-west of Turkey. They were identified through classical taxonomical methods by microscopic examination.

H. tubulosa, H. physodes, E. divaricata, P. furfuracea, P. sulcata and *Usnea* sp: Turkey, Kastamonu province, Kapaklı Village, 41.24492, 34.18330, G. Çobanoğlu.

2.4 Extraction of Lichen Samples

The experiment steps included washing, drying in air, weighing, pulverising by liquid nitrogen, adding acetone (ACS, ISO, Reag. Ph. Eur.), keeping in a dark place for 24 h followed by filtration through filter paper. Then, the evaporation of acetone in a rotary evaporator was performed and crude lichen acetone extracts were obtained (27).

2.5 Determination of Antibacterial Efficacies of Lichen Samples

The test isolate was grown on Tryptic soy agar media at 37°C for 24 h. The tests were performed in 96-well CELLSTAR®, F-bottom microplates with lid (Greiner Bio-One GmbH, Austria). Tryptic soy broth was added to each well and nine-fold serial dilutions of the acetone extracts of *H. tubulosa, H. physodes, E. divaricata, P. furfuracea, P. sulcata* and *Usnea* sp. were made. Final concentrations of all lichen extracts were 240 µg ml^{-1}, 120 µg ml^{-1}, 60 µg ml^{-1}, 30 µg ml^{-1}, 15 µg ml^{-1}, 7.5 µg ml^{-1}, 3.75 µg ml^{-1}, 1.9 µg ml^{-1} and 0.9 µg ml^{-1}. Overnight culture of the isolate was added to obtain a total volume of 100 µl with an optical density (OD) 600 nm of 0.01. The experiments included untreated and blank controls. The tests were performed in three replicates. Bacterial growth ratios at an OD 600 nm were measured using Cytation™ 3 Multi-Mode microplate reader (BioTek Instruments Inc, USA).

3. Results and Discussion

In the present study, Isolate 1, which was obtained from soak liquor samples collected from different tanneries in Istanbul Leather Organized Industrial Zone, Turkey, was identified by biochemical and molecular techniques. To our knowledge, there is no study on the antibacterial efficacies of lichen extracts against *E. durans* from soak liquor samples. For the first time, *H. tubulosa, H. physodes, E. divaricata, P. furfuracea, P. sulcata* and *Usnea* sp. acetone extracts were examined against *E. durans* isolated from soak liquor samples.

Isolate 1 was Gram-positive, oxidase and catalase-negative, protease and lipase positive. The degradative protease and lipase activities of bacteria have an important role in the production of high-quality leather. There are many studies focused on protease and lipase activities of halophilic, extremely halophilic and non-halophilic bacteria on hides or skins in the literature. McLaughlin and Highberger reported that bacterial strains with proteolytic activity were present in high percentages on salt-cured goat skins (31). The proteolytic and lipolytic activities of halophilic and extremely halophilic bacteria were also reported in previous studies. Birbir reported that 91% of 35 salt-cured skins had halophilic bacteria and 67% of 85 extremely halophilic bacterial strains had proteolytic activities (32). Bailey and Birbir

detected that 98% of 131 brine-cured skin samples had extremely halophilic microorganisms and 94% of 332 isolates from these samples showed proteolytic activity (12). Bitlisli *et al*. demonstrated that 53–74% of halophilic bacteria from salt-cured sheepskins had proteolytic activity and 47–62% of them had lipolytic activity (33). There are also several studies revealing the proteolytic and lipolytic activities of non-halophilic bacteria from soak liquor samples. Veyselova *et al*. showed proteolytic activity of some bacteria belonging to the genera *Enterobacter, Pseudomonas, Enterococcus, Lactococcus, Aerococcus, Vibrio, Kocuria, Staphylococcus* and *Micrococcus* and lipolytic activity of *B. licheniformis, B. pumilus, P. luteola* and *E. cloacae* from soak liquor samples (10).

In molecular analyses, the tested isolate was identified by comparative partial 16S rRNA gene sequence analysis with the sequences deposited in the GenBank® database *via* the BLAST® program. The Isolate 1 had similarities with *E. durans* CMGB-120 (99.86%, GenBank® accession number MF348232.1). The existence of *Enterococcus* species was previously reported from hides or skins in the leather industry (6, 34). It is well known that *Enterococcus* species are common in surface water, soil, vegetables and animal products and they are naturally commensal members of gut microflora of human and warm-blooded animals. *Enterococcus avium, E. casseliflavus, E. durans, E. faecalis, E. faecium* and *E. gallinarum* have been isolated from salted hide samples (34). Furthermore, despite increasing the concentration of antimicrobial agents containing didecyl dimethyl ammonium chloride from 0.4 g l^{-1} to 0.8 g l^{-1}, several bacteria including *E. avium* and *E. faecium* were reported from soak liquor samples (19). These results suggest that some *Enterococcus* species may come from salted hides and can survive in soak liquor samples even in the presence of antibacterial agents. Fluckey *et al*. isolated 279 *Enterococcus* isolates from faecal and hide samples. Among them, 169 isolates were detected to be *E. durans* by biochemical tests (35). *E. durans* is mostly found in pre-ruminant calves and young chickens and can survive in moderately harsh conditions such as various temperature ranges, pH degrees and salt concentrations as well as detergents (36–38). Similarly to our results, the proteolytic and lipolytic activities of *E. durans* were also demonstrated in previous studies. Aslan and Birbir detected that six *E. durans* isolates had proteolytic and lipolytic activities (34). In this regard, Isolate 1 may have the potential to cause several unwanted defects on finished products due to its enzymatic activities.

Antibacterial agents that are commonly used in the soaking process seem to be ineffective due to random or insufficient application and lead to antimicrobial-resistant bacteria in soak liquors (12, 19). From this point, we can suggest that *E. durans* from salted hides or skins could not be exterminated by curing methods and also in the soaking process despite the use of antibacterial agents. There are several studies focused on the determination of effective concentrations of several antimicrobial agents against various species of bacteria. Both the ineffectiveness of antibacterial agents in some cases and possible harmful and toxic effects for the environment and human health of some synthetic antimicrobial agents were emphasised in the literature (19, 21). In this respect, the need for safer, more ecological and effective materials has come into prominence for the leather industry. In the previous study, the potential antibacterial effects of acetone extracts of *H. physodes, E. divaricata, P. furfuracea* and *Usnea* sp. at the concentrations of 240 µg ml^{-1}, 120 µg ml^{-1}, 60 µg ml^{-1} and 30 µg ml^{-1} were demonstrated against *Bacillus toyonensis, B. mojavensis, B. subtilis, B. amyloliquefaciens, B. velezensis, B. cereus* and *B. licheniformis* which were isolated from soak liquor samples (28). In respect to these findings, we suggested that *H. tubulosa, H. physodes, E. divaricata, P. furfuracea, P. sulcata* and *Usnea* sp. acetone extracts may have antibacterial potential against *E. durans* which has protease and lipase activities.

According to our results, the acetone extracts of *P. sulcata* had no antibacterial effect at all tested concentrations against *E. durans* (**Figure 1**).

On the other hand, we observed a considerable antibacterial effect for the acetone extracts of *H. tubulosa* and *H. physodes* against *E. durans*. High inhibitory effects of these tested extracts for the growth of *E. durans* (above 50% inhibition) were detected at the concentrations of 240 µg ml^{-1}, 120 µg ml^{-1} and 60 µg ml^{-1} with inhibition ratios of 82.54%, 79.53% and 79.98% for *H. tubulosa,* and 86.8%, 78.2%, 77.75% for *H. physodes*, respectively (**Figures 2** and **3**).

The acetone extracts of *P. furfuracea* also had antibacterial effect against *E. durans* at the concentrations of 240 µg ml^{-1} and 120 µg ml^{-1} by the inhibition percentages of 80.63% and 85.2%. The other tested concentrations had also inhibitory effects on the tested bacteria but the inhibition ratios recorded were below 50% (**Figure 4**).

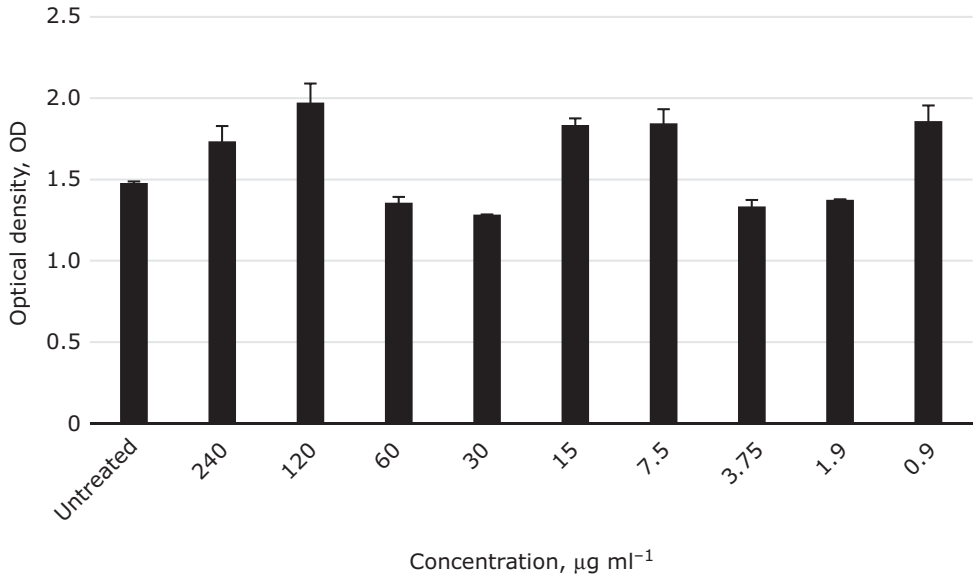

Fig. 1. Antibacterial effect of acetone extracts of *P. sulcata* against *E. durans* from soak liquor samples

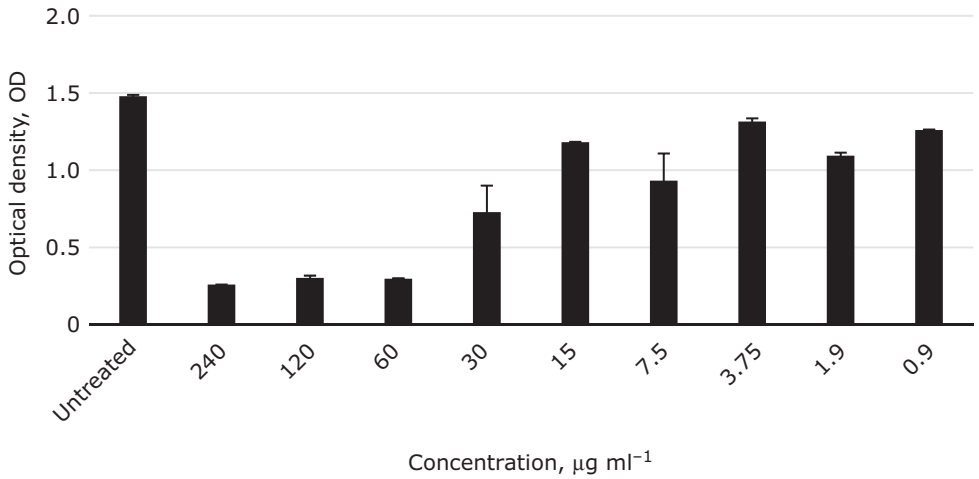

Fig. 2. Antibacterial effect of acetone extracts of *H. tubulosa* against *E. durans* from soak liquor samples

Potential antibacterial efficacy was also detected for the acetone extracts of *E. divaricata* against *E. durans*. At the concentration of 240 μg ml^{-1}, we detected 91% inhibition on the bacterial growth. Antibacterial effects were observed at the concentrations of 120 μg ml^{-1} and 60 μg ml^{-1} with inhibition ratios of 81% and 79% (**Figure 5**).

Usnea sp. acetone extract was determined to be the most successful among the tested lichen extracts. 240 μg ml^{-1}, 120 μg ml^{-1}, 60 μg ml^{-1}, 30 μg ml^{-1} and 15 μg ml^{-1} of the extracts belonging to *Usnea* sp. had an antibacterial effect above 80% inhibition. The inhibition ratios at these concentrations were similar and recorded as 88.7%, 84.2%, 92%, 87.8% and 89.5% respectively. Furthermore, a 58.1% inhibition ratio was noted for the concentration of 7.5 μg ml^{-1} (**Figure 6**).

All data showed that the acetone extracts of *H. tubulosa, H. physodes, P. furfuracea, E. divaricata* and *Usnea* sp. had potential antibacterial efficacies at varying concentrations against *E. durans*. *Usnea* sp. acetone extracts were found to have a stronger inhibitory effect on the bacterial growth of *E. durans*, even at a low concentration of 15 μg ml^{-1} (89.5% inhibition) compared to other extracts. These results emphasise the potential of lichens to be utilised as an antibacterial agent in the leather industry. Further studies are needed

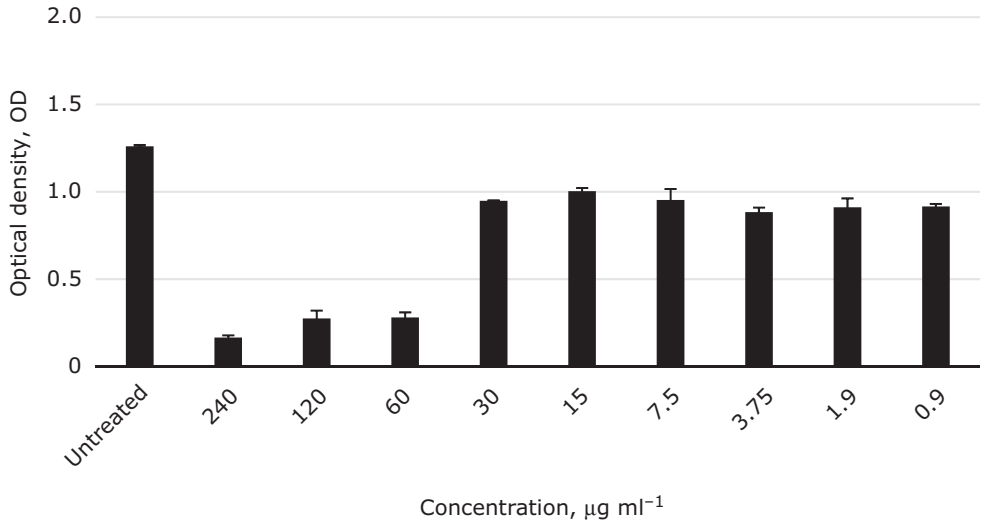

Fig. 3. Antibacterial effect of acetone extracts of *H. physodes* against *E. durans* from soak liquor samples

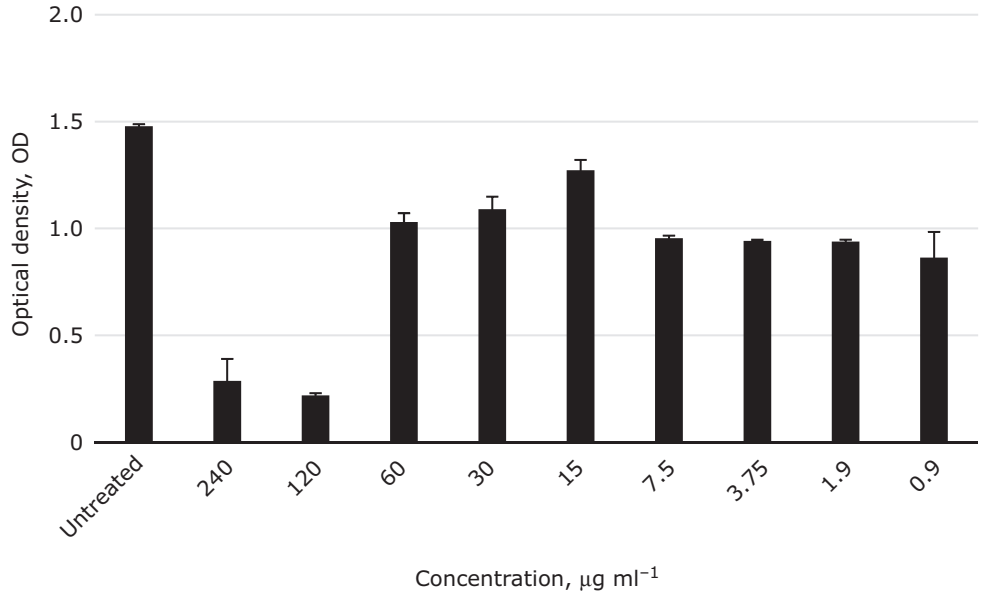

Fig. 4. Antibacterial effect of acetone extracts of *P. furfuracea* against *E. durans* from soak liquor samples

to detect potential compounds of these lichen species and then these compounds may be used in formulations in the industry.

4. Conclusions

In the leather industry, bacteria with proteolytic and lipolytic activities are important in terms of finished product quality. In this study, we tried to answer the question of whether acetone extracts of six lichen species (*H. tubulosa*, *H. physodes*, *P. sulcata*, *P. furfuracea*, *E. divaricata* and *Usnea* sp.) have antibacterial effects against *E. durans* with protease and lipase properties. Whereas *P. sulcata* did not have any antibacterial efficacy against *E. durans*, other tested extracts were successful depending on the lichen species and concentrations applied. The acetone extracts of *Usnea* sp. had the highest antibacterial efficacy. The potential antibacterial efficacies of several lichen species suggest that compound(s) extracted from lichens as natural resources may be used in the leather industry. We

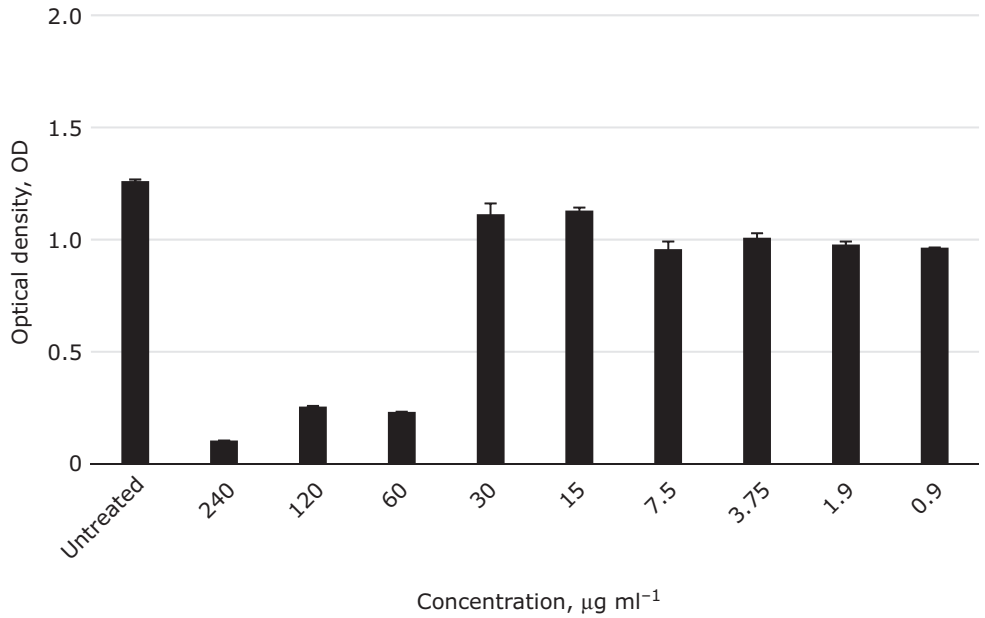

Fig. 5. Antibacterial effect of acetone extracts of *E. divaricata* against *E. durans* from soak liquor samples

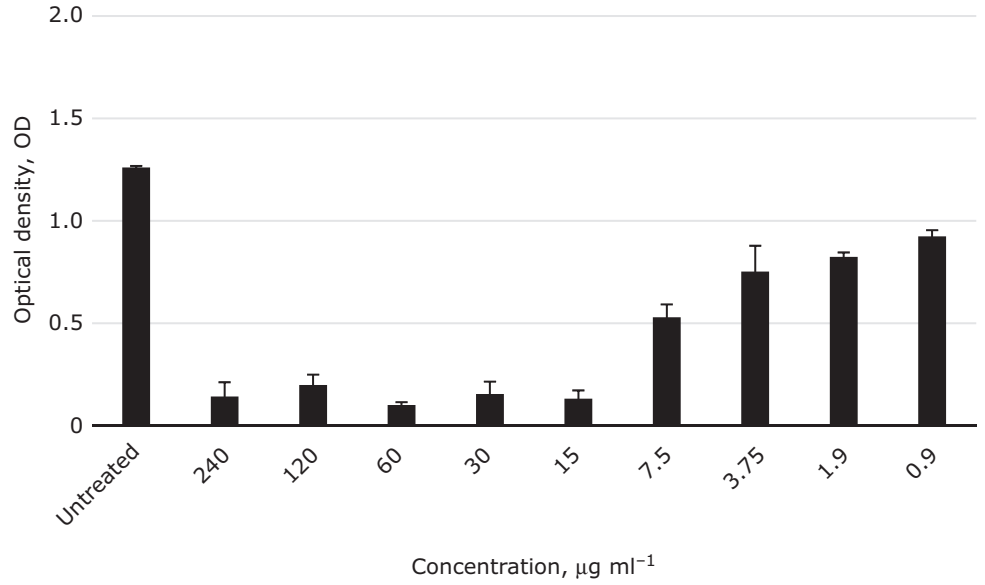

Fig. 6. Antibacterial effect of acetone extracts of *Usnea* sp. against *E. durans* from soak liquor samples

believe that more comprehensive studies about their unique chemical compounds will provide new insight to utilise them in this sector.

Acknowledgement

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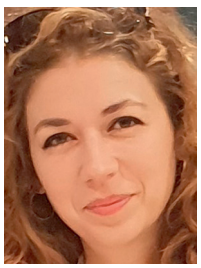

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