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RESEARCH ARTICLE / ARAŞTIRMA MAKALESİ

Antibacterial Effects of *Parmelia sulcata* and *Hypogymnia tubulosa* Acetone Extracts Against Isolates From Soak Liquors

Parmelia sulcata ve Hypogymnia tubulosa Aseton Özütlerinin Islatma Sıvılarından Elde Edilen İzolatlar Üzerine Antibakteriyel Etkileri

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Abstract
It is well known that there are halophilic or non-halophilic bacteria in salt, soak liquors, salted and soaked hides/skins in high numbers in the leather industry. These bacteria have several hydrolytic enzymes which cause irreversible defects on finished leather product. Although antimicrobial agents are utilized to control the bacterial population in the soaking process, these agents have not sufficient efficacy due to inadequate application of these agents or the presence of antimicrobial-resistant bacterial strains in soak liquors. In this respect, alternative agents or strategies may be helpful for controlling the bacterial population. For this purpose, *Bacillus toyonensis*, *B. mojavensis*, *B. subtilis*, *B. amyloliquefaciens*, *B. velezensis*, *B. cereus* and *B. licheniformis*, which were isolated from soak liquor samples of different tanneries in the previous study, were tested in the present study and the antibacterial effects of acetone extracts of *Parmelia sulcata* and *Hypogymnia tubulosa* were evaluated on these isolates. The pure cultures of the isolates were confirmed by colony morphologies on agar plates and Gram staining. Moreover, antibacterial activities for acetone extracts of *Parmelia sulcata* and *Hypogymnia tubulosa* against these isolates were tested at certain concentrations of 240, 120, 60 and 30 µg/ml. *Hypogymnia tubulosa* extracts were found to be more successful in comparison to the extracts of *Parmelia sulcata* on tested isolates. These tested lichen species can be used to control the population of bacteria in the soaking process and also to prevent potential defects on the hide/skin that may be seen in subsequent tanning processes due to the development of these bacteria.
Keywords: soak liquor, lichen, *Parmelia sulcata, Hypogymnia tubulosa*, acetone extract, antibacterial.

Öz
Deri endüstrisinde tuzda, tuzlanmış ve ıslatılmış büyükbaş ve küçükbaş hayvan derilerinde, ve ıslatma sıvılarında çok fazla sayıda halofil veya halofil olmayan bakterilerin bulunduğu bilinmektedir. Bu bakteriler bitmiş deri ürününde geri dönüşümü olmayan kusurlara neden olan birkaç hidrolitik enzime sahiptir. Islatma işleminde bakteri popülasyonu kontrol etmek için antimikrobiyal ajanların kullanılmasına rağmen, bu ajanların yetersiz uygulanmalarına veya ıslatma sıvılarında antimikrobiyallere dirençli bakteri suşlarının bulunmasına bağlı olarak yeterli etkinliğe sahip değillerdir.Bu bağlamda, bakteri popülasyonunu kontrol etmek için alternatif ajanlar veya stratejiler yardımcı olabilir. Bu amaçla, bir önceki çalışmada farklı tabakhanelerden toplanan ıslatma çözeltisi örneklerinden izole edilen *Bacillus toyonensis, B. mojavensis, B. subtilis, B. amyloliquefaciens, B. velezensis, B. cereus ve B. licheniformis* test edilmiştir ve *Parmelia sulcata* ve *Hypogymnia tubulosa*'nın aseton özütlerinin antibakteriyel etkileri bu izolatlar üzerinde değerlendirilmiştir. İzolatların saf kültürleri, agar plakaları üzerinde koloni morfolojileri ve Gram boyamaları ile doğrulanmıştır. Ayrıca, *Parmelia sulcata* ve *Hypogymnia tubulosa*'nın aseton özütlerinin bu izolatlar üzerine antibakteriyel aktiviteleri, 240, 120, 60 ve 30 µg/ml konsantrasyonlarında test edilmiştir. *Hypogymnia tubulosa* özütlerinin *Parmelia sulcata* özütlerine kıyasla test edilen izolatlar üzerinde daha başarılı olduğu bulunmuştur. Bu test edilen liken türleri, ıslatma işleminde bakteri popülasyonunu kontrol etmek için ve ayrıca ileriki tabaklama işlemlerinde bu bakterilerin gelişimine bağlı olarak görülebilecek deri üzerindeki potansiyel kusurları engellemek amacıyla kullanabilir.
Anahtar Kelimeler: ıslatma sıvısı, liken, *Parmelia sulcata, Hypogymnia tubulosa*, aseton özütü, antibakteriyel.

I. INTRODUCTION

The bacterial population subsequently changes after the flaying process and some species of bacteria easily colonize on hide/skin surfaces (1-3). This bacterial contamination may be originated from slaughterhouses, warehouses, or tanneries (4). The presence of major components such as water, proteins, and lipids in the

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structure of hides/skins, blood, dirt, and higher temperature, etc. provides excellent growth conditions for non-halophilic, salt-resistant or salt-tolerant bacteria, halophilic bacteria and extremely halophilic archaea (5, 6). It has been reported that moderately halophilic bacteria from salted goat skin secrete several enzymes such as protease, catalase, lipase, β-galactosidase, oxidase, urease, caseinase, amylase, lecithinase and cellulose (7). Especially proteolytic and lipolytic archaea may cause red heat, bad odor, pinpricks, disruption of collagen fibers, hair loosening, hair loss, hole formation, irreversible damage on grain surface, abraded appearance, uneven dyeing etc. As a result of these bacterial activities, the deterioration on hides/skins becomes inevitable and the leather quality is adversely affected (1, 6, 8-10). Therefore, adequate preservation methods against possible harmful bacterial activities during transportation and storage of the hides/skins have to be implemented.

Fresh hides/skins are traditionally preserved by the salt-or brine-curing method (1, 4, 6, 8). The cured raw hides/skins are soaked to remove excess salt, blood, soluble proteins, dirt, and manure in the soaking (first stage of tanning) process. The excessively prolonged soaking process results in harmful bacterial activities. The duration of soaking process is 24-36 h for dry salt-cured raw hides and 36-72 h for air-dried raw hides. This long soaking time can loosen hair follicles and cause hair loss which are unwanted situations for fur production (11). The effects of antibacterial agents in soak liquors may differ according to the type of microorganism. Many Gram positive and Gram negative bacteria may survive and grow during this process due to their resistance properties. Most strains may become resistant to these antibacterial agents due to prolonged utilization and in inadequate dosages (9, 12-14). Since antimicrobial agents may not always have the expected effect, novel agents with low toxicity, biodegradable, and biocompatible properties have to be investigated. As expected, natural antimicrobials derived from plants, animals, algae, and microorganisms, etc. are preferred for ecological and toxicological aspects. Plant based formulations and lichen derived extracts from *Pseudevernia furfuraceae* (L.) Zopf. have also been reported in the leather industry (15). Lichens, also called lichenized fungi, are symbiotic organisms between a fungus (mycobiont) and one or more green algae or cyanobacterium (photobiont). They produce various lichen secondary metabolites with low molecular weight (16, 17). The potential antibacterial effects of various lichen extracts against several bacteria species were reported in the literature (18-21).

There is a crucial need for controlling the bacterial population in the leather industry. For this purpose, lichen species with potential antibacterial properties may be an effective solution when compared to industrially utilized antimicrobial agents. The aim of this study was to investigate the antibacterial effects of acetone extracts of *Parmelia sulcata* and *Hypogymnia tubulosa* lichen species on eight isolates obtained from soak liquors collected from different tanneries.

II. MATERIAL AND METHODS

2.1. Bacterial strains

In the present study, *Bacillus toyonensis, B. mojavensis, B. subtilis, B. amyloliquefaciens, B. velezensis, B. cereus and B. licheniformis*, which were isolated from soak liquor samples of different tanneries in the previous study, were tested. The identification of the test organisms was performed by Gram Staining, oxidase and catalase tests, bacterial growth on several selective media, protease and lipase activities, and comparative partial 16S rRNA gene sequence analyses in the previous study. It was detected that test bacteria were Gram positive, rod-shaped, protease positive, and mostly catalase positive (22). The test isolates were stored at -80 ^{0}C. The pure cultures of test bacteria were confirmed by colony morphologies on agar plates as well as Gram staining, and then they were utilized in the experiments.

2.2. Lichen Samples

The lichen samples belonging to *Parmelia sulcata* and *Hypogymnia tubulosa* were collected from fir trees of Kastamonu province in the north-west of Turkey. They were identified through classical taxonomic methods by microscopic examination (23, 24). Voucher specimens were deposited with the lichen collection of Marmara University Herbarium (MUFE). *Parmelia sulcata* and *Hypogymnia tubulosa:* Turkey, Kastamonu province, Kapaklı Village, *41.24492, 34.18330,* G. Çobanoğlu.

2.3. Extraction of Lichen Samples

The lichen samples were washed, dried on air, and weighed. After the samples were taken into sterile bottles, acetone (ACS, ISO, Reag. Ph Eur) was added and kept in a dark place for 24 h followed by filtration through filter paper. The acetone was evaporated in a rotary evaporator and crude lichen acetone extracts were obtained (25).

2.4. Determination of antibacterial efficacies

Antibacterial tests were performed in 96-well microplates (Greiner Bio-One, Cell Star, F-bottom, with lid). Tryptic soy broth was added to each well and four-fold serial dilutions of the acetone extracts of *P. sulcata* and *H. tubulosa* were made. Final concentrations of both lichen extracts were 240, 120, 60, and 30 µg/ml. Overnight cultures of eight isolates were added to obtain a total volume of 100 µl with an OD 600 nm of 0.02. The experiments included untreated (the broth medium and test bacteria) and blind wells (only the broth medium). The tests were performed in three replicates. The inhibitory effect of

the acetone extracts on bacterial growth was evaluated at 20th hour using Cytation 3 multimode microplate reader (Biotek), by measuring the absorbance. The antibacterial effects of the extracts of *P. sulcata* and *H. tubulosa* against the test bacteria were compared with the untreated ones.

III.RESULTS

3.1. Colony Morphology

The colony morphology was concentric surfaced, undulate edged, and cream-white colored in isolate 1 (*B. tonoyensis*). Isolate 2 (*B. mojavensis*) had convex surfaced, undulate edged, and cream-white colored colonies. In isolates 3 (*B. cereus*) and 5 (*B. cereus*), colonies were large, flat surfaced, entire edged, dull and cream-white colored. The colony morphology in isolate 4 (*B. velezensis*) was observed as cream-white colored, rough and lobate edged colonies. The convex surfaced, opaque, lobate edged, rough and wrinkled colonies were recorded in isolate 6 (*B. licheniformis*).The colony morphology of isolate 7 (*B. amyloliquefaciens*) was detected to have rough, mucoid, cream-white colored colonies with irregular edges. The isolate 8 (*B. subtilis*) had opaque, convex surfaced, lobate edged, and rough colonies. The morphological appearances of eight isolates on agar plates are given in Figure 1.

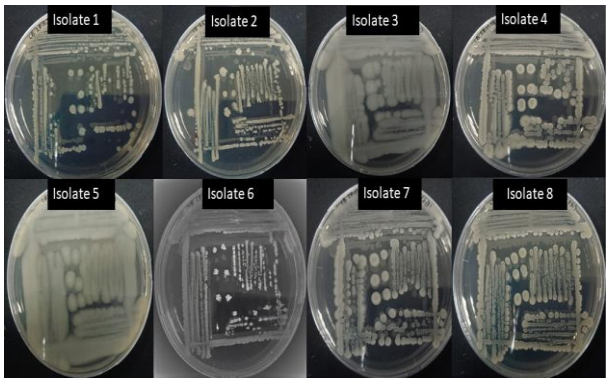

Figure 1. The morphological appearances of eight isolates on agar plates.

3.2. Gram Staining

The isolates were all Gram positive and rod-shaped. The microscopic appearances are given in Figure 2.

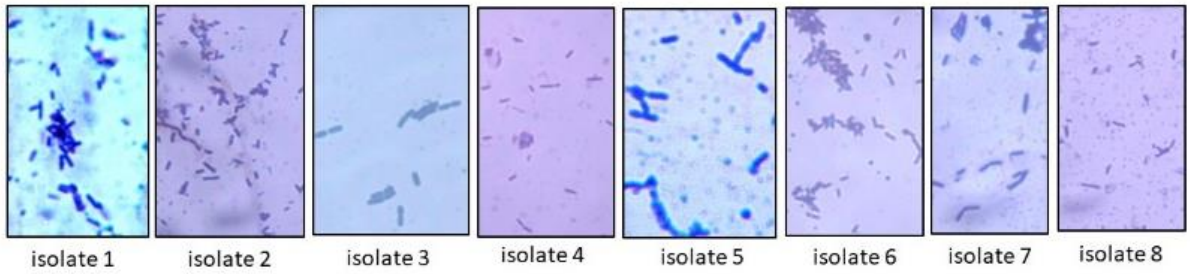

Figure 2. The microscopic appearances for Gram staining of eight isolates.

3.3. Antibacterial Efficacies of the Extracts

Compared to untreated groups, a great inhibition was detected in the bacterial growth of all isolates which were tested with the acetone extracts of *H. tubulosa* at the concentration of 240 µg/ml. The inhibition ratios were recorded as 80.24-88.65 % for this test concentration against eight test isolates. In this respect, we obtained similar inhibition ratios for the concentration of 240 µg/ml, and the highest antibacterial effect was observed in isolate 4 (*B. velezensis*). However, some differences were determined in other test concentrations depending on the isolates. The potential antibacterial effect was also detected at the concentration of 120 µg/ml in most of the isolates. The inhibition percentages for the isolates 3 (*B. cereus*), 4 (*B. velezensis*), 5 (*B. cereus*), 6 (*B. licheniformis*), 7 (*B. amyloliquefaciens*), and 8 (*B. subtilis*) were detected as 79.38, 72.44, 81.26, 82.29, 83.14 and 83.55, respectively. On the other hand, isolate 1 (*B. tonoyensis*) and isolate 2 (*B. mojavensis*) were not sufficiently inhibited by the extracts at the concentration of 120 µg/ml and inhibition ratios were recorded as 42.70 and 40.36 %, respectively. The extracts showed also antibacterial effects against isolates 6 (*B. licheniformis*), 7 (*B. amyloliquefaciens*), and 8 (*B. subtilis*) at the concentration of 60 µg/ml, while other isolates were not inhibited. The inhibition percentages of the isolates 6, 7 and 8 at the concentration of 60 µg/ml were calculated as 79.93, 84.38 and 84.16, respectively. There was no noteworthy effect at the concentration of 30 µg/ml. The percentages of inhibition for 30 µg/ml test group were varied between 23.01 and 6.59 except isolate 4 (*B. velezensis*). Based on these results, it was determined that the extracts had very high antibacterial effects at the concentration of 240 µg/ml against isolates 1 (*B. tonoyensis*), and 2 (*B. mojavensis*). There was no inhibition at the other test concentrations for these isolates. On the other hand, isolate 3 and 5 (*B. cereus*) and isolate 4 (*B. velezensis*) were also inhibited at the concentration of 120 µg/ml as well as 240 µg/ml. The isolates 6 (*B. licheniformis*), 7 (*B. amyloliquefaciens*), and 8 (*B. subtilis*) were highly inhibited at the concentrations of 240, 120 and

60 µg/ml but not effectively inhibited at 30 µg/ml. The data are given in Figure 3.

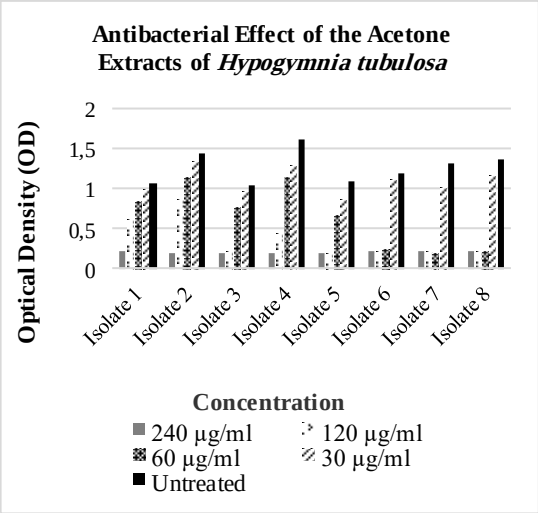

Figure 3. The antibacterial effects of the extracts against the isolates treated with acetone extracts of *H. tubulosa* at the certain concentrations of 240, 120, 60 and 30 µg/ml at 20th hour. Data are shown as optical absorbance over OD 600 nm.

The acetone extracts of *P. sulcata* were also tested against eight isolates. According to the data, these extracts were not effective when compared to the extracts of *H. tubulosa*. The extracts of *P. sulcata* had slightly inhibitory effects against the isolates 1, 3, and 5 in comparison with the untreated groups. The inhibition ratios at the concentration of 240 µg/ml were recorded as 46.69, 44.34 and 40.09 for these isolates, respectively. The inhibition ratios was detected similar in isolate 1 at the concentrations of 120, 60 and 30 µg/ml (32.09, 29.36, and 30.28 %, respectively). There was no inhibitory effect on other test isolates. The data are given in Figure 4.

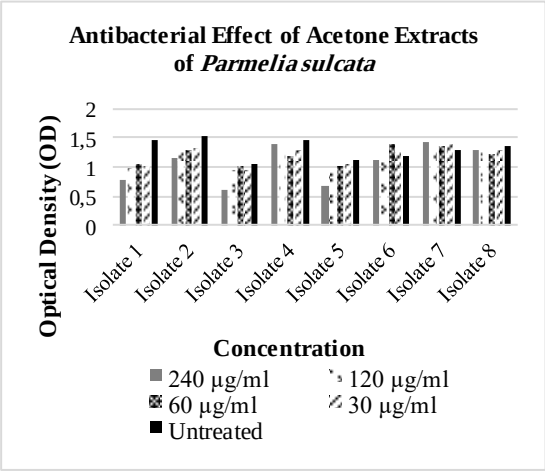

Figure 4. The antibacterial effects of the extracts against the isolates treated with acetone extracts of *P. sulcata* at the certain concentrations of 240, 120, 60 and 30 µg/ml at 20th hour. Data are shown as optical absorbance over OD 600 nm.

IV. DISCUSSION

In the leather industry, application mistakes in preservation and soaking processes can affect finished product quality seriously. Therefore, many criteria must be taken into consideration to produce the best quality and high added value leather. Whereas hide/skin preservation is generally provided by the application of salt, it has been suggested that salt may also cause bacterial contamination of hides/skins. In a study evaluating the bacterial numbers found in salt samples from Tuz Lake, Kaldırım, Kayacık Salterns, and Tuzköy Mine, high numbers of extremely halophilic archaea were reported (3, 26). Hide damages caused by proteolytic halophilic bacteria in salt samples were also recorded (1, 2, 10, 27-29). For this reason, several researchers have focused on controlling halophilic and non-halophilic bacterial population especially in salt, raw hides/skin, and soaking process. In previous studies, non-halophilic and halophilic bacteria and archaea were isolated from salted, and soaked animal hides and soak liquor samples (29-35). Inadequate salt-curing applications and soaking processes are considered to lead increased number of bacteria and also serious damages on leather due to bacterial activities.

Berber et al. (2010) reported high numbers of non-halophilic bacteria from soak liquor samples despite the utilization of antimicrobial agents. They evaluated the efficacy of antimicrobial agent (the active content, didesyldimethylammonium chloride) in soak liquors at a concentration of 0.4 g/l and they detected a high number of total non-halophilic, proteolytic and lipolytic non-halophilic bacteria in soak liquor samples. In the same study, the inhibition in bacterial growth was provided by doubling the concentration of the antimicrobial agent in soak liquor (36). Also, several antimicrobial agents were tested against various bacteria isolated from soak liquor samples in different concentrations (36, 37).

Although antimicrobial agents are applied in the soaking process, they seem to be not effective and bacterial population cannot be controlled. The ineffectiveness of antimicrobials may be due to the resistant bacteria since these bacteria can easily transfer their resistance genes via horizontal gene transfer. In this respect, alternative agents or strategies may be helpful. *B. toyonensis, B. mojavensis, B. subtilis, B. amyloliquefaciens, B. velezensis, B. cereus, and B. licheniformis* were isolated from soak liquor samples of different tanneries (22). In this study, the possible antibacterial effects of the acetone extracts of *P. sulcata* and *H. tubulosa* lichen species were evaluated against these isolates. It is well known that many biological activities (antimicrobial, antifungal etc.) of lichens have been known for many years. While many studies have reported that lichen substances are more effective against Gram positive bacteria, some lichen species have also been shown to

have antibacterial effects against Gram negative bacteria such as *Pseudomonas aeruginosa* and *Escherichia coli* (19, 38-42).

However, there are not many studies evaluating the efficacy of lichen extracts in the leather industry. Türkan et al. (2013) reported that acetone and chloroform extracts of *P. furfuracea* (L.) Zopf on raw skin and chrome-tanned leather samples against *Bacillus subtilis, B. cereus, Staphylococcus aureus, Escherichia coli, Enterococcus faecalis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Aspergillus niger, A. fumigatus, A. candidus, A. flavus, Penicillium jensenii, Geotrichum candidum and Candida albicans* (15). In the previous study, the antibacterial efficacies of the acetone extracts of *Hypogymnia physodes, Evernia divaricata, Pseudevernia furfuracea,* and *Usnea* sp. against *B. toyonensis, B. mojavensis, B. subtilis, B. amyloliquefaciens, B. velezensis, B. cereus, and B. licheniformis* at different concentrations were reported (22). Furthermore, the potential anti-biofilm properties of the acetone extracts of *Usnea* sp. were reported against biofilm–forming *B. subtilis, B. amyloliquefaciens,* and *B. velezensis* from soak liquor samples (43). In this meaning, the extracts and/or the constituents of other lichen species may also have potential effects against bacterial population in leather industry. There are many studies for several biological activities of *P. sulcata* and *H. tubulosa.* Altuner et al. (2011) examined antimicrobial activities of methanol extracts of *Letharia vulpine, P. furfurace* and *Evernia divaricata* collected from Kastamonu province against *Salmonella enterica, E.coli, Shigella flexneri* and *B. megaterium.* They did not observe any activity of *H. tubulosa* on tested microorganisms (44). On the other hand, Cansaran-Duman et al. (2010) tested three species of *Hypogymnia* for their antibacterial effects on some Gram positive and Gram negative bacteria and they observed highest activity in *H. tubulosa* extracts. Furthermore, *B. subtilis* was found to be most susceptible against acetone extracts of *H. vittata, H. physodes* and *H. tubulosa* species (45). In this study, considerable antimicrobial efficacy was also detected for *H. tubulosa* against test isolates from soak liquor samples. Similarly, the antibacterial efficacy of acetone extracts of *H. physodes* was demonstrated in the previous study (22). Therefore, it can be suggested that the species belonging to *Hypogymnia* may have potential antibacterial effects against *Bacillus* species from soak liquor samples. There is no study evaluating the antibacterial properties of *P. sulcata* against the bacterial population in the soak liquor. To our knowledge, *P. sulcata* has been firstly tested against *B.toyonensis, B.mojavensis, B.subtilis, B. amyloliquefaciens, B. velezensis, B. cereus and B.licheniformis* isolated from soak liquor samples of different tanneries. However, there are studies evaluating the antimicrobial effect of *P. sulcata* against several bacteria. Rankovic et al. (2007) reported the antimicrobial effect of acetone and methanol extracts of *P. sulcata* against several Gram positive and Gram negative bacteria except *S. aureus* (46). Moreover, Candan et al. (2007) showed antimicrobial activities of the acetone, chloroform, diethyl ether, and methanol extracts *of P. sulcata* collected from Eskişehir province against some Gram positive and Gram negative bacteria (47). On the contrary, *P. sulcata* was not successful against test isolates in this study.

While it was reported that both *H. tubulosa* and *P. sulcata* had antimicrobial activities against several bacterial species in several studies (39, 45-47), *H. tubulosa* was determined to have considerable antibacterial activity when compared to *P. sulcata* on tested isolates in this study. This result may be due to the differences in bacterial strains, applied extract concentrations and collection localities of lichen species. Considering these data, *H. tubulosa* extracts or their constituents may be utilized in the soaking process to control the bacterial population and also their potential defects on leather in further tanning processes. Since lichen extracts are natural and eco-friendly sources, they may be alternative antibacterial agents according to commonly used synthetic antimicrobial agents in the leather industry.

V. CONCLUSIONS

The bacterial population in soak liquor samples is one of the major problems in the leather industry. These bacteria cannot be eliminated easily by the antimicrobial application. This may be due to the antimicrobial resistance properties of some bacteria due to the ability to transfer resistance genes. For this purpose, alternative agents or strategies have to be investigated for controlling the bacterial population in soak liquors. In this study, the acetone extracts of *H. tubulosa* was determined to have antibacterial activities against *B. toyonensis, B. mojavensis, B. subtilis, B. amyloliquefaciens, B. velezensis, B. cereus* and *B. licheniformis* obtained from different soak liquor samples at the concentration of 240 µg/ml at the 20^{th} hour. On the other hand, the prominent antibacterial or inhibitory effect of *P. sulcata* extracts could not be detected on tested isolates. These data suggest that the acetone extracts of *H. tubulosa* may be utilized in the soaking process to control the bacterial population.

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