Examination of Antibacterial Effectiveness of Potassium Dimethyl-Dithiocarbamate Against Mix Population of Bacteria Isolated from the Salt-Pack Cured Hides

Meral Birbir[1], Nazlı Dölek[2], Pınar Çağlayan[1]

[1]Science and Letter Faculty, Biology Marmara University Istanbul, Turkiye, mbirbir@marmara.edu.tr

[2]Institute for Graduate Studies in Pure and Applied Science Marmara University Istanbul, Turkiye, nazli.dolek@gmail.com

[1]Science and Letter Faculty, Biology Marmara University Istanbul, Turkiye, pinar.caglayan@marmara.edu.tr

Abstract

The goal of the present study was to examine the antibacterial effectiveness of different concentrations of commonly used antibacterial agent containing potassium dimethyl-dithiocarbamate against mix population of *Enterobacter cloacae, Vibrio fluvialis, Pseudomonas luteola, Staphylococcus cohnii, Enterococcus faecium* and *Bacillus pumilus*. The antibacterial effect of different concentrations of the agent against the mix population of *Enterobacter cloacae, Vibrio fluvialis, Pseudomonas luteola, Staphylococcus cohnii, Enterococcus faecium* and *Bacillus pumilus*, which were isolated from salt-packed cured hides and identified with API test kits, was tested by the agar disk diffusion method on Nutrient Agar according to the guidelines of the National Committee for Clinical Laboratory Standards. The discs containing 7 µL of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1% (w/v) of the agent were prepared and placed on surface of Nutrient Agar inoculated with the mix population of bacteria (10^8 c.f.u./mL). Zones were detected around the discs containing all concentrations of the agent. Zone diameters increased proportionally to the concentrations of the agent. Inhibition zone diameters of ≥20 cm were observed at 0.8, 0.9 and 1% of the agent concentrations. Although inhibition zone diameter of 12 mm was observed around the disc containing 0.1% of the agent, inhibition zone diameter of 21 mm were seen around the disc containing 0.9-1% of the agent. As a conclusion, concentrations of 0.8, 0.9 and 1% of the test agent were proven effective against the mix population of test bacteria.

Keywords: Potassium dimethyl-dithiocarbamate, Antibacterial activity

1.Introduction

In the leather industry, the most important problem encountered is to conserve raw hide after the animal is slaughtered. The animals may be contaminated by the bacteria found in the air, in the soil and on animal's skin. Animal hides and skins contain large amounts of proteins and fat that bacteria can utilize. Proteolytic and lypolytic microorganisms found on raw hides may deteriorate the hides (Orlita 2004). In this respect, leather processing is very important for producing high quality leather products.

The fresh hides should be sent to a tannery to process immediately into leather. During salting and soaking processes as well as storage, a variety of microorganisms such as non-halophiles and halophiles may grow rapidly on the hides. Some of these microorganisms are pathogenic and harmful. If hide is not preserved after slaugthering process, it may be damaged by hydrolytic enzymes of bacteria. Therefore, short-term preservation of hides is imperative in tanneries.

The preservation methods known since the earliest times were formaldehyde tanning, drying and salting. In salt curing process, fresh hides are covered with salt after the animal is slaughtered. In addition to salt, naphthalene and boric acids are usually applied to hides. Some tanneries control bacterial activities on hides during storage; when bacterial activities are observed on these hides, salt and naphtalene are added to hides again. Sodium chloride and boric acid have bacteriostatic effect on microorganisms by reducing the skin's water content from 70% to 30% (Kanagaraj et al. 2000; Kanagaraj et al. 2001). Due to low water content of hides, it has been thought that putrefaction of collagen caused by bacterial activity is prevented by curing. In our study, it was proven that high numbers of extremely halophilic archaea and halophilic bacteria were present on the salted hides. It was found that total numbers of halophilic bacteria, proteolytic and lipolytic halophilic bacteria on the salted hide samples imported from England were 10^6-10^7 c.f.u./g, 10^5 c.f.u./g and 10^5-10^6 c.f.u./g, respectively (Yilmaz 2010). In the other study, the numbers of proteolytic and lipolytic extremely halophilic archaea were 10^2-10^6 c.f.u./g on the salted hides (Berber and Birbir 2010). In our previous study, despite curing hides with antibacterial agents, proteolytic and lipolytic bacteria and extremely halophilic archaea (10^5-10^6 c.f.u./g) were isolated in quite high numbers from the salted hides (Berber 2009). In another investigation, 10^4-10^8 c.f.u./g of bacteria were found on the cured hides treated with sodium chloride and boric acid (Aslan and Birbir 2011a). A total of 396 Gram-positive and 256 Gram-negative bacterial isolates were isolated and identified from the salted hides cured with sodium chloride and boric acid. The percentages of Gram-positive proteolytic, lipolytic, and both proteolytic and lipolytic isolates on these hides were 70%, 69%, and 57%, respectively (Aslan and Birbir 2011b). The percentages of Gram-negative proteolytic, lipolytic, and both proteolytic and lipolytic isolates on the hides were 68%, 52%, and 43%, respectively (Aslan and Birbir 2012). These studies showed that the curing methods, which are commonly applied to the hides, did not completely inactivate bacteria found on these salted hides. These results affirmed that salt curing method applied worldwide was not adequate to impede bacterial activities on the salted hides during storage.

Several antimicrobial agents and chemicals such as 1-2 dichlorobenzene, trichloro-S-triazinetrione, sodium sulphate, sodium bisulphite, acetic acid, alkyl phenol ethoxylates,

potassium dimethyldithiocarbonate, 2-(thiocyanomethylthio)benzo -thiazole, methylene bis (thiocyanate), sodium-orthophenylphenate, ortho-benzyl-para-chlorophenol, 5-chloro-2-methyl-4-isothiazolin-3-one, potassium dimethyldithiocarbamate, sodium salt of o-phenylphenate (Birbir and Bailey 2000), quaternary ammonium compounds (Bilgi et al. 2009), plus 2-bromo-2-nitropropane-1,3-diol (Muthusubramanian and Mitra 2006), antibacterial complex of copper (II) with benzothiazole (Haibin et al. 2008), bactericides containing silver particles (Yang et al. 2012) have been used in leather industries to prevent bacterial activities on hides. Plant based formulations and antibiotics have also been used such as *Acalypa indica* (Vijayalakshmi et al. 2009), *Aloe vera* (Bitlisli et al. 2010), *Azardirachta indica* (Preethi et al. 2010), *Lawsonia inermis* (henna) (Musa et al. 2011), *Liquidambar orientalis mill. var orientalis*, organosulfur compound (Bayramoğlu 2010), phytopreservative made from *Sesuvium portulacastrum* (Kanth et al. 2009), antibiotics, doxycycline HCl (Stockman et al. 2007), oregano essential oils. Phenol and 4-chloro-3-methyl (Bayramoğlu 2007) have been recommended by researchers. Potassium dimethyl-dithiocarbamate, quaternary ammonium compound containing 12.5% didecyl dimethyl ammonium chloride and 12.5% benzyl dimethyl ammonium chloride, organosulfur compound, phenol and 4-chloro-3-methyl are among the bactericides used in the Turkish Leather Industry. These antimicrobial agents are used to control and prevent bacterial growth and its negative effects on animal hides such as hair slip, discoloration of hides, odor, decomposition, deterioration and softening of hides.

The nonoxidizing chemicals are commonly used as antibacterial agent in many industrial applications (Frayne 2001). Potassium dimethyldithiocarbamate salt, which is a nonoxidizing chemical, is a broad spectrum antimicrobial. It is used in industrial fluids including air washing systems, water cooling systems, pulp and paper mills, metal working fluids, and brine solution. Moreover, the agent is used against bacteria, sulfate reducing bacteria, fungi and algae (Frayne 2001; Pera and Sharpley 1965). Its antimicrobial effect is due to interruption of cell metabolism and chelation of essential metallic ions (Frayne 2001).

The goal of the this study was to research antibacterial activity of potassium dimethyl-dithiocarbamate on bacteria isolated from the salted hides. Therefore, different concentrations of the antibacterial agent containing potassium dimethyldithiocarbamate were tested against mix population of *Enterobacter cloacae, Vibrio fluvialis, Pseudomonas luteola, Staphylococcus cohnii, Enterococcus faecium* and *Bacillus pumilus.*

2. Material and Methods

2.1. Test isolates and the antibacterial agent

A total of six isolates obtained from salt-pack cured hides were chosen for the experiment because they are the species mostly encountered on the salted hides. Three of these isolates are Gram negative (*Enterobacter cloacae, Vibrio fluvialis* and *Pseudomonas luteola*), two of Gram positive (*Staphylococcus cohnii* and *Enterococcus faecium*) and one of Gram positive endospore forming bacteria (*Bacillus pumilus*). All test isolates were isolated and identified in the previous studies (Aslan and Birbir 2011a; Aslan and Birbir 2011b; Aslan and Birbir 2012). API® 20E (bioMériux, Inc, France), API® 20NE (bioMériux, Inc, France),

API Staph (bioMériux, Inc, France), API 20 Strep (bioMériux, Inc, France) and API 50CH (bioMériux, Inc, France) test strips were used to identify Gram negative (*Enterobacter cloacae, Vibrio fluvialis* and *Pseudomonas luteola*) and Gram positive bacteria (*Staphylococcus cohnii, Enterococcus faecium* and *Bacillus pumilus*), respectively**.** The nonoxidizing test bactericide was obtained from Busan Industrial Chemical Materials Inc. Comp., Turkiye. Different concentrations of the test agent were prepared as 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1% (w/v).

2.2. Determination of protease activity.

Proteolytic activity of the isolates was screened on Tryptic Soy Agar containing 4% gelatine (Table 1). After 48 hours incubation of the test isolates at 37^{o}C, the plates were flooded with a saturated solution of ammonium sulfate. Clear zones around the colonies were taken as evidence of protease activity (Barnett and Venghaus 1988).

2.3. Determination of lipase activity.

Lipolytic activity of the isolates was examined on Tween 80 agar medium (Table 1). After the incubation of the test isolates at 37^{o}C for 24-48 hours, clear zones around the colonies were taken as evidence of lipase activity (Birbir et al. 2007).

2.4. Determination of antibacterial activity of Potassium dimethyl-dithiocarbamate

The test isolates (*Enterobacter cloacae, Vibrio fluvialis, Pseudomonas luteola, Staphylococcus cohnii, Enterococcus faecium* and *Bacillus pumilus*) were separately grown in Nutrient Broth (Merck, Darmstadt, Germany) overnight at 37^{o}C. After incubation period, each of these bacterial cultures was separately suspended in sterile physiological saline to a final cell density of 10^{8} c.f.u./mL. The colony forming units were determined by plate counting. Afterwards, the mixed culture of the test isolates was prepared from these physiological saline solutions.

The resistance or sensitivity of bacteria against potassium dimethyl-dithiocarbamate was evaluated by the disk diffusion method of Kirby and Bauer in accordance with the procedures recommended by National Committee for Clinical Laboratory Standards (Madigan et al. 2012; Bilgehan 2004). Test discs (6 mm) were prepared from Whatman No.1 filter paper and sterilized at 121°C for 20 minutes. Firstly, 100 µL of mixed culture of test bacteria adjusted to McFarland Turbidity Standard No.0.5 (10^{8} c.f.u./mL) were spread on Nutrient Agar and then 6-mm filter paper disk containing 7 µL of different concentrations [0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1% (w/v)] of the antimicrobial agent were placed on Nutrient Agar plates. The plates were incubated at 35°C for 24 hours. After incubation, the clear zones around the discs were measured.

3.Results and discussion

In the present study, *Pseudomonas luteola, Enterococcus faecium, Staphylococcus cohnii* and *Bacillus pumilus* showed both proteolytic and lipolytic activities, while *Vibrio fluvialis* and *Enterobacter cloacae* were found as protease positive and lipase negative (Table 1). The most common Gram-positive genera on the salted hides were *Staphylococcus* (115 isolates), *Bacillus* (111 isolates) and *Enterococcus* (75 isolates), while the most common

Gram-negative genera on the salt-pack cured hides were found to be *Enterobacter* (66), *Pseudomonas* (59) and *Vibrio* (32) (Aslan and Birbir 2011b; Aslan and Birbir 2012). These isolates which showed both proteolytic and lipolytic activities were in the highest numbers on the hides (Aslan and Birbir 2011b; Aslan and Birbir 2012). Hence, we used *Enterobacter cloacae, Pseudomonas luteola, Bacillus pumilus, Vibrio fluvialis, Enterococcus faecium* and *Staphylococcus cohnii* as test isolates in this study.

Table 1. Proteolytic and lipolytic activities of the test isolates

Isolates	Proteolytic Activity	Lipolytic Activity
Enterobacter cloacae	+	-
Vibrio fluvialis	+	-
Pseudomonas luteola	+	+
Enterococcus faecium	+	+
Staphylococcus cohnii	+	+
Bacillus pumilus	+	+

The antibacterial effectiveness of potassium dimethyldithiocarbamate against mixed bacterial population, as measured by disk diffusion method, is shown in Table 2. Zone sizes increased proportionally to the concentrations of the agent. Inhibition zone diameters of ≥20 mm were observed at 0.8, 0.9 and 1% of the agent concentrations. Although an inhibition zone diameter of 12 mm was observed around the disc containing 0.1% of the agent, an inhibition zone diameter of 21 mm was seen around the disc containing 0.9-1% of the agent. It is usually accepted that an inhibition zone of 20 mm is evaluated as effective concentration of antimicrobial agent against bacteria. The results can be interpreted as 0.8, 0.9 and 1% (w/v) of the test agent were found to be effective against mix population of the test bacteria (Table 2).

Table 2. *In vitro* antibacterial effectiveness of potassium dimethyl-dithiocarbamate against mixed population of Gram-negative, Gram-positive and Gram-positive endospore forming bacteria

Test bacteria*	Concentration of the test agent % (w/v)	Zone diameter range (mm)
Mixed culture of *Enterobacter cloacae, Vibrio fluvialis, Pseudomonas luteola, Staphylococcus cohnii, Enterococcus faecium* and *Bacillus pumilus*	0.1	12
	0.2	15
	0.3	15
	0.4	16
	0.5	16
	0.6	17
	0.7	18
	0.8	20
	0.9	21
	1	21

* The inoculum density of the test bacteria was 10^8 c.f.u./mL before experiment.

In another study undertaken with potassium dimethyl-dithiocarbamate containing 12.5% didecyl dimethyl ammonium chloride and 12.5% benzyl dimethyl ammonium chloride, mixed culture of *Bacillus licheniformis, Bacillus pumilus, Staphylococcus intermedius, Pseudomonas luteola, Enterobacter cloacae, Vibrio fluvialis* and *Enterococcus faecium* was completely inactivated with 0.297% (w/v) of the potassium dimethyl-dithiocarbamate (Veyselova et al. 2012).

If bacterial growth on salted hides cannot be prevented with effective treatment, bacterial activity will continue in soaking process. In this process, salted hides are soaked in clean water to remove left over curing salts, manure and dirt, and to increase the moisture so that the hide can be further treated. The soaking process is usually applied in two stages as presoaking and main soaking processes. Our previous studies proved that bacterial activity is still an important problem in the soaking process. A total of 7 presoak and 7 main soak liquors containing antibacterial agents were evaluated for bacterial population. These presoak and mainsoak liquors contained 10^4-10^5 c.f.u./mL and 10^6-10^7 c.f.u./mL, respectively (Birbir et al. 2008). Similar results were obtained from the analysis of 19 soak liquor samples treated with different antibacterial agents. The bacterial counts were 10^5-10^7 c.f.u./mL in these samples even if the antimicrobial agents were added (Berber and Birbir 2010). Furthermore, 29 of 34 soaked hide samples contained 10^7- 10^8 c.f.u./mL (Berber and Birbir 2010).

In another study, 10^4 c.f.u./mL of aerobic bacteria, 10^4 c.f.u./mL of proteolytic bacteria and 10^4 c.f.u./mL of lipolytic bacteria were found in the main soak liquor of sheep skins treated with 0.4% of bactericide containing Quaternary Ammonium Compounds and 0, 10 and 15% NaCl, respectively (Bilgi et al. 2009).

The presence of bacteria in main soak liqour in high numbers may be related with high organic content of the soak liquors and bacterial resistance to antibacterial agents. High organic content of soak liqours may affect the effectiveness of antibacterial agent adversely. Moreover, the bacteria may develop resistance to antibacterial agent and they can transfer resistance genes among themselves. Consequently, they can multipy in the presence of antibacterial agents in soak liquors (Birbir et al. 2008).

4.Conclusion

A concentration of 0.8, 0.9 and 1% of the test agent were found to be effective against mix population of *Enterobacter cloacae, Vibrio fluvialis, Pseudomonas luteola, Staphylococcus cohnii, Enterococcus faecium* and *Bacillus pumilus*. The test results affirmed that potassium dimethyldithiocarbamate has remarkable antibacterial capacity and can be used in brine curing and soaking process of hides in the leather industry to reduce the total bacterial counts and prevent bacterial deterioration. We also suggest that the antimicrobial capacity of chemical agents, used in the hide industry must be tested on the bacteria most commonly isolated from salted hides.

Acknowledgement

We wish to express our gratitude to Dr. Emel Aslan Con for isolation and identification of the test isolates. We thank Deteks Kimya San.A.S. for the gift of potassium dimethyl-dithiocarbamate.

References

Aslan, E., Birbir, M., 2011a, Examination of Efficiency and Sufficiency of Salt-Pack Curing Method, JSLTC, 95(3), 98-103p.

Aslan, E., Birbir, M., 2011b, Examination of Gram Positive Bacteria on Salt-Pack Cured Hides, JALCA, 106, 372-380p.

Aslan, E., Birbir, M., 2012, Examination of Gram Negative Bacteria on Salt-Pack Cured Hides, JALCA, 107(4), 106-115p.

Barnett, M.E., Venghaus, J.D., 1988, Microbiology Laboratory Exercises, Wm.C. Brown Publishers, Iowa, USA.

Bayramoglu, E., 2007, Unique Biocide for the Leather Industry, Ibid., 102, 347-352p.

Bayramoglu, E., 2010, Possibility of Using Sigla Tree (Liquidambar orientalis mill. var orientalis) Storax as Bactericide in the Soaking Float, Ibid.,105, 62-68p.

Berber, D., Birbir, M., 2010, Examination of Bacterial Populations in Salt, Salted Hides, Soaked Hides and Soak Liquors, JALCA, 105, 320-326p.

Berber, D., 2009, Tuzlarda ve Ham Derilerde Archaea ve Bacteria Populasyonlarının Fluorescence In Situ Hibridization (FISH) ile İncelenmesi, Doctoral Thesis, Institute for Graduate Studies in Pure and Applied Science Marmara University, Istanbul, Turkiye.

Bilgehan, H., 2004, Clinical Microbial Identification, Barıs Yayınları, Ankara, Turkiye.

Bilgi, S. T., Yapıcı, B. M., Yapıcı, A. N., 2009, Determination of Bacterial and Fungal Numbers in Foats of Pre-tanning Operations, Afr. J. Biotechnol., 8, 1602-1607p.

Birbir, M., Calli, B., Mertoglu, B., Elevi, R.B., Oren, A., Ogmen, M.N., Ogan, A., 2007, Extremely Halophilic Archaea from Tuz Lake, Turkey, and the Adjacent Kaldirim and Kayacik Salterns, World J. of Microbiol. and Biotechnol., 23, 309-316p.

Birbir, Y., Uğur, G., Birbir, M., 2008, Inactivation of Bacterial Population in Hide Soak Liquors via Direct Electric Current, J. Electrostat., 66, 355-360p.

Birbir, M., Bailey, D. G., 2000, Controlling the Growth of Extremely Halophilic Bacteria on Brine-cured Cattle Hides, J. Soc. Leather Technol. Chem., 84, 201-204p.

Bitlisli, B. O., Yasa, I., Aslan, A., 2010, Phsysical and Antibacterial Characteristics of Aloe vera Treated Split Suede Leather, JALCA, 105, 34-40p.

Frayne, C., 2001, The Selection and Application of Nonoxidizing Biocides for Cooling Water, The Voice of The Water Treatment Industry, New york.

Haibin, G., Wuyong, C., Changqing, Z., 2008, New Antibacterial Complex of copper(II) with Benzothiazole Derivative, JALCA, 103, 114-118p.

Kanagaraj, J., Babu, N.K.C., Sadulla, S., Rajkumar, V.S., Visalakshi, G., Kumar, N.K., 2000, Cleaner Techniques for the Preservation of Raw Goat Skins, J. Clean Prod., 9, 261–268p.

Kanagaraj, J., Babu, N.K.C., Sadulla, S., Rajkumar, V.S., Visalakshi, G., Kumar, N.K., 2001, A New Approach to Less-salt Preservation of Raw Skin/hide, J. Am. Leather Chem. Assoc., 95, 368–374p.

Kanth, S., Keerthi, S., Selvi, A., 2009, Studies on the Use of Sesuvium portulacastrum – Part II: Preservation of Skins, Ibid., 104, 25-32p.

Madigan, M.T., Martinko, J.M., Dunlop, P.V., 2012, Brock Biology of Micro-organisms, 13th ed., Pearson Education, Benjamin Cummings, USA.

Musa, A. E., Tamıl, A., Selvi, 2011, Evaluation of Antibacterial Activity of *Lawsonia inermis* (henna) Against Microbial Strains Isolated from Goatskin/Leather, Ibid., 106, 170-175p.

Muthusubramanian, L., Mitra, R. B., 2006, A Cleaner Production Method for the Synthesis of Bronopole. A Bactericide That is Useful in Leather Making, J. Clean. Prod., 14, 536-538p.

Orlita, A., 2004, Microbial Biodeterioration of Leather and Its Control: A Review, International Biodeterioration & Biodegradation, 53, 157-163p.

Pera, J.D., Sharpley, J.M., 1965, Process for the Control of Sulphate Reducing Bacteria, Buckman Laboratories, Inc., Memphis, 3, 198, 733.

Preethi, V., Rathinasamy, V., Kankan, N., 2010, Azodirachta indica: A Green Material for Curing of Hides and Skins in Leather Processing, Ibid., 101, 266-273p.

Stockman, G., Didato, D., Hurlow, E., 2007, Antibiotics in Hide Reservation and Bacteria Control, Ibid., 102, 62-67p.

Veyselova, C., Birbir, M., Berber, D., 2012, Quaternary Ammonium Compound as Bactericide in Hide Brine Curing and Presoaking Processes, JALCA, 107 (10) 336-341p.

Vijayalakshmi, K, Judith, R. and Rajakumar, S., 2009, Novel Plant Based Formulations for Short Term Preservation of Animal Skin. J. Sci. Ind. Res. India, 68, 699-707p.

Yang, W., Wang, X., Gong, Y., 2012, Preparation of Antibacterial Sheepskin with Silver Nanoparticles: Potential for use as a Mattress for Pressure Ulcer Prevention, JALCA, 107, 85-91p.

Yilmaz, P., 2010, Tuzlanmış Derilerden Ilımlı ve Aşırı Halofil Bakterilerin İzolasyonu ve Moleküler Yöntemlerle Karakterizasyonu, Master Thesis, Institute for Graduate Studies in Pure and Applied Science Marmara University, Istanbul.