Soaking with Storax- Possibility of Using Siğla Tree (*Liquidambar orientalis* Mill. var *orientalis*) Storax as Bactericide in the Soaking Float

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Abstract: The researches to limit the use of synthetic biocides for preventing microorganism formation on finished leather and to develop substitute products have recently gained pace. Especially, the researches have been directed towards the protective effects of natural bioactive materials. The antibacterial impact of storax against various bacteria has brought on the agenda the issue of searching its use also for the leather industry. In the present study, Sığla (*Liquidambar orientalis* Mill.var *orientalis*) storax was investigated for antibacterial activity in the soaking float. In this investigation, 1%, 2%, 5% of storax and 1% essential oil of *Origanum onites* mixed with the storax were tested for their antimicrobial activity in parallel with 50 % organosulfur compound as commercial bactericides commonly used in the leather industry. The results show that storax had an antibacterial effect in soaking with increasing concentrating rates. The bactericidal activity increased if mixed with the oil of oregano. Sığla Storax contained 1.39% essential oils and styrene accounts for 96.55% of this oil. These findings suggest that Sığla storax and oregano essential oil have synergic effects and the mixture of Sığla storax and oregano essential oil can be used as bactericidal agents in leather industry.
Key words: leather; soaking; storax; bactericide

1 Introduction

Soaking is the first process of rehydrating and cleaning hides. Due to extended times in soaking periods or when the number of bacteria with proteolytic activity increases very much, hides and skins need to protection against bacterial attacks. Currently synthetic preserving agents are used for this aim. However, the bactericidal agents that are currently used in the industry are generally harmful to human health and nature, and their use has been either restricted or banned in certain countries. For example, Pentachlorophenol (PCP) has been banned lately due to its toxicity problems despite the fact that it was in the past known as a common chemical widely used in control of bacterial and fungal contamination in leather industry.[5] The use of polyhalogenated phenolic compounds (TCP / TBP) has also been restricted in certain countries to that end.[6] The most recent regulation was reported by German authorities in the Blue Angel (RAL UZ 117): For Low emission upholstered furniture preserving agents must only be used for intermediates protection for transport and storage and the following threshold values apply for the finished leathers must be PCMC < 600 mg / kg, OIT < 250 mg / kg ,OPP < 1000 mg / kg , TCMTB < 500 mg/kg etc. [7] For this reason product formulations and application processes are under continuing development. Moreover alternative products are also being investigated.

The objective of this study is to determine whether the 100% natural products, namely Sığla storax, could be used as alternative bactericides in the leather industry. In this research, oregano essential oil is also used because it has been identified as an effective bactericidal agent in soaking process.[1,3] This study aims to test whether oil of oregano as a synergic impact on bacteria together with storax. Storax was also tested in the present research for its bactericidal activities against a commonly used antibacterial agent.

The species of Liquidambar, an important genus of Hamamelidaceae family, are known for their balsamic exudations. Of the species, Liquidambar orientalis Mill. is a native of Asia minor and has a local

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distribution in the southwestern coastal district of Türkiye, especially growing in Köyceğiz, Fethiye , Marmaris and Ula.[8,9] The balsam produced by injuring of the trees is known in commerce as Levant Storax. Balsam production from the living trees requires a special procedure of many stages.[9,10] Storax is a semi-solid, sticky material characterized by a balsamic and styrene-like odor; it is used in floral-type perfumes.[11]

Storax has been used for centuries in folk medicine for the treatment of some skin diseases, diseases of respiratory system and as a neurotonic drug.[9,10,12] Storax has antibacterial activity and the most sensitive bacterium to storax is B. cereus and for this reason it was reported that Sığla storax can be used for protection against bacteria as a topical antibacterial agents in some cases.[13] The researchers reported that *Bacillus subtilis, Bacillus cereus, Bacillus pumilis, Bacillus licheniformis, Bacillus megaterium, Bacillus sphaericus, Bacillus brevis, Bacillus laterosporus, Micrococcus luteus, Staphylococcus aureus, Micrococcus rubens, Kurthia variabilis, Pseudomonas aureginosa, Staphylococcus epidermidis,* and *Micrococcus candidus* were isolated from the raw hide.[14] Tab. 1 shows the inhibitory effect of sığla storax at different concentrations against bacteria and the bacteria which can grow in leather also marked.[13] From this results it seems that storax can be used in soaking process as bactericidal agents and this study has been designed accordingly.

Tab. 1 Inhibitory effect of Sığla storax at different concentrations against bacteria (diameter of inhibitation zone in mm)[13]

Test Bacteria	Different concentrations of the Sığla storax(%)				
	10	1	0.4	0.2	0.1
***Bacillus brevis**	**12**	-	-	-	-
***Bacillus cereus**	**16**	**13**	-	-	-
***Bacillus subtilis**	**10**	**8**	-	-	-
Corynebacterium xerosis		8	-	-	-
Enterobacter aerogenes		12	10	8	6
Enterococcus faecalis		13	-	-	-
Klebsiella pneumoniae	6	-	-	-	-
***Micrococcus luteus**	**14**	-	-	-	-
Mycobacterium smegmatis	11	-	-	-	-
Proteus vulgaris	12	8	6	6	-
***Pseudomonas aeruginosa**	**12**	-	-	-	-
Pseudomonas fluorescens	11	9	-	-	-
***Staphylococcus aureus**	**14**	-	-	-	-

-: not detectable

*: The bacteria isolated from hide[14]

2 Experimental

2.1 *Raw skin*

Native brand sheep (salted-dry) raw skins were selected for the investigation. From each skin, seven pieces (weighing about 100 grams) were carefully cut and used in soaking tests run in parallel in the following order; 1st. row contained a known 0.3% bactericidal agent (50% organosulfur compound), 2nd, 3rdand 4th soaking rows contained 1%, 2%, 5% storax, 5th row contained the oregano oil, 5th row contained mixture of 1% storax and 1% oregano essential oils and the 7th row was the control respectively. The test was repeated twice.

2.2 Identification of the essential oil content of sığla storax

The traditionally acquired sığla storax was taken from Dalaman Municipality, and its composition was examined. Traditionally, sığla storax is obtained with this procedure: The trunks of trees are injured in spring (early April) in order to obtain sığla storax. Beginning from July, the trunks are shaved by special knives and the balsam and barks are taken, these are soaked in boiling water for 10 to 30 minutes and then they are squeezed, separated from balsam barks and offered for sale.[9] Sığla balsam was distilled in Clevenger apparatus as described in the European Pharmacopeia for 3 hours, and the essential oil was obtained.[15]

2.3 Essential oil of storax composition analysis

The analysis to identify the compound of essential oil was conducted on HP 5973 selective mass detector and HP-INNOWax capillary column (60mx 032mm i.d., film thickness 0.5 µm) which were connected as combined to Hewlett-Packard (HP) 6890 gas chromatography. GC-MS identification was conducted through an ionization system with 70 eV ionization energy by using helium with 1 ml/min flow speed as the carrier gas. The furnace temperature was programmed from 70 ºC to 210 ºC/min. Injector temperature was 150 ºC. Essential oil sample was diluted with 1/100, h/h ratio ethyl acetate and injected as 1.0µl through auto-sampler in splitless mode.[16] The essential oil composition was identified by comparing the relative retention times of components and the mass spectrums of commercial standard essential oil components. The results were confirmed through computerized comparison of the components included in the data library of Wiley 275 L mass spectra. The relative quantities of compounds were calculated by the help of the areas of peaks included in the gas chromatogram.

2.4 Oregano essential oil and bactericidal agent

The essential oil; *Origanum onites* (Turkish oregano, potmarjoram) was purchased from a commercial company. The composition of the *Origanum onites* essential oil, which is used in the study, is explained in Tab. 2.[17] The storax and the oil were prepared in ethanol. 50% organosulfur compound was chosen as the control bactericidal agent commonly used in leather industry.

Tab. 2 Composition of oregano essential oil[17]

Constituent	***Origanum onites*[16]**
Carvacrol	84,48
Gamma-terpinen	2,58
P-cymene	2,01
Thymol	1,86
Linalool	1,64
Caryophyllene oxide	1,52
Beta-bisabolene	1,52
Caryophyllene	1,07
(+) - Borneol	0,79
Terpinen-4-ol	0,77
Alpha-terpinen	0,67
Myrcene	0,64
Alpha-thujen	0,46
Alpha-pinene	0,35
Beta-phellandrene	0,35
Linalylester	0,21
(+)-Aromadendren	0.21

2.5 Soaking Process

Throughout the experiments, all washing procedures were performed in sterile distilled water (DW). Pre-washing steps (for 1h) of the leather samples and the consecutive soaking steps (5 min. to 24h) took place in 1L volumes of sterile DW. The aim of the pre-washing process was to let the water diffuse through the hides, removing the substances like dirt, blood, conservation salt, dust etc. Necessary additions (levant storax, essential oil, mixture and the tester bactericide) were made prior to subsequent soaking steps.

2.6 Growth Media

PCA (Plate Count Agar-Oxoid) medium was used for total bacterial counts (aerobic mesophyllics). 1 ml aliquots were taken from each row of the 5 series of soaking waters after 5 min. and 24h respectively. The aliquots were further diluted (10^1 to 10^7) in sterile saline and 0.1ml samples in duplicates were poured into PCA medium (kept ready in liquid form at 55 C° in a water bath). The Petri dishes were incubated for 48 hrs at 37 C° and the bacterial counts were averaged and expressed as cfu/ml.

3 Results and discussion

The results of the GC-MS analysis (Fig. 1) indicated that the essential oil extracted from storax is composed of α-pinene (Rt:7.09) by 1.53%, β-pinene (Rt:8.11) by 0.36%, styrene (Rt:10.33) by 96.55%, 3-phenylpropanol (Rt:24.84) by 0.91% and 4-ethylphenol (Rt:27.68) by 0.64%. Harzemşah *et. al.* also identified as a result of their research that sığla storax is composed of styrene by 89.5%.[8]

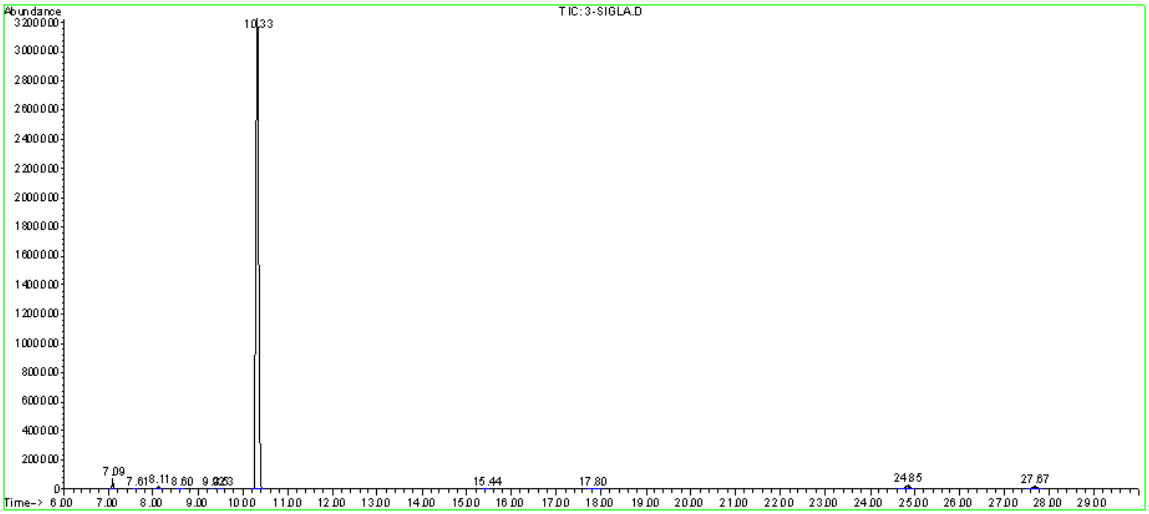

Fig. 1 GC-MS –Chromatogram of the Sığla (*Liquidambar orientalis* Mill.var *orientalis*) **storax**

Bacterial counts obtained from seven different soaking tests of the two sets with beginning and 24 h intervals have been averaged and are presented in Tab. 3. As can be seen from Tab. 3, although an antibacterial impact is not noticed when sığla storax is given to the soaking float by 1%, an antibacterial impact was identified when the dose was increased by 2 times and the impact increased significantly when the dose was increased by 5 times. It is thought that the composition of the active portion of sığla storax with antimicrobial impact has essential oil by 1.39%. In this case, when 5% sığla storax is used; it means approximately 0.069% sığla essential oil is actually used and it is more efficient during the processes than the bactericide with 50% organo sulfur which is used by 0.3%.

Tab. 3 Total bacterial counts averaged in the beginning and end of 24 hours soaking periods for six different skin samples

Biocides	Beginning (0 h)	24 h'
Control	$1.20x10^4$	$7.86x10^6$
Biocide B	$2.61x10^4$	$2.76x10^5$
0.o (1%)	$1.18x10^3$	$2.22x10^3$
L.o.(1%)	$6.04x10^3$	$1.01x10^7$
L.o.(2%)	$3.6x10^4$	$8.12x10^6$
L.o.(5%)	$5.14x10^3$	$3.51x10^3$
L.o.(1%)+ 0.o (1%)	$1.05\ x10^2$	$4.90\ x10^2$

On the logarithmic scale, the bacteria development in the soaking water at the beginning and 24-hours periods indicate that both the size and direction of the change at different times is not same for every group. Also, when the number of bacteria that developed during the soaking process was examined at the beginning of the process, it was indicated that each one yielded different values. In the control it is observed in *Liquidambar orientalis* (1) and *Liquidambar orientalis* (2) groups that the bactericide increases from the beginning of the process to the 24th hour, while the level remains unchanged in *Origanum onites(1), Liquidambar orientalis* (1)+*Origanum onites* (1) and *Liquidambar orientalis* (5) groups during this period. This can be considered as a meaningful group-time interaction ($p<0.05$).

In the beginning of the soaking process, the differences between groups were evaluated with Oneway Anova, while the differences between groups on the 24th hour were corrected according to the beginning values and evaluated with Ancova (Analysis of Covariance). As a result, although there was not any difference among the groups in the beginning ($p>0.925$), a meaningful difference was found among them on the 24th hour ($p<0.05$).

The differences among groups on the 24th hour are as follows: meaningful difference was found between the control and *Origanum onites(1), Liquidambar orientalis* (1)+*Origanum onites* (1) and *Liquidambar orientalis* (5) groups; between *Liquidambar orientalis* (1) and *Origanum onites(1), and Liquidambar orientalis* (1)+*Origanum onites* (1) and *Liquidambar orientalis* (5) groups; between *Liquidambar orientalis* (2), and *Origanum onites(1), Liquidambar orientalis* (1)+*Origanum onites* (1) and *Liquidambar orientalis* (5) groups ($p<0.05$).

As a result of the study, using the essential oil in sığla storax after distillation may sound more reasonable. However, the essential oil of sığla is not directly marketed in trade, and as it would mean extra cost and time, it is not considered as practical on the basis of the industry. Therefore, the essential oil amount and content of sığla storax was analyzed, but storax was used in combination with the essential oil of oregano. In this study, the most important advantage of storax use is considered as the facilitation of fleshing process in practice as it penetrates into the flesh layer of the skin. As for its use on fur processing, its impact on the hair should be researched.

Meanwhile, the strong antibacterial impact of *Origanum onites* was reconfirmed with this study and the mentioned impact increased further after its combination with 1% *Sığla storax*.[1,18] Here, the interesting point is, although the soaking float does not show an antibacterial impact when 1% storax is given, it increased the antibacterial impact of the oil of oregano when it was combined with the essential oil of oregano with this ratio. Quite interestingly, even 0.0139% of essential oil part of the 1% sığla storax added strengthens the antibacterial effect. As a hypothesis, this synergic effect can be explained as follows: Carvacrol is responsible for the biological activities of oregano such as antimicrobial activity.[19] Carvacrol, which is found by 84% in *Origanum onites*, exhibits a synergic interaction with the styrene, which accounts for 96.55% of the essential oil of sığla. And this increases the antimicrobial activity. Certain studies in the field of food products also support this thesis: it was reported that Styrene can be produced

naturally, usually by the decarboxylation of cinnamic acid, a common plant acid. Cinnamic acid is also used as an anti-bacterial agent in food products, and this has led to cases of food becoming contaminated with styrene, due to the activity of yeasts such as Ptchza *carsonii.*[20] Carvacrol, thymol and cinnamaldehyde in vapor phase showed highly potent antimicrobial activity against Gram negative bacteria (*Escherichia coli, Yersinia enterocolitica, Pseudomonas aeruginosa,* and *Salmonella choleraesuis*), Gram-positive bacteria (*Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus,* and *Enterococcus faecalis*), molds (*Penicillium islandicum* and *Aspergillus flavus*), and yeast (*Candida albicans*)[21,22] .

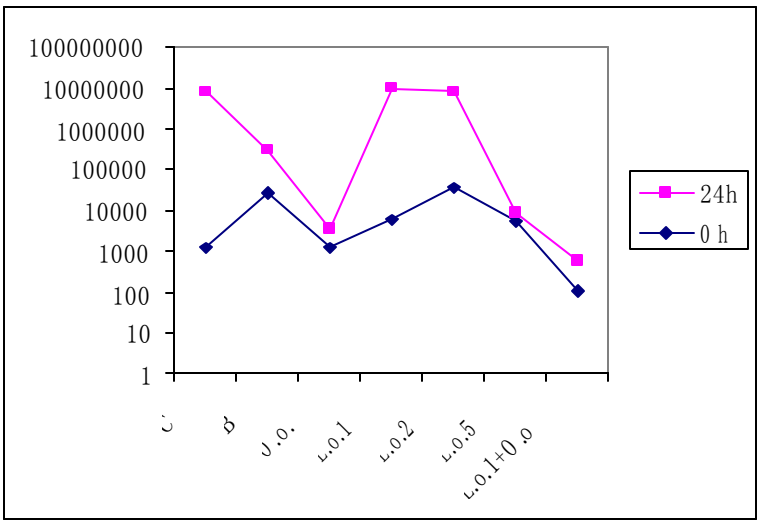

Fig. 2 Comparison of the total number of bacteria by beginning of the soaking and 24 hours soaking periods at semi-logarithmic scale

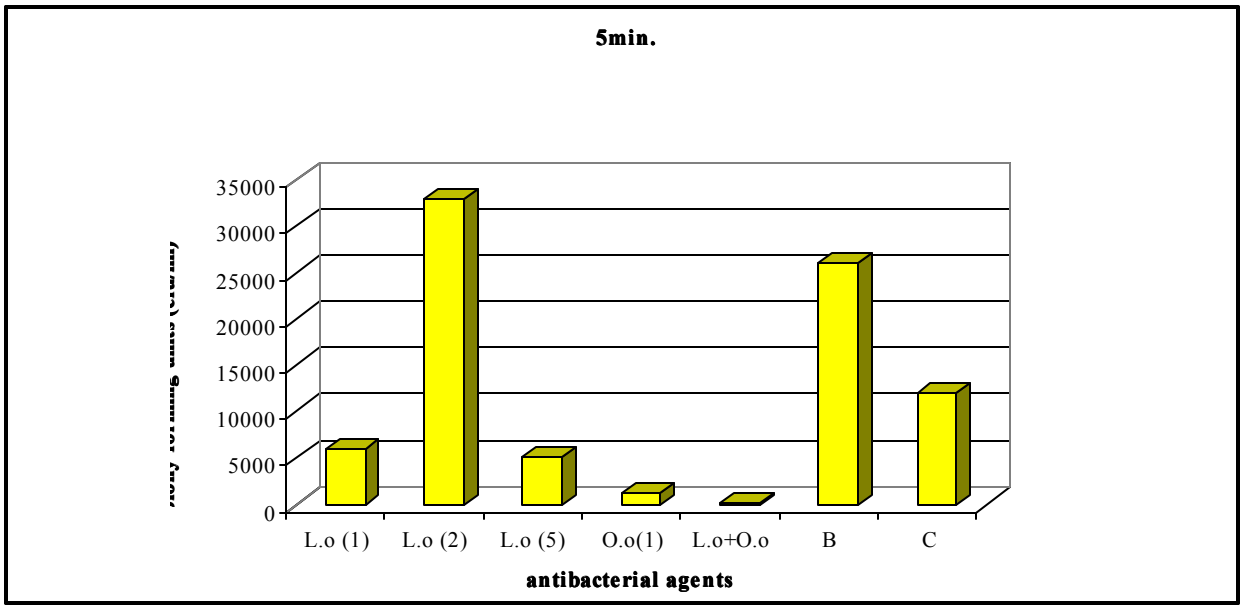

Fig. 3 Total number of aerobic bacteria at the end of 5 minutes soaking period
(B: 0.3% bactericide, L.o.(1): 1% storax (*Liquidambar orientalis*), L.o.(2).: 2% storax (*Liquidambar orientalis*), L.o.(5).: 5% storax (*Liquidambar orientalis*), O.o.: 1% *Origanum onites* oil, C: control , L.o+O.o: 1% *Origanum onites* oil +1% storax (*Liquidambar orientalis*)

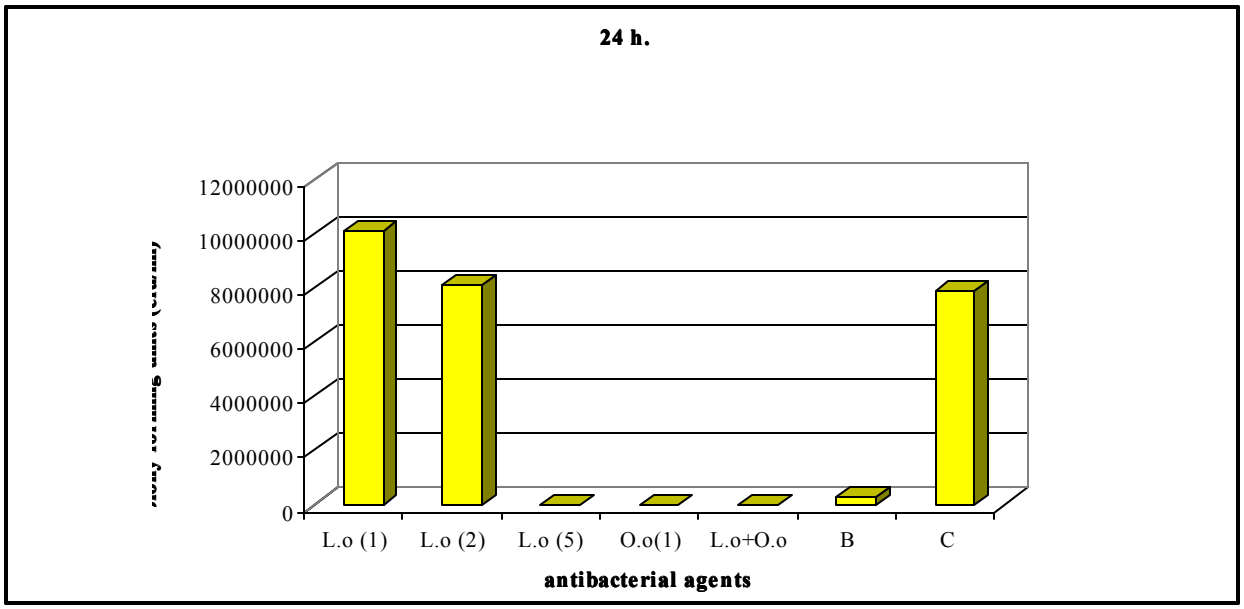

Fig. 4 The total number of aerobic bacteria at the end of 24 hours soaking period (B: 0.3% bactericide, L.o.(1): 1% storax (*Liquidambar orientalis*), L.o.(2).: 2% storax (*Liquidambar orientalis*), L.o.(5).: 5% storax (*Liquidambar orientalis*), O.o.: 1% *Origanum onites* oil, C: control , L.o+O.o: 1% *Origanum onites* oil +1% storax (*Liquidambar orientalis*)

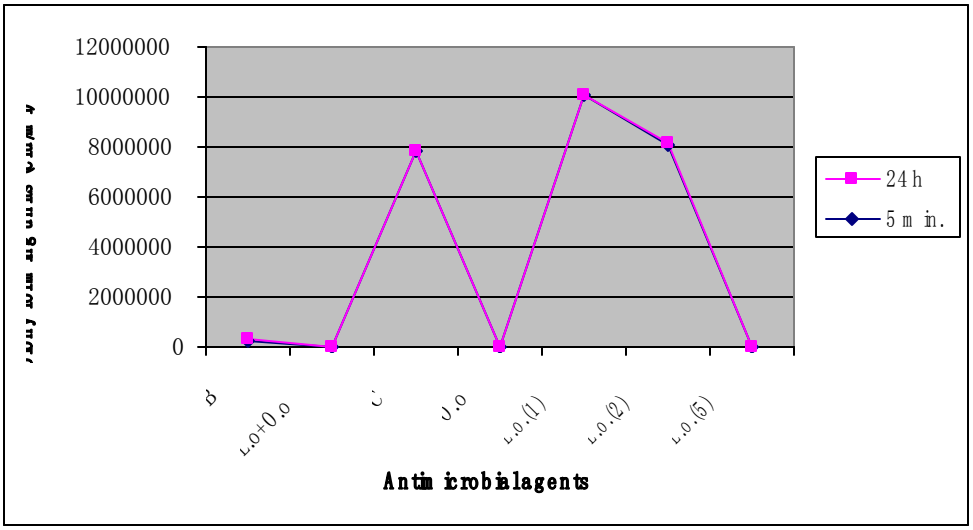

Fig. 5 Comparison of the total number of bacteria between the end of 5 min. and 24 h soaking periods

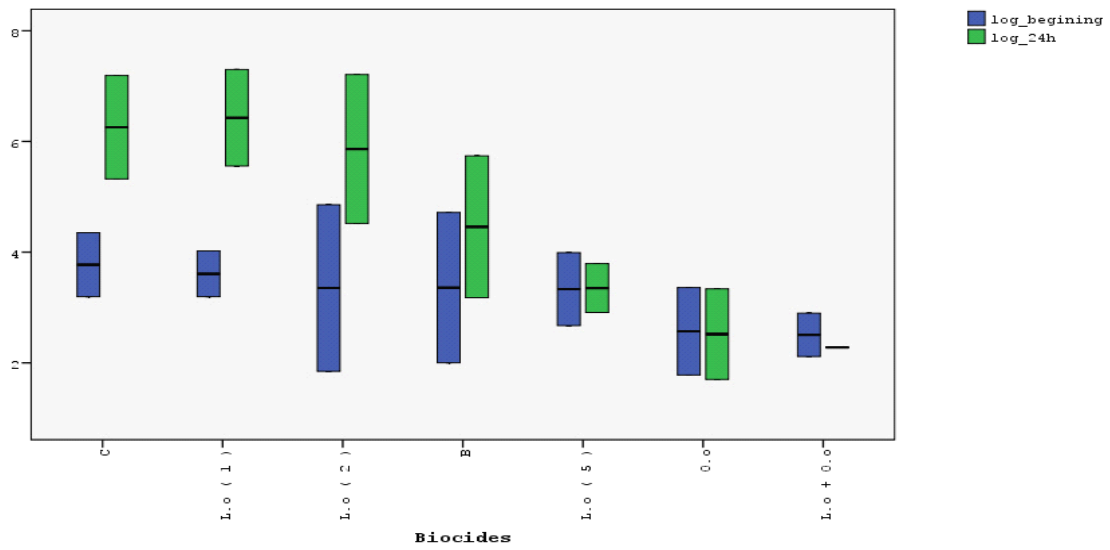

Fig. 6 Comparison of the total number of bacteria by the beginning and end of 24 hours soaking periods at logarithmic scale

4 Conclusions

As a result of the study, it can be concluded that the mixture of storax and oregano essential oils can be used as new types of bactericides which are not harmful to nature or human health due to their completely natural structure. Oregano essential oils display both antifungal and antibacterial activities and such properties are very important for their use in leather production.[1] Although the higher cost of natural components compared to synthetic biocides seems like a disadvantage, that disadvantage may be eliminated through the co-use of natural products with synergic effect. This study clarifies that the joint use of natural compounds with antibacterial properties increase the antibacterial effect; as a result, they can be used at lower rates. So, the input costs will decrease and the demand for such products will increase. The greatest advantage of these products in addition to being 100% natural is their beneficial effects rather than harmful effects on human health. It is thought that these existence of these components with antioxidant properties on the leather will eliminate free formaldehyde formation on the leather and our studies in this regard are currently continuing. The method is expected to find a significant place in leather industry in near future.

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