ECOLOGICAL AND INNOVATIVE FUNGICIDE FOR THE LEATHER INDUSTRY: ESSENTIAL OIL OF *ORIGANUM MINUTIFLORUM**

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ABSTRACT

Origanum minutiflorum (oregano) is an endemic species in Türkiye, from which essential oil is produced from its leaves and flowering tops by steam distillation. The antimicrobial activity of essential oil is known for years and many studies concerning its applications on different fields (medical science, pharmacy, food industry, etc.) have been carried out due to its advantages of being natural and nonmutagenic.

The objective of this study was to examine the applicability of *Origanum minutiflorum* essential oil as a fungicide against fungus that grows on leather during the pickling and tanning processes. In the study, 2-Thiocyano-methylthiobenzotiazole (TCMTB) and N-octyl-isothiazolinone (N-OITZ) containing commercial fungicides were also used as controls. During the microbiologic tests, the growth of mould species like *Aspergillus niger, Alternaria alternata, Penicillium rubrum* and *Trichoderma viride* that cause problems in leather industry were also investigated against this essential oil and the fungicides.

The results of this study show that oregano essential oil has antifungal activity and its effect improves with increasing concentration. Comparison of the experimental results reveals that leather treated with oregano essential oil is even more resistant against test moulds than leather treated with commercial fungicide at their recommended dosage.

ABSTRACTO

Origanum minutiflorum (orégano) es una especie muy extendida en Turquía, de la cual un aceite esencial es producido por destilación al vapor de sus hojas y capullos. La actividad antimicrobiana de este aceite esencial ha sido reconocida desde hace años y múltiples estudios sobre su aplicación en diferentes campos (medicina, farmacéutica, industria de alimentos, etc.) se han efectuado puesto tiene las ventaja de ser natural y no mutagénico.

El objetivo de este estudio fue examinar la aplicabilidad del aceite esencial del *Origanum minutiflorum* como fungicida contra los hongos que se desarrollan en los procesos de piquelado y curtido en el cuero. En el estudio, 2-Tiociano-metiltiobenzotiazola (TCMTB) y N-octil-isotiazolinona (N-OITZ) presentes en fungicidas comerciales se utilizaron también como controles. Durante las pruebas microbiológicas, el desarrollo de especies micóticas como *Aspergillus niger, Alternaria alternata, Penicillum rubrum* y *Trichoderma viride* que comunmente causan problemas en la industria del cuero, fue también investigado contra este aceite esencial como también en presencia de los fungicidas.

Los resultados de este estudio demuestran que el aceite esencial del orégano tiene actividad antimicótica y su efecto mejora al aumentar su concentración. Al comparar los resultados experimentales se demuestra que el cuero tratado con el aceite esencial del orégano es aun más resistente a los hongos que el cuero tratado con fungicida comercial a la dosis recomendada para la utilización los mismos.

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INTRODUCTION

Microorganisms are present in vast numbers in almost every environment and habitat on Earth. Microbes are found in Antarctica, on top of the tallest mountains, in the deepest parts of the oceans, and even miles down within the crust of the earth. Fungi are a group of eukaryotic microorganisms distinguished from plants by their lack of chlorophyll, differences in their cell walls, and by the fact that they are not truly multicellular. Fungi spores exist also in the air we breathe and sometimes in the water we drink. Together with the bacteria, many fungi play a major role as decomposers serving to bring about the rotting and decay of dead matter, and the recycling of this matter in the environment.[1] This can cause serious damages for pickled pelts and chrome tanned leathers during leather production, since leather is an organic material that consists of many nutrients for the fungi, which acquire the necessary nutrients for growth through absorbtion. The moulds and unicellular yeasts secrete enzymes into the surrounding environment breaking down (hydrolyze) complex organic compounds into simpler ones. As a result of this extracellular digestion, simpler compounds such as glucose and amino acids can be absorbed. Since the moulds are composed of numerous hyphae, the mycelium forms a tremendously large surface area for nutrient absorbtion.[1] Every mould species can produce different color of hyphae when they grow on the leather surface. For example, it was reported that *Aspergillus niger, Cladosporium sp.* and *Alternaria alternata* appear on the leather as black spots while *Trichoderma viride* appear as green spots.[2] The pigments produced by some moulds, which are virtually imposible to remove, can create problems with dye uptaken and cause fatty spues.[3] They may also generate a bad smell.

Fungal growth is influenced by many factors in the environment. Pickled and chrome tanned leather storage under humid conditions, high storage temparature and also the low pH value are the factors causing the growth of mould, besides the organic material of leather. Therefore, it has been considered that it would be appropriate to use fungicides in the above mentioned stages.[4] However, the chemicals used for this purpose are generally harmful to humans. A number of widely used fungicides may not be employed for wet blue in the future due to regulations or other restrictions.[5] Some microorganisms can develop resistance to microbicides as a result of restrictions and the use of less biocide than necessary.[6] Thus, leather market necessitates new biocides harmless to human health and natural life. In this study, a completely natural product was used as a fungicide in different percentages and it was compared with commercial fungicides.

The antimicrobial activity of essential oils and their derivatives was recognized long time ago. Ibn-i Sina was the first man, who invented the essential oils by steam distillation techniques and used (980-1037).[7] As essential oils and their components are natural products, they are becoming increasingly popular, and such antimicrobial agents are being used in many different sectors such as pharmacy, food industry, bakery, etc. In this study, essential oil of *Origanum minutiflorum,* which is an endemic species in Türkiye, was used.

Essential oil obtained from the oregano plant is produced by steam distillation of the leaves and flowering tops. Oregano oil contains four main groups of chemicals that contribute to its potent healing powers. Phenols, such as carvacrol and thymol, act as antiseptics; while terpenes that include pinene and terpinene display antiseptic, antiviral, anti-inflammatory and anesthetic properties. Linalool and borneol are two long-chain alcohols found in oregano oil, which add to the antiviral and antiseptic agents.[8] Many studies have been carried out to display the antimicrobial activity of oregano and two of its major components, carvacrol and thymol. Beuchat reported that the growth of the foodborne pathogen, Vibrio parahaemolyticus, was delayed by the presence of 100 ppm of the essential oils of oregano and thyme.[9] Shetty and Labbe reported that 150 ppm of either carvacrol or thymol inhibited the growth of E. coli 0157:H7 in tryticase soy broth at pH 6 (pH near that of meat).[10] Kim *et al.* also reported that carvacrol was the most antibacterial phenolic compound they tested against five foodborne pathogens (*Escherichia coli, E. coli* 0157:H7, *Salmonella typhimurium, L. monocytogenes* and *Vibrio vulnificus*). The paper disk assay was used to test each phenolic compound against the five foodborne pathogens. Carvacrol has been shown to have a larger zone of inhibition than the other phenolic compounds had at the same concentrations (citral, geraniol, terpineol, perillaldehyde, eugenol, linalool, and citronellal).[10] Some of the researchers also investigated the activity of essential oil of *Origanum minutiflorum* against *Aeromonas hydrophila, Bacillus amyloliquefaciens, B. brevis, B. cereus, B. subtilis, Corynebacterium xerosis, Enteroccus faecalis, Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Micrococcus luteus, Mycobacterium smegmatis, Proteus vulgaris, Staphylococcus aureus* and *Yersinia enterocolitica*

Figure 1. - The chemical structure of the fungicides that were used in the trials[15]

using a paper disc diffusion method and they reported that *Origanum minutiflorum* essential oils inhibited all bacteria at concentrations of 1/100 (v/v).[12] Oregano and light thyme essential oil, particularly when enhanced by agar stabilizer, may be effective in reducing the number or preventing the growth of *E. coli* O157:H7 in foods.[13]

During the soaking period, the antibacterial activity of *Origanum minutiflorum* was investigated and it was found that this essential oil also had antibacterial effect. This effect is higher than commercial bactericide, %7-25 phenol, 4-chloro-3-methyl, which is commonly used in the leather industry.[14]

The objective of this study was to determine whether the essential oil of *Origanum minutiflorum* can be used as an alternative to fungicides in the pickling and wet blue process, and to compare it with widely used commercial fungicides.

EXPERIMENTAL

Materials

Raw skin

In this study, dry salted Turkish domestic sheep skins were used in order to obtain pickled and chrome-tanned leather.

Fungicides

In these experiments, two different fungicides were used: N-octyl-isothiazolinone (N-OITZ) and 2-Thiocyano-methylthiobenzotiazole (TCMTB) based. The objective of this study was not to examine the MIC values of the commercially available products but to compare their performance at there recommended dosages with that of *Origanum multiflorum*. Therefore, the dosage indicated in the catalogues were used after confirmation from experts of the companies. Special care was paid in order to purchase recently produced materials containing active material.

TABLE I
Composition of the Essential oil of *Origanum minutiflorum*[17]

Constituent	weight percent
carvacrol	78.8
γ -terpinen	3.7
p-cimen	3.5
β-cariophillen	1.9
α-pinene	1.3
myrsen/α-phellandren	1.1
α-terpineol/borneol	1.1
thymol	1.1

Moulds

Species of mould used in this study are *Aspergillus niger, Alternaria alternata* (NRRL 10593), *Penicillium rubrum* and *Trichoderma viride* (NRRL 1608).

Essential oil

Essential oil of *Origanum minutiflorum*, which is an endemic species in Türkiye, was purchased from a commercial company. Although many compounds found in oregano oil have a combined effect, this effectiveness is mostly attributed to its primary ingredient, a phenol named carvacrol. When lab tested, carvacrol was found to be one of the most potent antibiotics known to science. In the tests done at Georgetown University, Oregano oil was poured on staphylococcus bacteria, which is responsible for a variety of severe infections and is becoming increasingly resistant to many antibiotics. They combined oregano oil with the bacteria in a test tube, and compared oregano oil's effects with those of standard antibiotics streptomycin, penicillin and vacnomycin. The oregano oil at relatively low doses was found to inhibit the growth of staphylococcus bacteria in the test tubes as effectively as the standard antibiotics did.[16] *Origanum minutiflorum* essential oil's composition and the percentage of its constituents are given in Table I.

Media

Malt Extract Agar (M.E.A) was used in keeping stocked cultures and obtaining fresh cultures in the experiments.

Methods

Obtaining the Leather Samples Used in the Experiment

Raw skins were processed so as to obtain two different groups. One group was processed in order to obtain pickled pelts and the other group was processed for obtaining chrome-tanned leathers, which were the main materials of the study. For all leather production, one standard garment leather processing method was used.

The first group of pelts were preserved by the pickling process. The features of the pelts are as follows:

1- Pickled pelt without fungicide (pH 1.5)
2- Preserved pickled pelt obtained by using N-octyl-isothiazolinone (N-OITZ)-based fungicide (0.02%, the amount advised by the chemical company)
3- Preserved pickled pelt obtained by using 2-Thiocyano-methylthiobenzotiazole (TCMTB) based fungicide (0.04 %, the amount advised by the chemical company)
4- Preserved pickled pelt obtained by using *Origanum minutiflorum* essential oils (3 different dosages: 0.5%, 1% and 2%).

Growth	Assessment	Rating
Specimen with growth	Inadequate	1
Growth on edge of specimen	Inadequate [with note: growth on specimen in (mm)]	2
Growth on cut edges	Still good (limit of efficacy)	2-3
No inhibition zone, no growth on the sample		
Specimen free of growth; Visible inhibition zone	Good [with note: size of inhibition zone in (mm)]	3

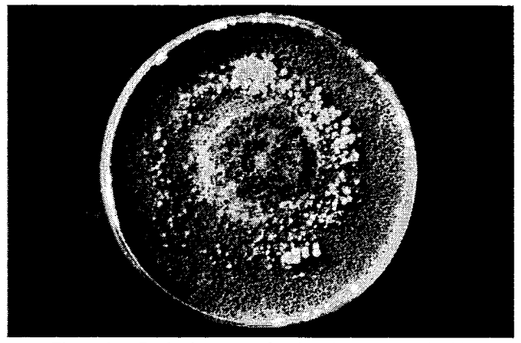

Figure 2. - *Trichoderma viride* - Specimen covered with growth (rating of 1)

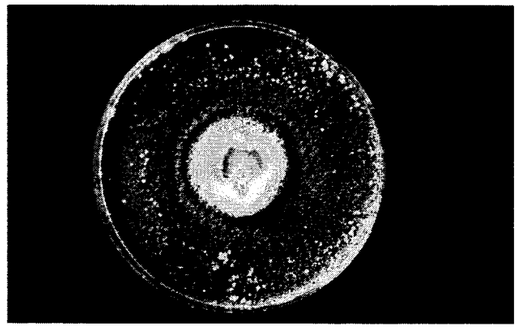

Figure 3. - *Trichoderma viride* - Growth on edge of specimen (rating of 2)

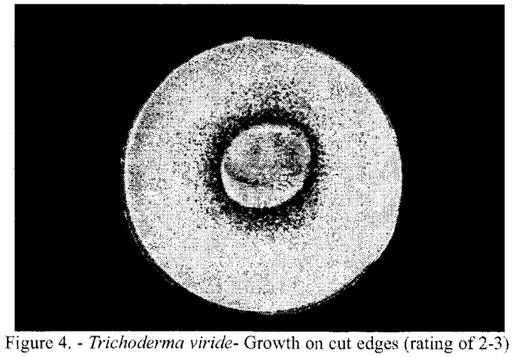

Figure 4. - *Trichoderma viride*- Growth on cut edges (rating of 2-3)

The second group of leathers, which was chrome-tanned, was primarily processed through a normal pickling process (pH 3).

5- Chrome tanned leather without fungicide.

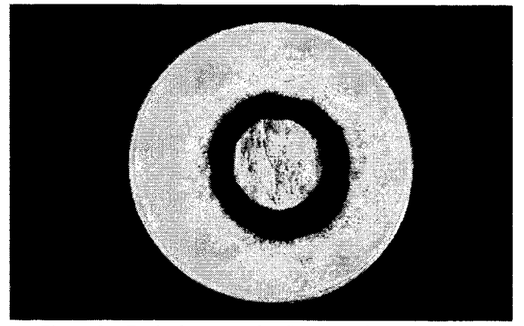

Figure 5. - *Trichoderma viride* - Specimen free of growth; visible inhibition zone - (rating of 3) Inhibition zone: Growth free zone around the specimen

6- Chrome tanned leather obtained by using N-octyl-isothiazolinone (N-OITZ) based fungicide (0.02 %, the amount advised by the chemical company)
7- Chrome tanned leather obtained by using 2-Thiocyano-methylthiobenzotiazole (TCMTB based fungicide (0.04 %, the amount advised by the chemical company)
8- Chrome tanned leather obtained by using *Origanum minutiflorum* essential oils (3 different dosages: 0.5%, 1% and 2%)

The leather samples used in the experiments were cut in a sterile room under sterile conditions. The laboratory punch was used to punch 3 circular specimens, measuring 3 cm in diameter, out of the leather samples to be tested. All equipments and tools were cleaned with ethanol and were flamed before and after changing samples. The leathers were processed with essential oil or biocide for 4 hours after being cut. Every trial was duplicated and repeated twice.

Activating Mould Species:

Mould species used in the experiments were horizontally prepared and separately incubated in the tubes with M.E.A., and fresh cultures were obtained through incubation at 27°C for a week.

Microbioiogical Test Standards:

ASTM D 4576-86 (Reapproved 1991) microbiological test method is used for mould growth resistance of wet blue and other leather samples. In this study, this test standard was applied.[18] Specimens of the leather were placed in petri-dishes on inoculated nutrient agar-medium. The specimens were evaluated for mould growth after 7, 10, 14,

TABLE II
Growth Ratings of *Trichoderma viride* on Pickled Pelts

Biocide	dosage, %	Number of Days 7	10	14	17	21	24	28
O. minutiflorum	0.5	3 (45 mm)	3	2-3	2 (5 mm)	1	1	1
	1	3 *	3	2-3	2 (10 mm)	2 (14 mm)	2 (14 mm)	2 (14 mm)
	2	3*	3*	3*	3*	3 **	3	2-3
N-OITZ	0.02	1	1	1	1	1	1	1
TCMTB	0.04	1	1	1	1	1	1	1
none	-	1	1	1	1	1	1	1

*no fungal growth on petri dishes ** Small growth near the edges of petri dishes

TABLE III
Growth Ratings of *Aspergillus niger* on Pickled Pelts

Biocide	dosage, %	Number of Days 7	10	14	17	21	24	28
O. minutiflorum	0.5	2-3	2 (2 mm)	1	1	1	1	1
	1	3 (40 mm)	2 (1mm)	1	1	1	1	1
	2	3	3	3	3	3	3	3 *
N-OITZ	0.02	2 (10 mm)	2 (10 mm)	2 (13 mm)	1	1	1	1
TCMTB	0.04	2 (13 mm)	2 (13 mm)	2 (14 mm)	1	1	1	1
none	-	1	1	1	1	1	1	1

*no fungal growth on petri dishes ** Small growth near the edges of petri dishes

TABLE IV
Growth Ratings of *Penicillium rubrum* on Pickled Pelts

Biocide	dosage, %	Number of Days 7	10	14	17	21	24	28
O. minutiflorum	0.5	3*	3*	3*	3*	3*	2-3	1
	1	3*	3*	3*	3*	3*	2-3	2-3
	2	3 *	3*	3*	3*	3*	3*	3**
N-OITZ	0.02	3*	3*	3	2-3	2-3	1	1
TCMTB	0.04	3*	3*	3*	3*	3*	3**	3**
none	-	3**	2-3	1	1	1	1	1

*no fungal growth on petri dishes ** Small growth near the edges of petri dishes

TABLE V
Growth Ratings of *Alternaria alternata* on Pickled Pelts

Biocide	dosage, %	Number of Days 7	10	14	17	21	24	28
O. minutiflorum	0.5	3*	3*	3*	3**	2 (1 mm)	2 (15 mm)	2 (15mm)
	1	3*	3*	3*	3*	3**	3**	2-3
	2	3 *	3*	3*	3*	3*	3*	3 *
N-OITZ	0.02	3*	3*	3*	3*	3 (35 mm)	2-3	2-3
TCMTB	0.04	3**	3**	3**	3 (45 mm)	3 (35 mm)	3 (32 mm)	2-3
none	-	2-3	2-3	2 (4 mm)	2 (15 mm)	2 (15 mm)	1	1

*no fungal growth on petri dishes ** Small growth near the edges of petri dishes

17, 21, 24 and 28 days of incubation. The extent of an inhibition zone around the specimens was visually graded using the rating scale shown above. Growth ratings are illustrated in figures 2, 3, 4, and 5.

Results and Discussion

The results of the experiments are reported in Tables II - IX. As can be clearly seen in the tables, 2 % essential oil has the best antifungal effect that is even better than the effect of

TABLE VI
Growth Ratings of *Trichoderma viride* on Wet Blues

Biocide	dosage, %	Number of Days 7	10	14	17	21	24	28
O. minutiflorum	0.5	3 (35 mm)	3 (35 mm)	2 (2 mm)	2 (7 mm)	1	1	1
	1	3 **	3**	3**	3**	3**	3**	3 (35 mm)
	2	3 *	3 *	3 *	3 *	3 **	3 **	3 **
N-OITZ	0.02	2 (7.5 mm)	2 (7.5 mm)	2 (10 mm)	2 (14 mm)	2 (14 mm)	2 (14 mm)	1
TCMTB	0.04	2 (1 mm)	2 (10 mm)	2 (10 mm)	2 (13 mm)	2 (14 mm)	2 (14 mm)	1
none	-	1	1	1	1	1	1	1

*no fungal growth on petri dishes ** Small growth near the edges of petri dishes

TABLE VII
Growth Ratings of *Aspergillus niger* on Wet Blues

Biocide	dosage, %	Number of Days 7	10	14	17	21	24	28
O. minutiflorum	0.5	3 (31 mm)	2-3	2 (3 mm)	2 (5mm)	2 (11mm)	1	1
	1	3 (35 mm)	3	3	2 (2mm)	2 (5mm)	2 (5mm)	2 (5mm)
	2	3 (50 mm)	3 (50 mm)	3 (50 mm)	3 (50 mm)	3 (40 mm)	3 (35 mm)	3(35 mm)
N-OITZ	0.02	2-3	2 (4 mm)	2 (10 mm)	1	1	1	1
TCMTB	0.04	2 (3 mm)	2 (5 mm)	2 (5 mm)	1	1	1	1
none	-	2 (2 mm)	2 (5 mm)	2 (10 mm)	2 (12 mm)	1	1	1

*no fungal growth on petri dishes ** Small growth near the edges of petri dishes

TABLE VIII
Growth Ratings of *Penicillium rubrum* on Wet Blues

Biocide	dosage, %	Number of Days 7	10	14	17	21	24	28
O. minutiflorum	0.5	3*	3*	3*	3*	3**	3**	3
	1	3*	3*	3*	3*	3*	3*	3*
	2	3 *	3*	3*	3*	3*	3*	3 *
N-OITZ	0.02	3*	3*	3*	3*	3 *	3**	3**
TCMTB	0.04	3*	3*	3*	3*	3*	3**	2-3
none	-	3**	3	3	3	3	3	3

*no fungal growth on petri dishes ** Small growth near the edges of petri dishes

TABLE VIII
Growth Ratings of *Penicillium rubrum* on Wet Blues

Biocide	dosage, %	Number of Days 7	10	14	17	21	24	28
O. minutiflorum	0.5	3*	3*	3*	3*	3	2-3	2 (5 mm)
	1	3*	3*	3*	3*	3*	3**	3 (32 mm)
	2	3 *	3*	3*	3*	3*	3*	3 *
N-OITZ	0.02	3*	3*	3*	3*	3 (40 mm)	2-3	2-3
TCMTB	0.04	3*	3*	3*	3*	3**	3**	3**
none	-	3**	3	2 (4 mm)	2 (4 mm)	2 (10 mm)	1	1

*no fungal growth on petri dishes ** Small growth near the edges of petri dishes

commonly used commercial fungicides at their recommended dosage. Even though these amounts were reported to be commercially appropriate, the results of our experiments revealed that the recommended dosage of the commercial fungicides was inadequate especially for *A. niger* and *T. viride.*

Even in the experiments carried out with *Penicillium rubrum* and *Alternaria alternata* test fungi, 1% of essential oil of *Origanum minutiflorum* had enough antifungal effect. Guynot *et al.* reported that essential oils of some oregano species have antifungal effect on Penicillium species and

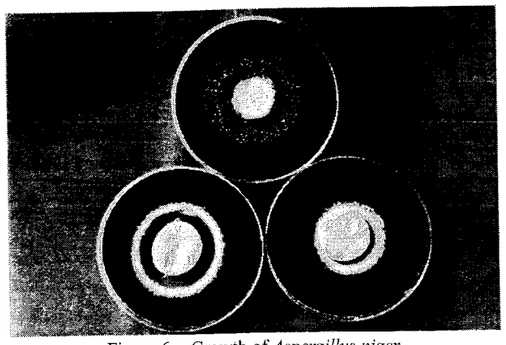

Figure 6. - Growth of *Aspergillus niger*

this information supported the results of our study.[19] The growth of *Botyrtis cinerea, Fusarium sp.* and *Clavibacter michiganensis* was completely inhibited by oregano, thyme, dictamnus and marjoram essential oils at relatively low concentrations (85-300 µg/ml).[20] In general, Gram-positive bacteria were more sensitive to inhibiton by plant essential oils than the Gram-negative bacteria.[21]

When all the test fungi behavior against antifungal agent was observed, it was found that each one of them displayed different responses. *Trichoderma viride* and *Aspergillus niger* were generally more resistant whereas *P. rubrum* and *A. alternata* were more sensitive.

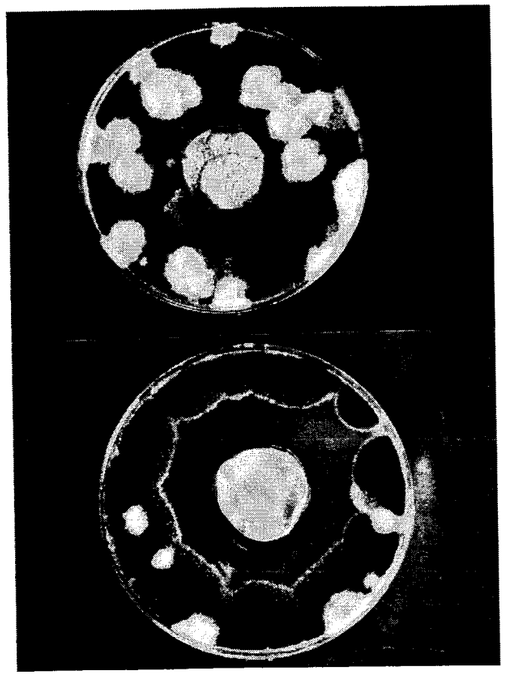

Figure 7. - Growth of *Alternaria alternata* with and without biocide

During the experiments with *Trichoderma viride*, it was detected that TCMTB and N-OITZ based fungicide were not effective enough on this fungi. Both pickled and wet blue leathers that contain these fungicides were covered with mould in a short period of time. The same situation was also observed for *Aspergillus niger*. Kennedy reported that there are two types of resistance: inherent and acquired. For example, the mould *Trichoderma viride* has inherent resistance against TCMTB.[22] This explanation is consistent with the results of our study.

Aspergillus niger is generally the most common fungi species in the tanneries.[2] Inhibition zone of this fungi on pickled pelts are given in Figure 6. In the above photo, which was taken on the 7th day of the experiment, the wet blue leather at the top consists of 0.02 % N-OITZ, the one on the left consists of 1% *Origanum minutiflorum*, and the one on the right consists of 0.5% *Origanum minutiflorum*. As for *P. rubrum*, the experiments in pickling stage revealed that N-OITZ based fungicide was not effective on this fungi. The growth of *Alternaria alternata* and the inhibition zone are given in Figure 7.

Economic consideration of the problem is of importance and some basic calculations should be made. The amount of oregano essential oil will be measured as 24 kg, if a thousand sheepskins weigh approximately 1200 kg and oregano oil is used at 2%. The additional cost of using oregano essential oil as fungicide is thought to be approximately 6$, if it is assumed that one coat is obtained from five skins and the price of oregano essential oil per kg is approximately 50$. This additional cost can be considered to be low for an ecologically produced leather coat. When customers, especially pregnant women are concerned, a leather coat with an additional cost of 6$ is worth purchasing compared to a potentially mutagenous and cancerous one.

CONCLUSIONS

As a result of this study, it was concluded that *Origanum minutiflorum* essential oil can be used as a new fungicide that does not give harm to nature or human health. Besides, unlike pharmaceutical antibiotics, essential oils do not create resistant strains of mutant bacteria.[23] Furthermore, it was reported that antimutagenicity of the oregano essential oil and carvacrol *in vitro* has pharmacological significance for the prevention of cancer.[24] Killing microorganisms with essential oils and aromatherapy is emphasized especially in the field of medicine. This method is expected to gain further significance in the leather industry in the course of time. It is significant for the future in the sense that it makes use of a natural product and causes less pollution, and its effectiveness does not decrease due to gradual increased resistance of microorganisms against many commercial biocides.

In addition to providing the pickled pelts and wet blues with antimicrobial property, other advantages of *Origanum minutiflorum* essential oil have been studied. The studies that focus on preventing Cr(VI) formation in leather and providing antimicrobial effects on the finished ones can be given as examples. The optimum antifungal effect of oregano essential oil on leathers was obtained at 2% based on ASTM D 4576/86 standards. However, the studies indicated that the commercial fungicides used at suggested amounts did not provide enough protection. At this point, it is possible to state that the fungicide manufacturers should continue working on research and development processes to provide enough protection with their products.

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