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ANTIBACTERIAL-TREATED TEXTILES WITH NATURAL ACTIVE COMPOUNDS

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In recent years, antimicrobial textiles have garnered significant attention for their potential to reduce infection transmission in medical and healthcare settings. This paper is focused on the assessment of antimicrobial activity of textile structures treated with essential oils (pine and eucalyptus essential oils) used for the undergarments of military personnel. For this purpose, three types of textile materials (polyester, cotton-polyester and cotton-elastane) were treated with a mixture of gum Arabic and pine essential oil and a mixture of gum Arabic and eucalyptus essential oil by exhaustion method using a Ugolini device. The antibacterial activity was assessed based on the standard SR EN ISO 20645/2005 Textile fabrics – Agar diffusion plate test, which revealed that the samples have antibacterial properties against *Escherichia coli* and *Staphylococcus aureus*. Cytotoxicity was evaluated by using cell cultures as models with perspectives of primary exposure to the action of the agents incorporated in the test preparations. Human cell lines that can be present in the subdermal space (wounds) were chosen, namely, fibroblasts (dermal fibroblast line ATCC PCS-201-041), human monocytes-macrophages (ATCC CRL 9855) and Jurkat T lymphocytes (ATCC TIB-152). Also, cellular viability, proliferation and cytotoxicity were evaluated by MTS and flow cytometry. It has been revealed that cell viability was not significantly affected and the sample shows no cytotoxicity.

Keywords: antimicrobial, military, essential oil, cytotoxicity, cellular viability

INTRODUCTION

The interest in antimicrobial textiles has constantly increased in the last decades, especially due to their everyday use in various fields: medicine and healthcare, clothing, sportswear and footwear, upholstery and furniture, food packaging, air and water purification systems, etc. Their ability to destroy or stop the multiplication of the microbial population defines their efficacy against bacteria, viruses, and fungi (Tanasa *et al*., 2023). Antimicrobial textiles play an important role in military clothing as many infections occur in hot and humid climates, while other conditions such as eczema are frequent in dry climates. There is a long history of skin-related medical conditions affecting the military population. It appears that approximately 27% of dermatological diagnoses registered in theatres of military operations are related to bacterial or fungal infection (Arcidiacono and Spitz, 2016). Various plant oils and extracts, such as pine, eucalyptus, rosemary and others, can naturally inhibit bacterial growth. Various plant extracts and essential oils can inhibit unpleasant sweat odours, reduce bacterial growth, possess antioxidant properties, and even serve as skin-lightening agents (Mikucioniene *et al*., 2024). Pine (*Pinaceae*) is one of the world's most significant sources of essential oils, containing over 50 constituents, with around ten being particularly important.

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The primary components of pine essential oil include pinene, camphene, carene, sabinene, terpinolene, myrcene, α-terpineol, caryophyllene, limonene, bornyl acetate, p-cymene, phellandrene, γ-terpinene, germacrene D, and spathulenol. According to Bhalla *et al*. (2013), pine essential oil enhances the activity of white blood cells, which are crucial for eliminating microbes from the body (Bhalla *et al.*, 2013). Terpenoids, which are the primary components of pine essential oil, possess antimicrobial, antiallergic, antifungal, antiviral, antispasmodic, and anti-inflammatory properties. These properties are beneficial in the prevention and treatment of numerous diseases, including cancer (Gheorghita *et al*., 2022). Eucalyptus (*Eucalyptus* spp.), a plant native to Australia, is primarily cultivated for its fast-growing wood and essential oil, which is used for various purposes. The essential oil, extracted from buds, leaves, bark, and fruits, possesses antiseptic, antibacterial, anti-inflammatory, antioxidant, and anticancer properties. Consequently, it is recommended for treating respiratory diseases such as flu, colds, and sinus congestion (Vecchio *et al*., 2016). The composition of eucalyptus oil varies with geographical location and seasons, which in turn affects its biological activity. The primary compounds of the essential oil, depending on the species, include eucalyptol, p-cymene, neo-isoeugenol, limonene, and spathulenol (Gheorghita *et al*., 2022).

MATERIALS AND METHODS

Three textile structures (P1-PES, P2-BBC/PES and P3-BBC/Elastane) were treated with 2 types of essential oils: pine essential oil (PG) and eucalyptus essential oil (EG) of 0.002% concentration and 1% gum Arabic by exhaustion method using an Ugolini device (drum 8L) with hydro module 1:10 (500 mL float). The treatment started from a temperature of 20°C, which was increased to 40°C with a gradient of 2°C/min. The samples were kept at this temperature for 30 min. The textile materials were washed for 30 min at a temperature of 30°C with Kemapon PC/LF solution. Afterwards, they were rinsed twice with hot water (30°C) and once with cold water (20°C) and dried at room temperature. Two treatment solutions with essential oils (pine essential oil and eucalyptus essential oil) of 0.002% concentration were obtained. Initially, a solution of 1% gum Arabic was made, in which the corresponding amount of essential oil dissolved in ethyl alcohol was added dropwise. The 3 samples of textile materials were treated by exhaustion with the 2 solutions obtained, for 30 min at a temperature of 40°C. After finishing the treatment, the samples were dried at room temperature for 24 hours.

The antibacterial activity was evaluated based on the standard SR EN ISO 20645/2005 Textile fabrics – Agar diffusion plate test. ISO 20645:2004 is applicable to testing finishes of hydrophilic, air-permeable materials or antibacterial products incorporated in the fibre. A minimum diffusion of the antibacterial treatment into the test agar is necessary with this procedure. In this procedure, textile samples are tested placed between two agar layers in Petri dishes. The lower layer corresponds to medium free of bacteria and the upper layer incorporates each of the two obligate bacteria a gram-positive bacterium *Staphylococcus aureus* and a gram-negative bacterium *Escherichia coli*. The nutrient media used was Nutrient Agar (NA) provided by Sanimed. After one day of incubation inhibition zone around sample is measured and according to its value sample is assessed as having good, moderate or insufficient antibacterial effect. The volume of agar was prepared for the bottom layer without bacteria, after which (10 ±0.1) ml was introduced into each sterilized Petri dish and the agar was allowed to solidify. The amount of gelatin for the upper layer was prepared and cooled to 45°C in a water bath. 150 ml of agar was seeded with 1 ml of bacterial working solution (1-5 x 10 8 cfu/ml). The container was vigorously shaken to distribute the bacteria evenly. (5±0.1) ml were introduced into each petri dish and the agar was allowed to solidify. The samples

were placed on the surface of the nutrient medium and then incubated at 37°C between 18h and 24h.

The evaluation is based on the absence or presence of bacterial growth in the contact area between the agar and the sample and on the appearance of a possible inhibition zone around the samples.

The width of the inhibition zone, i.e. the zone without bacteria near the edge of the sample, is calculated according to the following formula:

$$H= (D-d)/2 \quad (1)$$

H = inhibition zone, in millimeters

D = the total diameter of the specimen and the inhibition zone, in millimeters

d = diameter of the specimen, in millimeters.

Cell reactions were evaluated by using cell cultures as models with perspectives of primary exposure to the action of the agents incorporated in the test preparations. For this purpose, human cell lines that can be present in the subdermal space (wounds) were chosen, namely, fibroblasts (dermal fibroblast line ATCC PCS-201-041), human monocytes-macrophages (ATCC CRL 9855) and the Jurkat T lymphocytes (ATCC TIB-152). This test presents a higher degree of severity compared to the Skin Irritation test which is applied to dermal cells (healthy dermis). The use of these lines is by the regulations ISO 10993 5 2009, and with the regulations ISO 10993 (1, 6, 12,) and SR EN ISO/IEC 17025:2018.

Samples were prepared in culture medium (DMEM) not supplemented with serum, through an extraction/solubilization procedure from the collection surface, as follows:

- sample fragments (1 cm^2) were weighed on an analytical balance, in a 3 mL Eppendorf tube; a negative control was also used - support material without compounds;
- 2 mL of culture medium was added over each sample;
- samples were exposed to 3 repeated cycles, consisting of: 3 cycles of ultrasonication, frequency 40 kHz, maximum intensity, duration 30 minutes, on a Sonorex Digital 10P device; 3 vortexing cycles, at maximum intensity, 3000 RPM, on a VELP ZX4 vortex homogenizer.

The operation was repeated at 24-hour intervals, during which the samples were kept at room temperature in the extraction medium. Before exposing the preparations, an additional cycle of vortexing was performed.

The extracts were administered as such, in the amount of 30 µL/well. Cell cultures were inoculated into 96-well plates at an initial density of 10,000 cells/well and initially grown for 48 hours until the culture was 80% confluent. After this level was reached, the medium was removed and replenished with medium according to the cell type. 50 µL of the sample were administered in triplicate in each well, then incubated for 48 hours (incubator with 5% CO_2, 37^OC, humidity 90%). After incubation, the serum was collected and serum-free DMEM medium (180 microL) and MTS reagent (3 - (4,5 – Dimethylthiazol – 2 - yl) – 5 - (3 - carboxymethoxyphenyl) – 2 - (4-sulfophenyl) - 2H-tetrazolium) 20 µL were added. They were incubated for 3 hours as above, after which the optical density was measured in a microplate reader at a wavelength of 490 nm against a representative DMEM medium blank.

The cellular cytotoxicity stage can be approached in several ways to evaluate cellular viability, proliferation, cytotoxicity or cell lysis. To achieve this goal we used MTS assay and by flow cytometry technique, the evaluation of secondary necrosis (which appears at the end of the apoptosis phenomenon).

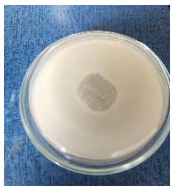
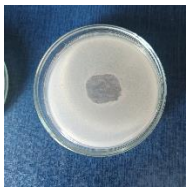
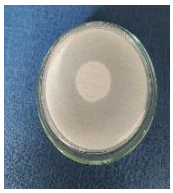
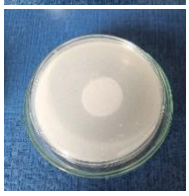
For the two assays, we used the cell line HUVEC (Primary Umbilical Vein Endothelial Cells; Normal, Human (PCS-100-010, ATCC, USA) cultivated in complete DMEM medium (10% fetal bovine serum, 1% penicillin/streptomycin solution and 1% l -glutamine) and incubated at 37°C, in an atmosphere supplemented with 5% CO_2. To carry out the tests, the

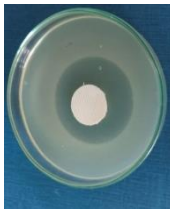
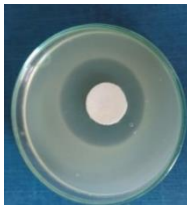
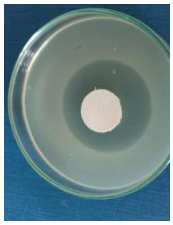
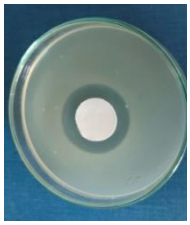
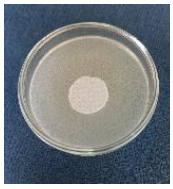
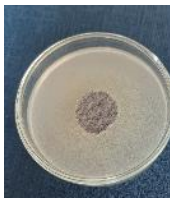
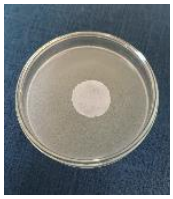
cells were multiplied and then placed in 6-well plates, at a concentration of 0.5 x 106/ml; after adhesion (24h), test agents were added. For MTS, the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, USA) was used. The CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, USA) was used for MTS. For flow cytometry analysis, the FACScan II cytometer (Becton Dickinson, USA) was used, using the apoptosis kit (BD Pharmingen™ Apoptosis Detection Kit - Becton Dickinson, USA) and analyzing the data with DIVa 6.2 software (Becton Dickinson, USA).

RESULTS AND DISCUSSIONS

The antimicrobial activity of treated textile materials has been tested according to SR EN ISO 20645/2005 – Determination of antibacterial activity – Agar diffusion plate test (Table 1), as mentioned above on Escherichia coli ATCC10536 (gram-negative) and Staphylococcus aureus ATCC 6538 (gram-positive). According to the obtained results, treated textile structures have higher antimicrobial activity (satisfactory effect) compared to the untreated samples. Cotton-based samples present better antimicrobial activity compared to polyester samples, due to cotton's better absorption (up to 27 times its weight). Although all samples showed good results, those treated with pine essential oil present a higher inhibition zone. The absence of multiplication – even without the inhibition zone – can be considered as a positive effect because the formation of such an inhibition zone can be prevented by a small diffusion of the active substance.

Table 1. Antibacterial efficiency of the treated textile materials according to SR EN ISO 20645/2005

Sample	Inhibition zone (mm) *Escherichia coli*	Inhibition zone (mm) *Staphylococcus aureus*	Description *Escherichia coli*	Description *Staphylococcus aureus*	Evaluation	
					E. coli	*S. aureus*
Untreated PES			Contamination	Contamination	Insufficient effect	Insufficient effect
Untreated CO/PES			Contamination	Contamination	Insufficient effect	Insufficient effect
Untreated CO			Contamination	Contamination	Insufficient effect	Insufficient effect

Sample	Inhibition zone (mm) *Escherichia coli*	Inhibition zone (mm) *Staphylococcus aureus*	Description *Escherichia coli*	Description *Staphylococcus aureus*	Evaluation	
					E. coli	*S. aureus*
PGP1	H= 14 (mm) 	H= 15 (mm) 	Inhibition zone > 1 mm, no multiplication	Inhibition zone > 1 mm, no multiplication	Satisfactory effect	Satisfactory effect
PGP2	H= 17 (mm) 	H= 18 (mm) 	Inhibition zone > 1 mm, no multiplication	Inhibition zone > 1 mm, no multiplication	Satisfactory effect	Satisfactory effect
PGP3	H= 12 (mm) 	H= 10 (mm) 	Inhibition zone > 1 mm, no multiplication	Inhibition zone > 1 mm, no multiplication	Satisfactory effect	Satisfactory effect
EGP1	No zone 	No zone 	No inhibition zone, no multiplication[c]	No inhibition zone, no multiplication[c]	Satisfactory effect	Satisfactory effect
EGP2	No zone 	No zone 	No inhibition zone, no multiplication[c]	No inhibition zone, no multiplication[c]	Satisfactory effect	Satisfactory effect
EGP3	No zone 	No zone 	No inhibition zone, no multiplication	No inhibition zone, no multiplication	Satisfactory effect	Satisfactory effect

c=Absence of multiplication - even without the inhibition zone – can be considered a positive effect because the formation of such an inhibition zone can be prevented by a small diffusion of the active substance

Viability tests were carried out, by applying the samples to the mentioned cultures, performing 3 sets of determinations for each sample. The results are presented in Fig. 1.

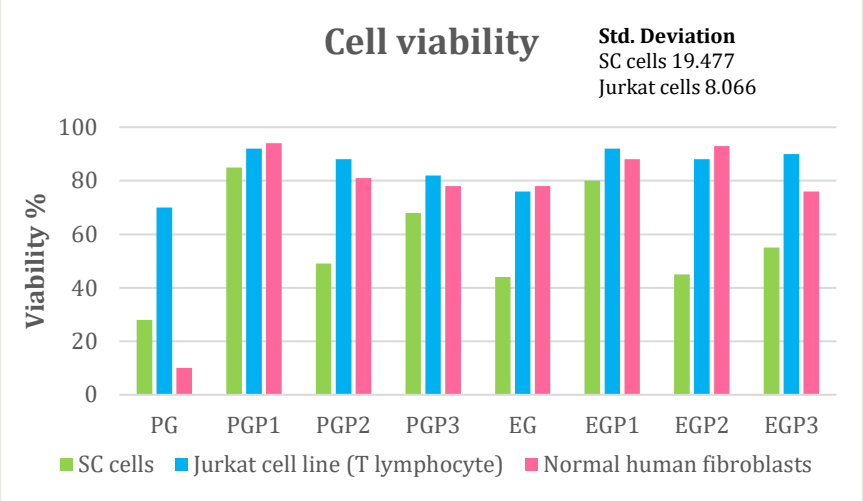


Figure 1. Cell viability – SC cells, Jurkat cell line, human fibroblasts

Cultures exposed to control sample PG showed a significant level of cytotoxicity (viability 28%), also control sample EG showed a level of viability of 43%. It should be noted that the viabilities recorded for the treated samples were systematically above the level of the controls, with the most remarkable effects for PGP1, and EGP1. In the case of the Jurkat line, a relative flattening of the results is observed, even if the control pieces showed higher cytotoxicity (viability 70, and 76% respectively), while the treated pieces resulted in viabilities between 88 and 92%, with the same maximum effects for PGP1, and EGP1.

For human fibroblast line ATCC PCS 201-041A, high cytotoxicity can be observed (only 10% viability for the PG control), and 48% viability for EG. Meanwhile, the treated samples showed low cytotoxicities, with viabilities ranging from 76-94%, again with the best results for PGP1 and EGP1, polyester samples. It should be noted that 100% polyester textile samples have the highest viability when tested on all three cell types.

For MTS and flow cytometry assays the cell line HUVEC (Primary Umbilical Vein Endothelial Cells; Normal, Human (PCS-100-010, ATCC, USA)) was used, cultivated in complete DMEM medium (10% fetal bovine serum, 1% penicillin/streptomycin solution and 1% l-glutamine) and incubated at 37°C, in an atmosphere supplemented with 5% CO_2.

In order to carry out the tests, the cells were multiplied and then arranged in 6-well plates, at a concentration of 0,5 x 10^6/ml; after adhesion (24h) the test agents were added. For MTS, the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, USA) was used.

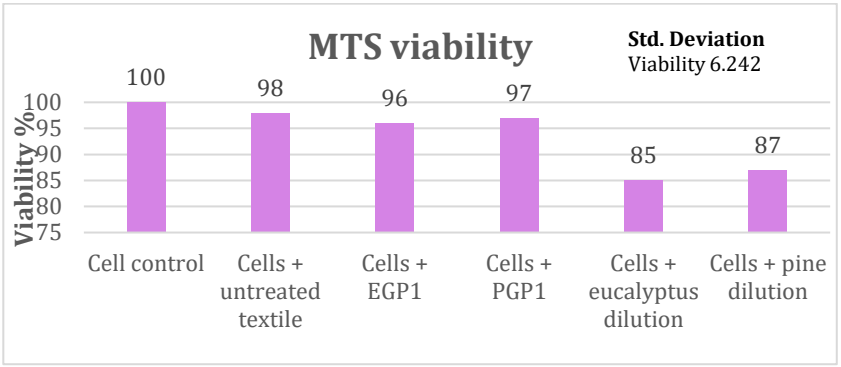


Figure 2. Determination of cell viability in the case of HUVEC cells treated with various possibly cytotoxic agents, using the MTS viability test

Fig. 2 presents the cell viability results obtained by MTS assay for the untreated textile support, the 100% polyester textile support treated with pine essential oil (PGP1) and the 100% polyester textile support treated with eucalyptus essential oil (EPG1). From the data analysis, it can be observed that cell viability was not significantly affected compared to the cell control (untreated cells).

For flow cytometry analysis, the FACScan II cytometer (Becton Dickinson, USA) was used, together with the apoptosis kit (BD Pharmingen™ Apoptosis Detection Kit – Becton Dickinson, USA) the data were analysed with DIVa 6.2 software (Becton Dickinson, USA).

Fig. 3 presents the results of cell viability and cell necrosis for the untreated textile support, the textile support of 100% polyester treated with essential pine oil (PGP1) and the textile support of 100% polyester treated with eucalyptus essential oil (EGP1).

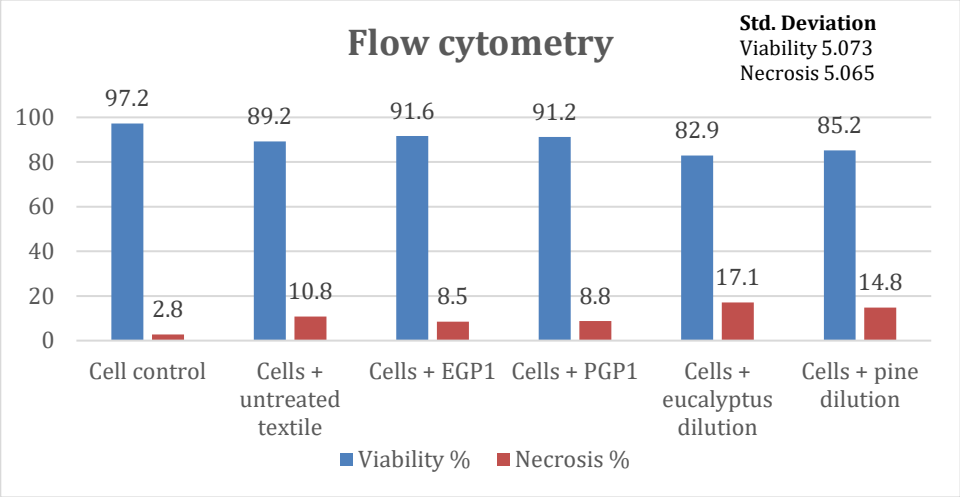


Figure 3. Determination of viability and necrosis in HUVEC cells using propidium iodide labelling of cells treated with possible toxic agents

From the data analysis, it can be observed that the level of necrotic cells does not vary significantly between the textile structure of 100% polyester treated with pine essential oil (PGP1) and the textile structure of 100% polyester treated with eucalyptus essential oil (8.8% and 8.5%) and does not show significant cytotoxicity. Necrotic cells level is very close to the control (untreated textile sample) – 10.8%.

CONCLUSIONS

The evaluation of antibacterial resistance according to SR EN ISO 20645/2005 standard demonstrated the effectiveness of treatments with essential oils. The results are considered "satisfactory" since no bacterial growth was observed beneath the samples. The *in vitro* testing of textile structures treated with pine (P) and eucalyptus (E) essential oils aimed to evaluate cell reactions using cell cultures (human cell lines that may be present in the subdermal space) as models with perspectives of primary exposure to the action of the agents incorporated in the test preparations. The use of these lines was in accordance with the regulations ISO 10993 5 2009, and with the regulations ISO 10993 (1, 6, 12,) and SR EN ISO/IEC 17025:2018. The experimental results showed that the treated textile supports present a biocompatibility between the limits of "moderately cytotoxic" to "practically non-cytotoxic". Considering that the main application is to ensure the isolation of possible open superficial lesions from pathogenic agents (especially pathogenic bacteria), it can be considered that especially the

products of 100% PES treated with pine and eucalyptus oils can be developed and applied as topical products. Also, by analyzing the data obtained for cell viability, by performing the MTS test, it was found that it was not significantly affected by the samples treated with essential oils. From the analysis of the results obtained by the LDH evaluation, it has been concluded that the level of necrotic cells does not vary significantly between the textile structure of 100% polyester treated with pine essential oil (PGP1) and the textile structure of 100% polyester treated with eucalyptus essential oil and does not show significant cytotoxicity. Necrotic cells level is very close to the control (untreated textile sample).

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