Eucalyptus-Enhanced Cotton: Pre-Treatment and Bioactive Coatings Strategies for the Development of Sustainable Textiles with Antimicrobial and Antioxidant Activities for Skin Applications

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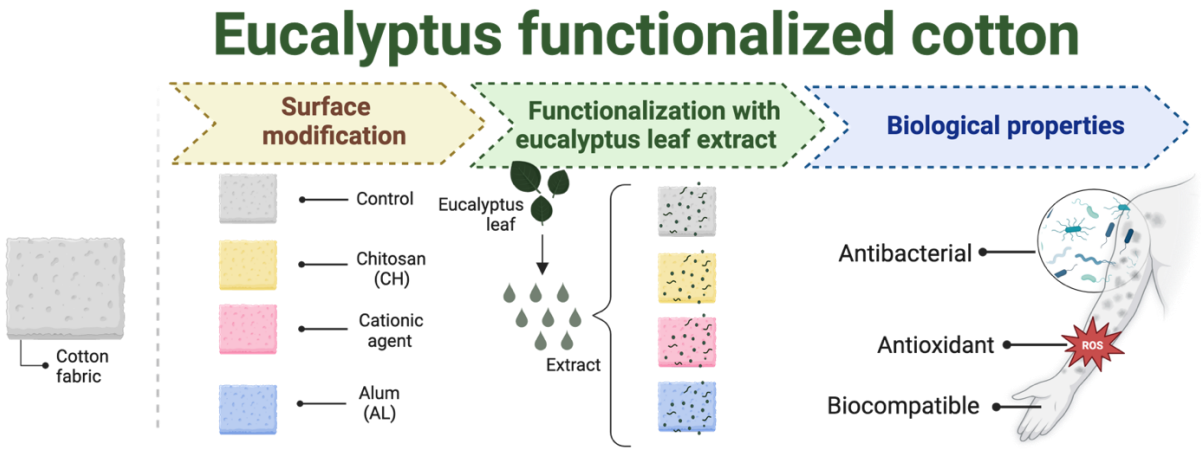
Graphical abstract



Keywords: bioactive textiles, bio-based coatings, plant-based textiles, antibacterial cotton, skin applications

Abstract

Eucalyptus essential oils and extracts are widely recognized for their antimicrobial, antioxidant, insecticidal, anti-inflammatory, and aromatizing properties, making them highly valuable across pharmaceuticals, cosmetics, and textiles. For the design of biomedical textiles, cotton is favored for its mechanical strength, porosity, and biodegradability, but its vulnerability to microbial action limits its applications. To address this issue, natural compounds like eucalyptus essential oils and extracts have been prioritized over synthetic agents to enhance its antimicrobial properties. While bioactive textiles using essential oils have been well-documented, the incorporation of eucalyptus leaf extracts with different surface modifications of cotton remains largely unexplored. In this line, this study investigated pre-treatment approaches to improve the uptake and uniformity of eucalyptus extract on cotton fibers. To achieve this, chitosan (CH), a cationic agent, and alum were applied to the cotton fabric to promote stronger electrostatic interactions and improve the binding of extract's bioactive components. The functionalized fabrics were tested for antimicrobial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Escherichia coli*, antioxidant properties, and cytotoxicity using human keratinocytes. Eucalyptus-functionalized cotton, particularly with CH pre-treatment, exhibited strong antibacterial activity against Gram-positive bacteria and enhanced antioxidant capacity in the DPPH assay. No cytotoxicity was detected with 8 hours of exposure, but potential effects was observed after 24 hours, indicating the need for further evaluation of long-term safety. These findings highlight the potential of eucalyptus-functionalized textiles for personalized clothing aimed at managing skin conditions linked to microbiota dysregulation, emphasizing the need for optimized functionalization and biocompatibility evaluation.

1. Introduction

Eucalyptus is a diverse genus with over 900 species that belong to the Myrtaceae family [1]. Worldwide eucalyptus species hold significant importance for their timber, pulpwood, and essential oils. Studies have shown that leaf/bark extracts and essential oils of eucalyptus, which are rich in volatile oils, exhibit a wide range of biological activities, including antimicrobial, insecticidal/insect repellent, antioxidant and anti-inflammatory properties [2-5]. These biological attributes make eucalyptus a valuable source for several commercial applications, namely pharmaceutics, cosmetics, textiles and the food industry [1, 2, 6, 7]. Despite their valuable properties and widespread utilization, vast quantities of eucalyptus leaves are wasted annually. These leaves are typically left to decompose on forest floors or discarded as by-products by timber and wood manufacturers, overlooking their significant potential for diverse applications that could support a circular economy.

The antimicrobial effects of eucalyptus are primarily attributed to bioactive compounds such as eucalyptol (1,8-cineole), a bicyclic monoterpene, along with other constituents [8]. These compounds are secondary metabolites produced by aromatic and medicinal plants, mainly derived from terpenoids, particularly monoterpenes. They encompass a diverse range of hydrocarbons and their oxygenated derivatives, including phenols, alcohols, ethers, esters,

aldehydes, peroxides, and ketones, which collectively contribute to the plant's antimicrobial properties. The antimicrobial mechanism of these volatile compounds involves disrupting microbial cell membrane integrity and inducing oxidative stress, thereby inhibiting the growth of several pathogenic microorganisms [8-10]. They interact with the lipid bilayer of bacterial membranes, altering its structure and increasing permeability. This disruption leads to leakage of intracellular components, including ions, nucleotides, and proteins, ultimately destabilizing and compromising bacterial viability.

Additionally, the polyphenols present in eucalyptus extracts exhibit strong antioxidant properties, playing a vital role in neutralizing free radicals and mitigating oxidative damage [2]. This antioxidant activity not only enhances the therapeutic potential of eucalyptus-derived compounds but also contributes to product stability by extending shelf life and reducing degradation caused by oxidative stress [11].

For these reasons, textile materials incorporating eucalyptus oils and extracts represent an emerging field in the development of bioactive textiles. These textiles leverage the natural properties of eucalyptus, offering various applications across different domains such as biomedical textiles, sportswear, home furnishings, and personal care products. In general, these products are designed to inhibit microbial growth, reduce odor in both human and textile, promote skin health and well-being [1, 9, 12, 13]. Additionally, the refreshing aroma of eucalyptus provides an extra sensory dimension, adding special value to these bioactive textiles by reducing stress, promoting relaxation, and aiding in better sleep. Furthermore, the use of eucalyptus supports the growing consumer demand for multifunctional, sustainable, and eco-friendly textile products [2, 6].

Among fabrics, cotton is the most used textile material for biomedical purposes, including healthcare staff clothing and accessories, bedding, therapeutic clothing, and wound dressings [6, 14-16]. Cotton exhibits very good mechanical properties, high porosity, permeability, water absorptivity, hydrophilicity, biodegradability, biocompatibility, and it is also easy to modify [16]. However, cotton provides an ideal surface for microbial attachment, growth, colonization and dissemination, representing a potential risk to human health and limiting its use in many biomedical applications [17].

To overcome these disadvantages, several approaches have been proposed for improving the antimicrobial properties of cotton-based materials for direct skin applications. However, many of these strategies rely on synthetic antimicrobial agents, namely silver, zinc, copper, titanium, quaternary ammonium compounds (QACs), and triclosan, which are incorporated through chemical coatings, nano/microparticles, plasma treatment, grafting [9, 18-20]. While these synthetic agents can provide strong antimicrobial effects, their widespread use raises environmental and health concerns, including potential toxicity, bioaccumulation, and antimicrobial resistance.

As a more sustainable and safer alternative, bio-based coatings have been explored for the development of bioactive cotton with natural antimicrobial agents, such as chitosan, nanocellulose, essential oils, and plant extracts [14, 17, 21-24]. These natural compounds not only offer broad-spectrum antimicrobial and anti-odor properties but also minimize the risk of adverse effects associated with synthetic agents. Additionally, bio-based approaches align with the growing demand for eco-friendly and biocompatible textile materials, ensuring both effectiveness and sustainability for textile functionalization.

These approaches have emerged as an easy and effective strategy for the design of bioactive textile materials with various biological proprieties, including antimicrobial, antioxidant, UV protection, and so on [24]. For instance, the literature highlights the benefits of surface modification on cotton-based materials, such as low cost, high homogeneity, and good affinity [14]. This strategy can be used to enhance the biological properties of those natural components in textile materials through simple modification processes.

Although some works have successfully developed bioactive textiles with antimicrobial properties, most efforts have been centered on incorporating essential oils, including those derived from eucalyptus [3-5, 25]. In contrast, only a few studies have explored the integration of eucalyptus leaf extracts into textile materials [1, 2, 13]. The bio-based coatings using natural components, such as essential oils and plant extracts, requires the establishment of strong chemical interactions with textile fibers to ensure these components remain securely bound and do not dissipate easily upon application [26]. However, research in this area remains limited and poorly documented, highlighting the need for further investigation to optimize functionalization strategies that enhance the uptake and uniform distribution of eucalyptus extract on cotton fibers.

Cellulosic fibers, namely cotton, acquire a negative surface charge in aqueous media due to the ionization of hydroxyl groups, inhibiting their interaction with natural extracts and dyes. In this study, chitosan was utilized as a pre-treatment because of its cationic properties, which make it an effective textile additive and finishing agent for enhancing color and functional durability, as is widely reported [27, 28]. Additionally, the commercial cationic agent and alum were applied to confer positive surface charges to the fibers, promoting the electrostatic interaction and coordination complex formation of between the natural extracts rich in phenolic hydroxyl groups and the fibers [29-31].

In this line, the current study aimed to explore the performance of different pre-treatment strategies, including chitosan, a commercial cationic agent, and alum (mainly potassium alum), prior to functionalizing the cotton textile with eucalyptus leaf extracts.

To evaluate the impact of these pre-treatments on the eucalyptus-functionalized cotton, antimicrobial assays were conducted against skin pathogenic bacteria (*Staphylococcus aureus* and *Escherichia coli*) and a common human skin commensal (*Staphylococcus epidermidis*). Additionally, the antioxidant properties of the eucalyptus-functionalized cotton were evaluated, and its cytotoxicity was assessed in contact with human skin cells. Cytotoxicity testing was performed using keratinocyte monolayers, as these cells represent the outermost layer of the skin and are the first to interact with textile materials. Both indirect and semi-direct exposure models were employed to ensure a comprehensive assessment.

2. Materials and Methods

2.1 Preparation of eucalyptus leaf extracts

An ultrasound assisted extraction (UAE) protocol was applied to ground eucalyptus leaves (≤1 mm), using a hydroethanol mixture (50:50) as extraction solvent. To ensure a 3% (*m/v*) extraction, 400 mL of solvent were added to 12 g of plant residue. The mixture was subjected

to ultrasound treatment, using a Hielscher Ultrasonics GmbH UIP1000hdT device (Teltow, Germany), equipped with a titanium Hielscher sonotrode BS4d22 (frontal area: 3.8 cm^2). The system operated at 20 KHz, with a rated power of 1000 W, and was programmed to work at an amplitude of 60 µm for the duration required ensure an energy density of 6.6 kJ/L, after which the probe automatically stops. Subsequently, the mixture was placed with stirring on a heating plate at 50 °C for 30 minutes. Afterward, it was centrifuged at 9000 rpm for 10 minutes, using a Thermo Scientific™ Sorvall™ LYNX 6000 centrifuge (Waltham, MA, USA), and filtered through a 5 µm - 13 µm filter to separate the extract from the extracted residue. Extracts were stored at -60 °C and sheltered from the light.

2.2 Fabric surface modification and functionalization of cotton with eucalyptus extracts

The functionalization with eucalyptus extract was performed on 100% cotton knitted fabrics, characterized by a thickness of 0.494 mm and a grammage of 164 g/m². The cotton fabrics were initially washed at 60 °C for 60 minutes in a domestic laundry machine, using a non-ionic detergent (1 g/kg of textile) to remove possible contaminants introduced during the manufacturing process. Three different pre-treatments of the cotton fabrics were explored to improve the adherence of the eucalyptus extract to the fabric. For this, different pre-treatment agents were applied to confer a positive charge to the textile surface, namely two natural agents, potassium alum (Sameca PQ, Portugal) and chitosan (Primex, Canada) and a synthetic one, a commercial cationic agent.

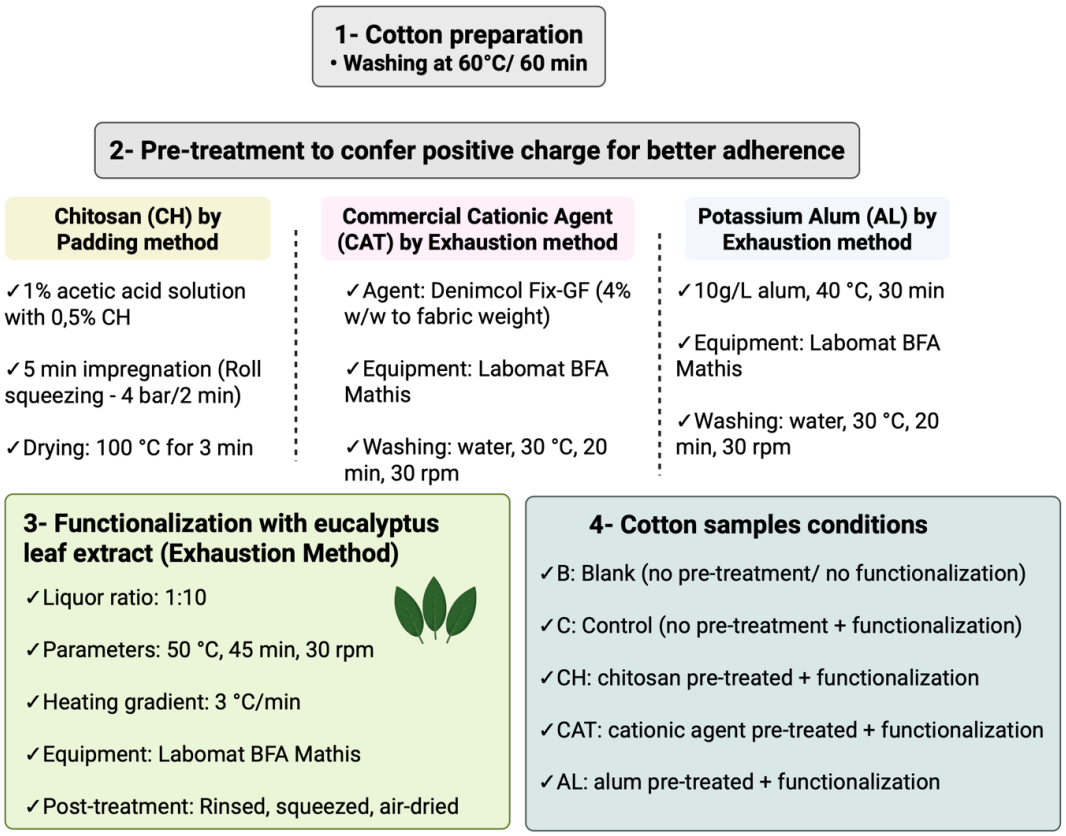
The pre-treatment of the cotton fabrics with alum and the commercial cationic agent was performed using the exhaust method with a Labomat BFA Mathis (Mathis AG, Germany). The commercial cationic agent, Denimcol Fix-GF (CHT Group, Germany), commercialized to improve the fixation of anionic dyes by adding positive charges onto cellulosic fibers, was applied according to the manufacturer´s recommendations. Briefly, the agent was applied at a 4% (*w/w*) to the fabric weight in a 1:50 bath (*w/v*) at pH 9, for 20 minutes at 50 °C. The pre-treatment with potassium alum was also carried out by exhaustion using a solution with a concentration of 10 g/L of alum, in a 1:50 bath (*w/v*), for 30 minutes at 40 °C. After the pre-treatment, the fabrics were washed with water at 30 °C for 20 minutes at 30 rpm in a Labomat BFA Mathis (Mathis AG, Germany).

The pre-treatment with chitosan was applied by padding using a Foulard Mathis HVF 27291 (Mathis AG, Germany). This process consisted of impregnating the fabric for 5 minutes in a 1% (*v/v*) acetic acid solution containing 0.5% (*w/v*) chitosan. After that, the fabrics were squeezed through the rolls, with a pressure of 4 bar and a speed of 2 m/min. Finally, the fabric was dried for 3 minutes at 100 °C and cured for 5 minutes at 150 °C, to enhance the binding efficiency to the cotton fibers [32, 33].

The functionalization of the pre-treated cotton fabrics with eucalyptus extracts was performed by exhaustion in a Labomat BFA Mathis (Mathis AG, Germany). A liquor ratio of 1:10 of the eucalyptus leaf extract solution was applied, with the temperature raised to 50 °C at a heating gradient of 3 °C/min, for 45 minutes at 30 rpm. Additionally, a cotton sample without pre-treatment (control) was functionalized according to the previously mentioned conditions. To ensure correlation of results, the same batch of eucalyptus leaf extract was used for all functionalization conditions. The functionalized samples were rinsed with water, squeezed, and

left to air-dry at room temperature (Scheme 1). This step was carried out to preserve properties of the hydroethanolic extract, which contains volatile bioactive compounds susceptible to degradation or evaporation at elevated temperatures. Furthermore, since the textiles are usually stored and used under ambient conditions, drying at room temperature more accurately reflects their real-world usage environment.

The different conditions of textile treatments were identified as follows: B -Blank textile (cotton without pre-treatment and without functionalization), C – Control textile (cotton without pre-treatment and functionalized with eucalyptus leaf extract; CH - cotton pre-treated with chitosan and functionalized with eucalyptus leaf extract; CAT – cotton pre-treated with the commercial cationic agent and functionalized with eucalyptus leaf extract; AL – cotton pre-treated with alum and functionalized with eucalyptus leaf extract. Afterwards, to conduct the assays and prevent contamination, all the textiles were cut into squares (± 1.5 cm in length), round-shaped (± 3 cm diameter) and sterilized using UV light for 20 minutes on each side.



Scheme 1: Procedure for the preparation of cotton pre-treated and functionalized with eucalyptus extract.

2.3 Antimicrobial assays

2.3.1 Bacterial strains

Suspensions of *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (DSH 20044), and *Escherichia coli* (ATCC 8739) were prepared in Tryptic Soy Broth (TSB, Biokar Diagnostics). These suspensions were incubated at 37 °C for 24 hours. A sterile loop was used to extract a small sample from each bacterial suspension, which was then streaked onto Tryptic Soy Agar (TSA, Biokar Diagnostics). The streaked plates were incubated under optimal conditions until visible colonies formed. Subsequently, a single isolated colony from each plate was selected and inoculated into TSB, followed by incubation at 37 °C for approximately 24 hours. Fresh inoculum for each bacterial strain was prepared from the previous inoculum for all assays. The bacterial concentration, i.e., colony forming unity (CFU)/mL of each inoculum was determined during the mid-log growth phase using densitometry and CFU counting to ensure accurate bacterial concentrations for the antimicrobial assays.

2.3.2 Antimicrobial qualitative assay of the bioactive cotton samples

The antibacterial testing procedure adhered to the guidelines of ISO 20645:2004, with slight modifications. Briefly, 1 mL of each bacterial inoculum (concentration approximately 8 log CFU/mL) was added to a flask containing 150 mL of TSA heated to 45 °C. To cultivate a bacterial lawn, 5 mL of the bacterial suspension was evenly spread over 10 mL of pre-solidified TSA. After 10-15 minutes, three textile pieces per condition were aseptically placed on the agar surface using sterile tweezers. The agar plates were then incubated at 37 °C for 2 24 hours. After 24 hours, bacterial growth around and beneath the textile samples was assessed according to ISO 20645:2004 standards. To ensure reproducibility and accuracy, each condition was tested in triplicate. After removing the textile samples, the plates were further incubated for an additional 24 hours without any textile presence to assess residual antimicrobial effects.

2.3.3 Antimicrobial quantitative assay of the bioactive cotton samples

To evaluate bacterial adhesion to the cotton samples, we followed the methodology previously described [34] with some modifications. Briefly, the procedure described above for the antimicrobial qualitative assays was used. After 24 hours of incubation, each textile sample was retrieved from the agar plate and immersed in 2 mL of sterile Ringer solution (Biokar Diagnostics). The samples were then vortexed five times for approximately 5 seconds to detach the bacterial cells from the textiles. The resulting supernatant was serially diluted up to 10^{-5} and plated onto TSA. After an additional 24 hours of incubation at 37 °C, the colonies were counted, and the bacterial count was expressed as log CFU/ mL. This process allowed for the quantification of viable bacteria that had adhered to the textile materials.

2.4 Antioxidant evaluation of the cotton samples

The antioxidant activity of the functionalized cotton samples with eucalyptus extracts was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays. Samples of each textile, approximately 1 mg in weight, were cut and placed into 2 mL Eppendorf tubes. Subsequently, 2 mL of DPPH solution in methanol and 2 mL of ABTS solution were added to the respective Eppendorf tubes.

For the DPPH assay, the samples were incubated for 30 minutes, while for the ABTS assay, the incubation time was 6 minutes. After incubation, the absorbance of the samples was measured using a spectrophotometer at wavelengths of 517 nm for DPPH and 734 nm for ABTS. Both assays were conducted at room temperature and in the dark. All analyses were performed in triplicate. The radical scavenging activities were expressed as percentage of inhibition and calculated according to the following equations:

% DPPH inhibition = [(A0-AS/A0] ×100/mass of textile sample (1)

% ABTS inhibition = [(A0-AS)/A0] ×100/mass of textile sample (2)

where A0 is the absorbance of DPPH solution and AS is the absorbance of textile sample.

2.5 Skin cell line and cell culture conditions

The immortalized human keratinocyte cell line (HaCaT) was obtained from Cell Lines Service (Oppenheim, Denmark). Upon thawing, the cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose), which was supplemented with 10% Fetal Bovine Serum (FBS, BioWest) and 1% Penicillin-Streptomycin-Fungizone solution (Pen-Strep, Lonza). The keratinocyte cell line was maintained in T75 flasks at 37 °C in a humidified atmosphere containing 5% CO_2 for the entire duration of the experiments.

2.6 Cytotoxicity evaluation: indirect and semidirect methods

Indirect Method: To assess cytotoxicity using an indirect method, we followed the modified methodology described by Singh et al. [35]. Briefly, textile samples were cut into round discs (approximately 3 cm in diameter) and sterilized using UV light for 20 minutes on each side. To prepare the leachate, all the cut and sterilized textile samples were then incubated with 3 mL of DMEM (containing high glucose, FBS, and Pen-Strep) at 37 °C in a humidified atmosphere with 5% CO_2 for 24 hours. After incubation, the textile samples were removed, and the leachate medium was collected for falcons of 15 mL. Simultaneously, HaCaT were seeded onto a 96-well plate at a density of 1×10^5 cells/mL and the cells were allowed to attach and grow overnight at 37 °C in a humidified atmosphere with 5% CO_2. Following the 24-hour incubation, the growth medium from the HaCaT was discarded, and 100 µL of each leachate solution was added to each well. The plates with leachate solutions were then incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 8 and 24 hours.

Semi-direct Method: To assess cytotoxicity using a semi-direct method, HaCaT were seeded into a 24-well plate at a density of 1×10^5 cells per well and allowed to adhere overnight at 37 °C in a humidified atmosphere with 5% CO_2. After 24 hours, the growth medium was removed and replaced with fresh medium. Subsequently, a transwell insert (BRAND) containing a membrane with 1.2 cm diameter and pore sizes of 8 µm was placed into each well. A textile sample of 1 cm^2 was placed inside each transwell insert above the cell's monolayers, ensuring no direct contact with the cells. A positive control, consisting of cells in culture

medium without exposure to the cotton fabric, was also included. The plates were then incubated for 8 and 24 hours at 37 °C in a humidified atmosphere with 5% CO_2.
After 8 and 24 hours of incubation for both indirect and semi-direct methods, the metabolic activity of HaCaT was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Following incubation, the leachate solution, textile samples, inserts, and culture medium were removed. Fresh DMEM containing MTT solution (5 mg/mL) was added, and the cells were incubated for 3–4 hours at 37 °C in a CO_2 incubator. Subsequently, the medium was discarded, and formazan crystals were solubilized by adding 100 µL of DMSO to each well of the 96-well plates and 500 µL to the 24-well plates. The plates were gently agitated for 10 minutes at room temperature to ensure complete dissolution. Absorbance was then measured at 570 nm using a microplate reader (Synergy 4, Biotek).
Cell viability was calculated as a percentage of the control. Each condition was tested in triplicate in three independent experiments for both the indirect and semi-direct methods.
Using the same experimental approach described for the cytotoxicity assay, a Live/dead was conducted to evaluate the viability of keratinocytes following semi-direct exposure to the cotton samples. Keratinocytes were then seeded and exposed to the cotton samples under the same conditions as previously mentioned. After 24 hours of incubation, the cell monolayers were gently washed with PBS and subsequently stained with 10 µl of Calcein AM (1 µM solution) and 1 µl of propidium iodide (PI, 50 µg/mL) in 1000 µl of PBS. The staining was performed at 37°C in a 5% CO2 humidified atmosphere for 15 minutes. Following incubation, cells were washed with PBS, maintained in PBS, and immediately analyzed by fluorescence microscopy (Axio Imager 2, Zeiss) to visualize viable (green) and dead (red) cells.

2.7 Scanning electron microscope (SEM)

To evaluate bacterial presence on the various bioactive cotton samples, qualitative antimicrobial assays were conducted following the same methodology outlined in section 2.3.2 (Antimicrobial Qualitative Assay). After 24 hours of incubation with the bacterial strains, the cotton samples were carefully retrieved from TSA plates. Subsequently, the samples were fixed using 2.5% glutaraldehyde and subjected to a dehydration process via a graded ethanol series (10% to 100%), with each step lasting 10 minutes. To ensure the complete drying of the samples, they were left to dry overnight. For analysis, the textile samples were mounted on observation pins using double-sided adhesive carbon tape (NEM tape, Nisshin, Japan). The samples were then sputter-coated with a 9 nm - 12 nm layer of gold/palladium (Polaron, Germany) and examined using a Phenom Pro G6 SEM (Thermo Fisher Scientific, Eindhoven, Netherlands) at 5 kV with the Backscattered Electron Detector (BSD).

2.8 Statistical analysis

Data were analyzed using the GraphPad Prism 10 software and are presented as means ± standard deviation (SD). For quantitative assessments of antimicrobial activity, antioxidant capacity, and cytotoxicity, multiple comparison tests were performed using one-way or two-way ANOVA. Statistical significance was defined as follows: $^{*,\#} = p < 0.05$, $^{**,\#\#} = p < 0.01$, $^{***,\#\#\#} = p < 0.001$, $^{****,\#\#\#\#} = p < 0.0001$. Asterisks (*) indicate statistically significant

differences between blank cotton and other samples conditions, while hashtags (#) indicate differences between untreated cotton (control) and pre-treated/functionalized cotton samples.

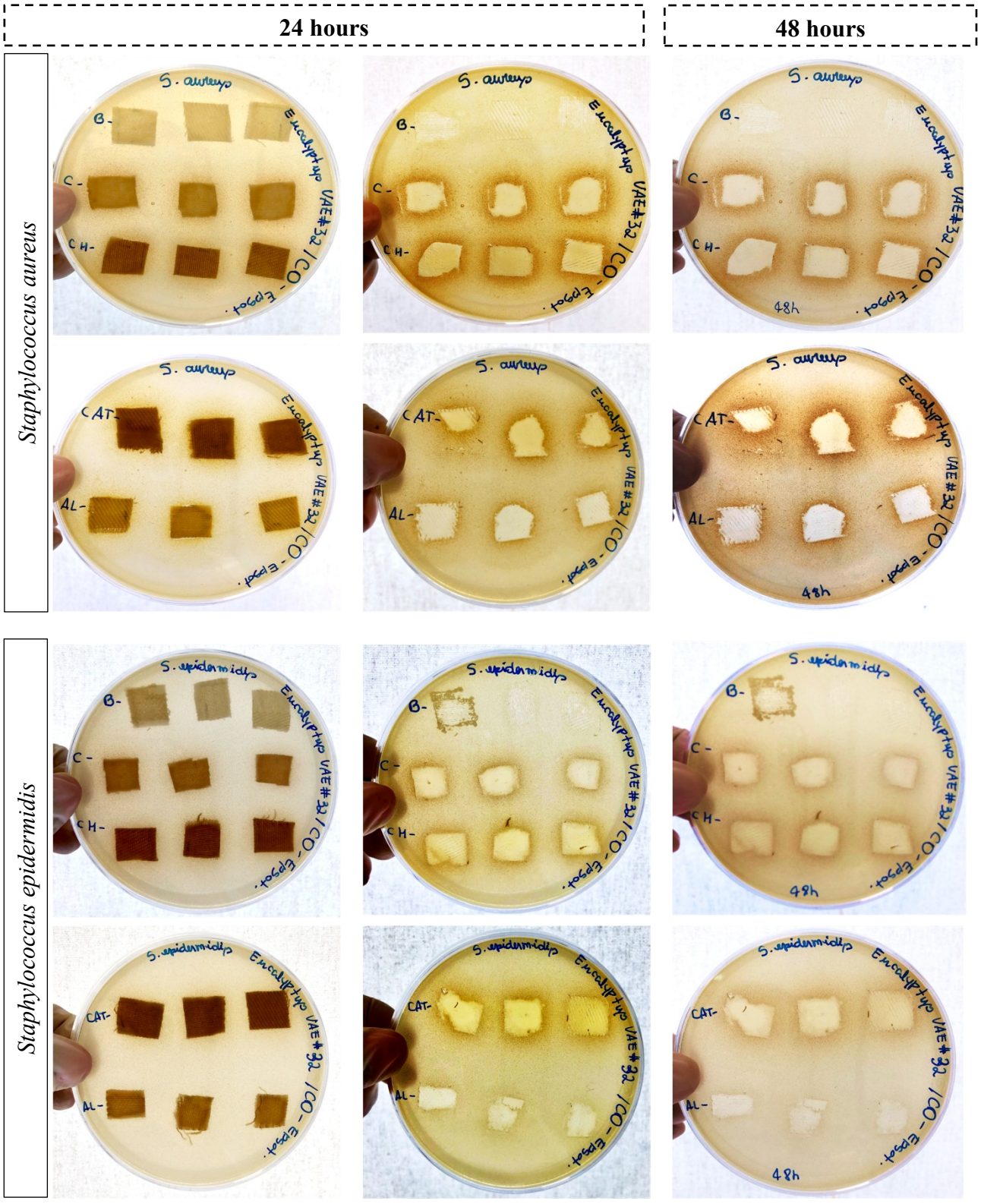
3. Results and Discussion

3.1 Evaluation of the antibacterial activity of cotton fabrics functionalized with eucalyptus leaf extracts

To evaluate the antimicrobial activity of the cotton fabrics functionalized with eucalyptus leaf extract, the ISO 20645:2004 standard was followed. The test microorganisms were selected according to the standard guidelines and included pathogenic bacteria commonly found on human skin, namely *S. aureus* and *E. coli*, respectively Gram-positive and Gram-negative bacteria. Additionally, *S. epidermidis*, a non-pathogenic Gram-positive bacterium and a key component of the human skin microbiota, was also included.

TSA plates were inoculated with the bacteria and tested with cotton fabrics either untreated or functionalized with eucalyptus extracts containing different pre-treatments. After 24 hours of contact, the samples were assessed for antimicrobial activity by examining the presence of an inhibition zone around the textile sample and the absence of bacterial growth under the textile samples (Figure 1).

Before the removal of the textile samples, all tested bacteria exhibited uniform growth with no signs of inhibition zone. This was evidenced by the absence of clear zones and any significant color changes on the TSA plates for all textile samples labeled B, C, CH, CAT, and AL. However, after the textile samples were removed, visible bacterial growth was observed under the untreated textiles (B - blank cotton samples) for both Gram-positive bacteria, indicating that these textile samples exhibited no antibacterial effect (Figure 1). In contrast, the textiles functionalized with eucalyptus extracts labeled as C, CH, CAT, and AL showed a noticeable absence of bacterial growth, particularly against Gram-positive bacteria, with the most pronounced effect observed in *S. aureus*. This suggests strong antibacterial activity of the eucalyptus-treated fabrics, especially against *S. aureus*. Nonetheless, after 24 hours of exposure, no antibacterial effect was observed in *E. coli* for any of the textile samples (B, C, CH, CAT, and AL). This suggests that cotton fabrics functionalized with eucalyptus extracts do not exhibit antibacterial activity against this Gram-negative bacterium, even when pre-treated with CH, CAT, or AL before functionalization (Figure 1).


24 hours
48 hours
Staphylococcus aureus
Staphylococcus epidermidis
S. aureus
S. epidermidis
B-
C-
CH-
CAT-
AL-
Eucalyptus VAE#32/CO-Epox.
48h

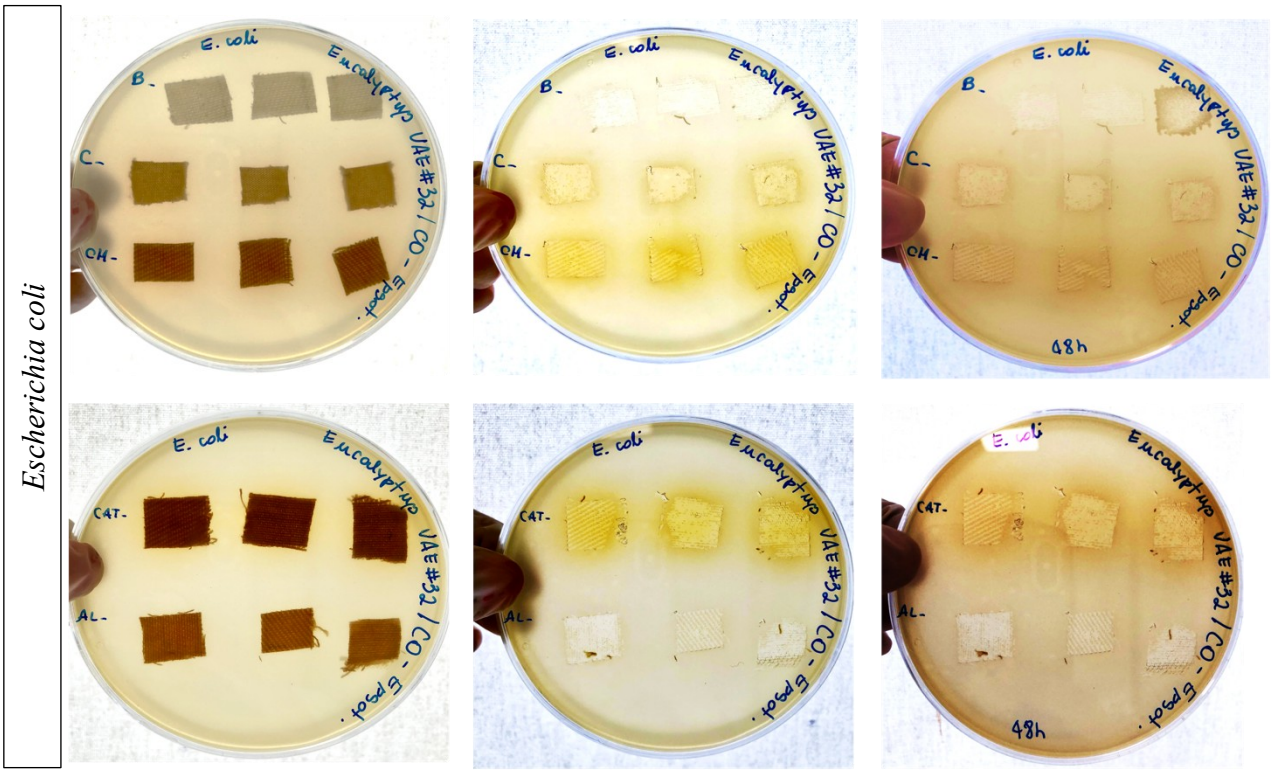


Figure 1. Results of the qualitative antimicrobial assay performed in *S. aureus*, *S. epidermidis* and *E. coli*. TSA plates containing bacteria inoculum and eucalyptus functionalized cotton fabrics after 24 hours of contact are shown. Plates on the left (before the removal of the textile samples) show no inhibition zone around all the textile samples. Plates at the center show the absence of bacterial growth under the textile samples. Plates on the right were incubated for more 24 hours in the absence of textile specimen. The textiles samples are classified as **B** – blank cotton (cotton without any pre-treatment and without functionalization; **C**- cotton control (cotton without any pre-treatment and with functionalization with eucalyptus extract); **CH** – cotton pre-treated with chitosan and functionalized with eucalyptus extract; **CAT** – cotton pre-treated with commercial cationic agent and functionalized with eucalyptus extract; **AL** – cotton pre-treated with alum and functionalized with eucalyptus extract.

After 48 hours of incubation, TSA plates with an additional incubation of 24 hours without textile samples, the antibacterial effect against both Gram-positive bacteria remained strong in cotton samples functionalized with eucalyptus leaf extracts (C, CH, CAT, and AL) as evidenced by the absence of bacterial growth in the textile zones (Figure 1). In contrast, the untreated cotton sample (B) showed noticeable signs of bacterial growth. For *E. coli*, visual growth was observed on all cotton textile samples, demonstrating the same lack of antibacterial effect as seen after 24 hours (Figure 1).

To further validate these results, the attached and viable bacteria were quantified on various cotton fabrics - both non-functionalized and functionalized with eucalyptus leaf extracts - using the methodology described by Ivankovic et al. 2022, and visualized the bacterial adherence through SEM. This additional evaluation was crucial, as interpreting the qualitative antimicrobial test according to ISO 20645:2004, can be subjective in some cases. For this reason, the agar diffusion analysis was investigated to better understand the interaction between bacteria and the different cotton fabrics, both with and without eucalyptus leaf extract functionalization, as well as in the absence and presence of textile surface modification. This

investigation involved counting the viable cells that remained attached to the textiles after 24 hours of incubation on TSA plates inoculated with bacteria.
In line with the previous qualitative evaluation, the quantitative analysis further highlighted the greater sensitivity of Gram-positive bacteria to cotton functionalized with eucalyptus leaf extracts compared to Gram-negative bacteria (Figure 2). A significant reduction in the number of viable and attached Gram-positive bacteria was observed in the presence of the functionalized cotton (C - control, CH, CAT, and AL), particularly when compared to their respective blank textile samples (B). Notably, for *S. aureus*, the combination of surface modification with CH and functionalization with eucalyptus leaf extract significantly increased the antimicrobial effect, resulting in the lowest bacterial counts on the textile samples. For *S. epidermidis*, a substantial reduction in viable bacteria was also observed when eucalyptus functionalization was applied, both with and without surface modification, with no significant difference between those conditions. In contrast, *E. coli* showed no reduction in viable bacteria when exposed to cotton functionalized with eucalyptus leaf extract, compared to the untreated textile. This indicates that the functionalized cotton, even with eucalyptus leaf extracts, does not have any antibacterial effect on *E. coli*, regardless of surface modification (Figure 2).

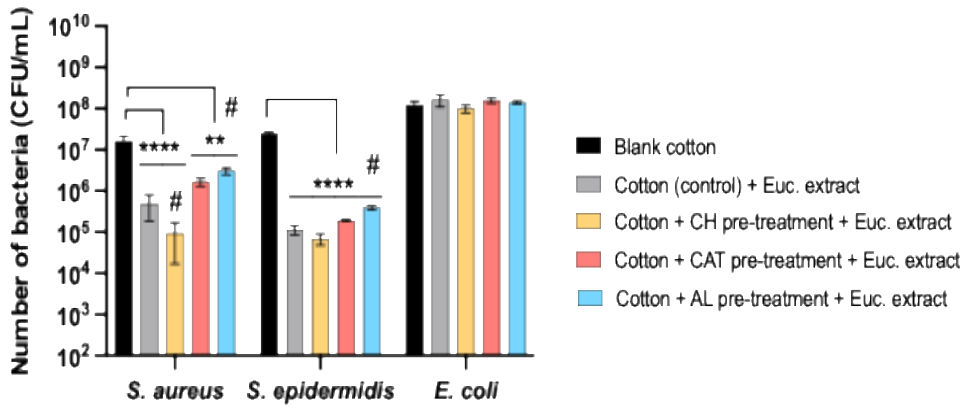


Figure 2. Determination of the number of viable and attached bacteria on textile samples in study. The number of viable S. *aureus*, *S. epidermidis*, and *E. coli* adsorbed onto the textile specimens after 24 hours of contact in the agar diffusion test are represented. Data are reported as the mean of three textile sample/ condition. Statistically significant differences: * indicates the differences compared to the Blank cotton used as control, while # indicates statistical difference in pre-treated cotton samples compared to untreated cotton (control).

To further validate and understand these findings, the presence of different bacterial species was assessed on the various cotton samples using SEM analysis (Figure 3). The SEM images revealed a high number of bacteria adhered to untreated (blank) cotton, which lacked any surface pre-treatment or functionalization with eucalyptus extract. Furthermore, the bacteria appear to exhibit a greater tendency to aggregate on cotton textiles. These results confirm that both Gram-positive and Gram-negative bacteria strongly adhere to cotton fabrics without eucalyptus extract. In fact, cotton fabrics, composed of cellulose polymers, inherently facilitate bacterial adherence and are more susceptible to microbial attack in the absence of plant extracts [36]. This can be attributed to the more hydrophilic characteristics and low surface energy of the

substrate, provided by the hydroxyl groups on its surface, as well as low contact angle and low surface energy of the bacteria, as reported by [37-39]. However, a visible reduction in the number of adherent Gram-positive bacteria was observed on all cotton samples functionalized with eucalyptus extract compared to blank cotton. This reduction was even more pronounced for cotton pre-treated with chitosan, consistent with the results of the antimicrobial quantitative assay. On the other hand, no differences were observed for Gram-negative bacteria between blank cotton and cotton with eucalyptus extract, independently of the pre-treatments surface. Interestingly, this result demonstrates the capacity of *E. coli* to adhere and grow in the cotton fibers even in the presence of eucalyptus extracts. The images revealed the formation of a bacterial layer on the textile. No bacterial aggregates were observed, indicating that this type of bacteria exhibits a stronger affinity for the tested textiles.

Taking all these findings into account, it is evident that cotton fabrics functionalized with eucalyptus leaf extract exhibited significant antibacterial activity against *S. aureus* and *S. epidermidis*. These two Gram-positive bacteria exhibited a significant reduction in antibacterial activity across qualitative (absence of bacterial growth in the fabric), quantitative (decreased viable bacterial counts), and SEM analyses in all cotton samples functionalized with eucalyptus leaf extracts. Interestingly, the antibacterial effect was more pronounced in both Gram-positive bacteria, particularly when the fabric was pre-treated with chitosan prior to functionalization, also confirmed by SEM analysis. This outcome may be attributed to the synergy between chitosan, an antimicrobial agent, and the bioactive compounds present in eucalyptus extracts, especially phenolic compounds and terpenoids known for their antibacterial properties [4, 14, 40].

The strong antibacterial effect against *S. aureus* aligns with previous studies that have demonstrated the efficacy of plant extracts, including eucalyptus extracts, as antimicrobial coatings for textiles [1, 6, 12, 36]. Although some studies have also demonstrated the susceptibility of *S. epidermidis* to plant extracts, such as eucalyptus, the studies involving textile functionalization with plant-based antimicrobial agents focus on targeting pathogenic skin Gram-positive bacteria, particularly *S. aureus* [1, 6, 12, 36]. Therefore, there is no information about the effect of such materials on skin commensal bacteria, namely *S. epidermidis*.

In contrast, the functionalized cotton fabrics with eucalyptus leaf extracts did not present antibacterial effect against *E. coli*. This result is consistent with previous studies that have shown Gram-negative bacteria often exhibit greater resistance to plant-based antimicrobials. This is attributed to their unique cell wall architecture, particularly the presence of an outer membrane rich in lipopolysaccharides, which acts as a barrier to the penetration of hydrophobic compounds, such as those found in eucalyptus extract, namely α-terpineol and para-isoproplyphenol [10, 41, 42]. The absence of antibacterial activity against *E. coli* across all tested samples suggests that the concentration of bioactive compounds with antibacterial properties in eucalyptus extract incorporated into cotton may be suboptimal, insufficient to penetrate the bacterial outer membrane, or ineffective by intrinsic resistance mechanisms. These may include the action of efflux pumps (e.g., AcrAB-TolC), which actively expel antimicrobial agents from the cell, as well as the presence of enzymes and porin channel modifications that further restrict compound uptake [43].

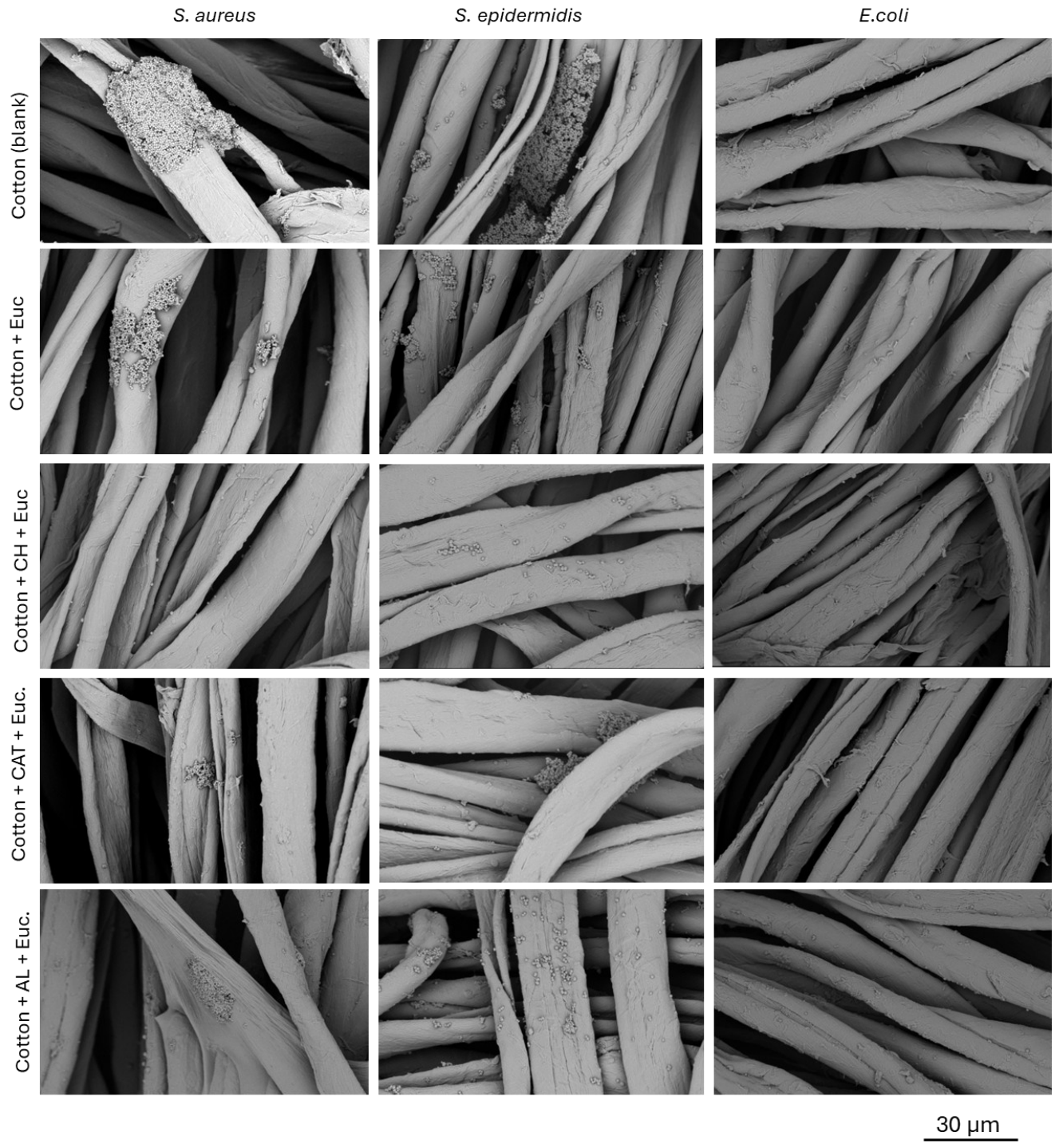


Figure 3. SEM images of non-functionalized cotton and cotton functionalized with eucalyptus leaf extract after 24 hours of contact with *S. aureus*, *S. epidermidis*, and *E. coli*. The images illustrate the presence and density of each bacterial species on the different cotton samples. The images highlight variations in bacterial adhesion and interaction based on the functionalization and surface pre-treatment. Magnification of the images: 5000x.

This outcome is in line with the findings of previous studies where fabrics coated with eucalyptus extracts did not exhibit antibacterial activity against *E. coli* [1, 36]. Nonetheless, the findings presented here highlight the potential application of eucalyptus leaf extract as an effective antimicrobial treatment for cotton fabrics, particularly against Gram-positive bacteria

commonly associated with skin infections and hospital-acquired infections. The pronounced antibacterial activity observed against Gram-positive bacteria, especially *S. aureus*, suggests that eucalyptus-coated cotton could hold promise for clinical applications, such as therapeutic textile designed to target skin microbiota dysregulation conditions caused by these bacteria, or in the development of personal hygiene products [9, 44] . In contrast, the lack of antibacterial activity against *E. coli* raises important considerations regarding the broad-spectrum effectiveness of these bioactive textiles. In this line, future research should explore synergistic combinations of eucalyptus extract with potent antimicrobial agents effective against Gram-negative bacteria or investigating strategies to enhance the permeability of Gram-negative outer membrane – such as the use of permeabilizing agents or nanocarrier systems - to improve the antibacterial activity of eucalyptus-derived compounds.

In this line, a notable synergistic approach demonstrated that a CVC fabric treated with a colloidal zinc solution combined with plant extracts, namely carrot and orange peel, achieved a 99.17% reduction rate in *E. coli* [45] . The accumulation of zinc ions disrupts the bacterial cell wall integrity and facilitates the penetration of bioactive compounds from the plant extracts, enhancing their antimicrobial effectiveness. Another promising strategy involves incorporating eucalyptus extracts into metallic nanoparticles (NPs). Numerous studies have explored the synthesis of NPs from plant extracts using metals (silver and gold) and metallic oxides (iron, zinc, and zirconium) to develop multifunctional materials for biomedical applications, including wound healing [19, 46, 47]. These studies highlight that due to the nanometric size and increased surface area, these NPs can effectively disrupt the bacterial membrane of Gram-negative bacteria, facilitating penetration and inducing intracellular damage. Their antibacterial activity is attributed to multiple mechanisms, including oxidative stress induction, the release of metal ions (e.g., Ag^{+}), which can inactivate essential biological components (DNA, and proteins), and non-oxidative pathways [48]. Consequently, incorporating eucalyptus extracts into NPs for bioactive textile coatings could synergistically enhance their antimicrobial efficacy, providing a promising strategy to target Gram-negative bacterial associated to skin infections and to advance textile-based healthcare applications.

3.2 Evaluation of the antioxidant properties of cotton fabrics functionalized with Eucalyptus leaf extracts

To evaluate the antioxidant potential of cotton functionalized with eucalyptus leaf extract, *in vitro* assays such as DPPH (which primarily assesses hydrophobic antioxidant activity) and ABTS (which detect hydrophilic antioxidant activity) were performed. Notably, all cotton samples treated with eucalyptus leaf extract exhibited antioxidant activity, even without any pre-treatment (control), as compared to the untreated cotton (blank cotton without any pre-treatment and no functionalization with eucalyptus extracts) (Figure 3).

All samples of functionalized cotton with eucalyptus extracts exhibited significant DPPH inhibition per milligram of textile ($p < 0.001$). Among them, cotton pre-treated with CH demonstrated the highest antioxidant activity compared to the untreated control (blank cotton). Furthermore, among the different pre-treatments, only CH showed a statistically significant

difference compared to the cotton control (cotton functionalized with eucalyptus extract with no pre-treatment) ($p < 0.01$). Conversely, the ABTS scavenging assay revealed that the cotton (control) had the highest antioxidant potential compared to the pre-treated cotton with CH, CAT, and AL (Figure 3).

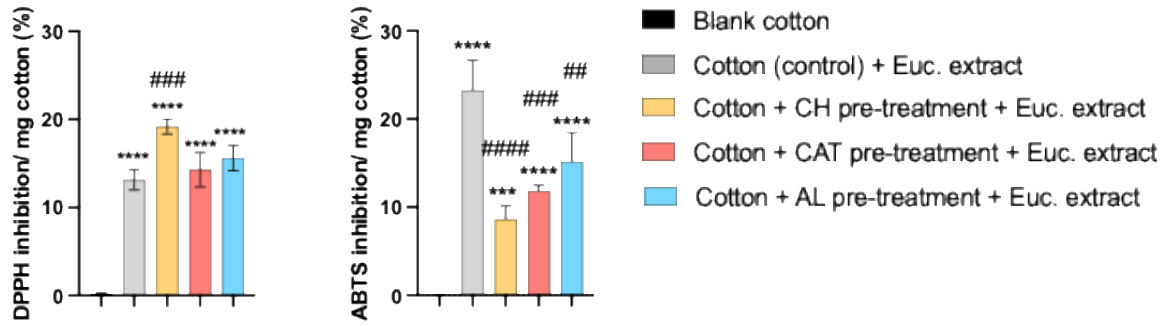


Figure 3. Determination of the antioxidant activity of the cotton functionalized with extracts of eucalyptus leaf. Percentage of DPPH and ABTS inhibition per mg of textile sample is shown. * Indicates statistical difference compared to blank cotton, and # indicates statistical difference of pre-treated cotton samples compared to the untreated cotton (control).

The DPPH and ABTS scavenging assays are well-established methods for evaluating antioxidant capacity, and the outcomes presented can be correlated with known properties of eucalyptus extracts, which are rich in polyphenols and flavonoids, compounds widely recognized for their antioxidant activities [7, 49].

The significant DPPH inhibition across all functionalized cotton samples is consistent with previous research demonstrating the potent antioxidant properties of eucalyptus leaf extracts [49]. For instance, Park et al. [50] reported that eucalyptus leaves contain high levels of phenolic compounds, which are effective in neutralizing free radicals, as seen in the DPPH assay. This suggests that the incorporation of eucalyptus extracts into cotton fabric successfully imparts these antioxidant properties, even without additional pre-treatments. However, the superior antioxidant activity of CH-pre-treated cotton in the DPPH assay could be attributed to the potential enhancement of extract absorption or binding facilitated by chitosan. In this line, a similar study showed that chitosan can synergistically enhance the antioxidant activity of functionalized textiles by increasing the surface area available for interaction with antioxidant agents present in the plant extracts [51].

Interestingly, the ABTS assay results, where the control cotton (cotton functionalized with no pre-treatment) exhibited higher antioxidant potential than pre-treated samples, suggest that certain pre-treatments and mordants may alter the accessibility or reactivity of antioxidant compounds in eucalyptus extract with ABTS radicals. This could be explained by the findings of Prior et al. [52], who noted that different antioxidants might interact differently with DPPH and ABTS radicals depending on their molecular structure and the matrix in which they are

embedded. Pre-treatments like CH, CAT, and AL might have modified the properties of the cotton surface, potentially reducing the availability of active sites for ABTS radical scavenging. Overall, this study demonstrates that eucalyptus leaf extract effectively enhances the antioxidant capacity of cotton fabrics, with CH pre-treatment showing the most significant improvement in DPPH scavenging activity. In contrast, the variability observed in the ABTS assay underscores the complexity of antioxidant interactions and the critical influence of textile surface modifications. These findings highlight the pivotal role of fabric pre-treatment in modulating the bioactive of functionalized textiles. Specifically, surface modifications, such as CH application, appear to facilitate stronger binding between the cotton fibers and the bioactive compounds in eucalyptus extract, potentially leading to more stable and long-lasting functionalization. Nonetheless, further investigations are necessary to confirm the durability and long-term performance of the functionalization under real-world conditions.

3.3 Biocompatibility

Keratinocytes, the predominant cell type in the human epidermis, play a critical role in maintaining the skin's barrier function and protecting the body from external threats, including environmental factors and pathogenic microorganisms [53]. Given that keratinocytes are the primary cells in direct contact with textile materials, we utilized a 2D keratinocyte model to evaluate the biocompatibility of the different non-functionalized and functionalized cotton samples with eucalyptus extracts.

To assess the biocompatibility of the cotton samples under study, we employed the MTT assay, a well-established method for evaluating potential cytotoxicity of biomedical devices, including textile materials, using both indirect and semi-direct methods (Figure 4).

Firstly, keratinocytes were exposed to leachate solutions from the cotton samples for 8 and 24 hours. The 8-hour exposure period simulates a prolonged textile usage scenario, such as during sleep, which typically takes between 7 to 9 hours. The 24-hour exposure period mimics extended usage conditions, such as those encountered with hospital bedding. This duration is sufficient to provide meaningful data without being confounded by other potential skin reactions, such as irritation, infection, or unpleasant odors, which might develop with even longer exposure times.

After 8 hours of incubation with the leachates, a slight decrease in keratinocyte viability was observed compared to cells maintained only with complete media and without cotton fabric, which served as the positive control for cell growth (Figure 4A). This decrease was observed in keratinocytes in the presence of both blank cotton (without eucalyptus extracts) and cotton with eucalyptus leaf extracts. However, for cells exposed to leachates from cotton pre-treated with CH, CAT, and AL and functionalized with eucalyptus leaf extract, a significant reduction in cell viability was noted, though this reduction is not considered cytotoxic. In contrast, after 24 hours of incubation with the leachates, a significant decrease in cell viability was observed across all cotton samples, including the untreated (blank) cotton. However, this effect was most pronounced in keratinocytes exposed to leachates from cotton samples functionalized with eucalyptus leaf extracts, with cell viability dropping below 20%. These results suggest a

potential cytotoxicity effect of eucalyptus extract-functionalized cotton leachates on human skin cells during prolonged exposure. Here, the observed cytotoxicity may be attributed to the high concentration of bioactive compounds released from the functionalized cotton samples during the leachate preparation process, i.e., 24 hours. Consequently, prolonged exposure to these leachates negatively impacts the viability of keratinocytes.

To better understand the real impact of the different cotton samples and to closely mimic the interaction between human skin and textiles, we evaluated the cell viability of keratinocytes in contact with the different cotton samples using a semi-direct method (Figure 4B). Unlike direct methods - where biomedical devices, including textile materials, may trap, damage, or detach cells in a 2D monolayer, leading to unrealistic data - semi-direct methods provide a more reliable alternative to direct methods [54]. In fact, the semi-direct method with cell culture monolayer incubated *in situ* in the presence of transwells containing biomedical devices, prevents the material from causing physical damage to cells and provides more accurate insights into cell-material interactions [54].

In this sense, after 8 hours of *in situ* incubation with transwells (Figure 4B), it was observed a slight decrease in keratinocyte viability in the presence of all cotton samples compared to cells maintained in complete media with no fabric (the positive control for cell growth). Interestingly, cotton fabrics pre-treated with AL and functionalized with eucalyptus extracts showed no significant reduction in cell viability. However, all other cotton samples functionalized with eucalyptus extracts, including those without any pre-treatment, and pre-treated with CH and CAT, exhibited a significant decrease in keratinocyte viability. Despite this reduction, the overall biocompatibility of the eucalyptus extract-functionalized cotton was considered good, as they maintained high cell viability (approximately >80%) with no cytotoxic effects after 8 hours of incubation.

After 24 hours of incubation, similar results were observed, with a more pronounced decrease in keratinocyte viability for all eucalyptus-functionalized cotton samples, except for cotton pre-treated with AL. These findings suggest that, after 24 hours of semi-direct contact, most eucalyptus-functionalized cotton samples exhibit moderate cytotoxic effects, reducing cell viability to approximately 50% viability. Such outcome may pose limitations for long-term applications involving prolonged skin contact. To validate these findings, a live/dead fluorescence assay was performed using the same semi-direct methodology to assess cell morphology and distinguish between viable and non-viable cells after 24 h of exposure. While brightfield images revealed comparable cell morphology across all conditions (Figure 5), fluorescence microscopy clearly demonstrated an increased proportion of red-stained (non-viable) cells, particularly in the CH-treated group. In contrast, the cotton control, CAT-treated, and AL-treated samples retained a predominantly green-stained (viable) cell population (Figure 5), supporting their comparatively lower cytotoxicity. These observations further underscore the potential cytocompatibility concerns associated with certain eucalyptus-functionalized textiles, especially in the context of long-term skin contact.

Together, these results provide important insights into the biocompatibility of both non-functionalized and eucalyptus-functionalized cotton samples, with and without different surface pre-treatments when using indirect and semi-direct methods. The slight decrease in keratinocyte viability observed after 8 and 24 hours of exposure to blank cotton using both indirect and semi-direct methods, indicates a minimal, non-cytotoxic impact of cotton on

human keratinocytes. This finding is consistent with previous studies that have reported a good interaction between untreated cotton textiles and human skin cells under short- and long-term exposure [55]. However, the more pronounced decrease in cell viability after exposure to leachates from textiles functionalized with eucalyptus leaf extract and pre-treated with CH, CAT, and AL suggests a more complex interaction. Although a significant reduction in viability was noted, it did not reach cytotoxic levels within the 8 hours timeframe. This could be indicative of a transient stress response in the keratinocytes, correlated to the high concentration of bioactive compounds in the eucalyptus leaf extract. These compounds, such as terpenes and phenolic acids, possess both antimicrobial and anti-inflammatory properties, but their effects on human cells can vary depending on the cell type, concentration, and time of exposure [56].

This may explain the significant shift observed with extended exposure, where leachates from all eucalyptus-functionalized cotton samples led to a substantial decrease in keratinocyte viability, indicating potential cytotoxic effects after 24 hours. This finding is consistent with previous research suggesting that while natural extracts, including eucalyptus, can offer antimicrobial benefits, they may also induce cytotoxicity in human cells if not properly formulated, if exposure is excessively prolonged, or when cells are subjected to leachate containing higher concentrations of the bioactive compounds [57, 58]. The use of CH, CAT, and AL as pre-treatments did not alter the release of eucalyptus extract compounds from cotton, showing similar cytotoxicity to the cotton control, i.e., fabric without pre-treatment but functionalized with eucalyptus leaf extract. In fact, the enhanced cytotoxicity in the eucalyptus-functionalized textiles after 24 hours of leachates exposure could be attributed to the higher concentration of bioactive compounds from the eucalyptus extract present in the leachates that can induce cumulative stress on the keratinocytes and reduce cell viability.

Like the indirect method, no cytotoxic effect is observed when keratinocytes are in semi-direct contact for 8 hours with eucalyptus-functionalized textiles, regardless of the pre-treated surface. However, after 24 hours of semi-direct contact, all eucalyptus-functionalized cotton samples exhibited moderate cytotoxic effects, with keratinocyte cell viability reduced to approximately 50%. This outcome can be attributed to the gradual release of bioactive components from the functionalized textiles into the surrounding medium during *in situ* interaction with keratinocytes for 24 hours. These results were made possible by the appropriate selection of the porous membrane size of 8 μm in the transwell system, which allowed the bioactive compounds released from the functionalized cotton to pass through the membrane and interact with the keratinocytes monolayer. This is further supported by the evaluation of cell viability using transwells with a 0.4 μm pore membrane (Supplementary Material, S1). After 24 hours of semi-direct contact, keratinocytes exhibited more than 80% cell viability for all cotton samples functionalized with eucalyptus extract. This high viability indicates that the small pore size of the transwell membrane effectively limits the diffusion of most bioactive compounds released from the eucalyptus-functionalized textiles, preventing them from reaching the surrounding medium and interacting with the cells. As a result, bioactive compounds are unable to impact keratinocyte viability.

Based on these results, the semi-direct contact test can also be a valuable approach for evaluating the cytotoxicity of bioactive materials, including textiles. However, a key limitation of this test lies in the need to appropriately match the pore size of the membrane with the size

of the leachable substances [54]. The membrane pore size must exceed the size of the leachable substances to prevent false-negative results for the cytotoxicity evaluation.

Many studies have developed bioactive textiles using plant extracts, such as eucalyptus, which exhibit antimicrobial, antioxidant, anti-UV, and biodegradable properties [1, 2, 6, 12-14, 36], however, most of these studies do not evaluate the cytotoxicity of these materials on human cells, even when designed for skin applications. Although our study demonstrates that eucalyptus-functionalized cotton fabrics are safe for direct contact with human skin for up 8 hours, the limited number of comprehensive cytotoxicity assessments in the field of bioactive textiles incorporating plant extracts underscores the urgent need for standardized methodologies addressing both short- and long- term exposures. To address this gap, extensive investigation is necessary to evaluate critical parameters, including optimal surface pre-treatments protocols by fabric, the most effective plant extraction methods, and the biological effects of eucalyptus-functionalized fabrics on human skin cells, namely keratinocytes and dermal fibroblasts - across a range of concentrations and exposure durations. These studies are essential to identify the ideal extract concentration that ensures a balance between antimicrobial and antioxidant efficacy and skin biocompatibility across different conditions.

In this line, while the bioactive compounds in eucalyptus extracts provide antimicrobial and antioxidant properties, their use at high concentrations or over prolonged exposure periods may compromise cell viability and cause skin irritation. Therefore, achieving a safe and effective balance between biological activity and biocompatibility is essential for advancing these materials toward clinical applications. Achieving this balance requires systematic dose-response studies to identify the minimum effective concentrations, careful selection of extracts based on their phytochemical composition and compatibility with human skin cells, and adherence to standardized testing protocols, such as those outlined in ISO 10993, to ensure both safety and reproducibility.

Moreover, our findings also underscore the critical importance of optimizing several experimental conditions—particularly the selection of transwell pore size in semi-direct exposure models and the duration of contact—when developing textiles designed for close and prolonged interaction with human skin. Addressing these parameters is essential for ensuring that next-generation bioactive textiles are not only functionally effective but also safe for human use. Future research should also focus on advanced delivery strategies, such as encapsulation or controlled-release systems, to mitigate initial cytotoxicity while preserving long-term bioactivity.

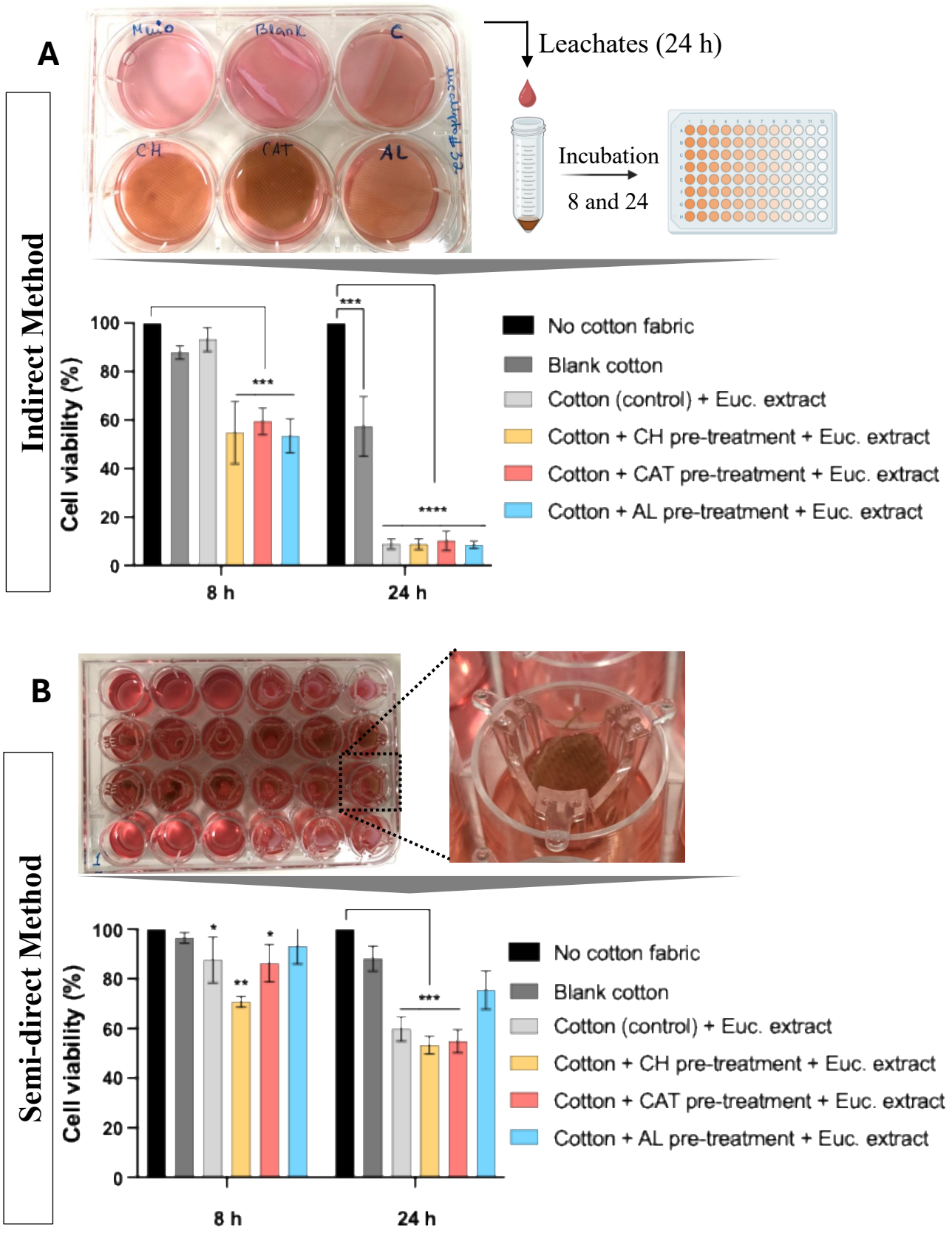


Figure 4. Cell viability of keratinocytes in the absence and presence of cotton samples (non- functionalized and functionalized with eucalyptus leaf extracts) using indirect and semi-direct methods. **A)** Schematic representation of the indirect method, where leachates were prepared from all cotton samples in a 6-well plate after 24 h of incubation. The cell viability of keratinocytes exposed to these leachates was assessed after 8 and 24 hours of incubation. **B)** Schematic representation of the semi-direct method, where cotton samples were placed in inserts

with an 8 µm pore membrane in a 24-well plate to evaluate cytotoxicity. The cell viability of keratinocytes exposed to the cotton samples was assessed after 8 and 24 hours of incubation. **No cotton fabric** - keratinocytes monolayer maintained only with media, without any cotton sample; **Blank cotton** - cotton without any pre-treatment and without functionalization; **cotton control + Euc extract** - cotton without any pre-treatment and with functionalization with eucalyptus extract; **cotton + CH pre-treatment + Euc extract** – cotton pre-treated with chitosan and functionalized with eucalyptus extract; **cotton + CAT pre-treatment + Euc extract** – cotton pre-treated with commercial cationic agent and functionalized with eucalyptus extract; **cotton + Al pre-treatment + Euc extract** - cotton pre-treated with alum and functionalized with eucalyptus extract. *Indicates statistical difference compared to keratinocytes cultured with any cotton fabric (black bars).

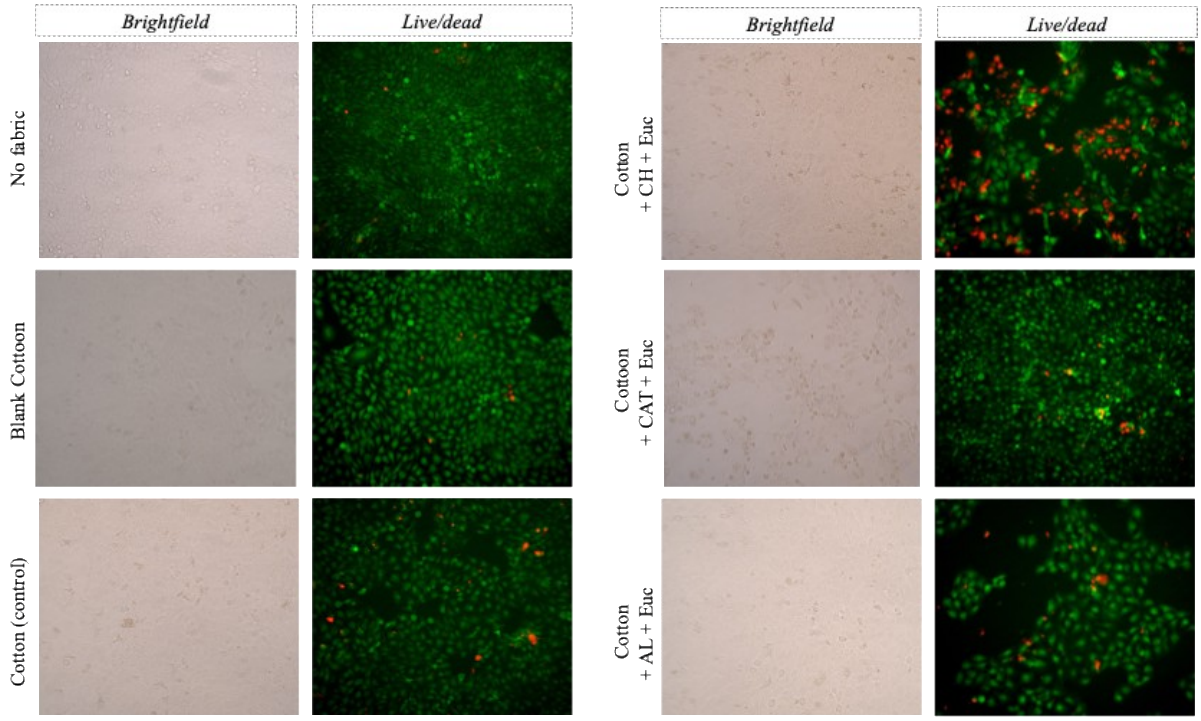


Figure 5: Representative brightfield and fluorescence from the live/dead assay of cotton fabrics functionalized with eucalyptus leaf extracts. Human keratinocytes were incubated for 24h in semi-direct contact with different samples of eucalyptus functionalized cotton fabrics. Live cells are stained green, and dead cells are stained red. Scale bar = 100 µm.

4. Conclusion

Natural extracts, such as eucalyptus, hold immense potential for the development of bioactive textiles. Nevertheless, energy, economic, and environmental considerations present significant challenges for industrial scaling up of typical solvent extraction technologies. The integration of novel technologies in industrial set ups, such as UAE, in industrial processes can substantially enhance extraction yields while reducing energy consumption and costs associated with solvent use and chemical waste disposal.

Bioactive textile materials, with antimicrobial and antioxidant properties, should balance their effects on skin pathogenic bacteria and the commensal skin microbiota members ensuring beneficial outcomes for skin health. For instance, eucalyptus-functionalized cotton exhibits

strong antibacterial activity against Gram-positive bacteria, making it suitable for personalized clothing to address patients with skin conditions linked to microbiota dysregulation, such as those caused by *S. aureus*.

Additionally, eucalyptus leaf extract enhances the antioxidant capacity of cotton fabrics, with certain pre-treatment methods, like CH application. These properties open the door to versatile applications in healthcare, cosmetics, sportswear, protective clothing, and everyday apparel, offering benefits such as wound healing, oxidative stress mitigation, and skin health promotion. However, several challenges must still be overcome before industrial scale implementation, including the durability of the functionalized textiles and their shelf life. Additionally, regulatory and certification barriers, necessary to validate the claimed properties of the textiles, must be established and approved to ensure consumer protection.

Eucalyptus-functionalized cotton has shown no cytotoxic effects up to 8 hours of exposure, but the safety of extended use remains a critical concern that requires thorough investigation. Optimizing pre-treatment strategies, such as the application of AL on the cotton surface, could reduce potential cytotoxicity and enhance the long-term safety and effectiveness of these materials. This highlights the importance of a comprehensive approach in developing bioactive textiles with biocompatible properties. For this, key factors that must be carefully considered include the type and concentration of plant extracts used, the biofunctionalization processes employed, the pre-treatment surface of fabrics, the methodologies for cytotoxicity evaluation, the specific human cell types tested, and the duration of exposure. These considerations are particularly crucial for bioactive textiles intended for prolonged and close contact with human skin.

In conclusion, future research should prioritize rigorous biocompatibility assessments using human skin cells to fully unlock the potential of bioactive textiles. Such evaluations are essential to ensure both the efficacy and safety of these materials for short- and long-term skin applications. By addressing these challenges, it will be possible to develop high-performance textiles with tailored functionalities that meet the evolving demands of both consumers and industries, while maintaining a strong commitment to skin health and safety.

Declarations

Author contributions

CSO carried out the antimicrobial, antioxidant and cytotoxicity assays and analyzed the data. CSO also contributed with conceptualization, literature investigation, writing-original draft, writing-review, and editing. FC, DM, TM, JM, and JASAO contributed with the eucalyptus leaf extraction, pre-treatment of cotton surface, textile functionalization, writing and editing of the Material and Methods. BGB carried out the SEM analysis and editing of the Material and Methods. JASAO and CJS revised and validated the manuscript. FKT contributed with writing-review, editing and validation. All authors read and approved the submitted version.

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Data availability

All the data are available in the main text. Additionally, any data in this study can be obtained from the corresponding authors upon reasonable request.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Supporting Information

Figure S1-Cell viability of keratinocytes evaluated after 24 h of incubation using a 24-well plate with transwell inserts (0. 4 μm pores) to assess the cytotoxicity of non-functionalized and eucalyptus functionalized cotton samples via using a semi-direct method.

Figure S2 – FTIR analyses of cotton textiles functionalized with eucalyptus extracts.

Table S1 – EDX analyses of cotton textiles functionalized with eucalyptus extracts.

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