Fabrication, characterization and bioevaluation of novel antimicrobial composites based on polycaprolactone, chitosan and essential oils

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Abstract
The continuously spreading use of implantable medical devices (IMDs) in all fields of medicine increased the risk of microbial colonization leading to difficult to treat infections. The objective of this study was to obtain and characterize by physico-chemical and biological methods, new biopolymeric composites with anti-adherence properties based on polycaprolactone (PCL) dopped with chitosan (CS) and essential oils (EOs) as antimicrobial agents, for further use in the manufacture or coating of IMDs.

The EOs were extracted from cinnamon (CIN), coriander (COR) and tea tree (TEA), and were characterized by GC-MS. Biopolymeric composite films (PCL/CS, PCL/CS/EOs) were obtained by solvent evaporation in two variants and further characterized by DSC and FTIR techniques.

The in vitro antimicrobial activity of EOs was determined by the minimum inhibitory concentration (MIC) assay in liquid medium, towards Gram-positive and Gram-negative bacterial strains. The anti-adherence activity of films was analyzed against Staphylococcus aureus ATCC 29213 by viable cell counts assay. The cytotoxicity of the obtained films was assessed using the HT29 cell line. The results of this study suggest that CIN EO strongly inhibited the growth of all tested bacterial species, followed by TEA and COR EOs. From all tested composites, only PCL/CS/CIN exhibited a significant anti-adherence effect.

Keywords: biopolymeric composite, antimicrobial activity, cytotoxicity, anti-adherence

1. Introduction
Adhesion of microorganisms on the surface of medical devices is considered an essential step in the pathogenicity of infectious diseases [1]. When polymeric medical devices are implanted into the human body, they become easily colonized by microbial cells [2-4], causing devices-related infection, responsible of high rates of morbidity and mortality, and thereby significantly increasing health care costs [2, 5-9]. Prevention of such infections remains a major challenge to deliver quality medical care. In this regard, biomaterials industry
has focused on the design of anti-infective polymers, which are usually impregnated or mixed with different antimicrobial agents [2, 8, 10-13]. Composite systems are becoming more and more popular in biomedical applications because of the desired properties obtained by the combination of their constituents. From this perspective biocompatible polymeric matrices are one of the most frequently used for the development of implantable medical devices (IMDs).

Polycaprolactone (PCL), one of the earliest polymers synthesized by the Carother’s group in the early 1930s, is a hydrophobic, semi-crystalline substance with low melting point (59–64°C [14, 15]. PCL is soluble in chloroform, dichloromethane, carbon tetrachloride, benzene, toluene, cyclohexanone and 2-nitropropane at room temperature. It has a low solubility in acetone, 2-butanol, ethyl acetate, dimethylformamide and acetonitrile and is insoluble in alcohol, petroleum ether and diethyl ether [14, 16]. Due to their appropriate physico-chemical properties, PCL biomaterials have been proposed for multiple applications over the last two decades (sutures, wound dressings, fixation devices, contraceptive devices, dentistry), drug delivery and tissue engineering (scaffolds, bone, cartilage, tendon and ligament, cardiovascular, blood vessel, skin, nerve) [14, 17, 18].

Enzyme-embedded PCL-based coating, co impregnated with antibiotic (gentamicin sulfate) exhibited antibacterial properties against Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus [19].

Biodegradation of PCL is slow in comparison to other polymers, so it is suitable for long-term delivery approaches, extending over a period of more than 1 year. PCL also has the ability to form compatible blends with other polymers, which can affect the degradation kinetics, facilitating tailoring to achieve the desired release profiles [14, 20-22].

Chitosan (CS) is a copolymer of N-acetyl glucosamine and glucosamine units. CS is a unique basic polysaccharide with high molecular weight that occurs in nature in the cell walls of some fungi, insects, etc. It is a positively charged polysaccharide prepared by the N-deacetylation of chitin with 40-50% aqueous alkali at 120-160°C [23]. CS is hydrophilic and soluble in weak aqueous acids (pH<6.3) [24, 25]. Chitosan is biodegradable, biocompatible and non-toxic, properties which are appropriate for many applications in the pharmaceutical and biomedical field, such as the controlled release of drugs, space filling implants and wound management [23].

Chitosan and its derivatives have attracted a great interest due to their antimicrobial properties. Allan and Hardwiger (1979) reported for the first time the antimicrobial activity of chitosan [26, 27]. A recent study, in which PCL / CS polymers blends were investigated for their antimicrobial properties has shown that blends based on low molecular weight chitosan exhibited a pronounced inhibitory effect [28].

Chitosan and polycaprolactone are biodegradable biomaterials approved by Food and Drug Administration (FDA) [29].

In order to overcome some of the PCL properties drawbacks, such as high price and long biodegradability cycles, it is blended with a cheaper biodegradable natural polymer, such as starch, cellulose, or chitin and chitosan [30].

Also, the bioactivity of PCL can be enhanced when combined with natural polymers. The hydrophilic nature of CS will enhance its wettability and permeability, with a consequent acceleration of the PCL hydrolytic degradation [31]. The PCL / CS polymer blends reported in different studies were obtained by polymers bending in solution as well as by the melting technique.

The combination of these two polymers leads to high porosity, hydrophilicity and high mechanical strength [32, 33]. Also, by combining the synthetic polymers with natural polymers the biocompatibility can be increased [34].
The cytocompatibility of PCL was improved by the immobilization of chitosan rich in NH$_2$ groups onto the PCL membranes surface [35].

Essential oils (EOs) are composed of a very complex mixture of volatile molecules that are produced in the secondary metabolism of aromatic and medicinal plants. The main group of EOs is composed of terpenes and terpenoids and other aromatic and aliphatic constituents, all characterized by a low molecular weight [36]. The chemical composition, the quality and the quantity of EOs are influenced by many factors, such as: the soil composition, plant organ, vegetative cycle phase and climate [37-40]. The hydrophobicity is a very important characteristic of EOs and of their components, enabling them to partition the lipids of the bacterial cell and mitochondrial membranes, disturbing the cell structures, rendering them more permeable and causing metabolic disturbances, leading to microbial cells death [41, 42]. This indicates that minor or even trace EOs elements may be critical for the antimicrobial activity. The complexity of EOs prevents bacteria to develop tolerance and resistance [42, 43].

The usage of EOs to combat hospital-acquired infections and to control epidemic multi-resistant bacteria, such as methicillin-resistant Staphylococcus aureus shows promising results. The EOs of eucalyptus, tea tree, thyme white, lavender, lemon, lemongrass, cinnamon, grapefruit, clove bud, sandal wood, peppermint, kunzea and sage, proved to be active against problematic microorganisms, like methicillin-resistant Staphylococcus aureus (MRSA), Streptococcus and Candida strains [44].

The antimicrobial activity and applications of nanocomposite coatings based on PCL and thymol have been reported [45].

Taking into account the consecrated antimicrobial properties of EOs, we have focused our attention to obtain biomaterials incorporating cinnamon EO, coriander EO and tea tree EO as antimicrobials agents.

The objective of this study was to obtain new antimicrobial composites based on polycaprolactone (PCL), chitosan (CS) and essential oils (EOs) and to characterize them by physico-chemical and biological methods. The chemical composition of the essential oils (CIN, COR and TEA) was investigated by GC-MS. The antimicrobial activity of CIN, COR and TEA essential oils was tested against *Staphylococcus aureus* ATCC 29213, *Enterococcus fecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 8730 and *Klebsiella pneumoniae* IC 134202 bacterial strains. The chemical structure of antimicrobial composites was evidenced by fourier transform infrared spectroscopy (FTIR). Thermal analysis was performed by differential scanning calorimetry (DSC). Antibacterial adherence and cytotoxicity tests were used to prove the use of antimicrobial composites for the biomedical field.

### 2. Materials and Methods

#### 2.1. Materials

PCL was obtained from Sigma-Aldrich (M$_w$ = 45,000, Milwaukee, Wisconsin USA). Chitosan was obtained from Sigma-Aldrich (Iceland); Cyclohexanone (extra pure), was purchased from Scharlab S.L (Barcelona, Spain). The cinnamon essential oil (CIN), coriander essential oil (COR) and tea tree essential oil (TEA) were supplied by Cozac Plant SRL (Bucharest, Romania). Tryptic Soy Broth (TSB) medium was acquired from Biomedics (Madrid, Spain); Plate Count Agar (PCA) medium was obtained from Oxoid (England); Dimethylsulphoxide (DMSO) solvent was purchased from Sigma-Aldrich; Phosphate Buffered Saline (PBS) was obtained from Biochrom. The bacterial strains were provided by...
the collection of the Department of Microbiology of the Faculty of Biology (University of Bucharest, Romania).

The HT-29 ATCC HTB-38™ cells were used in the evaluation of cytotoxicity. The cells were maintained as an adherent culture in Dulbecco’s Modified Essential Medium (DMEM) (Sigma, USA) supplemented with 10% heat-inactivated (FBS) fetal bovine serum (Sigma, USA) at 37°C, 5% CO₂, in a humid atmosphere.

2.2. Preparation of antimicrobial composites films

Biopolymeric composites films (PCL/CS, PCL/CS/EOs) were prepared by solvent evaporation at room temperature conditions. PCL film was used as control. EOs were added to the PCL solution (10% w/v) prepared in cyclohexanone and stirred magnetically for 5 min at 40°C and 400 rpm for obtaining of homogeneous dispersions. Then the CS solution (1% w/v prepared in acetic acid aqueous solution1% v/v) was added, followed by magnetic stirring for 5 min at 40°C and 400 rpm. The mixtures were molded in Petri glass dishes and kept at room temperature conditions for seven days for the solvent evaporation. The proportion of the compounds PCL/CS/EOs was 4: 1: 0.2 and respectively 4: 1: 0.04. The proportion for the PCL/CS was 4: 1, as shown in Table 1.

### Table 1. The composition of biopolymeric composites films

<table>
<thead>
<tr>
<th>Code Sample</th>
<th>Composition, volume ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>PCL/CS = 4:1</td>
</tr>
<tr>
<td>PCCI₁</td>
<td>PCL/CS/CIN = 4:1: 0.2</td>
</tr>
<tr>
<td>PCT₁</td>
<td>PCL/CS/TEA = 4:1: 0.2</td>
</tr>
<tr>
<td>PCC₁</td>
<td>PCL/CS/COR = 4:1: 0.2</td>
</tr>
<tr>
<td>PCCI₂</td>
<td>PCL/CS/CIN = 4:1: 0.04</td>
</tr>
<tr>
<td>PCT₂</td>
<td>PCL/CS/TEA = 4:1: 0.04</td>
</tr>
<tr>
<td>PCC₂</td>
<td>PCL/CS/COR = 4:1: 0.04</td>
</tr>
</tbody>
</table>

2.3. Gas chromatography/mass spectrometry (GC-MS) analysis of essential oils (EOs)

The GC–MS analysis was carried out using an Agilent 6890 N gas chromatograph interfaced with an Agilent mass selective detector 5975B (Agilent Technologies, USA). Oven temperature program: 100–320°C, at 10°C/min (39 min analysis times); the injector parameters were: initial temperature 250 °C, pressure 6.89 psi, split ratio 2:1, carrier gas helium. A HP-5MS 5% phenylmethylsiloxane capillary column was used for the separation of the sample components with the following characteristics: max. temperature 325°C, nominal length 30.0 m, nominal diameter 250.0 μm, nominal film thickness 0.25 μm, initial flow 1.0 mL/min. The mode of operating was constant flow. Interface temperature was 280°C; standard electronic impact (EI), MS source temperature: 230°C; MS quadrupole temperature: 150°C; mass scan range: 29 – 850 amu at 70 eV, 1 μL of sample was injected into the system.

The constituents of the EOs were identified by comparing their mass spectra obtained with those in the mass spectra library (NIST, Wiley). For quantification purposes, area percentages were obtained by integration of chromatograms [46].

2.4. Differential Scanning Calorimetry (DSC) measurements

The DSC tests were performed using a METTLER-TOLEDO apparatus DSC-823° model, using STAR® software, version 9.10, and previously calibrating with indium standard.
The weighted samples were sealed in 40 µl aluminum crucibles with a small hole in the lids, and the first heating curves were measured from room temperature to 100°C at a heating rate of 10°C/min, as first heating. The melting temperature and the enthalpies of melting (ΔH) of the samples were determined. The melting region in this experiment was defined by onset temperature (T_on) and maximum melting temperatures (T_m).

2.5. Fourier Transform Infrared Spectroscopy (FTIR) measurements
The functional groups present in the prepared films were identified by FTIR using a Spectrum BX Spectrometer. The spectra were recorded in the range of 400 to 4000 cm⁻¹ with a resolution of 4 cm⁻¹.

2.6. EOs Minimum Inhibitory Concentration (MIC) assay
MIC tests were carried out according to [47] by using 96 multi-well plates. The stock solutions of EOs were transferred into the first well, and serial microdilutions were performed by using TSB medium. Microbial suspensions of 0.5 McFarland density (1:5 volume/well) were added to the wells and the plates were incubated at 37°C for 24 h. The experiments were carried out using DMSO as solvent. MIC was defined as the lowest concentration of EOs at which the microorganisms did not demonstrate visible growth in the liquid medium.

2.7. Anti-adherence test
The bacterial anti-adherence effect was demonstrated using 24 multi-well plates. Standard pieces of 1cm x 1cm of PCL, PCL/CS and PCL/CS/EOs were sterilized by exposure to direct UV light for 30 min on both sides and distributed in 24 multi-well plates (one per well). The Staphylococcus aureus ATCC 29213 suspension of 0.5 McFarland density was diluted 1:100, for reaching an inoculum with density of 10⁵–10⁶ (CFU/ml). S. aureus inoculums prepared in TSB medium were added in each well, to completely cover films pieces. Samples were incubated for 24h at 37°C. The inhibitory effect of staphylococcal colonization on the surface of films was analyzed by viable cell count assay. After the incubation period, biopolymeric films pieces were gently washed with sterile PBS for not removing the bacterial cells adhered on films surface and placed in 2 ml Eppendorf tubes containing 1000 µl PBS. Samples were centrifuged for 5 min at 10000 rpm, followed by 30s vortexing in order to disperse the bacterial cells on films surfaces, and ten-folds microdilutions were performed from the obtained suspensions. Three replicates of each dilution were plated on PCA medium for viable cell counts assay and the Colony Forming Units (CFUs) were calculated [48-50].

2.8. Cytotoxicity test and cell cycle analysis
5x10⁵ HT29 cells were seeded in 3.5-cm diameter wells, in RPMI medium supplemented with 10% FBS, 400 IU/ml penicillin and 200 µg/ml streptomycin. HT29 were incubated for 24 h at 37°C in humidified and 5%CO₂ atmosphere. The sterile film pieces of 0.7cm x 0.7cm were added over the monolayer, and then incubated for another 24 h and 48h, in the same conditions. The effect of samples on HT29 cell was observed using inverted microscope Zeiss Observer D1 and was evaluated by Trypan Blue Exclusion method. The cells were trypsinised, centrifuged for 6 minutes at 1200 rpm and resuspended in 1 mL PBS. 50 µL of cell suspension was stained with 50 µL Trypan blue solution (0.4%), for 1 minute, and the mixture was placed in a haemocytometer and viable and non-viable cells were counted. Each experiment was repeated three times. The rest of harvested cells were fixed in cold ethanol (70%) and stored at – 20°C overnight. The samples were then centrifuged, washed with PBS, re-suspended in 100 µl PBS, treated with 1 mg/mL RNase A at 37°C for 15 minutes, and stained with 100 µg/mL propidium iodide for 1 h at 37°C. DNA content of cells was
quantified on a Beckman Coulter EPICS XL flow cytometer and analyzed using FlowJo 8.8.6 software (Ashland, Oregon, USA).

3. Results and discussion

3.1. Chemical composition of EOs

The GC-MS technique revealed the presence of 12 substances in the composition of cinnamon EO, of which 10 could be identified. The chromatogram revealed the cinnamaldehyde to be the major compound, with a relative abundance of 86.27% followed, in smaller amounts, by o-methoxycinnamic aldehyde (2.57%), and cinnamic acid (1.93%), as well as by other compounds, in percents under 1%.

Several reported studies have shown that CIN EO are very complex mixtures of compounds and many variations have been found in their chemical composition [51], but of these, cinnamaldehyde is the main component, with percentages varying from 79.38% [51-55] to 41.62% [41].

For coriander EOs, the chromatogram showed 24 components from which 22 compounds were identified. The main compounds identified in tested COR EO was linalool (61.30%), and in smaller percentages, α-pinene (7.38%), DL-limonene (5.80%), camphor (4.82%), α-terpinenyl acetate (2.15%), geranyl acetate (1.78%), linalool oxide (1.45%), and others. Our results are in good agreement with the literature data, stating that coriander seeds oil is rich in linalool (60-70%) [56]. Zorca et al. (2006) identified 45.31% linalool as major component of coriander oil obtained by hydrodistillation and 72.10% linalool for the oil obtained by supercritical CO2 extraction [57]. Nazrul et al. (2009) reported linalool (37.65%) as major component from seeds oil of coriander from Bangladesh [58], while other authors report 69.60% linalool [59].

In case of the tested tea tree EO, 29 components were detected, from which 27 were identified. Terpinen-4-ol was found in the highest amount (28.87%) followed by γ-terpinene (16.00%), α-terpinene (9.83%), DL-limonene (8.99%), p-cymene (6.51%), α-pinene (5.88%), α-terpinolene (4.23%), trans-caryophyllene (3.12%) and other compounds in smaller percentages. Other GC-MS performed tests have been shown that terpinen-4-ol represents the majority chemical component of TEA, with a percentage of 39.8% [60] and respectively, 41.6% [61].

3.2. DSC measurements of the biopolymeric films

Melting temperature and heat of fusion (ΔH) determined from the DSC termograms of the samples (Figure 1 and Figure 2) are summarized in (Table 2).

![DSC termograms of biopolymeric composites films](image_url)
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Figure 2. DSC termograms of biopolymeric composites films: a) PCL(film); b) PCL (granules); c) PC; d) PCC2; e) PCCI2; f) PCT2

Table 2. DSC characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Code sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCL (granules)</td>
</tr>
<tr>
<td>ΔH (J/g)</td>
<td>67.07</td>
</tr>
<tr>
<td>Tm (°C)</td>
<td>56.69</td>
</tr>
<tr>
<td>Tm (°C)</td>
<td>63.46</td>
</tr>
</tbody>
</table>

Data from Table 2 and Figures 1 and 2 reveal that PCL film recorded the melting temperature (Tm) at 67.02 °C and the enthalpy of melting (ΔH) at 71.2 J/g, respectively. PCL/CS composite shows lower Tm than PCL pure, due to the hydrophilic character of CS, that likely leads to poor adhesion with the hydrophobic PCL. The lower Tm recorded for PCL/CS/EOs in ratio 4:1:0.2 v/v % in comparison with PCL film (by about 2–5 °C) is due to the lower melt viscosity of chitosan and EOs while the higher ΔH (by about 12 - 27 J/g) is caused by the higher crystallinity of the composites. We can suppose here that EOs can act like plasticizers. It is proposed that this is also a result of less space available for molecular motion within this composite. Instead, the values of Tm in the composites PCL/CS/EOs in ratio 4:1:0.04 v/v % increased (by about 2 °C) while the ΔH decreased (by about 7 J/g) than PCL film. This implies a decreased in crystallinity with decrease of EOs content, probably caused by chitosan movement of the polymer segments and thereby making them more easily to arrange the polymer chain. This melting behavior shows that PCL/CS/EOs composites in the ratio 4:1:0.2 are easier to process than those in ratio 4:1:0.04.

3.3. FTIR measurements of the biopolymeric films

IR absorbance spectra of PCL, PC, PCC1, PCCI1, PCT1, PCC2, PCCI2 and PCT2 biopolymeric films are presented in Figures 3 and 4.

The spectra clearly suggest the presence of the various functional groups corresponding to the raw materials used to obtain the biopolymeric films by solvent evaporation.

The PC spectra show the characteristic absorption bands at 1148 and 841 cm⁻¹ attributed to the glycoside bond specific to chitosan. These bands are also present in all of the biopolymeric investigated composites films. In other studies the bands assigned to the glycoside bond of chitosan were reported at 890 and 1147 cm⁻¹ [62], 905 and 1157 cm⁻¹ [63], as well as 897 and 1154 cm⁻¹ [64].

Also, the amine N-H symmetrical vibration band reported in the IR spectrum for chitosan at 1650 cm⁻¹ [65] can be attributed to the bands 1685, 1698, 1689, 1675, 1679, 1669, 1681 cm⁻¹, confirming the presence of chitosan in all the investigated samples.
The characteristic absorbance band of ester in PCL film was shown at 1739 cm\(^{-1}\). All the investigated biopolymeric composites films presented bands at 1755 cm\(^{-1}\) (PC), 1764 and 1742 cm\(^{-1}\) (PCT\(_1\) and PCT\(_2\)), 1765 and 1757 cm\(^{-1}\) (PCCI\(_1\) and PCCI\(_2\)), 1756 and 1764 cm\(^{-1}\) (PCC\(_1\) and PCC\(_2\)). In the case of PCL, the ester bands were identified in other studies at 1750 cm\(^{-1}\)[66], 1734 cm\(^{-1}\)[62] and 1724 cm\(^{-1}\)[67].

![Figure 3](image1.png)

**Figure 3.** FTIR spectra of PCL/CS/EOs composites in the ratio 4:1:0.2 in comparison to PCL and PC

![Figure 4](image2.png)

**Figure 4.** FTIR spectra of PCL/CS/EOs composites in the ratio 4:1:0.04 in comparison to PCL and PC

In the case of the samples with incorporated EOs, which have chemical groups similar to those of PCL and CS, it can be observed a difference between the bands in the regions 2800-3000 cm\(^{-1}\) and 1650-1800 cm\(^{-1}\). The modifications in those regions are attributed to the essential oil incorporation into the samples.

### 3.4. Quantitative assay of the Minimum Inhibitory Concentration (MIC) of EOs

Antimicrobial activity of essential oils was confirmed by the MICs data. Microbial susceptibility to the tested oils is shown in Table 3. CIN EO showed the highest antimicrobial activity against both Gram positive and Gram negative bacteria, followed by TEA and COR EOs. The MICs values confirm the strong inhibitory effect of CIN EO on a broad spectrum of bacterial strains. Similar studies have also reported the strong inhibitory effect of CIN EO against *Staphylococcus aureus*, *Escherichia coli*, including *E. coli* O157:H7, *Campylobacter jejuni*, *Listeria monocytogenes*, *Salmonella typhimurium*, *MRSA*, *Streptococcus pyogenes*, *Bacillus cereus*, *Bacillus subtilis* and *Klebsiella pneumoniae* [41, 51, 68-70].

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Table 3. Minimum Inhibitory Concentration (MIC) of cinnamon, coriander and tea tree EOs.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Cinnamon EO (CIN)</th>
<th>Coriander EO (COR)</th>
<th>Tea tree EO (TEA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 29213</td>
<td>0.39</td>
<td>6.25</td>
<td>3.13</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> ATCC 27853</td>
<td>0.19</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td><em>Escherichia coli</em> ATCC 8730</td>
<td>0.39</td>
<td>25</td>
<td>6.25</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> IC 134202</td>
<td>0.78</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td><em>Enterococcus fecalis</em> ATCC 29212</td>
<td>1.56</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>

The antimicrobial activity of the tested COR EO was better against the *S. aureus* ATCC 29213. Another study also reported that COR EO exhibited a broad spectrum inhibitory effect, *S. aureus* ATCC 25923 being the most sensitive, while *P. aeruginosa* ATCC 27853 was more resistant [71]. In case of TEA EO, the results revealed that *S. aureus* and *E. coli* were more susceptible to the EO action. Similar MIC values were obtained for COR and TEA EOs on *P. aeruginosa*, *K. pneumoniae* and *E. fecalis* (Table 3).

Numerous studies have shown that the CIN EO broad spectrum antimicrobial activity is attributed to cinnamaldehyde. The lipophilicity or hydrophobicity and the chemical structure of the main compounds, such as the presence of functional polar groups and aromaticity have an important role in antimicrobial activity [51, 72, 73], these characteristics enabling the partition among the lipids of the bacterial or fungal cell membrane and mitochondria, disturbing the cellular structures and rendering them more permeable, which will lead to cell death [51, 74]. So, the antibacterial activity of cinnamaldehyde can be due to the lipophilicity of phenylpropanoids, which can penetrate the microbial membrane, react with the membrane enzymes and proteins as well as with the phospholipids bilayer, causing the impairment of microbial enzymatic systems and/or of the genetic material functionality [51, 68, 70]. Singh et al., (1995), cited by Sheng-Yang Wang et al., (2005) have shown that cinnamaldehyde is a strong antifungal agent [75, 76].

The essential oil of *Melaleuca alternifolia* (tea tree oil) consists largely of cyclic monoterpenes of which about 50% are oxygenated and about 50% are hydrocarbons. It exhibits a broad-spectrum antimicrobial activity that can be principally attributed to terpinen-4-ol [77-79].

The strong antimicrobial activity of cinnamon essential oil tested in this work could thus be attributed to the major product cinnamaldehyde (86.27%) and the antimicrobial activity of tea tree essential oil could be associated with the terpinen-4-ol (28, 87%). The presence of these chemical compounds was confirmed by GC-MS analysis.

However, it must be taken into account that the antimicrobial activity of the EOs is due to complex interactions between individual components that lead to an overall higher activity, as also demonstrated for COR EO as compared to its main constituent (linalool) [71, 80].

### 3.5. Anti-adherence properties of the obtained biopolymeric films

In this study, we have also tested the anti-adherence activity of antimicrobial composites based on polycaprolactone, chitosan and essential oils, against *Staphylococcus aureus* ATCC 29123 using viable cell counting assay. It was observed that the antimicrobial composite PCCI1 totally reduced (CFUs/ml = 0) the viable staphylococcal cells adhering to the tested surfaces, when comparing with other composites, and with control (PCL film). The other composites did not exhibit any anti-adherence activity (Table 4).

Table 4. CFUs of *Staphylococcus aureus* ATCC 29213, calculated after viable cell counts assay by removing cells adhered on films surfaces at 24h.
These observations are consistent with the results of MIC, in other words, the anti-adherence activity against *Staphylococcus aureus* ATCC 29123, is due to the influence of EOs (CIN, COR and TEA). The quantitative assay (MIC) showed that CIN EO strongly inhibited *S. aureus* growth at very low MICs values, while TEA and COR EOs exercised inhibitory effect against *S. aureus* at higher MICs. Therefore, increasing staphylococcal adhesion observed in case of the PCCI2 composite, as compared to PCCI1 could be explained by the low amount of EOs incorporated into the polymeric material, unable provide an inhibitory effect and anti-adherence effect respectively, probable hypothesis in the case of composites based on COR and TEA EOs.

### 3.6. Cytotoxicity assay results

Originally biocompatibility referred to the ability of a material to induce an appropriate host response in a specific application. *In vitro* biocompatibility, or cytotoxicity, is generally evaluated through cell cultures systems. Hence, biocompatibility is a factor that must be considered before the selection of polymers to be used in the design of medical devices [14].

The obtained results showed that after 24h, although the morphology of HT29 cells developed under the films pieces was altered compared with control cells and the rest of the cells from the well (Figure 5), a > 90% cellular viability was registered for the majority of film pieces. However, in case of PCCI1 and PCCI2 the area with affected morphology was higher, and the viability decreased to nearly 80%. PCCI1 and PCCI2 pieces films increased cell mortality when were maintained in HT29 cells for 48h reaching 50% and respectively 60%.

![Figure 5](image-url). Morphology of HT29 cells after 24 h, analyzed by Optical Inverted Microscope (OIM): A) control HT29 cells; B) cells treated with PCL; C) cells treated with PC; D) cells treated with PCC; E) cells treated with PCC; F) cells treated with PCCI; G) cells treated with PCC; H) cells treated with PCT; I) cells treated with PCT (x200).
These effects were confirmed by the appearance of a subG0 peak in cell cycle analysis, correlated with apoptosis (Figure 6). The peak was strongly increased in the case of PCCI1 and PCCI2. In addition, the PCCI1 and PCCI2 films modified the percent of cells in different cell cycle phases. In case of PCCI2 we noticed a slight increase in the S and G2 phases; these values were double in case of PCCI1 comparing to HT29 control cells.

Therefore, the alteration of cells morphology after 24 h of contact of HT29 cells with the films pieces, the decrease of cell viability by 50-60% after 48h for HT29 cells maintained in the presence of PCCI1 and PCCI2 films pieces, the presence in both cases of a subG0 peak in cell cycle compared with control cells as well as modification the percent of cells in different cell cycle phases exercised by the PCCI1 and PCCI2 films suggest that the biopolymeric films exhibit cytotoxic effect.

![Flow cytometry diagrams of cell cycle analysis of the HT29 cells treated with biopolymeric films pieces: A) PCL film; B) PC film; C) PCC1 film; D) PCC2 film; E) PCCI1 film; F) PCCI2 film; G) PCT1 film; H) PCT2 film; I) control HT29 cells.](image)

**Figure 6.** Flow cytometry diagrams of cell cycle analysis of the HT29 cells treated with biopolymeric films pieces: A) PCL film; B) PC film; C) PCC1 film; D) PCC2 film; E) PCCI1 film; F) PCCI2 film; G) PCT1 film; H) PCT2 film; I) control HT29 cells.

**Conclusions**

We report the fabrication of new antimicrobial and biocompatible composites based on polycaprolactone (PCL), chitosan (CS) and essential oils (EOs) and their characterization by physico-chemical and biological methods. The obtained results suggest that the composites with the formulation PCL/CS/CIN (4:1:0.2) were resistant to staphylococcal cells adhesion.
The cytotoxic nature of these composites eliminating their applicability as biomaterials, but they could be represent an interesting approach for the food packaging and surfaces industry.

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