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ORIGINAL ARTICLE

Development of bacterial cellulose incorporated with essential oils for wound treatment

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Abstract

Bacterial cellulose (BC) is promising as a wound dressing because it is non- toxic and maintains moisture in the wound. Although BC does not have antimicrobial activity, its structure allows the incorporation of antimicrobial compounds such as essential oils (EOs). This study aims to associate BC with rosemary, clove, eucalyptus, ginger, lavender and lemongrass EOs to obtain wound dressings. The Gas Chromatography-Mass Spectrometry and Fourier Transform Infrared Spectroscopy analyses showed characteristic compounds of EOs in the incorporated membranes. These compounds reduced the thermal stability of most samples due to their different degrees of volatility. The Scanning Electron Microscopy indicated that the EOs filled the membrane pores and coated the cellulose fibers. Samples incorporated with clove, ginger and lemongrass EOs inhibited *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* due to the presence of eugenol and citral. The results confirmed the incorporation method's effectiveness, maintaining the composition and antimicrobial characteristics of the EOs.

Keywords: *Bacterial cellulose, essential oils, incorporation, wound dressings.*

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1. Introduction

Traditional methods of treating chronic wounds include dressings that can interfere with the healing process since constant dressing changes are made, causing pain and discomfort to the patient and delaying tissue reconstitution[1]. As an alternative to traditional dressings, a new generation of functional dressings has been developed seeking to prevent infections and improve wound healing, with properties such as maintaining wound moisture, removing exudates and antimicrobial control. In that regard, biopolymer-based dressings may be a good choice for treating these wounds[1,2]. Of the polymers used for this purpose, bacterial cellulose (BC) stands out because it has characteristics that meet the requirements of an ideal dressing. BC is synthesized by bacteria, such as those of the genus *Komagataeibacter*, is biodegradable, has high purity, high mechanical resistance, biocompatibility and has a reticulated structure that serves as a barrier against pathogenic microorganisms. BC has a structure similar to the skin's extracellular matrix, creating and maintaining a humid environment for the wound due to its high hydrophilicity and also serving as a barrier against UV radiation[3-5]. However, BC alone cannot inhibit the growth of pathogenic microorganisms, which reduces its effectiveness as a dressing for contaminated wounds. Nonetheless, antimicrobial agents can be incorporated into its structure to improve its biological properties. In this sense, essential oils (EOs) become a good option for this purpose, as they have several applications in the pharmaceutical industry and medicine due to their antimicrobial, antioxidant, and anti-inflammatory properties, among others, arising from the presence of bioactive compounds, such as phenols and terpenes, in its structure. In addition, EOs are advantageous over synthetic antimicrobial agents because they are widely available natural compounds with low toxicity[6–8], making the material promising for application in tissue regeneration. In that regard, some EOs that can be used for the production of biocomposites include rosemary (REO), clove (CEO), eucalyptus (EEO), ginger (GEO), lavender (LEO) and lemongrass (LGEO)[8-12] because, in addition to their antimicrobial properties, they stimulate tissue regeneration and exert a healing effect on individual organs and systems[13].

In this context, the present work aims to associate BC with these EOs to develop a material for use in the biomedical area as a dressing with a high healing capacity.

2. Materials and Methods

2.1 Bacterial cellulose biosynthesis

BC membranes were synthesized by the bacteria *Komagataeibacter hansenii* (ATCC 23769) in a culture medium adapted from Hestrin and Schramm[14]. This step was carried out using 50 mL ®Falcon tubes with 20 mL of culture medium, incubated at 30 °C in static condition for 12 days. Then, the BCs were treated with a 0.1 M NaOH solution at 80 °C for 1 h to remove bacteria. Subsequently, the membranes were washed with distilled water repeatedly until the pH of the washing water was neutralized[15]. Finally, the BC membranes were sterilized and submitted to the incorporation processes with the EOs and, subsequently, to the analysis of incorporated EO content, Total Phenolic Concentration (TPC), High Resolution Scanning Electron Microscopy (SEM), Thermogravimetric Analysis (TGA), Fourier Transform Infrared Spectroscopy (FTIR) and antimicrobial activity.

2.2 Incorporation of essential oils

The EOs were purchased from the company Phytoterapica (Solua Comercial LTDA). According to the manufacturer, all EOs were obtained by steam distillation. Following the methodology adapted from Albuquerque[16], BC membranes were immersed in 150 mL of ethanol at 60 °C for 10 min. Afterward, each BC was immersed in a solution containing 3.0 g of EO in 20 mL of ethanol and heated at 60 °C until the solution evaporated. Finally, the BCs incorporated with the EOs were lyophilized and stored for analysis.

2.3 Incorporated essential oil content

The composition of EOs and the actual amounts of EOs incorporated into BCs were determined by Gas Chromatography-Mass Spectrometry (GC-MS). Each sample was immersed in 20 mL of dichloromethane for 24 h to extract the EOs[17], while pure EOs were diluted 1:100 with dichloromethane. The analysis was conducted in Agilent GC:7890A, MS:5975C equipment, and the injection volume was 1 µL in Split-Splitless mode. The column was HP- 5ms (30 m x 250 µm x 0.25 µm). The carrier gas was helium with a flow rate of 1.2 mL/min; the injector temperature was 280 °C. The initial oven temperature was 50 °C with an isotherm of 2 min, then increased to 300 °C at 10 °C/min and maintained at that temperature for 5 min. The interface temperature was 300 °C, the ion source temperature was 230 °C, and the electron impact ionization was 70 eV. Mass spectra were analyzed in SCAN mode.

2.4 Total phenolic content (TPC)

Phenolic compounds are involved with the functional properties of herbal medicines[18]. TPC was evaluated in EOs before and after incorporation into BC membranes by the turbidimetry technique using the Folin-Ciocalteau reagent adapted from Waterhouse[19]. The TPC was expressed in mg of gallic acid equivalents (GAE) per g of dry sample, determined by the equation of the straight line of the calibration curve of gallic acid (0.05-0.5 mg/mL).

2.5 Structural characterizations

The characterization of the functional groups of the EOs, the BC and the incorporated BCs was carried out by FTIR in Perkin Elmer Frontier equipment. 32 scans were performed per sample, from 650 to 4000 cm^{-1}, with a resolution of 2 cm^{-1}, in attenuated total reflectance (ATR) mode. To evaluate the influence of the addition of the EOs on the thermal stability of the BC, TGA was used, conducted in TGA-Q50 equipment (TA Instruments), where the samples were heated from 25 to 1000 ºC at 10 ºC/min, under an inert atmosphere (N2). The samples were observed in SEM in a JEOL equipment, model JSM-6701F. The samples were coated with gold, and the analysis was conducted with 5 kV of accelerating voltage.

2.6 Antimicrobial activity

The incorporated EOs and BCs had their inhibitory capacities evaluated against *Candida albicans* (ATCC 10231), *Escherichia coli* (ATCC 8739) and *Staphylococcus aureus* (ATCC 6538) by the disc diffusion test adapted from Kirby & Bauer (1966)[20]. A statistical analysis of the inhibition zones was performed using the Tukey's Method with 95% confidence.

3. Results and Discussions

3.1 Incorporation of essential oils

Images of BC membranes incorporated with REO (BC/R), CEO (BC/C), EEO (BC/E), GEO (BC/G), LEO (BC/L) and LGEO (BC/LG) are shown in Figure 1. All samples had a sticky appearance and characteristic aromas and colors of each oil.

3.2 Incorporated essential oil content

The main components of EOs detected by GC-MS in the samples are presented in Table 1. The compound with the highest concentration in REO and EEO was eucalyptol, corroborated by the literature[21,22]. The concentrations in ppm of the BC/R and BC/E compounds were low concerning the other samples, which may be due to the greater volatilization of these EOs and their components during incorporation into the BC. Moreover, camphor and α-pinene were identified for REO and limonene for EEO. For CEO, eugenol, the major compound of clove[23], and β-caryophyllene were identified. The incorporated sample maintained a high concentration of eugenol; however, β-caryophyllene was not identified. The GEO showed the greatest variety in its composition, where β-caryophyllene, neral and geranial (citral) were identified with the highest percentage, in addition to ar-curcumene, zingiberene and β-sesquiphelandrene, corroborated by the literature[24,25]. The compounds with the highest concentrations in BC/G were ar-curcumene and β-sesquiphelandrene, possibly due to greater volatilization of the other compounds during incorporation. LEO and LGEO presented two major compounds each: linalool and linalyl

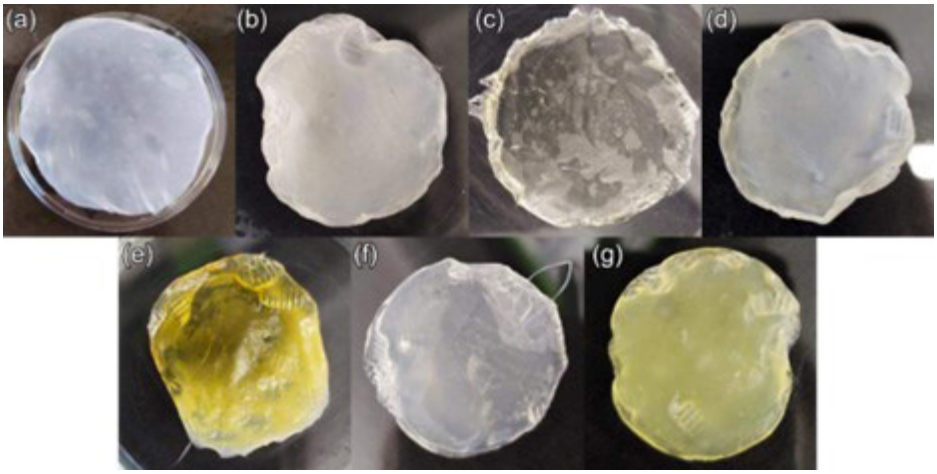

Figure 1. Physical aspects of (a) BC, (b) BC/R, (c) BC/C, (d) BC/E, (e) BC/G, (f) BC/L and (g) BC/LG.

Table 1. Main components of EOs detected by GC-MS.

Components	Sample	Concentration (%)	Sample	Concentration (ppm)
Eucalyptol	**REO**	72.222	**BC/R**	4.640
Camphor		13.366		258.133
α-pinene		4.105		6.164
Eugenol	**CEO**	86.809	**BC/C**	76.860
β-caryophyllene		11.397		nd
Eucalyptol	**EEO**	89.303	**BC/E**	4.220
Limonene		8.259		4.120
Ar-curcumene	**GEO**	2.215	**BC/G**	364.440
Zingiberene		3.651		102.840
β-sesquiphelandrene		1.484		232.440
Neral		6.882		117.360
Geranial		9.536		151.200
β- caryophyllene		10.709		93.240
Linalool	**LEO**	45.298	**BC/L**	126.880
Linalyl acetate		35.844		126.980
Neral	**LGEO**	30.292	**BC/LG**	60.360
Geranial		41.572		85.680

nd = not detected.

acetate, and neral and geranial, respectively, which agree with the literature[26,27]. The BC/LG sample kept geranial as the compound with the highest concentration, and BC/L had a very close concentration between its two compounds, suggesting a greater volatilization of linalool. All tested EOs showed chemical compositions similar to those found in the literature but with variations in the concentration of the compounds. The chemical composition of essential oils is quite variable, as it depends on the EOs extraction method and time, type and concentration of solvents, the species involved, the climatic conditions and the soil where the plant was grown, among others[28,29].

3.3 Total phenolic content

The TPC results determined by the turbidimetry technique using the Folin-Ciocalteau reagent are presented in Table 2.

Table 2. Total phenolic content of the samples.

Sample TPC (mg GAE/g EO)	Sample	TPC (mg GAE/g EO)
REO $0.093 \pm 0.007^{e, f}$	**BC/R**	$0.088 \pm 0.006^{e, f}$
CEO 5.656 ± 0.083^{a}	**BC/C**	2.316 ± 0.021^{b}
EEO $0.083 \pm 0.028^{e, f}$	**BC/E**	$0.033 \pm 0.002^{e, f}$
GEO 0.758 ± 0.104^{c}	**BC/G**	0.277 ± 0.016^{d}
LEO $0.142 \pm 0.036^{d, e}$	**BC/L**	0.002 ± 0.001^{f}
LGEO 0.639 ± 0.077^{c}	**BC/LG**	$0.147 \pm 0.013^{d, e}$

Different letters indicate differences in columns ($p \leq 0.05$).

The CEO has the highest concentration of total phenolics among the tested EOs due to the high percentage of eugenol in its composition. GEO and LGEO presented values close to each other. The other EOs had low TPC values, emphasizing REO

and EEO, with the lowest TPC among the selected EOs. There was a significant decrease in the TPC of the incorporated BCs, which may be related to the volatilization of these components at 60 °C in the incorporation step and in the lyophilization step, related to the operating pressure and processing time during the process since a decrease in the drying chamber pressure reduces the lyophilization time, but increases the release of volatile compounds[30]. The BC/L sample stands out, which had a significant decrease in TPC, being the sample with the lowest observed value. The TPC analysis includes phenolic acids, flavonoids, isoflavonoids, lignans, stilbenes and other polyphenols. The variation in TPC values obtained reflects the diversity of phenolic compounds and their ability to reduce the Folin-Ciocalteau reagent. The differences observed between the results of the work and those obtained in the literature are justified due to the factors that influence the composition of the EOs, as discussed in the previous item[28,31].

3.4 Structural characterizations

3.4.1 Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra and the main bands obtained from the samples of BC, EOs, and incorporated BCs are presented in Figure 2.

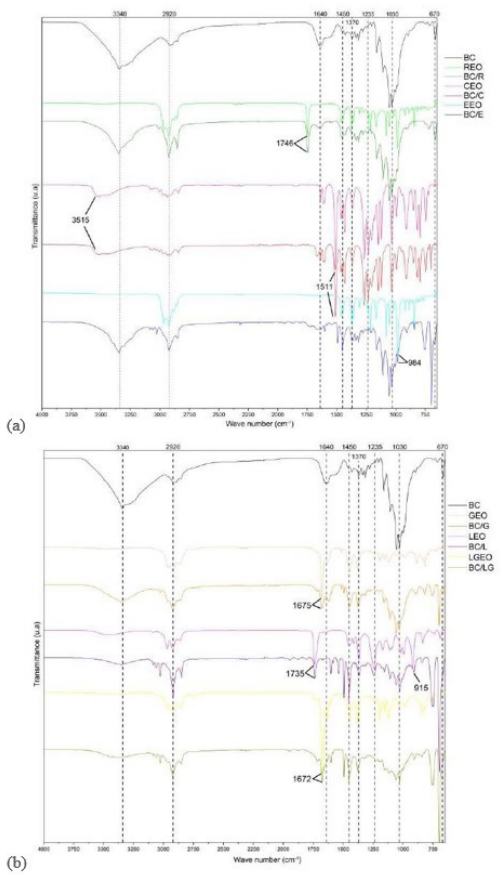

Figure 2. FTIR spectra of samples: (a) comparison between BC and samples incorporated with rosemary, clove and eucalyptus and (b) comparison between BC and samples incorporated with ginger, lavender and lemongrass.

The BC sample showed high-intensity bands at 3344 cm^{-1}, attributed to the axial stretching of hydroxyl groups present in organic compounds[32]. Other bands referring to the presence of OH groups were observed at 1205 and 664 cm^{-1}, referring to in-plane binding and out-of-plane angular deformation, respectively[3]. Bands referring to the C-H bonds of methyl groups were observed at 2896 and 895 cm^{-1} due to stretching and angular deformation, respectively[33]. Furthermore, several bands attributed to the stretching of C-O groups were observed, such as those at 1336, 1161, 1055 and 1032 cm^{-1}[33-35]. Moreover, the band at 1641 cm^{-1} stands out, which, in the case of BC, refers to the angular deformation of water, demonstrating its hydrophilicity[3]. In the incorporated samples, several bands related to EOs appeared, such as the C-H stretching band in vinyl groups at 3088 cm^{-1}, attributed to the presence of linalool and linalyl acetate in the BC/L sample[36,37]; around 1735 cm^{-1}, referring to the stretching of C=O groups for BC/R, BC/L and BC/LG samples, derived from camphor, linalyl acetate and citral (neral and geranial), respectively[37-39]; around 1675 cm^{-1} for BC/G and BC/LG, attributed to citral[40]. Bands referring to the eugenol of the BC/C sample were observed at 3515 and 1511 cm^{-1}, attributed to the stretching of O-H bound to benzene and stretching of C=C of the aromatic ring, respectively[41,42]. Bands were observed at 985 cm^{-1}, referring to out- of-plane symmetric deformation of CH2, which, in the case of samples incorporated with rosemary and eucalyptus, can be attributed to the presence of eucalyptol[38].

3.4.2 Thermogravimetric analysis (TGA)

Figure 3 shows the TG and DTG curves of the samples, while Table 3 shows the thermal events of the sample obtained from these curves.

The samples showed three stages of thermal degradation. Pure BC obtained a thermal degradation profile characteristic of this material, where cellulose degradation occurred at a maximum degradation temperature (TPEAK 2) of 332 °C[43]. The BC/R had a greater mass loss in the second stage of thermal degradation, suggesting that the REO decomposition may be co- occurring with the BC. Furthermore, there was an increase of 69 °C in the TPEAK 2 compared to the BC, demonstrating an increase in the thermal stability of the membranes, suggesting a better interaction between REO compounds and BC. BC/C showed the most significant change in the thermal degradation profile compared to pure BC. A greater mass loss (84.39%) occurred in the first degradation stage, attributed to the volatilization of water, ethanol and eugenol, CEO's major component, which occurs between 100 and 270 °C and may represent a higher percentage by mass of the sample[44]. The BC/G and BC/LG samples showed a profile similar to that of BC/C, with the most significant mass losses occurring in the first stage of degradation, corresponding to the evaporation of water, ethanol, and volatile components of the EOs, such as citral, present in both EOs and cellulose degradation[40,45]. BC/E and BC/L showed similar thermal degradation profiles; however, the greatest mass loss of both samples occurred in the third stage of degradation, demonstrating that the degradation of cellulose and carbonaceous residues occurred during all stages. Notably, the phenolic compounds of the EOs have different degrees of volatility, causing a decrease in thermal

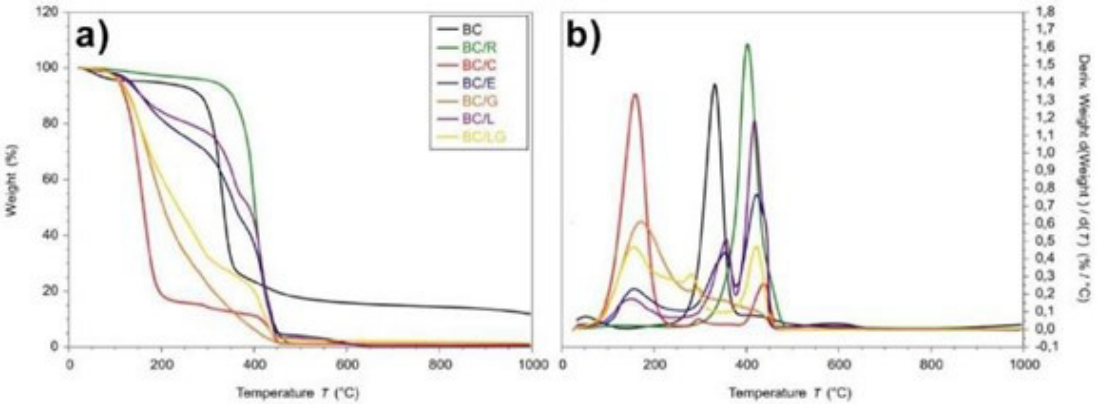

Figure 3. Curves (a) TG and (b) DTG of the samples.

Table 3. Sample thermal events obtained from the curves TG/DTG.

Sample	M1 (%)	TPEAK 1 (°C)	M2 (%)	TPEAK 2 (°C)	M3 (%)	TPEAK 3 (°C)	Residue (%)
BC	4.48	56	74.14	332	9.15	594	11.86
BC/R	3.13	115	95.46	401	0.92	560	0.43
BC/C	84.39	159	4.01	296	10.68	437	0.89
BC/E	26.37	155	30.19	353	42.79	423	0.40
BC/G	71.81	170	16.21	296	11.49	471	0.49
BC/L	19.37	156	28.66	355	51.37	417	0.44
BC/LG	51.42	153	22.01	282	25.21	424	1.40

stability. Thus, it is possible that the greater mass losses in the last stage of most samples, compared to the BC, may be due to the losses of these various compounds under high temperatures by evaporation and chemical decomposition[46].

3.4.3 High-resolution electron microscopy (SEM)

The morphologies of pure BC and incorporated BCs were observed by SEM (Figure 4).

The micrograph of pure BC (Figure 4a) shows the typical structure of this material, consisting of tangled nano and microfibrils arranged randomly and with pores[47,48]. The BC/R (Figure 4b) presented a more homogeneous and smooth surface, demonstrating that the EO filled the membrane pores. The morphology shows some points with roughness, particulate matter, and REO droplets on the membrane surface.

Similar morphology to that of BC/R was observed for samples of BC/G (Figure 4e), with regions of greater roughness and some reliefs, and BC/L (Figure 4f), where fibers can be seen at different points. BC/C (Figure 4c) and BC/LG (Figure 4g) samples maintained the tangled fiber structure of pure BC, however, with greater thickness and roughness. In this case, the EOs might have coated the BC fibers. Finally, the BC/E (Figure 4d) presented a region resembling the BC/R, BC/G and BC/L samples with a homogeneous surface; however, it also has a region with greater roughness. These two regions are separated by an interface with irregularities, which may be related to the difference in BC membrane thickness in these regions. The interaction between BC and added materials can occur through adherence (physical form) or by the hydrogen bonds between the hydroxyls of the BC with the added materials (chemical form)[49]. The interaction between hydroxyl groups causes a decrease in porosity and roughness[50]. This behavior was observed for BC/L, where it is possible to assume that there was an interaction between the linalool hydroxyl and the BC hydroxyls. The same was not observed for BC/C, where the interaction between BC and eugenol hydroxyl maintained the fibrillar morphology of the sample. The other samples do not have hydroxyls in the structure of their main components; in this case, it is assumed that the interaction between the BC and the EOs occurred by adherence.

3.5 Antimicrobial activity

Figure 5 shows the disc diffusion test plates with the inhibition zones formed by the samples of REO (R), CEO (C), EEO (E), GEO (G), LEO (L) and LGEO (LG) at 15%; and by BC samples incorporated with their respective EOs (BC/R, BC/C, BC/E, BC/G, BC/L and BC/LG) against *C. albicans, E. coli* and *S. aureus*, while the diameters of the inhibition zones of the samples with the statistical analysis are shown in Figure 6. Jiang et al.[51] demonstrated the antimicrobial activity of REO from *Rosmarinus officinalis* against *C. albicans, E. coli* and *S. aureus*. However, it is noteworthy that this same EO, when incorporated into BC (BC/R), presented a zone of inhibition with an average diameter of 8.67 mm against *S. aureus* (Figure 5c). These results suggest lower absorption of EO by the filter paper used in the test concerning the amount absorbed by BC, in addition to insufficient concentration to inhibit *E. coli* and *C. albicans.*

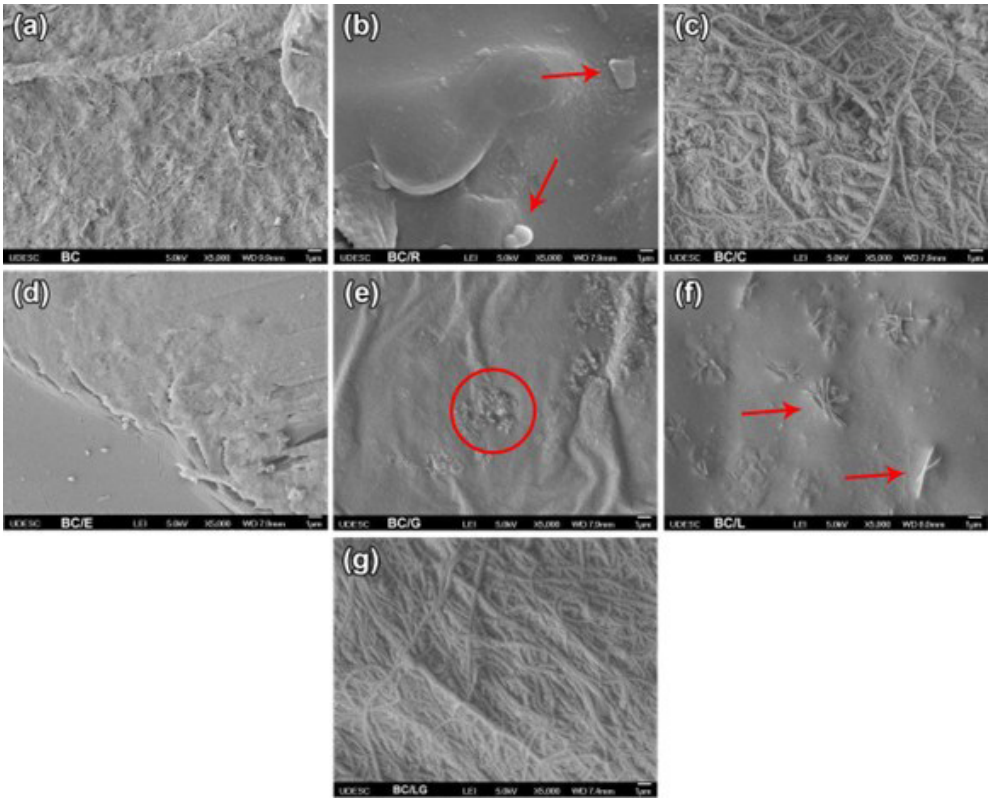

Figure 4. SEM micrographs (a) BC, (b) BC/R, (c) BC/C, (d) BC/E, (e) BC/G, (f) BC/L and (g) BC/LG.

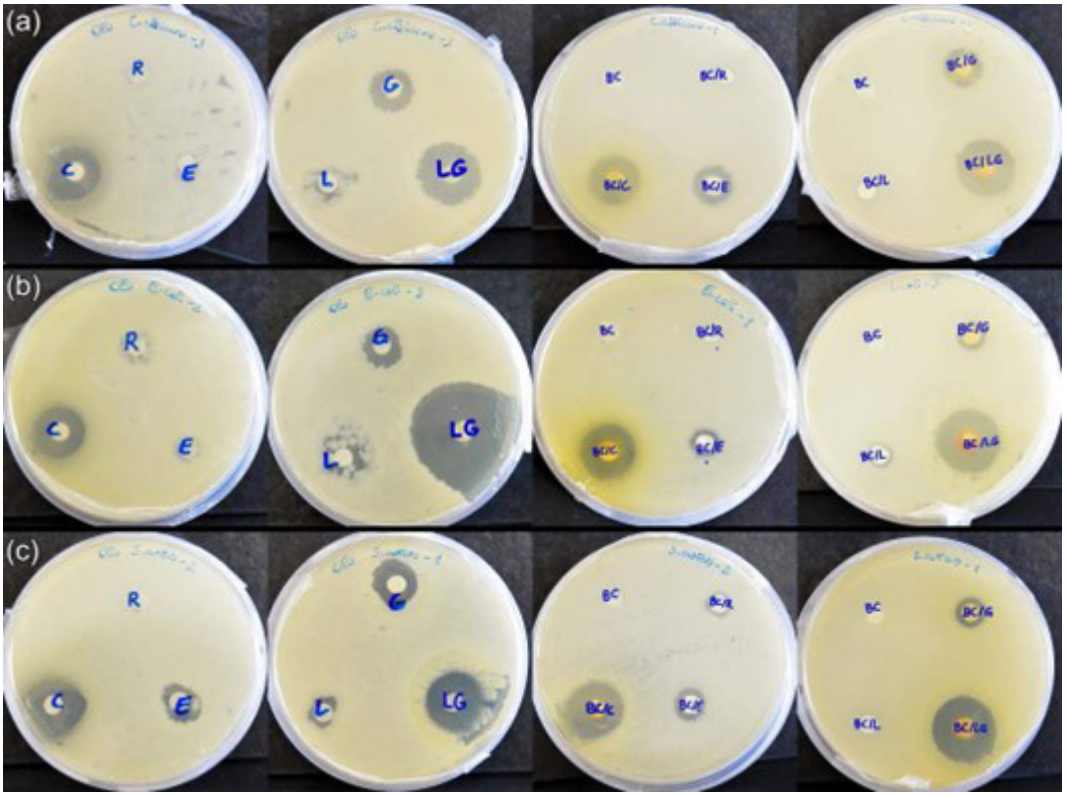

Figure 5. Disc diffusion test of the samples for (a) *C. albicans*, (b) *E. coli* and (c) *S. aureus*.

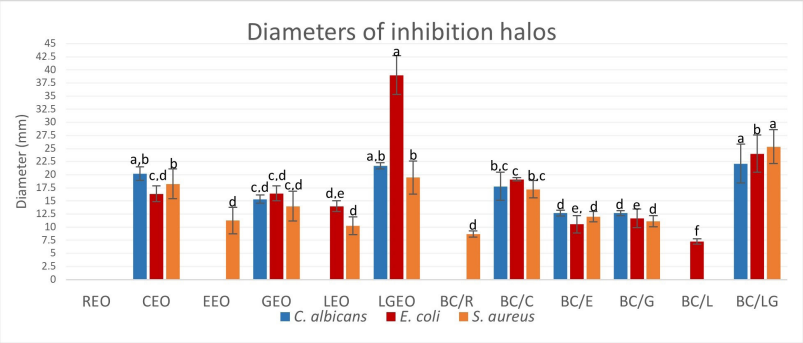

Figure 6. Diameters of inhibition zones (n = 3). The letters above the bars correspond to the statistical analysis by Tukey's Method with 95% confidence.

The literature[52-54] demonstrates the antimicrobial activity of EEO and LEO against *C. albicans, E. coli* and *S. aureus*. However, in this experiment, the samples showed irregular inhibition zones for *E. coli* and *S. aureus,* and there was no inhibition for *C. albicans* (Figure 5), which may be due to the low concentration of EO (15%), which was not enough to inhibit the yeast. Conversely, the BC/E sample had inhibition zones against all test microorganisms. The BC/L sample maintained antimicrobial activity against E. coli but with a decrease in the inhibition zone from approximately 14 mm (LEO on filter paper) to 7.25 mm (BC/L). The BC/R and BC/E samples showed a similar concentration of eucalyptol in their composition (item 3.2); however, the antimicrobial activities were different, which may be related to the different morphologies of these samples (item 3.4.3). In this case, the roughness of the BC/E may have favored the release of EEO constituents, causing greater inhibition of the test microorganisms. The same did not occur with BC/R, which had a smoother surface and filled pores, which may have hindered the release of antimicrobial compounds. On the other hand, CEO, GEO and LGEO showed inhibitory effects for all test microorganisms. With few exceptions (Figure 6), the membranes incorporated with these EOs had a slight decrease in the average diameter of the inhibition zones, suggesting minor incorporation or less diffusion of the microbial agents in these incorporated BCs. The literature proves the inhibitory action of these oils against *C. albicans, E. coli* and *S. aureus*[55-58]. The components of EOs, such as phenols and terpenes presented in GC-MS analysis, have hydroxyl groups that affect the cells of microorganisms by several mechanisms, which include disturbance of the enzymatic system, damage to the genetic material of the microorganism and reactions with the phospholipid membrane, resulting in the release of cell constituents and consequent death of microorganisms[6,59]. Nonetheless, the antimicrobial activities of EO depend on their chemical composition, which depends on several other factors (item 3.2), and, together with the type of antimicrobial test used and the strains of microorganisms used, can generate different performances in the study and validation of antimicrobial activity[60]. These factors explain the low performance of REO, EEO and LEO. However, the antimicrobial activity results of the samples were positive and demonstrated that the incorporation of EOs into the BC structure was effective, maintaining its antimicrobial characteristics in most cases.

The results obtained for the antimicrobial test are related to the results of the TPC analysis since the EOs that presented the highest TPC values had the greatest antimicrobial activity. In this case, samples with CEO showed higher TPC due to the presence of eugenol in its composition. However, the best result observed for antimicrobial activity was for samples with LGEO, demonstrating the inhibition capacity of terpenes (citral and geranial, for example), which were not significantly detected in the TPC analysis but were confirmed by GC-MS analysis. Thus, considering the compounds identified in the GC-MS, it is possible to state that the compounds with the most significant antimicrobial power were eugenol, present in CEO, and citral, present in GEO and in greater quantity in LGEO.

4. Conclusions

The EOs were successfully incorporated into the BC matrix, confirmed by GC-MS and FTIR analyses, which identified the compounds of the EOs in the incorporated BCs, and by SEM analysis, where it was possible to observe that CEO and LGEO coated the cellulose fibers, maintaining a morphology similar to the original. In contrast, the other EOs infiltrated the pores of the cellulose membrane, filling them and giving the samples a distinct morphology. The compounds of the EOs caused a reduction in the thermal stability of the BC, but this does not affect its use as a wound dressing since this application will not expose the polymer to high temperatures. CEO, GEO and LGEO showed the best antimicrobial activities due to the presence of eugenol and citral mainly. These characteristics were maintained after incorporation into BC membranes, demonstrating a synergistic effect obtained from the incorporated BCs that combined the intrinsic characteristics of BC with the antimicrobial properties of EOs.

5. Author's Contribution

- **Conceptualization** – Sandro Rogério Kumineck Junior.
- **Data curation** – Sandro Rogério Kumineck Junior.
- **Formal analysis** – Sandro Rogério Kumineck Junior; Giannini Pasiznick Apati.
- **Funding acquisition** - Ana Paula Testa Pezzin.

• **Investigation** – Sandro Rogério Kumineck Junior; Victória Fonseca Silveira.

• **Methodology** – Sandro Rogério Kumineck Junior; Ana Paula Testa Pezzin.

• **Project administration** – Ana Paula Testa Pezzin; Flares Baratto-Filho.

• **Resources** – Ana Paula Testa Pezzin; Flares Baratto-Filho; Andréa Lima dos Santos Schneider.

• **Software** – NA.

• **Supervision** – Ana Paula Testa Pezzin; Flares Baratto-Filho.

• **Validation** – Sandro Rogério Kumineck Junior; Victória Fonseca Silveira; Ana Paula Testa Pezzin; Denise Abatti Kasper Silva.

• **Visualization** – Sandro Rogério Kumineck Junior; Ana Paula Testa Pezzin; Michele Cristina Formolo Garcia; Denise Abatti Kasper Silva.

• **Writing – original draft** – Sandro Rogério Kumineck Junior; Ana Paula Testa Pezzin.

• **Writing – review & editing** – Sandro Rogério Kumineck Junior; Ana Paula Testa Pezzin; Michele Cristina Formolo Garcia.

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