ENHANCING TISSUE ENGINEERING: INCORPORATION ESSENTIAL OILS INTO BACTERIAL CELLULOSE MEMBRANES

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ABSTRACT

Bacterial cellulose (BC) is an extracellular linear homopolymer of glucose with great mechanical properties, such as porosity, high water content,as well as excellent biocompatibility. Due to these properties, BC is widely used in tissue engineering, and one alternative to enhance its biological properties is to incorporate essential oils into the BC membrane. *Copaifera officinalis* (Copaiba) oil is widely used in the cosmetic and pharmaceutical sectors due to its health benefits, including its antioxidant potential and tissue regeneration potential. *Mauritia flexuosa* oil (Buriti) is another essential oil of interest due to its beneficial adhesion properties and its ability to promote tissue regeneration, offering significant advantages in the wound healing process. Additionally, it is an economical and sustainable product. Thus, the objective of the study was to produce bacterial cellulose membranes incorporating essential oils extracted from copaiba and buriti plants, aiming to study cell viability using L929 cell line (mouse fibroblasts). CB membranes were produced by incorporating essential oils *in situ* and characterized by microscopy and cell viability assays. The results demonstrated that the membrane with copaiba essential oil showed higher cell viability and proliferation, suggesting a promising potential for applications in the biomedical field and tissue engineering.

Keywords: Murine fibroblasts. Copaiba. Buriti. Cell culture.

1 INTRODUCTION

Bacterial cellulose (BC) is a linear and extracellular homopolymer of glucose linked by β-1,4, synthesized by specific bacteria that attract researchers from various fields of knowledge such as biotechnology, pharmacy, and primarily tissue engineering[1]. BC possesses very interesting properties such as its excellent biocompatibility, water retention capacity, flexibility, mechanical properties, and a large number of hydroxyl groups (characteristics more similar to native tissues)[2] Furthermore, it is possible to further enhance the properties of BC by incorporating materials such as essential oils, including lavender, lemongrass, copaiba, geranium, ginger, and pink pepper, among others[3.] Essential oils are highly promising as natural actives to add properties to BC, such as anti-inflammatory, cell proliferation properties, among others[3]. *Copaifera officinalis* (Copaiba) is rich in sesquiterpenes, particularly β-caryophyllene, which gives it anti-inflammatory properties. Additionally, copaiba oil is widely used in the cosmetic and pharmaceutical sectors due to its aromatic properties and health benefits, including its antioxidant capacity and potential contribution to tissue regeneration[4].

Another essential oil with very interesting properties is *Mauritia flexuosa* (buriti), which contains significant amounts of carotenoids, vitamin E (alpha-tocopherol), and unsaturated fatty acids such as oleic and linoleic acids. These characteristics confer potential therapeutic benefits to buriti oil, evidencing effects that promote advantages in the wound healing process[5].

The objective of this study was to produce composite membranes of bacterial cellulose, incorporating essential oils from copaiba and buriti plants *in situ*. Simultaneously, the research aimed to conduct *in vitro* assays using the L929 cell line, comprising murine fibroblasts. These assays were conducted to evaluate the cell viability of the incorporated membranes and their cell proliferation capacity, thus providing relevant data and information about the potential and applicability of these materials for biomedical purposes and tissue engineering.

2 MATERIAL & METHODS

Production of bacterial cellulose (BC)

The bacterial cellulose (BC) membranes were produced utilizing the bacterium *Gluconacetobacter hansenii*, a gram-negative, non-pathogenic rod-shaped bacterium, strictly aerobic and known for producing cellulose membrane[6-11]. The culture medium used for BC production consisted of a Manitol formulation prepared with the following composition: peptone (0.6 g), yeast extract (1 g), mannitol (5 g) in 200 mL of distilled water. The pH of the medium was 6.5 without the need for additional adjustments. Subsequently, the culture medium was sterilized in a vertical autoclave for 20 minutes at 121 °C and 1 atm, ensuring its sterility and proper preparation for BC membrane production. For membrane production, an inoculum with *G. hansenii* bacteria was prepared, and in this study, the incorporation of copaiba and buriti essential oils was conducted. The incorporation method involved adding the essential oils during the membrane production process (*in situ*). For this purpose, membranes containing copaiba and buriti essential oils separately were produced. The concentration of oil in all productions was maintained at 2%.

After the inoculum production, the membranes were produced in 96-well ELISA plates and incubated in an oven at 37 °C for a period of 10 days. The produced membranes underwent a purification process using a 0.1M NaOH solution, where they were

kept at 50 °C for 24 hours. After purification, they were repeatedly washed with distilled water until reaching pH 7, and then sterilized in an autoclave for 20 minutes at 121 °C and 1 atm.

Cell viability

This study investigated the cell viability of the L929 cell line (murine fibroblasts) according to ISO 10993-5[10-9]. The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% Pen/Strep, with 50,000 cells plated on each membrane in petri dishes. After 4 hours of incubation at 37 °C for cell adhesion, DMEM medium was added, and the plates were kept in an incubator at 37 °C for 24 hours. Cell viability was evaluated at 1, 3, 6, and 9 days using the MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]. The membranes were incubated with the MTS reagent at 37 °C for 2 hours and 30 minutes, and the absorbance of the supernatant solutions was measured using spectroscopy at 490 nm.

Morphology

The morphology of cells cultured on the membranes was evaluated using optical microscopy at the predetermined times established in the study. For this purpose, the Olympus CX21 microscope with a 40X objective was used. This analysis allowed for the observation of morphological characteristics of the cells, such as adhesion and potential cell proliferation.

3 RESULTS & DISCUSSION

Cell viability

The main objective of the MTS assay was to evaluate the cell viability of cells in contact with pure membranes and membranes produced with essential oils *in situ*, investigating whether the incorporation of these oils influenced cell adhesion and proliferation. The results indicated that cell viability significantly increased after 24 and 72 hours in the presence of copaiba essential oil (CB-COP); however, after the 72-hour period, the sample showed no statistically significant difference compared to the control group (CB). Regarding the membrane incorporated with buriti essential oil (CB-BUR), there was no statistically significant difference in cell viability after 24 hours. However, after 72 hours, the sample exhibited a considerable decrease in cell viability, a trend that persisted until the ninth day of analysis (Fig.1).

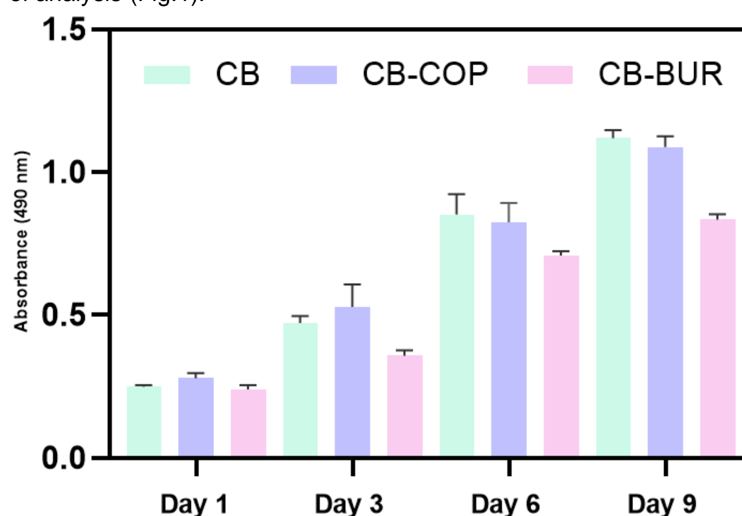

Figure 1 Cellular viability MTS assay on pure cellulose membranes (CB) and those incorporated with copaiba (CB-COP) and buriti (CB-BUR) essential oils, evaluated after 1, 3, 6 and 9 days of cultivation**.** The graphs were constructed using the GraphPad Prism 10 software[12].

Optical Microscopy

The membranes with adhered cells were cultured in DMEM medium in 48-well ELISA plates at the predetermined times. Images were captured using a 40X objective and processed in the ImageJ software. The results show that there was no cell spreading in the first days of analysis, indicating cell aggregation. However, it was possible to identify cell proliferation between 24 and 72 hours in the presence of copaiba essential oil (CB-COP), with a lack of proliferation in the membrane incorporated with buriti essential oil (CB-BUR) after 72 hours (Fig. 2e-f, j). On the sixth day of evaluation, the CB-BUR sample showed a significant difference compared to the pure samples and those incorporated with copaiba essential oil (CB and CB-COP), Indicating low cellular interaction with the biomaterial. Conversely, the CB and CB-COP samples showed similar morphologies (Fig. 2, c-g). On the ninth day, a complete change in cell morphology was observed in all samples, with particular emphasis on the low cell count in the CB-BUR sample (Fig. 2l).

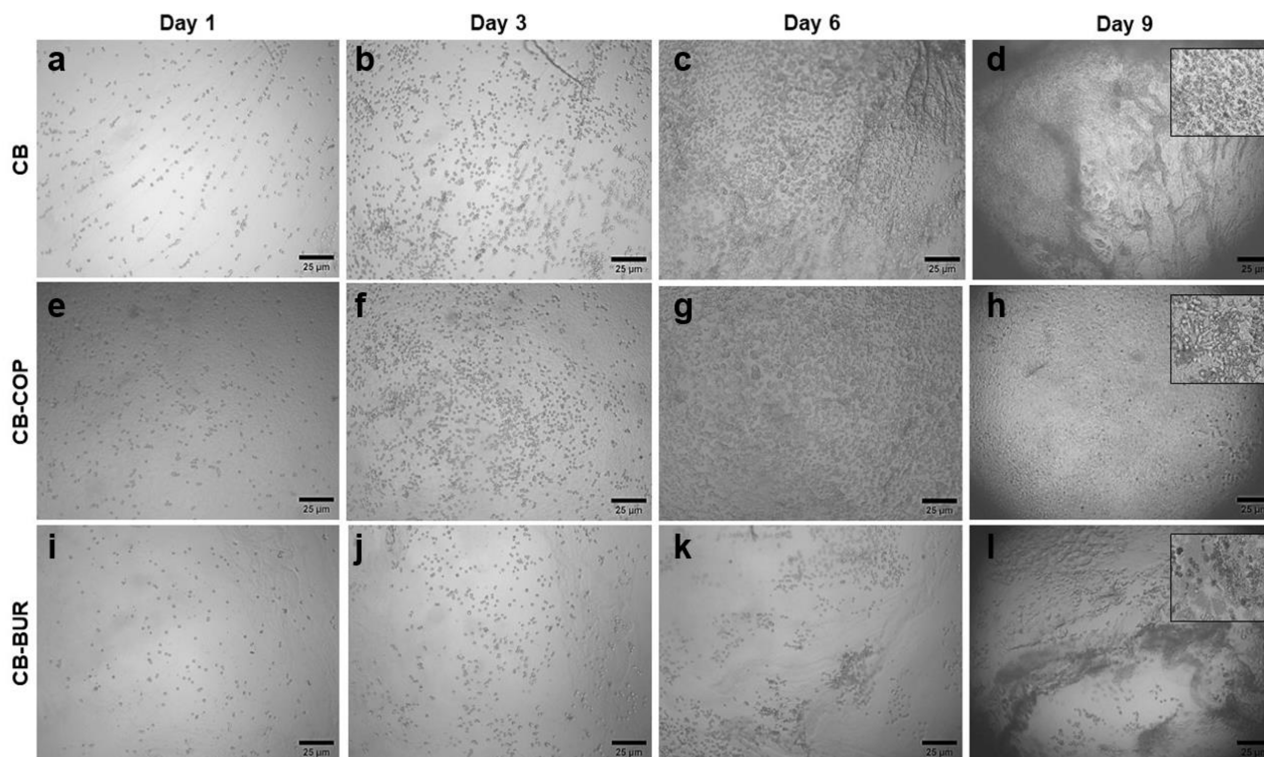

Figure 2 Optical microscopy of bacterial cellulose membranes**.** Pure CB (a, b, c, d), CB incorporated with copaiba essential oil (e, f, g, h), CB incorporated with buriti essential oil (i, j, k, l).

4 CONCLUSION

Based on the observations and quantitative data from the MTS assay, we can conclude that the membrane incorporated with copaiba essential oil (CB-COP) provides a favorable environment for cell adhesion, indicating cell-biomaterial and cell-cell interactions. On the other hand, the membrane incorporated with buriti essential oil (CB-BUR) showed significantly lower cell adhesion, especially after 6 and 9 days. This conclusion is supported by the cell viability data obtained from the MTS assay, which indicates that the CB-COP sample maintained stable levels of cell viability over time. In contrast, the CB-BUR sample showed a decrease in cell viability after the sixth day of observation. It is crucial to emphasize that none of the tested samples induced cellular toxicity, highlighting the biocompatible potential of the studied membranes. These results suggest that the *in situ* incorporation of copaiba oil has potential applications in tissue engineering and biomedical purposes. As future perspectives, collagen production in the produced membranes will be analyzed.

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