POLYLACTIC ACID (PLA) SCAFFOLD FOR CONTROLLED RELEASE OF ESSENTIAL OIL OF CANELO *DRIMYS WINTERI*: CONTROL OF PHYTOPATHOGENS IN FRUITS

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ABSTRACT

We have designed and prepared polylactic acid (PLA)-based porous support for the controlled release of essential oils from *Drimys winteri*, common name Canelo (CEO). The chemical composition of the essential oil was characterized by HS/GC-MS. The interaction with the PLA-based porous support was determined by FTIR-ATR spectrophotometry. Our results showed that the manufactured PLA support allows the controlled release of essential oils over time, at 21 °C. The PLA/essential oil support showed a non-contact antifungal activity against *B. cinerea*. In addition, pure compounds (standards) detected in the gas phase of CEO essential oil were independently studied to identify which of these molecules is responsible for the growth control action of the fungus. In conclusion, the PLA/CEO porous support is an alternative to protect against infections such as *B. cinerea*, offering a potential use of this strategy to preserve fresh functional foods in the post-harvest stage, as an atmosphere modifier.

Keywords: *Canelo (Drimys winteri) essential oil, PLA support, non-contact antimicrobial activity, Botrytis cinerea*.

1. INTRODUCTION

Polylactic acid (PLA) supports are ideal materials for biological applications [1]. It is used in 3D printing as a biodegradable thermoplastic [2], it is used to manufacture structures by fusion technique. It is a polymer with potential applications in biomedicine and bioplastic [3]. Porous PLA supports are mainly used as scaffolds for cell growth in bone and tissue repair [4]. The use of PLA support is associated with the manufacture of active containers that contain essential oils, manufacturing films with antimicrobial properties [5]. For this, essential oils, natural antibacterial compounds, antioxidants are used for the development of smart containers [6]. With the incorporation of these compounds in the polymeric matrix, the desired activity is enhanced as antimicrobial or antioxidant properties, however, the percentages of these additives must be low in the order of 1-9%, to preserve the mechanical and processability properties of the polymer [7].

Organic compounds such as carvacrol, clove essential oil, propolis, green tea extracts, thyme, mint oils, cinnamon, oregano, rosemary, limonene are incorporated [8]. The design of porous support allows to control parameters such as porosity, pore type and size [9]. The Techniques used for pore design are freeze-drying [10] and salt leaching [11]. Nowadays, scaffolds are prepared by 3D printing mainly for biomedical applications [12]. Substances are incorporated into these scaffolds for controlled release, such as drugs [13], antibacterial substances [14], and growth factors [15]. These additives do not affect the mechanical properties of the scaffold at low concentrations and provide flexibility to the material [16].

The use of essential oils for the control of pathogens such as the gray rot fungus *Botrytis cinerea* has been reported in literature [17]. However, the complete fraction is used by direct contact between the essential oil and the pathogen [18]. Microencapsulation of essential oils is an alternative to improve the efficiency of oil release by contact [19]. Authors such as Niu Xiaodi [20] postulate that the best way to control the pathogen is the use of essential oil without contact with the product, that is, they use the volatile fraction of the essential oil.

This study presents the results of the study of essential oil from the native cinnamon tree *Drimys winteri* (CEO), provided by the company AMPE spA and the results of tests against *B. cinerea* in a model without direct contact between the essential oil and the fungus. For this purpose, an experiment was designed to use the most volatile fraction of the essential oil for tests against the pathogen.

A porous PLA scaffold was used as support for the essential oil, which contains the essential oil. This support allows the controlled release over time of the volatile compounds of the essential oil. The essential oil was characterized using HS-GCMS, static headspace for the detection of volatile compounds. Based on the results of the analysis, the main compounds in the most volatile fraction were identified. With this information, tests were carried out without contact with B. cinerea, between the essential oil and the pathogen. The minimum inhibitory concentration MIC was determined.

A second aspect explored in this research is to clarify whether any of the molecules present in high concentrations in this complex mixture [21], are responsible for controlling the fungus. For this, high purity standards of molecules detected in the volatile fraction were used and the tests were repeated only with these compounds. In this way we can determine whether the action against the pathogen is collaborative between molecules or of a key molecule.

2. MATERIALS AND METHODS

High purity CEO was obtained from the company AMPE Spa., located in Puerto Montt, Chile, (41°35'48.5"S, 72°42'20.6"W), isolated by steam distillation of fresh leaves of the tree. The pure oil was analyzed with HS/GC-MS using a gas chromatograph coupled to an Aguilent 7890B-5977A mass spectrometer and equipped with an Aguilent 7697A Headspace autosampler. The capillary column used is an HP-5 MS UI of (60 m x 0.25 mm x 0.10 µm film thickness). The sample is introduced into 20 mL vials which are heated at 140 °C for 15 minutes. Then, the gas phase is injected into the GC-MS. Injection time is 1 minute. The initial oven temperature of the column is 80 °C for 5 minutes, and then it was raised to 160 °C at 5 °C x min-1 for 5 minutes. Then, it was heated to 300 °C for 10 °C x min-1. The ionization energy was 70 eV. Fragments with masses of the order of 25 to 450 m/z were recorded. Volatile compounds were identified based on comparison of their retention indices and mass spectra from an online database of the National Institute of Standards and Technology library (NIST). The compounds were quantified using the relative areas of the maximum and the areas of the internal standards.

The preparation of the porous Polylactic Acid (PLA) supports (NatureWorks, Ingeo™ Biopolymer 2003D) is carried out based on (Pankongadisak P. et al., 2024). PLA is dissolved in dichloromethane (20% w/v) with magnetic stirring (CIMAREC, BARNSTEAD, USA). The diameter of sieved NaCl particles p.a. (MERCK) is 100 to 250 µm. These were added to the previously prepared PLA

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solution at (60% w/v). Pour it into a 20 x 30 cm Teflon-coated mold. The solvent then evaporated at room temperature for 5 days. The salt particles are dissolved in ultrapure water for 2 days and the water is changed every 12 hours. After the NaCl leaching step, the porous support is dried at 50°C for 48 hours. The morphology of the support is characterized using a scanning electron microscope, SEM (Jeol JSM-6610LV, USA). The porosity of the PLA support was calculated using the method described in the literature [22].

ATR-FTIR analysis was performed on a spectrophotometer (Shimadzu IRAffinity-1S, Japan) with QATR-10 diamond crystal accessory. All spectra are performed in the range 3600 to 400 cm^{-1} and the average of 40 scans is reported. The resolution used is 4 cm^{-1}

The PLA supports were sized in discs with a diameter of 2 cm and a thickness of 3.0 ± 0.25 mm. To determine the amount of oil that is released from the PLA supports at 21°C, a gravimetric analysis was performed. Using an analytical balance (Sartorius, M-Power AZ-214, 0.1 mg, USA), the essential oil was incorporated into the porous support using a 100 µL micropipette (ONILAB, 100 µL, China). The amount of essential oil released as a function of time is reported in relative % and calculated as described by Naidu [23].

A *B. cinerea* isolate was selected to evaluate the non-contact antimicrobial activity of the PLA/CEO porous support. A strain with strong spoilage potential was previously isolated and identified from rotting blueberries [24]. *B. cinerea* was incubated in 5 ml of PDA at 28 °C with shaking for 24 hours. The strain suspension was diluted in freshly prepared PDA to 1x 10^{5} CFU/mL. The isolate was grown on potato dextrose agar (PDA) at 28 °C. Spore suspensions were prepared at the final concentration of 1x10^{6} conidia/mL. All antimicrobial assays were carried out with the same diameter of *Botrytis cinerea* inoculum (5 mm), in 15 mL of PDA agar. Additionally, all plates had a porous PLA backing adhered to the inner center of the top lid with double-sided adhesive tape. A positive control of tea tree oil was used [25]. Each test was performed in triplicate. MIC and MFC values are reported in (µL/mL) where the volume of air corresponds to that of the Petri dish volume (80 mL).

The MIC and minimum fungicidal concentration (MFC) of Canelo essential oil (CEO) incorporated into the PLA support against *B. cinerea* were determined as described by Tian [26] with some modifications. To determine MFC, PDA samples were extracted without observing visible B. cinerea growth and inoculated onto potato dextrose agar (PDA) at 28 °C for 72 h. A porous PLA support was used with a specific volume of CEO. The volumes for CEO are: 1, 5, 10, 25, 50 µL. This porous support is attached onto the upper dish lid of the petri capsule. In the lower lid, a 5 mm diameter disc of *B. cinerea* is inoculated in the center of the capsule containing PDA agar (Figure 1). The plates were sealed with parafilm and incubated at 21°C for 7 days. The control treatment is similar except the PDA/*B. cinerea* plate has a porous support without essential oil. The diameter of the *B. cinerea* colony was measured during the 7 days.

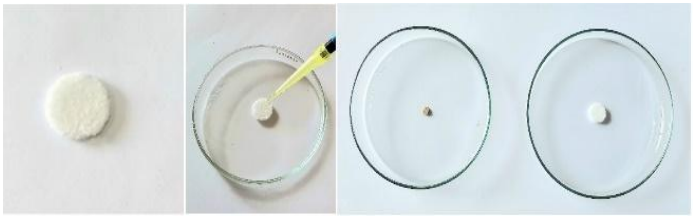

Figure 1. Experimental setup for testing the release of essential oils from porous PLA supports for the release of volatiles without direct contact between the essential oil and the fungus *B. cinerea*.

The assays were performed in triplicate. Data was analyzed using SPSS 22 software. A non-parametric test for multiple comparisons (Kruskal-Wallis) was used. Significant differences between values were determined using Duncan's multiple range test ($p < 0.05$), graphs were constructed using Origin 2018.

3. RESULTS

The analysis of the CEO by HS/GC-MS gas chromatography-mass spectroscopy allowed to identify 14 compounds of CEO and its 3 main ones represent 96.43% that correspond to: α-Pinene (58.47%), the (-)-β-Pinene (36.94%) and (+)-Limonene (1.02%).

The supports are prepared by dissolving 21 g of PLA in 100 mL of CH_2Cl_2 at room temperature with continuous stirring, 105 g of NaCl are added with a particle size ranging from 100 to 250 µm. Stirring is carried out for 30 minutes, then pouring into a 20 x 30 cm Teflon-coated mold. Drying is carried out at room temperature for 5 days. After drying, the salt particles are dissolved in deionized water for 2 days and the water is changed every 12 hours. After leaching of the NaCl, the porous support is dried at 50°C for 48 hours, obtaining a porous sheet with an average thickness of 3.0 ± 0.25.

Table 1. Chemical composition of the CEO *Drimys winteri* leaves essential oil phase gas.

Peak	RT	Compound	% (Relative area)	Identification
1	9.79	*Cyclofenchene*	0.14	MS
2	9.80	*α-Thujene*	0.15	MS
3	10.26	***α-Pinene***	58.47	MS
4	10.79	*Camphene*	0.50	MS
5	11.82	***(-)-β-Pinene***	36.94	MS
6	12.48	*β -myrcene*	0.67	MS
7	12.87	*α -phellandrene*	0.50	MS
8	13.06	*3-Carene*	0.32	MS
9	13.31	*α-Terpinene*	0.15	MS
10	13.60	*β-Cymene*	0.34	MS
11	13.72	***(+)-Limonene***	1.02	MS
12	13.78	*Eucalyptol*	0.51	MS
13	14.78	*γ-Terpinene*	0.17	MS
14	15.75	*(+)-4-Carene*	0.15	MS

RT- retention time (min); % - considering detected compounds; MS - mass spectra. Compounds written in bold correspond to the most abundant compounds detected in phase gas the essential of *Drimys winteri* oil (CEO).

The morphology of the support is characterized by using a scanning electron microscope (SEM) Jeol JSM-6610LV (Figure 2).

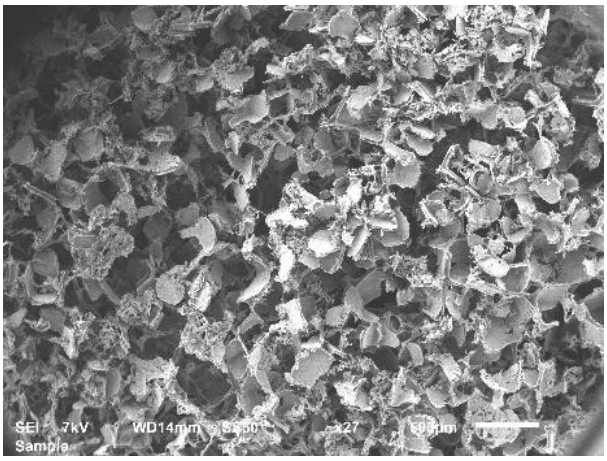

Figure 2. Micrograph of porous PLA supports at x 27 magnification.

The porosity of the PLA scaffold was calculated using the method described in the literature [27]. This method uses the displacement of a liquid to determine the porosity. Hexane is used as a non-wetting liquid 5 measurements are taken and averaged and reported as a percentage of porosity. The average result for this support is 85.33 ± 4.20 % porosity for PLA.

The characterization was performed using a Shimadzu IRAffinity-1S FTIR-ATR spectrophotometer, with a QATR 10 accessory. The results presented in (Figure 3) show the prepared porous PLA support, and the PLA support with CEO.

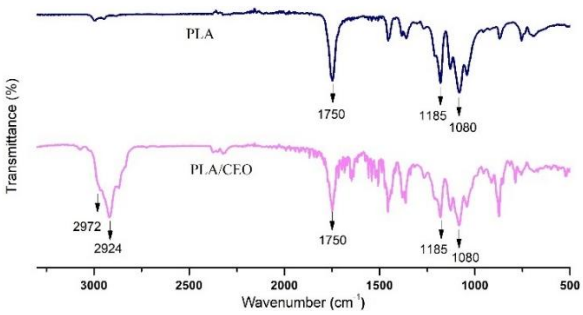

Figure 3. FTIR-ATR spectral analysis of porous PLA and PLA/CEO supports containing *Drimys winteri* CEO essential oil.

The signals appearing at 1750 cm^{-1} corresponding to C=O stretching vibrations of the ester group, the bands at 1185 and 1080 cm-1 corresponding to stretching of the -C-O- bond of the -CH-O- and -CO-O- groups respectively [28]. In the case of PLA/CEO, the PLA support vibrations already observed, in addition to characteristic vibrations of the majority compounds identified in the mixture, such as alpha-pinene and beta-pinene, vibration at 2972 and 2924 cm^{-1} corresponding to stretching and deformation of the methyl and methylene groups [29].

The mass loss tests due to evaporation as a function of time, of the CEO oil, supported on PLA sponges (Figure 4), the tests are carried out at a constant temperature of (21°C), constant oil volume of (100 µL).

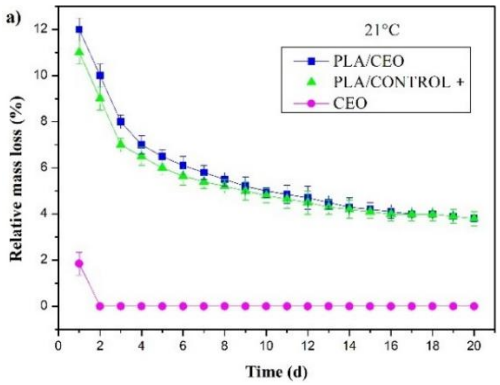

Figure. 4. Evaporation mass loss for PLA/CEO support; PLA/Control + and CEO on glass, at 21°C.

It is observed that the loss of mass due to evaporation of the essential oil for day 1 is 88% due to evaporation, then on day 2 to 20 a control in the mass loss is observed, this allows a controlled release over time.

The objective of the in vitro tests is to demonstrate that the porous PLA supports work for the controlled release over time of CEO essential oil with fungal activity against the fungus *B. cinerea*.

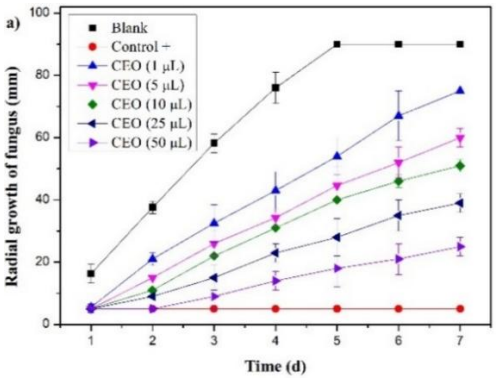

Figure 5. Effect of volatile compounds of PLA/CEO on the growth of *B. cinerea*. Non-contact assay at different concentrations of CEO.

A decrease in the growth of *B. cinerea* is observed as the volume of CEO increases. The greatest decrease in growth is observed at a volume of 50 µL of the essential oil. At this concentration, no MIC is observed for the non-contact assay.

The MIC for CEO essential oil without contact was determined at 250 µL per assay, corresponding to 3.125 µL/mL. However, it was not possible to determine the MFC, since the assays to determine its botrycidal action were not positive at concentrations above 250 µL.

The micellar growth of the *B. cinerea* strain was studied with pure standards present in Canelo essential oil CEO. Two aliquots of 10 and 25 µl were used on the PLA scaffold to evaluate the inhibition capacity of *B. cinerea*. Linalool, α-pinene, β-pinene and Eucalyptol were analyzed (Figure 6). It was identified that the linalool standard inhibited micellar growth. Significant differences in micellar growth were observed increasing to concentrations of 10 µl, observing significant differences only between the means of the standards ($F_{(6,324)} = 284.4$; $P = 0.001$) (Figure 6). An increase in the growth of B. cinerea over the blank was observed in the presence of α-pinene and β-pinene when a 10 µl aliquot was applied between days 3 to 5, stimulating micellar growth. Regarding the effect of the concentrations on the growth of the mycelium in the different treatments, significant differences were observed ($F_{(4,324)} = 594.5$; $P = 0.001$), where micellar growth was lower in the concentrations of 25 µl in α-pinene, β-pinene and Eucalyptol. It was observed that the Eucalyptol standard has a fungistatic capacity against B. cinerea, delaying its growth until the 6th and 7th day to complete the Petri dish.

A significant inhibition of micellar growth was observed in the test with Linalool at 10 µl, with significant differences compared to the other concentrations ($F_{(56,324)} = 3.4$, $P = 0.001$). It was observed that at 5 µl the volatiles of linalool present fungistatic capacity of the B. cinerea strain, but when evaluating its fungicidal capacity, it showed growth in PDA culture medium on the second day of exposure.

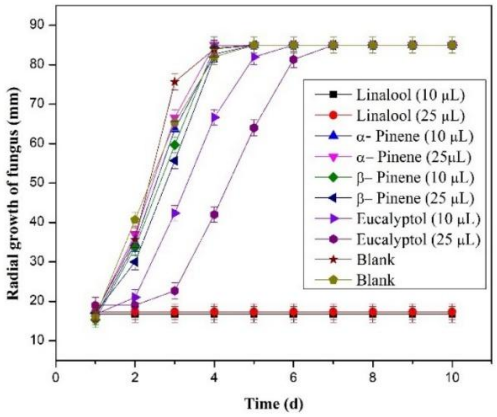

Figure 6. Effect of volatile compounds of PLA/standard on the growth of *B. cinerea*. Non-contact assay at different concentrations of standard.

4. DISCUSION

Regarding the characterization of the volatile fraction of the CEO essential oil, 14 compounds were identified, 3 of them in higher quantities, these will be tested in pure form as a standard in the test against *B. cinerea*. The porosity of the PLA scaffold prepared by salt leaching is determined at 85.33 ± 4.20 %, its morphology presents open and interconnected pores, which allows it to be used as a support for essential oil. The tests of mass loss of the essential oil by evaporation at 21°C show us that a high percentage of the oil is lost on the first day, however, on the second day, evaporation stabilizes, and a controlled release occurs. This behavior is similar for the CEO as for the essential oil of the tea tree (positive control) [30].

The non-contact tests of the essential oil of Canelo *Drimys winteri* CEO against *B. cinerea* showed a moderate action in the control of the pathogen, forcing an increase in the amount of essential oil to achieve its control. It can be observed in Figure 5 that as the volume of CEO essential oil increases, the growth halo is reduced, generating inhibition in its growth.

Using the same experimental conditions, pure compounds (standards) detected in the volatile fraction of CEO were tested by static HS-GC-MS analysis. The compounds Linalool, α-pinene, β-pinene, and eucalyptol were tested, testing volumes of 10 and 25 µL. It is observed that on the third day of testing, the halo begins to grow again, however, the compound Linalool surprises with a high action of controlling the growth of the fungus, which does not show growth during the test.

CONCLUSION

In summary, the porous PLA scaffold allows time-controlled release of CEO. Release evaluation studies of the PLA/CEO scaffold using the inverted Petri dish method showed controlled release of CEO and inhibition of *B. cinerea* growth over the time of the experiment. FTIR-ATR verified the presence of active CEO compounds on the surface of the PLA scaffold. SEM studies showed the morphology of the prepared porous PLA scaffold. Antimicrobial testing demonstrated that the PLA/CEO scaffold allows slow release of antifungal volatiles over time, extending the antifungal efficacy of CEO at high concentrations. Non-contact treatment of PLA/CEO showed moderate antifungal effects at low concentrations. The use of this type of PLA/CEO scaffolds with high concentrations of essential oil is a possible viable strategy for long-term food preservation for use as an atmosphere modifier in fruit preservation packaging.

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