



Bioactivity and action mechanism of green propolis against *Pythium aphanidermatum*

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Abstract: We have established how natural compounds from green propolis collected by the species *Apis mellifera* act against the growth of *Pythium aphanidermatum*. On the basis of mass spectrometry (Q-ToF MS), we determined that Artepillin C, the major constituent of green propolis, underlies the effect and displays activity against *P. aphanidermatum* at a minimal inhibitory concentration of 750 $\mu\text{g}\cdot\text{mL}^{-1}$. Biophysical studies based on model membranes showed that this inhibitory effect may be linked with a membrane-related phenomenon: Artepillin C increases the permeability of membranes with relatively high fluidity in their lateral structure, a feature that is in line with the lipid composition reported for the cytoplasmic membrane of *P. aphanidermatum*. Therefore, the present study supports the use of the effective and inexpensive green propolis to control the impact of the dangerous phytopathogen *P. aphanidermatum* on agriculture.

Key words: green propolis, Artepillin C, fungicide, *Pythium aphanidermatum*, model membranes.

INTRODUCTION

Brazilian food security and environmental sustainability are globally important. Brazil is one of the three largest world producers and exporters of sugar, coffee, orange juice, soybean, beef, tobacco, ethanol, and broiler chicken. This country

has one of the greatest biodiversity reserves in the globe as well as several farmers with strong entrepreneurial capability (Pereira et al. 2012, The Economist 2012). According to the latest report by the Brazilian Association of Collective Health (ABRASCO) (Carneiro et al. 2015), economic growth has encouraged the use of pesticides to expand cultivated land. Between 2000 and 2012, this practice has increased by more than 162 percent in Brazil. About 16% of the pesticides employed

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in the Brazilian agriculture consist of fungicides, which pose many problems related to antimicrobial resistance (Dasgupta et al. 2001, Price et al. 2015). Furthermore, overexposure of the population to the synthetic chemicals present in pesticides affects the human health negatively – such chemicals are toxic and highly persistent in the environment, and they can enter the food chain (Kaur and Garg 2014).

In an attempt to diminish the environmental impact of currently available fungicides, many researchers have been searching for naturally occurring bioactive compounds that act differently from commonly known antifungals. Propolis is a resinous product consisting of compounds that bees collect from the vegetation; e.g. phenolic acids, terpenoids, caffeic acids, and flavonoids (Carvalho et al. 2011, Franchin et al. 2018). These compounds are processed by the salivary enzymes of the bees, to protect their colonies against the attack of pathogens (Bonvehí and Gutiérrez 2012, Carneiro et al. 2015, Mirzoeva et al. 1997). Depending on the region where the bees collect the propolis constituents, they might display different repertory of biological actions. For instance, it is believed that terpene-rich propolis comes from *Araucaria* species, since several terpene derivatives are found in their resins, acting in a defense response against bark beetles, fungi, bark wound sealing, disruption of insect development and attraction of herbivore predators (Bankova et al. 1998, Perotti and Fett-Neto 2015).

Among all the types of Brazilian propolis, the propolis produced by the species *Apis mellifera*, known as green propolis and collected from *Baccharis dracunculifolia* plant exudates, is one of the most studied worldwide. This propolis has many pharmacological properties, such as antibacterial, antifungal, and antioxidant actions (Bastos et al. 2008, Lotfy 2006, Pazin et al. 2017, Sawaya et al. 2009). However, the agricultural benefits of green propolis remain to be explored. Studies have shown that phytochemical compounds like the ones found

in green propolis can interact with and modify the structure of the biomembranes of target cells, leading membrane proteins to lose their functions and reflecting a large repertoire of biological effects (Rempe et al. 2017).

Oomycetes comprise several genera that contain important plant pathogens. For example, *Pythium* spp. includes a group of pathogens that damage crops to a large extent, leading to loss of millions of dollars (Rai et al. 2018, van West and Gow 2003). *Pythium aphanidermatum* is an aggressive false-fungal species that is highly resistant to fungicides and which causes pre- and post-emergence damping off in vegetable crops such as potato, tomato, beet, cucumber, and pepper (Garibaldi et al. 2013, Owen-Going et al. 2003, Postma et al. 2013, 2000, Sabbagh et al. 2018, Strange and Scott 2005). Rapid germination of sporangia followed by immediate infection makes management of Oomycetes very difficult (Nelson 1987, Osburn et al. 1989, van West et al. 2003, Whipps and Lumsden 1991). Vegetables and field crops affected by *Pythium* diseases are considered an important limiting factor to the successful cultivation of crop plants worldwide.

The full potential of the biological control of Oomycete pathogens has not been explored yet. Oomycetes differ from fungi in various aspects, including the absence of ergosterol in the lipid composition of the cytoplasmic membrane. This feature makes the application of some fungicides to combat Oomycetes useless: most fungicides, like the azole family of antifungals, act by interacting with or inhibiting ergosterol (Cheng et al. 2004).

In this study, we report observation of the deleterious effect of green propolis against *P. aphanidermatum*. We have identified the major constituent of this natural product, Artepillin C, and we have drawn conclusions on the action mechanism of this bioactive compound. We have analyzed how Artepillin C interacts with model membranes that mimic fungal and Oomycete cytoplasmic

membranes. To the best of our knowledge, this is the first report that has suggested the potential use of green propolis as an anti-phytopathogen compound against *P. aphanidermatum*.

MATERIALS AND METHODS

SOURCE AND COLLECTION

Green propolis was collected from colonies of *Apis mellifera* honeybees (March, 2012) located in Bambuí (20°1'0''S, 45°58'0''W - Minas Gerais State, Brazil). The ethanolic extract of propolis (EEP) was obtained by adding 300 g of crude propolis (compound + beeswax) extracted from the colonies to 1000 mL of ethyl alcohol (70%), which was followed by maceration in the dark for two months. After this period, the extract was filtered through Whatman filter paper, to give a hydro-alcoholic solution. Next, 40 mL aliquots of this solution were collected and placed in 50 mL wide-mouth flasks. The open flasks were transferred to an oven to eliminate the alcohol. After stabilization of the resin mass, the flasks were sealed and kept at -20 °C for further use.

BIOGUIDED FRACTIONATION

Initially, 1.0 g of EEP was dissolved in methanol/water (90:10, v/v) and subjected to liquid/liquid partitioning with 150 mL of hexane (three times), to afford 165.95 mg of hexane extracts. Then, 57 mL of water was added to the remaining partition (methanol/water, 90:10, v/v), to make it 70:30 (v/v) methanol/water. Next, the latter solution was partitioned with 150 mL of chloroform (three times), to yield 762.36 mg of chloroform extracts. The remaining methanol was removed by rotary evaporation, which was followed by addition of 100 mL of ethyl acetate, to afford 66.27 mg of ethyl acetate extracts. The water in the extract was eliminated by rotary evaporation, to give 5.42 mg of extracts. The chloroform extracts were separated on a 30 cm x 5 cm Silica gel (Merck) column and

eluted at 1 mL/min by using solvents of increasing polarity (hexane; hexane/dichloromethane [50:50 and 25:75]; dichloromethane; dichloromethane/methanol [75:25; 70:30; 60:40; 50:50]). The fractions were monitored by thin layer chromatography (TLC) on 10 x 10 cm Alugram silica gel plates (Macherey-Nagel) developed with dichloromethane/hexane/methanol (65:30:5, v/v/v). After the plates were developed and dried, they were visualized under UV light at 254 and 365 nm, and then similar fractions (10 mL each) were combined, to afford eight major fractions. The mass yield of each fraction was determined, and their biological activities (at concentrations of 5 mg.L⁻¹) were tested against the phytopathogen *P. aphanidermatum*.

BIOLOGICAL ACTIVITY

The EEP was assayed against the phytopathogen *P. aphanidermatum*. The tested microorganism was obtained from the EMBRAPA Culture Collection of Microorganisms of Agricultural and Environmental Importance. The antimicrobial activity of EEP was evaluated by the agar cup disc diffusion method (Bauer et al. 1966). To this end, 5 mg.mL⁻¹ EEP and its bioactive fractions were tested against *P. aphanidermatum* until the bioactive compound was found. After that, the bioactive compound was tested against the microorganism at concentrations ranging from 50 to 10 mg.mL⁻¹. More specifically, each disc containing a total volume of 10 µL of the active compound solution diluted in methanol was incubated at 28 °C for four different times (17, 20, 24, and 30 h). The minimal inhibitory concentration was defined as the lowest concentration of the compound that maintained inhibition of microorganism growth at 30 h. Culture medium grown without the addition of the tested substance served as negative control. The experiments were performed in triplicate.

Q-ToF MS ANALYSIS OF THE PROPOLIS FRACTION SAMPLES

Samples containing the propolis fractions were directly infused into a hybrid quadrupole orthogonal time of flight (Q-ToF) mass spectrometer, Synapt, Waters. Electrospray Ionization (ESI) in the negative mode was used as the ionizing source, and the software used for acquisition control and data treatment was MassLynx, version 4.1 (Waters). The capillary and cone voltages were set to 3,000 and 20 V, respectively. The nebulization gas was maintained at a flow rate of 500 L.h⁻¹ and at a temperature of 450 °C. The cone gas was maintained at a flow rate of 20 L.h⁻¹, and the source temperature was set to 105 °C. MS data were collected for *m/z* values ranging from 70 to 1,000 Da. The scan time was 0.5 s, and the inter-scan delay was 0.02 s over an analysis time of 13 min. The [M-H]⁻ ion of the Artemillin C standard arose at *m/z* [299.1646] for the syringe pump operating at 5 µL.min⁻¹. All the MS data were collected with the LockSpray system to ensure mass accuracy and reproducibility.

VESICLE PREPARATION AND CONFOCAL FLUORESCENCE MICROSCOPY

Giant Unilamellar Vesicles (GUVs), used as model membranes, were prepared by the electroformation method developed by Angelova and Dimitrov (Angelova and Dimitrov 1986). A homemade temperature-controlled chamber was used as described previously (Husen et al. 2012). Briefly, 4 µL of the desired lipid stock solution (0.2 mg.mL⁻¹) in chloroform was spread on each platinum electrode. The chamber was then placed under vacuum for at least 1 h, to remove any remaining trace of organic solvent. To perform leakage studies, fluorescent dyes were incorporated into the lumen of the GUVs according to Ambroggio et al. 2005a, b. For this purpose, solutions of sucrose/AlexaFluor-488 10 µM with an overall osmolarity of 200 mOsM were equilibrated to temperatures above the lipid phase transition and added to the chamber covering the platinum electrodes (0.4 mL

final volume). The platinum wires were connected to a function generator (Digimess FG 100) after addition of the buffer, and a low-frequency alternating field (sinusoidal wave function with a frequency of 10 Hz and amplitude of 1 V) was applied for 120 min. The AC field was turned off, and the GUVs were harvested from the chamber. To remove the fluorescent solution from outside the vesicles, the GUVs were washed several times with an iso-osmolar solution consisting of glucose in 15-mL centrifuge tubes. Aliquots of GUVs suspended in glucose (0.3 mL) were added to an eight-well plastic chamber (Ibidi, Munich, Germany). Due to the different densities of the sugar solutions inside and outside the vesicles, the GUVs precipitated at the bottom of the chamber, which facilitated observation in the inverted confocal microscope (Ambroggio et al. 2005a, b). The chamber had been placed in an inverted confocal microscope (Zeiss LSM 510 META, Jena, Germany) for observation. The excitation wavelength was 488 nm for AlexaFluor-488. Either Artemillin C methanol solution or pure methanol 1% (v/v) was injected into the chamber, and a time-series scan was initiated to monitor the GUVs. The experiments were performed in triplicate. After the injections, several vesicles were monitored as a function of time in each experiment. All the leakage experiments were analyzed by taking the change in fluorescence intensity as the average gray value measured for each micrograph of AlexaFluor-488 inside the GUVs. The ImageJ software was used for this measurement. The negative control comprised samples without Artemillin C.

RESULTS

BIOLOGICAL ACTIVITY OF PROPOLIS COMPOUNDS AGAINST *P. aphanidermatum*

First, we investigated whether the ethanolic extract of propolis (EEP) inhibited the phytopathogen *P. aphanidermatum* and verified good activity. We then used a bioassay-directed fractionation to separate the EEP molecules that were bioactive

against the phytopathogen. Compounds separated in chloroform were the most active against *P. aphanidermatum*. The flowchart in Figure 1 illustrates the bioguided assay processes.

The chloroform fraction, which was the most active against the target phytopathogen, was further separated on a chromatographic column, to afford eight subfractions. These subfractions were also assayed against *P. aphanidermatum*. Q-ToF MS indicated the presence of a major compound [M-H], m/z 299.1641, in the bioactive fraction, identified as Artepillin C.

Artepillin C at concentrations ranging from $50 \mu\text{g}\cdot\text{mL}^{-1}$ to $1 \text{ mg}\cdot\text{mL}^{-1}$ was tested against *P. aphanidermatum*. Solutions of the compound were placed into agar cup discs containing the phytopathogen. Analysis performed after 17 hours indicated resistance to *P. aphanidermatum* diffusion near the region between 50 and $125 \mu\text{g}\cdot\text{mL}^{-1}$. Three hours later, only regions with concentrations higher than $250 \mu\text{g}\cdot\text{mL}^{-1}$ were able to restrain the growth of *P. aphanidermatum*. This activity persisted for up to 24 hours for the concentrations 250 and $500 \mu\text{g}\cdot\text{mL}^{-1}$ and for up to 30 hours for concentrations above $750 \mu\text{g}\cdot\text{mL}^{-1}$, defined as the minimum inhibitory concentration (Figure 2).

INTERACTION OF ARTEPILLIN C WITH MODEL MEMBRANES

Confocal fluorescence microscopy was used to investigate permeability changes in model membranes. We are aware that DOPC-GUVs have a simple composition, but they offer a plausible model that represents the major class of lipid species present in an Oomycete cytoplasmic membrane; i.e., DOPC-GUVs contain high levels of unsaturated lipids but no sterols. We have also tested another GUV composition containing sterols in an attempt to mimic fungal membranes. More specifically, we used a mixture of DOPC, DPPC, and cholesterol at a molar ratio of 23:47:30. In this latter model, liquid ordered and liquid disordered

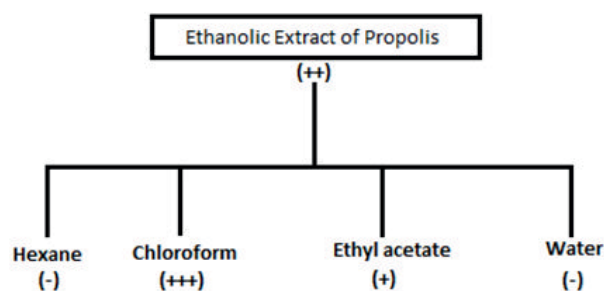


Figure 1 - Representative flowchart of the fractionation process of the ethanolic extract of propolis (EEP) with solvents of different polarities and activities of the fractions against *P. aphanidermatum*. (-) no activity; (+) weak activity; (++) good activity; and (+++) high activity against *P. aphanidermatum*.

phases coexist at room temperature (Husen et al. 2012), which implies a substantial increase in the membrane order parameter (or a decrease in the overall membrane fluidity). Figure 3 shows that addition of Artepillin C diminished the initial fluorescence intensity observed in the lumen of the DOPC- GUVs over time. This indicated that the dye leaked from the DOPC-GUVs to the outer solution, as previously observed in the case of antibiotic peptides (Ambroggio et al. 2005a, b). Hence, Artepillin C enhanced the permeability of the bilayer. Similar experiments performed with the GUVs consisting of DOPC/DPPC/Cholesterol (23:47:30 mol/mol) clearly showed that Artepillin C did not affect the permeability of the latter vesicles at all (Figure 3a, b).

DISCUSSION

The propolis produced by the species *Apis mellifera*, known as green propolis and collected from *Baccharis dracunculifolia* plant exudates has many pharmacological properties, such as antibacterial, antifungal, and antioxidant actions. Therefore, it is a potent natural product for the biological control of phytopathogens in agriculture. We observed that the ethanolic extract of propolis (EEP) presented activity against the phytopathogen *P. aphanidermatum* and a bioassay-directed fractionation on the EEP showed that compounds

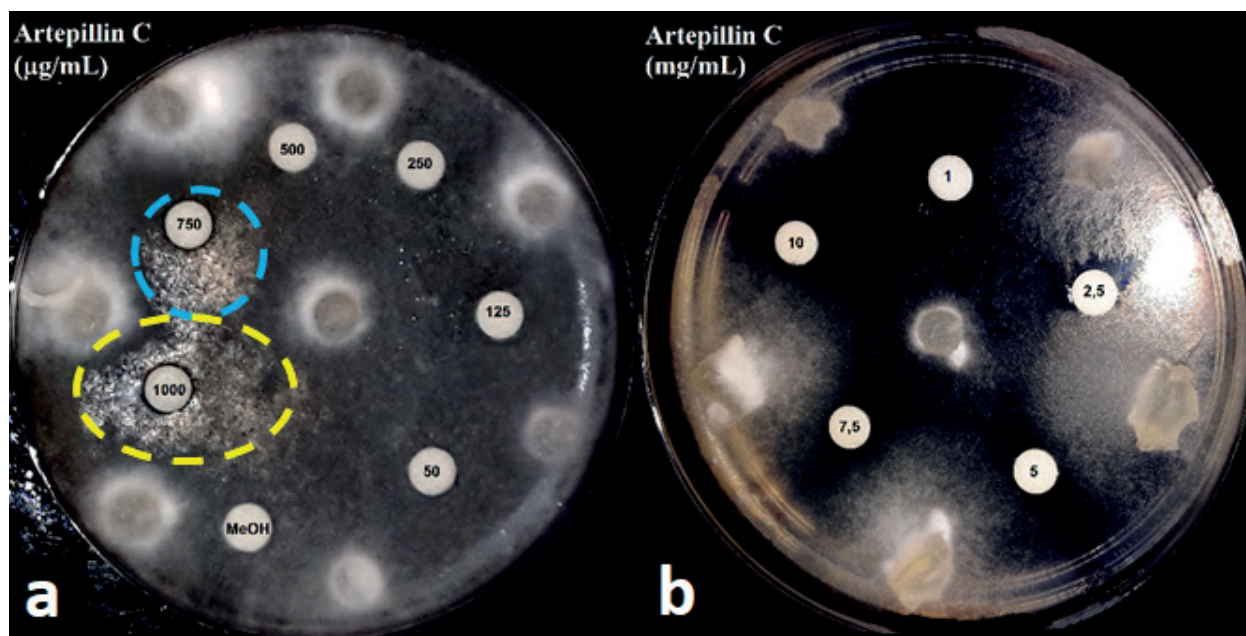


Figure 2 - Analysis of *P. aphanidermatum* growth in agar cup discs after 30 hours in the presence of Artepillin C. **a)** Artepillin C concentrations ranging from 50 to 1000 $\mu\text{g}\cdot\text{mL}^{-1}$ and methanol (MeOH) as negative control (blue and yellow dashed circles represent the inhibition zone for 750 and 1000 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively); **b)** Artepillin C concentrations ranging from 1 to 10 $\text{mg}\cdot\text{mL}^{-1}$.

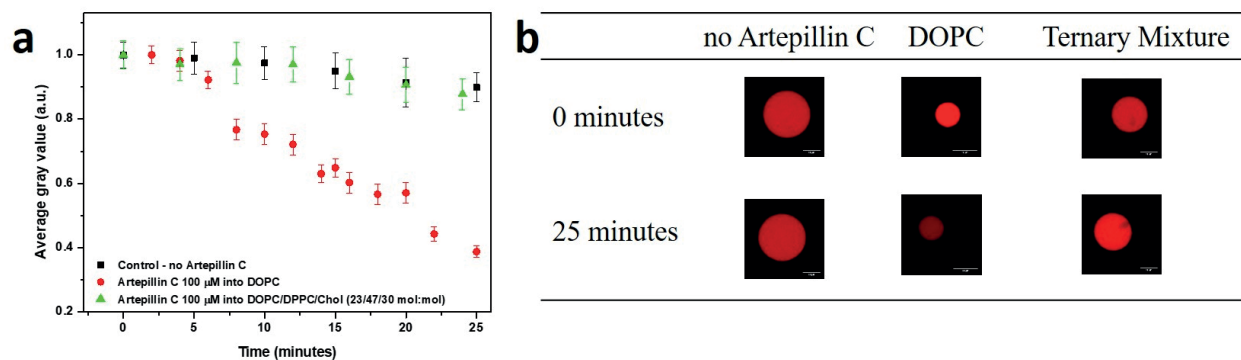


Figure 3 - **a)** AlexaFluor-488 release kinetics from inside the GUV membrane models after insertion of Artepillin C, as evaluated by confocal fluorescence microscopy. Black squares: control – no Artepillin C; red dots: 100 μM of Artepillin C into fluid-phase GUV formed of DOPC; green triangles: 100 μM Artepillin C into liquid-ordered/liquid-disordered GUVs consisting of a ternary mixture of DOPC/DPPC/cholesterol (23/47/30 mol:mol:mol). **b)** Representative images of DOPC-GUVs and GUVs consisting of a ternary mixture of DOPC/DPPC/cholesterol (23/47/30 mol:mol:mol) at the initial (0 minutes) and final (25 minutes) fluorescence intensity analyses.

separated in chloroform were the most active against *P. aphanidermatum*. The chloroform fraction was separated on a chromatographic column, and Q-ToF MS identified Artepillin C as a major compound in the bioactive fraction. The tests of Artepillin C against *P. aphanidermatum* demonstrated resistance to *P. aphanidermatum* diffusion depending on the concentration of the

compound. The activity persisted for 30 hours at concentrations above 750 $\mu\text{g}\cdot\text{mL}^{-1}$, defined as the minimum inhibitory concentration.

Green propolis displays a large spectrum of pharmacological properties and, particularly, it is one of the most famous propolis in the world due to the high amount of cinnamic acid in its composition, especially Artepillin C. This compound has

molecular formula $C_{19}H_{24}O_3$, low molecular weight (300.39 $g \cdot mol^{-1}$), and bears two prenylated groups bound to a phenyl group (Figure 4).

Artepillin C, identified as the major compound of green propolis, underlies many biological effects against pathogens of different species; moreover, it displays antioxidant, anti-inflammatory, and antitumor activities (Kimoto et al. 1998, Paulino et al. 2008, Shimizu et al. 2004). To the best of our knowledge, no research has tested Artepillin C or green propolis as potential anti-phytopathogen compounds. A previous study has shown that the structure of Artepillin C favors its interaction with amphiphilic aggregates such as cell membranes. This interaction is mainly favored by the presence of two prenylated bonds in Artepillin C, which enhance the hydrophobicity of this bioactive compound (Pazin et al. 2017). It is important to notice that the hydrophobic effects of Artepillin C depends on the protonation state of its carboxyl group, which could improve its partition to the lipid environment (Camuri et al. 2018). Such affinity allows the compound to modify the structure of the biomembranes of target cells, possibly affecting membrane proteins function and inducing a large repertoire of biological effects. Here, it is worth noting that the membrane of *P. aphanidermatum* consists of saturated and mainly of unsaturated lipids; sterols are absent (Avis and Belanger 2001) and, interestingly, leakage effects was observed only for the model that contains the major class of lipid species present in the *P. aphanidermatum* membrane. The different effects observed for the two tested GUV compositions could be related to the structure of the membranes, which in turn influenced the partition of the active compound to the membrane: a pure liquid disordered phase should favor partition of the active compound compared to a more rigid membrane, enhancing the effect of the target compound on the permeability of the membrane.

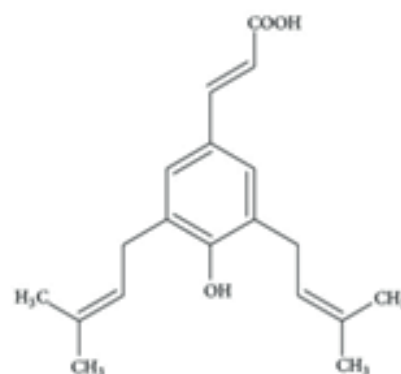


Figure 4 - Molecular structure of Artepillin C.

Avis and Bélanger (2001) have investigated how the antifungal fatty acid *cis*-9-heptadecenoic acid acts against different fungal groups. In contrast to some specific antifungal compounds that interact with membrane sterols, *cis*-9-heptadecenoic acid specifically acted on *P. aphanidermatum*, which contains little or no sterol and has unsaturated lipids in the cytoplasmic membrane. The authors concluded that the high content of unsaturated lipids in the membrane of *P. aphanidermatum* enhanced the deleterious effect of *cis*-9-heptadecenoic acid, which promoted higher bilayer fluidity and increased the permeability of the membrane. Remarkably, our findings pointed to a similar action mechanism for Artepillin C: the presence of unsaturated lipids and the absence of sterol in DOPC-GUVs enhanced the effect of Artepillin C on the membrane – membrane permeability changed and probably caused release of intracellular materials and cytoplasmic disintegration of mycelia and spores.

Strategies to combat phytopathogen species belonging to the Oomycete Class have not received much attention, and few studies have been conducted in this field. Our results herein indicate that green propolis might be an advantageous ecological alternative for the biological control of *pythiaceus* species. The obtained data suggest that green propolis is an effective and inexpensive alternative to control the dangerous phytopathogen

P. aphanidermatum by small farming communities and in large plantations.

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AUTHOR CONTRIBUTIONS

WMP – Conceptualization, Methodology, Data curation and Writing; SNS, SCNQ and LAB – Methodology, Data curation and Writing; AEES and ISM – Resources, Funding Acquisition; ASI – Conceptualization, Methodology, Data curation, Writing and Supervision.

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