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In vitro studies of Brazilian propolis against a phytopathogen agent: analyzing bioactivity and mechanism of action in model membranes

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Resumo
Estudos de agregados anfifílicos, tais como vesículas fosfolipídicas, são extremamente importantes para entender as membranas celulares, estruturas que envolvem células e constituídas principalmente por lipídios e proteínas, mantidos juntos por interações não covalentes. Diferentes fármacos agem em células alvo modificando propriedades estruturais de membranas, combatendo diversos tipos de doenças. As própolis brasileiras são produtos fitoterapêuticos atraentes, devido ao seu grande espectro de ações preventivas e tratamento de doenças, apresentando propriedades antimicrobianas, antitumorais e antioxidantes. Neste estudo foram efetuadas análises por bioensaios da ação de quatro tipos de própolis brasileiras contra o pseudofungo *Pythium aphanidermatum*, um fitopatógeno que ataca e apodrece raízes e frutos de diferentes tipos de cultivos vegetais. A interação entre a própolis que demonstrou maior bioatividade com membranas modelo constituídas por vesículas unilamelares zwitteriônicas e aniônicas, foi estudada através de medidas de fluorescência (fluorescência resolvida no tempo e imagem de tempo de vida de fluorescência). Observamos que os compostos presentes na própolis com maior bioatividade apresentam alta interação com as estruturas anfifílicas nano-organizadas, com grandes modificações em suas propriedades físico-químicas e estruturais.
Palavras-chave: própolis brasileiras; membranas modelo; fluorescência; FLIM; bioensaios.

Abstract
Studies of amphiphilic aggregates, like phospholipid vesicles, are extremely important to understand cell membranes, structures that involve cells and constituted mainly of lipids and proteins, held together by non-covalent interaction. Different drugs act in target cells modifying structural properties of membranes, during the action against many types of diseases. Brazilian propolis are attractive phytotherapeutic products due to their wide spectrum of preventive actions and treatment of diseases, showing antimicrobial, antitumoral and antioxidant activities. In this study, we performed bioassays to investigate the action of four types of Brazilian propolis against the pseudofungus Pythium aphanidermatum, a phytopatogen which attacks and rots roots and fruits of different types of vegetation. The interaction of the most bioactive propolis with model membranes made of zwitterionic and anionic unilamellar vesicles was studied by means of fluorescence measurements (time-resolved fluorescence and fluorescence lifetime image miscroscopy). We observed that compounds present in the most bioactive propolis present high interaction with nano-organized amphiphilic structures greatly modifuing their physicochemical and structural properties.
Keywords*: Brazilian propolis; model membranes; fluorescence; FLIM; bioassays.*

1. Introduction

Brazilian propolis are attractive products due to their wide spectrum of preventive actions and treatment of diseases, showing antimicrobial, antitumoral and antioxidant activities[1,2,3]. Propolis is a mixture of bees wax and resinous compounds which bees collect from the vegetation and process through their salivary enzymes[4]. The resinous compounds include bioactive molecules that protect bees hives against pathogens that destroy them. Among these compounds, the main classes of molecules forming propolis are phenolic acids, terpenoids and flavonoids, like kaempferol and quercetin[5,6]. It is not known if there is synergy between propolis compounds, but different plant sources available for the bees to collect resinous compounds result in diverse propolis-types and, as a consequence, the formed products have activities dependent on their constituents[7,8].

The mechanism of action of the different types of propolis is not yet elucidated, but studies showed that bioactive phytochemical compounds as found in the Brazilian propolis, interact with biomembranes of target cell displaying a wide repertory of biologic action[9,10]. In this study, we performed bioassays to investigate the action of four types of Brazilian propolis against the pseudofungus *Pythium aphanidermatum*, a phytopatogen which belongs to Oomycetes class known for their attacks in different types of plants, for instance rotting roots and fruits of tomato, beet, cucumber and pepper. Moreover, we examined by fluorescence lifetime measurements the interaction of the most bioactive propolis in those assays with model membranes, showing that compounds present in this product have high interaction with nano-organized amphiphilic structures, like lipid vesicles. The obtained results are important to understand the molecular mechanisms underlying the biological action of propolis.

2. Materials and Methods

Phospholipids DMPC (1,2-dimiristoyl-sn-glycero-3-phosphocholine), DMPG (1,2-dimyristoyl-sn-glycero-3-[phospho-rac-glycerol]) and DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) were purchased from Avanti polar Lipid (Alabaster, AL, USA). Ethanolic extracts of propolis collected by Brazilian bees were obtained at Ribeirão Preto Medical School, University of São Paulo (Ribeirão Preto, SP, Brazil).

Fluorescent probe 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide ($DiIC_{18}$) and others reagents, used without additional purification, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Aqueous solutions were prepared using dust free Milli-Q water (18.2 MΩ·cm). The phosphate buffer was used at the concentration of 10 mM, adjusted with NaOH to reach pH 7.4.

To investigate the bioactivity of propolis against the phytopatogen agent, we performed agar diffusion test, using paper discs (diameter ¼ IN) autoclaved and impregnated with 5 µL of a 5 mg/mL solution of the extracts against 5 mm disks of phytopathogen spread in the Potato Dextrose Agar (PDA) disk, 28º C, over a period of 24 h. After this period, we observed if there were formations of inhibition halos.

Large unilamellar lipid vesicles (LUVs) were prepared from solutions of DMPC and DMPG lipids in chloroform at the desired concentration. Dry films were obtained by solvent evaporation under a N_2 flow and elimination of the remaining organic solvent traces under reduced pressure. Multilamellar vesicles were prepared by addition of Milli-Q water onto the films and LUVs were obtained with further extrusion, passing the MLVs suspension through a 0.1 µm pore polycarbonate membrane. Giant unilamellar vesicles (GUVs) of DOPC were formed by electroformation method, as described elsewhere, placing a lipid suspension between two conducting glass plates (ITO) connected to an alternate tension generator (2 Volts, 10 Hz), during 60 minutes, at room temperature[11].

Fluorescence intensity decays and fluorescence lifetime imaging microscopy (FLIM) measurements were recorded in a time-resolved confocal fluorescence miscroscope, MicroTime 200 (PicoQuant), using a picosecond laser pulse with wavelenghts 375 nm and 530 nm to excite the resinous compounds and the fluorescent probe, respectively. The laser was reflected with a dichroic beam splitter centered into an inverted Olympus IX 71 microscope base. The samples were placed in a special 20x20 mm cover slip (Knittel glass, Germany) and excited when the light was focused by a 60x water immersion objective, numerical aperture 1.2. Fluorescence emission was collected with the same objective, passing through dichroic filter to remove scattered light before reaching a pinhole for confocal detection. Long pass filters were also added in the optical path to guarantee only fluorescence emission, detected by single avalanche photo diode (SPAD) using time-correlated single photon counting (TCSPC) method. All images were obtained in a 10^{-15} L volume, and the films were scanned in the X-Y plane by a piezoscanning through the excitation focus, in a fixed Z. Data were processed and images were obtained by an operating software of the system (SymPhoTime, PicoQuant), and its resolutions were maintained at 256 x 256 pixels. Analyses of fluorescence decay profiles were carried out by non-linear least-squares formalism and the quality of the fit was analysed from the reduced-χ^2 values and the residuals distribution. Intensity I(t) decays were fitted to multi-exponential curves:

$$I(t) = I_0 \sum_i \alpha_i e^{\frac{-t}{\tau_i}} \qquad (1)$$

3. Results

Bioassays were performed to investigate the potential of the ethanolic extracts of four different types of Brazilian propolis against the phytopathogen *Pythium aphanidermatum*. The analysis of diffusion of the phytopathogen in the PDA disk demonstrated that an inhibition halo was created around the filter with extract 2, indicating that this type propolis has the highest bioactivity among all studied extracts against the microorganism (Table 1).

Table 1. Bioactivity of Brazilian propolis against phytopathogen

Propolis Extracts	Bioactivity
1	+
2	+++
3	-
4	-

(-) no activity; (+) low activity; (+++) high activity

From time-resolved fluorescence experiments, the fluorescence intensity decay of the resinous compounds in propolis extract 2 was obtained, in three different conditions: in phosphate buffer and in the presence of DMPC (zwitterionic) and DMPG (anionic) vesicles (Figure 1). The decay profiles were best fitted to multi-exponential curves. From the parameters obtained from the fitting of the curves (Table 2), it was verified that in the presence of both DMPC and DMPG vesicles, there is an increase in the average fluorescence lifetime, compared to the result obtained for the system where the propolis extract 2 is inserted into phosphate buffer solution and, for both lipid suspensions, the obtained values were approximately the same, around 3.2 ns.

Fluorescence lifetime images were obtained to observe damages in the morphologic structure of unsaturated giant unilamellar vesicles (DOPC 1mM), caused by the compounds of propolis ethnolic extract 2. The fluorescence intensity is attributed to the presence of the fluorescent probe $DiIC_{18}$ onto the lipid bilayer structure (Figure 2A).

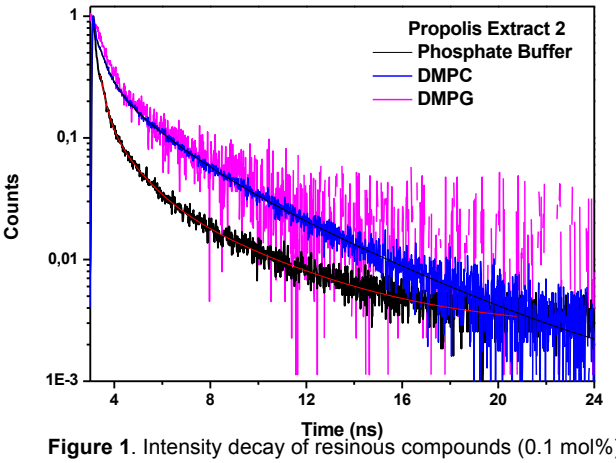

Figure 1. Intensity decay of resinous compounds (0.1 mol%) in phosphate buffer, DMPC (0.5 mM) and DMPG (0.5 mM) vesicles in phosphate buffer 7.4.

Table 2. Intensity decay parameters (lifetimes τ_i, fluorescence contribuition $\%_i$), obtained from experimental intensity profiles measured for resinous compounds (0.1 mol%) in phosphate buffer, DMPC (0.5 mM) and DMPG (0.5 mM) vesicles in phosphate buffer 7.4. Estimated deviations are 2%

Propolis Extract 2	τ_1 (ns)	$\%_1$	τ_2 (ns)	$\%_2$	τ_3 (ns)	$\%_3$	τ_{avg} (ns)
Buffer	0.33	76.7	-	-	2.24	23.3	1.62
DMPC	0.32	40.0	1.40	32.2	4.22	27.8	3.21
DMPG	0.59	77.2	-	-	4.50	22.8	3.29

In the experiment, the compounds of the propolis ethanolic ethanolic extract were added in a solution of dimethyl sulfoxide (DMSO) and added to the suspension of DOPC vesicles (2% v/v). A negative control was measured adding DMSO without the presence of ethanolic extract of propolis 2 (Figure 2B), showing that the solvent does not change properties of the biomembranes.

Figure 2C shows morphologic changes in the structure of the GUV caused by the addition of 1 $\mu g \cdot mL^{-1}$ of the ethanolic extract of propolis 2 in the lipid suspension. Thus, following the interaction with lipid bilayers, the extract compounds promote drastic changes in their structural properties. As shown by lifetime scale, there was an increase of average fluorescence lifetime of probe $DiIC_{18}$, indicating a loss of fluidity of the vesicle.

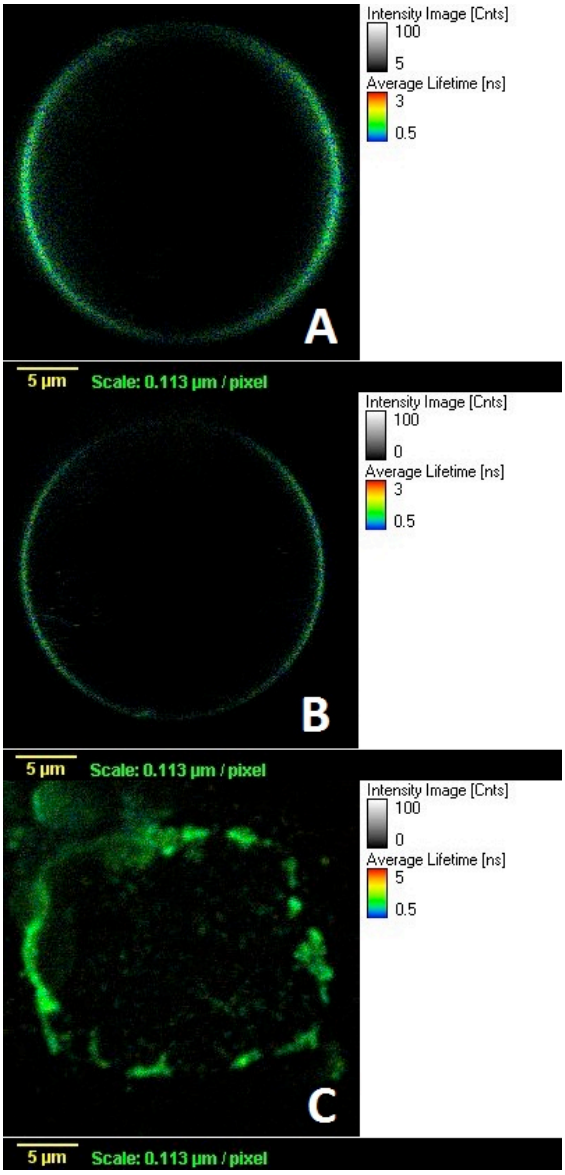

Figure 2. Micrographs of Giant Unilamellar Vesicles (GUVs) of DOPC with fluorescent probe ($DiIC_{18}$) in Milli-Q water, obtained by Fluorescence Lifetime Imaging Microscopy (FLIM). A) Pattern GUV; B) GUV + 2% (v/v) of DMSO; C) GUV + 1 $\mu g.mL^{-1}$ of propolis extract 2. All micrographs have 29x29 μm^2 of dimension).

4. Discussion and Conclusion

Propolis are collected by bees to protect their hives against different micro-organism. In this study, we investigated the bioactivity of Brazilian propolis from different bee species by the inhibition of the phytopathogen *Pythium aphanidermatum* of Oomycetes class. Analysis of diffusion of the

micro-organism in PDA disk showed the highest activity for propolis extract 2, while others products either did not show bioactivity or presented very low inhibition of the phytopathogen. From these results, interactions of propolis compounds of the extract 2 with lipid bilayers were studied by fluorescence techniques. It was observed an increase in fluorescence average lifetime when zwitterionic and anionic vesicles formed of DMPC and DMPG, respectively, were added to the buffer solution containing the propolis ethanolic extract. This increase indicates that the contribution of non-radiative processes decreases due the non-polar environment around the molecules in the presence of the amphiphilic aggregates, originated from their insertion into the lipid bilayers, either zwitterionic or anionic. The increase in lifetime in DMPC vesicles is similar to that in DMPG vesicles, showing that there is no significant contribution of anionic charges in the system, indicating the predominance of hydrophobic effects in the interaction of the compounds with lipid bilayers.

Micrographs obtained from FLIM reveal that compounds present in propolis extract 2 interact strongly with DOPC giant vesicles, changing the fluidity of the lipid bilayers proved for the increasing in the fluorescence lifetime of $DiIC_{18}$, and, furthermore, modifying structure and disrupting the DOPC vesicles.

The observation of interactions between drugs with model membranes are important to verify if there are physicochemical changes and structural damages in the membranes of target cells, that are fundamental structures to maintain the functionalization of proteins and enzymes. Previous studies have shown that some drugs do not need specific markers or receptors to recognize cells and to attack them, exerting the biological action through their interactions with biomembranes.

It was verified in this study the potential of the propolis compounds against the *phytopathogen Pythium aphanidertaum* which rot roots and fruits of many types of vegetation. The compounds interact and disorganize model membranes composed of zwitterionic and anionic lipids, showing that this can be an important mechanism of action of propolis in membranes of target cells related to their activity against diseases.

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References

1. Borges, K.S., et al., Antiproliferative effects of Tubi-bee propolis in glioblastoma cell lines. Genetics and Molecular Biology, 2011. 34(2):310-314.
2. Sawaya, A.C.H.F., I.B.D. Cunha, and M.C. Marcucci, Analytical methods applied to diverse types of Brazilian propolis. Chemistry Central Journal, 2011. 5:27.
3. Gregoris, E. and R. Stevanato, Correlations between polyphenolic composition and antioxidant activity of Venetian propolis. Food and Chemical Toxicology, 2010. 48(1):76-82.
4. Bonvehi, J.S. and A.L. Gutierrez, The antimicrobial effects of propolis collected in different regions in the Basque Country (Northern Spain). World Journal of Microbiology & Biotechnology, 2012. 28(4):1351-1358.
5. Carvalho, A.A., et al., In vivo antitumoural activity and composition of an oil extract of Brazilian propolis. Food Chemistry, 2011. 126(3):1239-1245.
6. Park, Y.K., S.M. Alencar, and C.L. Aguiar, Botanical origin and chemical composition of Brazilian propolis. Journal of Agricultural and Food Chemistry, 2002. 50(9):2502-2506.
7. Scheidt, H.A., et al., Investigation of the membrane localization and distribution of flavonoids by high-resolution magic angle spinning NMR spectroscopy. Biochimica Et Biophysica Acta-Biomembranes, 2004. 1663(1-2):97-107
8. Ota, A., et al., Interactions of p-coumaric, caffeic and ferulic acids and their styrenes with model lipid membranes. Food Chemistry, 2011. 125(4):1256-1261.
9. Sun, Y., et al., The bound states of amphipathic drugs in lipid bilayers: Study of curcumin. Biophysical Journal, 2008. 95(5): 2318-2324.
10. Sun, Y., et al., Interaction of Tea Catechin (-)-Epigallocatechin Gallate with Lipid Bilayers. Biophysical Journal, 2009. 96(3):1026-1035.
11. Angelova, M., Dimitrov, D. Liposome electroformation. Faraday Discussions of the Chemical Society , 1986. 81:303-311.

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