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NOTA CIENTÍFICA

Propolis extract significantly reduces microbial load in fresh bee pollen for human consumption

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Abstract

We evaluated propolis as a preservative of fresh bee pollen for human consumption. An in vitro quantitative assay was performed to determine the antimicrobial activity of a propolis ethanolic extract against cultivable heterotrophic mesophilic bacteria, molds and yeasts in bee pollen for 22 days. The chemical characteristics of the propolis extract were also determined. From the results of the quantitative antimicrobial assay, none of the control treatments exhibited an inhibitory effect similar to that of the ethanolic extract of propolis. This is one of the first studies that combine both bee products, propolis and pollen, to improve the preservation of fresh bee pollen, avoiding the drying process. The results of the in vitro assay were promising, as the antimicrobial action of propolis ethanolic extract against bacteria, molds and yeasts from fresh bee pollen was excellent.

Keywords: antimicrobial activity, bee pollen, microbiological quality, propolis.

El extracto de propóleos reduce significativamente la carga microbiana en el polen apícola fresco para consumo humano

Resumen

En este trabajo se propuso evaluar el uso del propóleos como conservante del polen apícola fresco destinado al consumo humano. Se realizó un ensayo cuantitativo *in vitro* para determinar la actividad antimicrobiana de un extracto etanólico de propóleos sobre bacterias mesófilas heterótrofas cultivables y mohos y levaduras de polen apícola durante 22 días. También se determinaron las características químicas del extracto de propóleos. Los resultados del ensayo antimicrobiano cuantitativo mostraron que ninguno de los tratamientos de control mostró un efecto inhibidor similar al extracto etanólico de propóleos. La actividad antimicrobiana se debió a la presencia de compuestos bioactivos del propóleos y no al alcohol presente como disolvente. Este es uno de los primeros trabajos que presentan la combinación de ambos productos apícolas, propóleos y polen, con el fin de mejorar la conservación del polen apícola fresco evitando el proceso de secado. Los resultados del ensayo *in vitro* fueron alentadores ya que la acción antimicrobiana del extracto etanólico de propóleos contra bacterias y mohos / levaduras del polen apícola fresco fue excelente.

Palabras clave: actividad antimicrobiana, calidad microbiológica, polen apícola, propóleos.

Honey bees (*Apis mellifera* L.) collect pollen from different plant sources for brood rearing. During collecting trips, they pack floral pollen grains into pollen loads, mixing them with nectar and saliva and then transporting it to hives (Thakur & Nanda, 2020). Beekeepers collect part of this product using bee pollen traps and then are processed (Margaoan *et al.*, 2010). The final product is commercially traded as bee pollen (BP), considered one complete natural human consumption food. It can be listed as a functional food, which resembles traditional food with demonstrated physiological and therapeutic benefits such as

improving health and reducing disease risk (Thakur and Nanda, 2020).

Fresh pollen contains 20-30 % moisture in nature and has a water activity from 0.66 to 0.82. It is rich in proteins, lipids, and sugar. Due to these chemical characteristics, several microorganisms in this product from natural habitats and human manipulation may proliferate. Also, chemical and enzymatic reactions that degrade this product may occur (Margaoan *et al*., 2010). In that sense, drying is the most common way of dehydration before commercialization. However, the temperatures used in this step must be rigorously controlled to avoid the denaturation of bioactive compounds and preserve BP's biological properties (De-Melo *et al*., 2015; De-Melo *et al*., 2016). The hygienic quality of BP was established by the Argentine Food Code (AFCode): the maximum level allowed for molds and yeasts is 100 colony-forming units (CFU) g^{-1} of BP and 150 x 103 CFU g^{-1} for non-pathogenic aerobic microorganisms, respectively. Therefore, natural products such as propolis have emerged as new technologies for post-harvest storage, shelf-life extension, and improvement of food quality (Barrera Bello *et al*., 2012).

Propolis, another bee product, is a highly complex resinous substance collected by honey bees from buds and tree leaves, mixed with pollen and enzymes. It contains a wide variety of chemical compounds depending on the origin of the samples, which is strongly related to the flora surrounding the hive. The main polyphenols are flavonoids (quercetin and pinocembrin), accompanied by phenolic acids and their esters. The antibacterial and antifungal properties of propolis are the most popular and extensively investigated biological activities (Bankova *et al*., 2014). Currently, propolis is the object of many studies as a natural food preservative (Barrera Bello *et al*., 2012; Khalaf *et al*., 2014). However, as far as we know, the effect of propolis as a preservative for fresh BP has not yet been reported.

In this context, we propose to evaluate propolis as a natural preservative of BP intended for human consumption. Thus, this study aimed to determine the antimicrobial activity *in vitro* of a propolis ethanolic extract (PEE) over fresh BP which has not been dried. A quantitative assay was performed using fresh BP and propolis collected from a central region of Argentina. Some chemical characteristics of the PEE, including the total phenolic and flavonoid content, were also determined.

The BP sample used in this study was harvested from one hive located in Hilario Ascasubi (south of Buenos Aires province, Argentina) (39°23' 33"S and 62°37'4"W) using external pollen traps in March 2017. This BP sample was composed of approximately 500 pollen loads and stored in a jar with an airtight seal in a refrigerator (±5 °C) for one week until the assay was done. On the other hand, the sample of raw propolis was also collected in March 2017 with propolis traps from an apiary located in Villa Manzano (Río Negro province, Argentina) (38°40′48″S and 68°12′58″W). This product was stored in a sterile bottle, frozen at -15±2 °C, and protected from light until use.

The PEE was prepared as described by Cibanal *et al*. (2019) by dissolving 10 g of raw propolis sample in 100 mL of ethanol 96 % (v/v). The chemical analyses were performed to characterize PEE according to the Norma Argentina IRAM-INTA 15935-2 (Bedascarrasbure *et al*., 2006). The absorption spectrum was determined by scanning the diluted extract (1:10000) in a UV spectrophotometer (Agilent Cary 60 UV-Vis) at 240-420 nm. Dry residue (mg mL^{-1}) free of volatile substances was obtained by heating 20 mL of PEE in an oven at 100 °C for 24 h and placed in a desiccator until room temperature was reached. The total phenolic content was determined by the Folin-Ciocalteu method, and results were expressed in terms of gallic acid equivalents (g GAE 100 g^{-1} dry propolis extract). The total flavonoid content was analyzed by the spectrophotometric method based on aluminum complex formation and expressed as quercetin equivalents (g QE 100 g^{-1} dry propolis extract). All determinations were carried out in triplicate.

The antimicrobial activity of PEE against microorganisms from fresh BP loads was tested in a quantitative *in vitro* assay using a specific culture media for culturable heterotrophic mesophilic bacteria (CHMB) and for molds and yeasts (MY) for 22 days (Baldi *et al*., 2004). Briefly, three treatments were carried out: a control with only BP, no additives (C); a solvent control consisting of BP loads soaked with 5.00 µL of ethanol 96 % (EC); and BP loads soaked with 5.00 µL of PEE (PEE). Three replicates per treatment for each type of microorganism were performed. Each replicate consisted of five plates with 20 BP loads per plate (100 BP loads per replicate) soaked with the corresponding treatment. CHMB was studied in Plate Count Agar (Britania®, Argentina) with nystatin (Sigma, Germany) at 35 °C, while MY were counted in Sabouraud Dextrose Agar (Bioclar, Argentina) at 25 °C. The results were expressed as the number of BP loads (out of 20) with visible colony-forming units of CHMB and MY every 48 h for 22 days.

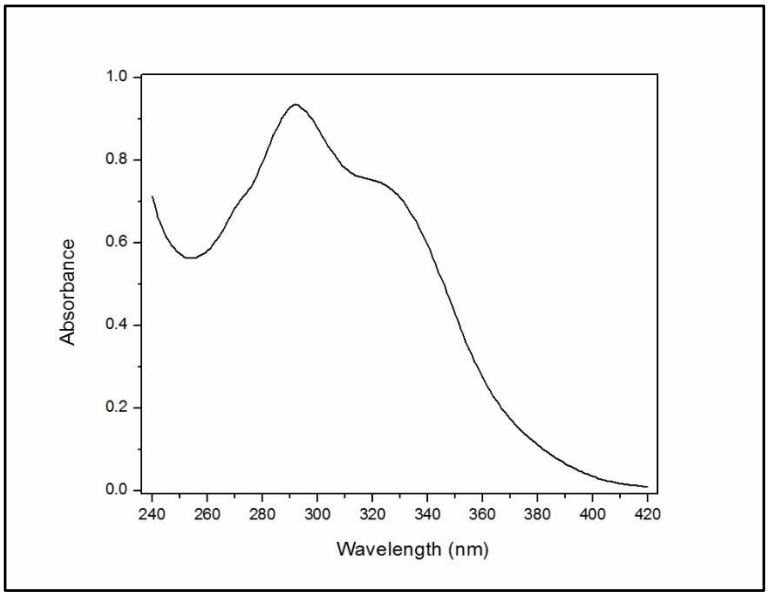

Figure 1. UV spectrum of propolis ethanolic extract (PEE) (range: 240 to 420 nm) obtained from a raw propolis sample from Río Negro province (Argentina).

Preliminary assays were done to test volumes of 5.00 and 7.50 µL of PEE, and no significant differences in microbial growth were observed (data not shown).

The antimicrobial assay was conducted following a fully randomized model. Data from the number of BP loads with visible colony-forming units of CHMB and MY were analyzed statistically with a one-way analysis of variance. When a significant F-value was detected, means were compared with two tests: Fisher's Least Significant Difference (LSD) ($p<0.05$) and Bonferroni ($p\leq0.01$). Infostat software (Balzarini *et al*., 2011) was used for statistical analyses.

In recent years, the demand of natural foods has increased rapidly so as the number of scientific research on BP (Végh *et al*., 2021). Accordingly, our research focused on fresh BP intended for human consumption by evaluating the antimicrobial activity of PEE against CHMB and MY from fresh BP. The chemical characterization of PEE was included since the antimicrobial activity of propolis depends, to a large extent, on their composition (Bankova *et al*., 2014) and helped us to explain outcomes from our antimicrobial trial.

The chemical characterization of PEE guarantees a good quality product and a reasonable degree of antibacterial activity (Baldi *et al*., 2004). As shown in Fig. 1, the PEE UV-Vis absorbance spectrum shows absorption bands between 270 and 330 nm. As reported by Park & Ikegaki (1998) who evaluated different ethanolic and water extracts, the appearance of absorption bands at 270-330 nm was attributable to the presence of polyphenols and flavonoid compounds. On

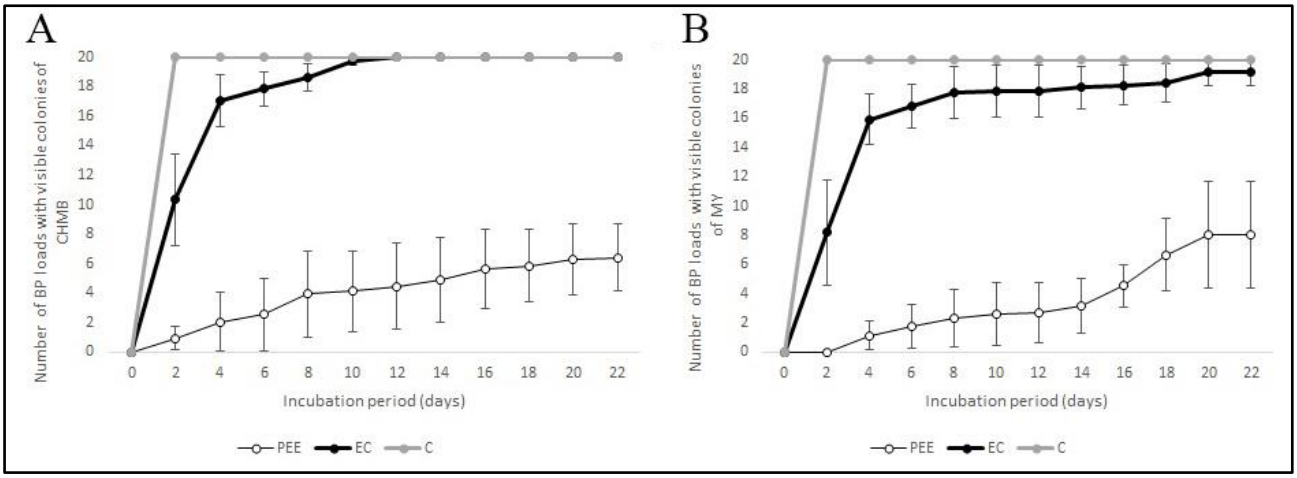

Figure 2. Antimicrobial activity of propolis ethanolic extract (PEE) against culturable heterotrophic mesophilic bacteria (A) and molds and yeasts (B) from fresh bee pollen (BP) loads.

Note. Three treatments: control (only BP) (C); a solvent control consisting of BP soaked with 5.00 µL of ethanol 96 % (EC); and BP loads soaked with 5.00 µL of propolis ethanolic extract (PEE).

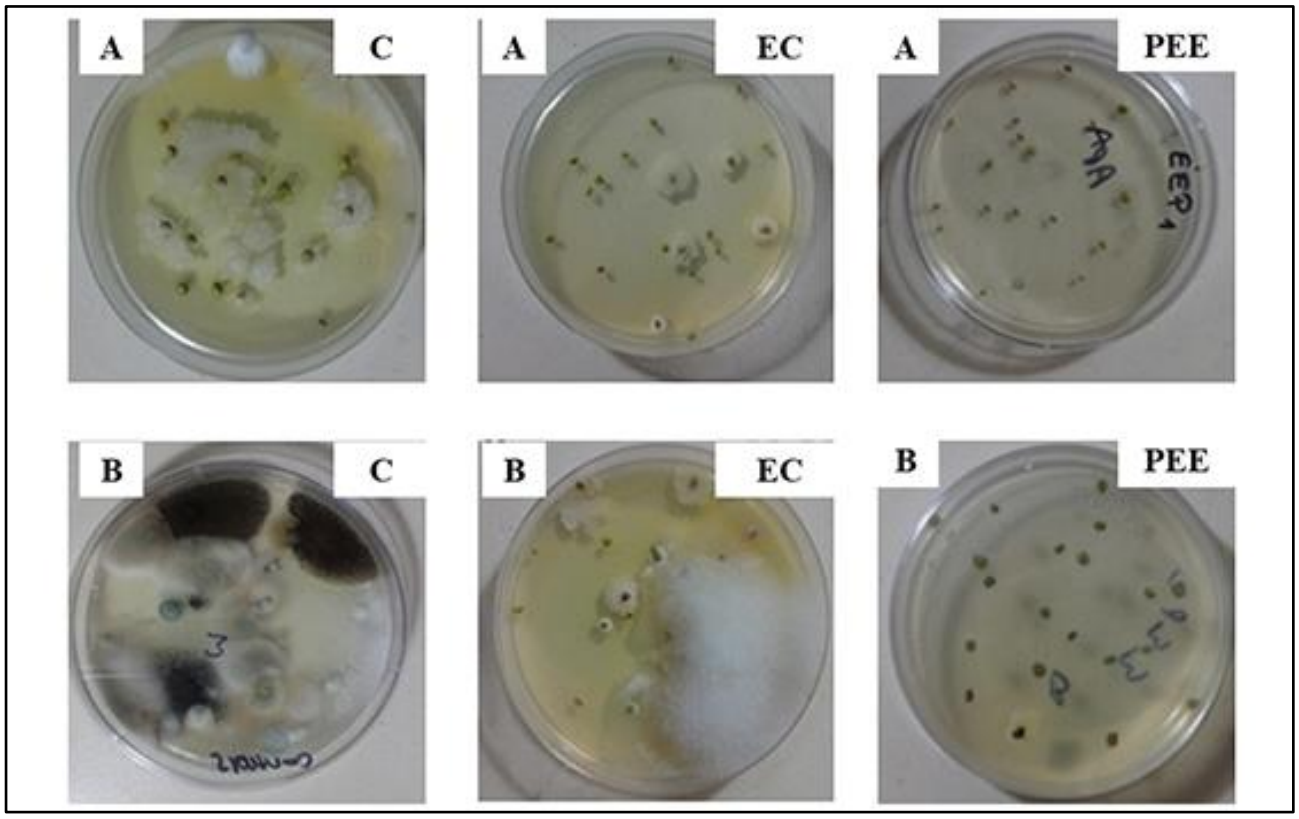

Figure 3. Assay of the antimicrobial activity of propolis ethanolic extract (PEE) against culturable heterotrophic mesophilic bacteria (A) and mold and yeast (B) over fresh bee pollen (BP) for 22 days.

Note. Three treatments: a control without aggregates (only BP) (C); a solvent control consisting of BP loads soaked with 5.00 µL of ethanol 96 % (EC); and BP loads soaked with 5.00 µL of propolis ethanolic extract (PEE).

the other hand, the extract's dry residue percentage was 12.52 ± 0.08 g 100 mL^{-1}, while the total polyphenol and flavonoid contents were 27.10 ± 0.32 g GAE 100 g^{-1} and 8.86 ± 0.03 g QE 100 g^{-1}, respectively. The evaluated parameters presented admissible values according to the Argentinian regulation (Norma Argentina IRAM-INTA 15935-2) and were consistent with other Argentinean propolis characterizations (Agüero *et al*., 2010; Chaillou & Nazareno, 2009).

The quantitative *in vitro* assay results are shown in Fig. 2. Statistical analyses showed significant differences between treatments after two days of incubation. In PEE treatment, only 30 % (6 BP loads) presented CHMB growth (Fig. 2A), and 40 % (8 BP loads) presented MY (Fig. 2B) after 22 days. Fifty percent of the BP from EC treatment and almost all of the BP from treatment C showed CHMB development after 48 h of incubation (Fig. 3A). Similar results were observed in both control treatments for MY (Fig. 3B). PEE treatment did not reach these percentages either after 48 h of incubation (Fig. 3) or during the whole incubation period.

Neither of the control treatments (C and EC) showed an inhibitory effect against CHMB and MY similar to the PEE treatment (Figs. 2; 3). Thus, these data confirmed that the antimicrobial activity was due to the presence of bioactive compounds in the alcoholic extract from the propolis (PEE) and not to alcohol inhibition (alcohol present as a solvent in the extract), as previously described in several studies (Cibanal *et al*., 2019; Fernández *et al.,* 2019; Gallez *et al.,* 2014).

As previously mentioned, propolis is studied as a natural preservative for food. The employment of different coatings containing propolis over papaya fruits (*Carica papaya* L. cv. Hawaiian) (Barrera Bello *et al.*, 2012), Gouda cheese (Khalaf *et al.,* 2014), raspberries (*Rubus idaeus* L) (Moreno *et al*., 2020) and as a preservative for fruit juices (Chang *et al*., 2021) has been reported. The authors observed that propolis presented excellent antimicrobial activity against total bacteria, coliform, molds, and yeasts in all cases. Likewise, the *in vitro* results of this study were in accordance with those authors, as PEE was an excellent preservative during the whole assay avoiding microbial growth, CHMB, and MY, over fresh BP (Figs. 2; 3).

As far as we know, regarding the production process of BP, this is the first study that presents the combination of propolis and BP to improve the quality and the conservation conditions in replacement of the drying process. De Melo *et al*. (2015) have deeply studied

different conditions to process BP and its consequences over many properties of this food. They demonstrated that drying BP in an electric oven at 42 °C negatively influenced antioxidant, anti-inflammatory, and antimicrobial properties. They also observed that drying BP diminished the levels of the total phenolic content. These results reinforced our idea of using propolis as a natural preservative, eluding the drying step through the production process of BP.

Moreover, Végh *et al*. (2021) suggested in their review that attention must be paid to BP's microbiological quality, especially when considering fresh bee pollen. Some environmental microorganisms, such as lactic acid bacteria, *Enterobacteriaceae*, yeast, and molds, could be present in relevant concentrations, possibly leading to the alteration or the accumulation of toxic metabolites, such as mycotoxins or biogenic amines. Therefore, our *in vitro* assay results demonstrated the excellent antimicrobial power of PEE against microbiological contamination.

The present research regarding propolis as a preservative for BP intended for human consumption is preliminary; additional experiments must be conducted to determine sensory properties and other physicochemical parameters in fresh BP with propolis as a preservative.

DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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