Journal of Apicultural Research

ISSN: 0021-8839 (Print) 2078-6913 (Online) Journal homepage: https://www.tandfonline.com/loi/tjar20

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To cite this article: C. Letullier, A. Manduchet, N. Dlalah, M. Hugou, S Georgé, J. M. Sforcin & N. Cardinault (2019): Comparison of the antibacterial efficiency of propolis samples from different botanical and geographic origins with and without standardization, Journal of Apicultural Research

To link to this article: https://doi.org/10.1080/00218839.2019.1681117

Published online: 05 Nov 2019.

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Journal of Apicultural Research, 2019
https://doi.org/10.1080/00218839.2019.1681117

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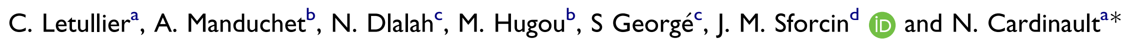

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(Received 28 March 2018; accepted 10 May 2019)

Propolis is a resinous product made by bees and has attracted the attention of researchers for decades due to its numerous biological properties (antimicrobial, antioxidant, anti-inflammatory, immunomodulatory, etc.). Studies have demonstrated that polyphenols may be involved in such effects, although each propolis sample has its own phenolic profile which is related to its botanical origin. Several studies have investigated the biological efficiency of different kinds of propolis extracts obtained from distinct botanic sources, using different extraction ratios and types of solvent, that complicates or even makes it impossible to objectively compare the results and to remove irrelevant information from it, in order to achieve a better understanding, a proper control, and reproducibility of the biological effects, aiming its use in human and animal medicine. The aim of this study with a theoretical approach was to determine whether the interpretation of results of the antibacterial activity of three propolis extracts from different botanical origins could be the same or not before standardization. The three samples of propolis (poplar propolis and red and green propolis) were extracted identically and their antibacterial activity was tested against *Streptococcus agalactiae* (Gram+) and *Escherichia coli* (Gram-). Our data clearly showed that interpreting the magnitude of efficiency of the extracts was completely different when they were standardized or not regarding their total polyphenol content. This conceptual work demonstrates the need of standardizing propolis extracts before testing their biological activities.

Keywords: Propolis; biological efficiency; polyphenols; standardization

Introduction

Honey bees, whose existence dates back more than 80 million years, have known how to adapt themselves throughout time to become a long-lasting species capable of living in any housing environment on the planet. To do this, bees have made an intrinsic "chemical weapon of defense" by stimulating its own immune system and an extrinsic one by being capable of fighting pathogenic microorganisms: propolis or "bee glue." Propolis is a sticky resinous material made by bees from various parts of plants (resins, leaves, buds, exudates) present in the collection zone. Resins are mixed with wax and enzyme-enriched salivary secretions of the bees that become propolis. Its color may vary from green to red and up to dark brown depending mainly on the botanical species, which are the source plants of propolis for the worker bees (Salatino, Fernandes-Silva, Righi, & Salatino, 2011; Sforcin, 2016)

It has been demonstrated that the presence of specific plants in a given geographical region establishes a constant and unambiguous preference for making propolis. For this reason, different types of propolis from specific botanical origins have been already defined, such as the propolis from poplar, the ones from *Baccharis dracunculifolia* and from *Dalbergia ecastaphyllum*, among others. Since resin sources of propolis are different, the chemical constituents that define it are also different among propolis samples, and it is therefore impossible to speak about propolis as a unique or universal product (Bankova, 2005; Popova, Trusheva, & Bankova, 2017; Toreti, Sato, Pastore, & Park, 2013).

The various resins collected by bees are secreted by plants to protect them from microorganisms. Bees make and use propolis with the same aim as the plants: to protect themselves. For thousands of years, the observations made by humans have allowed us to use this product for the same protective properties. Thus, the story of the use of propolis in folk medicine has been built on this property but since the 1990s, modern science has shown many other pharmacological properties such as antioxidant, immunomodulatory, antitumoral, anti-inflammatory, anti-ulcer, organ protective, and so on (Lotfy, 2006; Sawicka, Car, Borawska, & Nikliński, 2012; Sforcin, 2007; Vit et al., 2015). Scientific interest in propolis and its potential biological effects has greatly increased; however, scientists have not forgotten that propolis is not a product with a constant chemical composition. So, a kind of "cartography" linking certain types of propolis with specific botanic origins to their chemical constituents and biological properties has been established. Its chemical composition revealed the presence of various compounds such as flavanones,

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flavones, and phenolic acids and their ester derivatives in poplar propolis, prenylated *p*-coumaric acids, and diterpenes in propolis from *Baccharis* or isoflavonoids in propolis from *Dalbergia* (Bankova et al., 2016b).

Different samples of propolis with different chemical constituents may exert the same biological activities while different mechanisms of action may be involved, but the quantity and/or the quality of active compounds responsible for these activities remain a crucial and fundamental issue in the advance of the understanding the health effects of various propolis. In order to obtain relevant elements to decide what is the most appropriate type of propolis for which indication and which dose is essential to be accepted in both medical and food industry, several authors have already argued the strict need to develop studies from standardized propolis extracts according to its botanical origin and chemical composition (Bankova et al., 2016a; Molnar et al., 2017). In this work, we investigated whether the standardization of the extracts of propolis before testing them was of a crucial issue in the interpretation of the results. Thus, in a completely conceptual approach, a comparison was established regarding the antibacterial efficiency of three propolis samples from different phytogeographical sources (one from the Northern Hemisphere and two from the Southern Hemisphere) and extracted in the same way, but not standardized or preliminary standardized on the basis of their active compounds (total polyphenols) content.

Materials and methods

Propolis samples

Three propolis samples with a well-identified botanical origin have been chosen for this study: a propolis sample produced from poplars and harvested in the Lot et Garonne region in France, a green propolis sample produced from *B. dracunculifolia* collected in the Minas Gerais region in Brazil, and a red propolis sample harvested in the Alagoas region in Brazil whose vegetal source is *D. ecastaphyllum*.

Preparation of ethanolic extracts of propolis

Raw propolis samples were ground to a fine powder. Next, 30 g was mixed with 100 ml 70% ethanol and stirred for 48 h at room temperature shielded from the light. After extraction, the mixture was filtered. Ethanolic extracts of propolis (EEP) were subjected to the dosage of the total polyphenols.

Dosage of total polyphenols

Total polyphenols content was measured according to the method described by Bankova et al. (2016a). The results were expressed in mg/100 mL of alcoholic extract of propolis as pinocembrin/galangin equivalent for poplar propolis and as gallic acid equivalent for *B. dracunculifolia* and *D. ecastaphyllum* propolis. Methanolic solutions of pinocembrin/galangin (2/1) are used for calibration of poplar propolis (Popova et al., 2004) and methanolic solutions of gallic acid are used for calibration of for *B. dracunculifolia* and *D. ecastaphyllum* propolis. For the standard, a blank tube was prepared with 100 μl of 70% ethanol and 100 μl of standard mix of diluted concentrations and 1.5 mL of distilled water was added with 0.4 mL of Folin–Ciocalteu reagent. The tubes were vortexed and 0.6 ml of Na_2CO_3 20% and 2.4 mL of distilled water were added and mixed. The tubes were incubated at 50 °C for 15 min. After cooling at room temperature, absorbances were measured at 760 nm. For the samples, EEP were diluted 1/10 with 70% ethanol before analysis. 100 μL of diluted extracts of propolis was placed into tubes and the procedure was the same for the standard.

Bacteria strains

A Gram + and a Gram- strain (*Streptococcus agalactiae* and *Escherichia coli*, respectively) were provided by the collection of the Microbiology Department of the Agricultural School of St. Livrade (France). *S. agalactiae* was incubated in trypticase casein soy (TCS) agar while *E. coli* was incubated in nourishing ordinary agar (NOA). Bacterial inoculum was prepared by transplanting colonies in TCS broth and incubating at 37 °C for 24 h. Successive dilutions were made as well as a surface count to determine the colony-forming units (CFU) into the culture medium to obtain a final concentration of approximately 10^5 CFU/mL.

Microbiological assays

Determination of the minimal inhibitory concentration

The minimal inhibitory concentration (MIC) for both strains was determined for the three extracts of propolis. Decreasing concentrations of each EEP were tested ranging from 1% to 0.01% for *S. agalactiae* and from 12% to 1% for *E. coli*. After inoculation of 10^5 CFU/ml in medium containing different concentrations of EEP for 24 h at 37 °C, 100 μl was removed and inoculated in a Petri dish for CFU counting after 24 h. MIC was determined as the last concentration with no visible bacteria growth.

Survival curve

The survival curve of Gram + and Gram- bacteria was performed in order to observe the antibacterial kinetics of the respective MIC of the three EEP during 8 h. Each strain was cultivated in the same conditions as previously described in the presence of the MIC of each EEP. A sampling of broth was taken every hour during 8 h and inoculated in a Petri dish for 24 h at 37 °C to determine the number of live colonies.

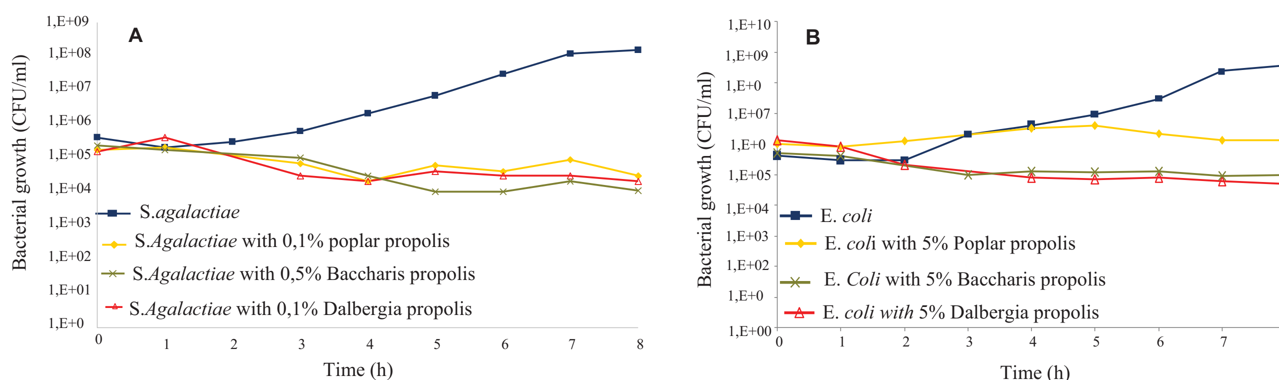

Figure 1. Kinetic follow-up of anti-*S. agalactiae* (A) and anti-*E. coli* (B) activity of three ethanolic propolis extracts applied at their respective CMI.

Results

Regarding *S. agalactiae*, the MIC for both EEP from poplar and *D. ecastaphyllum* samples was 0.1% while the one for *B. dracunculifolia* extract was 0.5%. On the other hand, the MIC value for *E. coli* was 5% for all EEP and clearly higher than those obtained for the Gram + bacteria.

The kinetic curve of the growth of *S. agalactiae* and *E. coli* is presented with or without propolis extracts in Figure 1(A,B). A bacteriostatic effect of the three EEP may be seen after incubation of the bacteria with their respective MIC for 8 h. The EEP from poplar seemed to be less efficient toward *E. coli*.

Although the three propolis samples originated from different botanical origins but extracted by the same procedure, their total polyphenols contents were very different. The extract of poplar propolis exhibited a higher concentration of polyphenols than the extract of *Baccharis* and *Dalbergia* propolis ($9.9 > 7.1 > 3.6$ g/dL, respectively).

In order to compare the effect of these different EEP, it is important to guarantee that the treatments were administered in a common base of comparison to all the treatments. Since the three EEP presented different concentrations in total polyphenols content varying from 3.6 to 9.9 g/dL, the growth kinetic of the strains was analyzed after incubation with the same concentration of polyphenols. Thus, the two most concentrated EEP (poplar and *Baccharis*) were diluted in order to obtain an identical concentration of total polyphenols to that from *Dalbergia* extract. Figure 2(A) shows the kinetic follow-up of the growth of *S. agalactiae* incubated with 0.1% of diluted extract of poplar propolis, 0.5% of diluted extract of *Baccharis* propolis, or 0.1% of initial extract of *Dalbergia* propolis. Although the *Dalbergia* extract exerted only a bacteriostatic action, it was more efficient toward *S. agalactiae* than poplar and *Baccharis* extracts. Similar results were obtained with *E. coli* (Figure 2(B)).

Discussion

Propolis antibacterial activity is one of the main biological properties widely documented in literature (Sforcin, Fernandes, Lopes, Bankova, & Funari, 2000). Although this activity is no longer contested in a broader sense, several studies carried out so far do not allow an appropriate comparison of the various extracts of propolis used in these studies, nor does a predictive rule arise concerning the antibacterial activity of propolis as the modes of obtaining the extracts, the antibacterial tests used, the modes of expression of this activity are very variable. The purpose of this conceptual work was to estimate if a biological activity such as the antibacterial one, determined with standardized or non-standardized extracts, would allow a relevant, objective, and reproducible interpretation among various samples of propolis stemming from the same or different studies.

In this study, two bacteria strains were used: a Gram+ (*S. agalactiae*) and a Gram- one (*E. coli*). First, the data revealed that the three extracts of propolis were clearly more effective (from 10 to 50 times superior) against Gram + bacteria than against *E. coli*. These results are in agreement with those found in literature (Boufadi et al., 2014; Sanpa et al., 2017; Seidel, Peyfoon, Watson, & Fearnley, 2008), since there is a coherence of results between all the studies showing that the extracts of propolis are always more efficient against Gram + than Gram- bacteria. Nevertheless, there are only a few studies dealing with the mechanisms of action by which propolis could inhibit bacterial growth and the reason some of them are more resistant to its extracts. Oryan, Alemzadeh, and Moshiri (2018) reported that there have been no controlled studies comparing the mechanisms of action of propolis against Gram + or Gram- strains. Propolis activity against Gram + bacteria may be attributable to flavonoids, aromatic acids, and esters present in the samples, and some hypothesis indicated an increased permeability of cell membrane which would affect the ATP synthesis, the transport, and the motility (Mirzoeva, Grishanin, & Calder, 1997), or an inhibition of the cellular division or an inhibition of the synthesis of proteins of Gram + bacteria (Takaisi-Kikuni & Schilcher, 1994). On the contrary, the resistance of Gram- bacteria could be

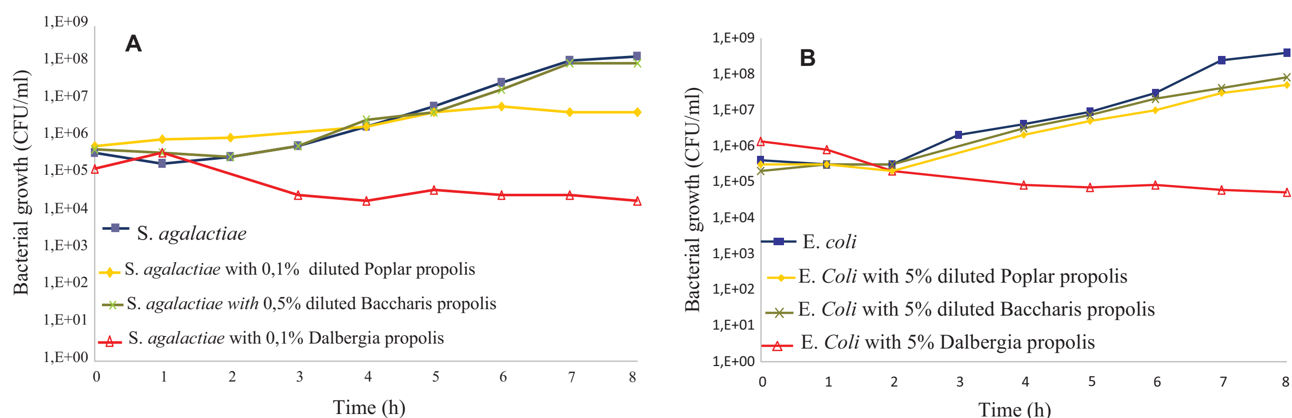

Figure 2. Kinetic follow-up of anti-*S. agalactiae* (A) and anti-*E. coli* (B) activity of three ethanolic propolis extracts at the same concentration in total polyphenols and applied at their initial CMI.

ascribed to the presence of efflux pumps preventing the intracellular entrance of active constituents of propolis (Garedew, Schmolz, & Lamprecht, 2004).

The antibacterial activity was estimated through diverse parameters such as the determination of the diameter of the inhibition zone or MIC determination. The latter can itself be submitted to several modes of expression, such as percentage (volume of propolis extract/volume of broth), mass of propolis/volume of broth, or in concentration, but in the latter, it was never clearly indicated to what this concentration corresponds to: dry resin mass after extraction or mass of raw propolis or something else. Here, in the first set of experiments, the MIC of each extract of propolis was calculated by the liquid dilution method, allowing to obtain the concentration responsible for the bacteriostatic effect after 8 h. MIC results expressed in % volume led us to conclude that EEP of poplar and *Dalbergia* were five times more efficient than *Baccharis* extract regarding the inhibition of *S. agalactiae* growth (0.1 vs. 0.5% v/v). Hence, on the basis of this data and those from many other articles, we would be tempted to establish a classification of increasing order of efficiency to fight against this bacterium: *Baccharis* $<$ poplar $=$ *Dalbergia*. However, the comparison of these three EEP showed no differences against *E. coli*.

Streptococcus agalactiae is not frequently used in antibacterial assays compared to *Staphylococcus*. There are numerous studies with the three types of propolis investigated in this article and the MIC of poplar sample against this bacteria has been reported between 0.2 and 6.25 mg/mL (Boisard et al., 2015; Dolci & Ozino, 2003; Jug, Zovko Kon, & Kosalec, 2014) while Ramanauskiene, Inkeniene, Petrikaite, and Briedis (2013) obtained 0.17 µg/mL (or a factor 1000 of variation). Using propolis from *Baccharis*, a MIC between 0.2 and 0.4 µg/mL has been reported by Salomao et al. (2008) while Fernandes Junior et al. (2005) reported a $CMI_{90} = 0.4\%$ v/v. Concerning the propolis from *Dalbergia*, some researchers obtained a MIC ranging from 50 to 200 µg/mL (Bueno-Silva et al., 2013; Bueno-Silva, Marsola, Ikegaki, Alencar, & Rosalen, 2016) whereas others reported an IC50 between 6 and 25 µg/mL (Monzote et al., 2012). Based on these few non-exhaustive results found in literature but fairly representative, one may identify the very large heterogeneousness of the modes of expression of the results of antibacterial activity. It can be also noticed that the results concerning the poplar propolis revealed a very large variability while it was much more restricted for the two other samples. Finally, by taking only the weakest results, we could be tempted to generalize that the propolis from *Dalbergia* was less effective than the other two propolis against *Staphylococcus* (0.2 vs. 0.2 vs. 50 µg/ml).

Is it correct to give this type of interpretation once the antibacterial activity depends on active compounds contained in these types of propolis? It is accepted that these active molecules belong to the superfamily of polyphenols and to their different subclasses. Moreover, bees have colonized all ecosystems of the earth and consequently there is no universal propolis because it is produced from various available resins in the site of collection. However, in a given geographic area, bees have a preference for specific botanical sources to make their propolis. These propolis samples have the advantage of having a specific chemical fingerprint of the plant sources (Cardinault, Cayeux, & Percie du Sert, 2012). It is on this basis of specificity that some researchers have for about ten years begun to claim the need for a standardization in order to improve its credibility and its accessibility in both the medical and food industry. Taking into account the method of standardization reported in the review by Bankova et al. (2016a), we determined the total polyphenol concentrations in the three extracts of propolis. The levels measured showed that the extract of propolis from *Dalbergia* is 2 and 3 times less concentrated than the propolis extracts from *Baccharis* and poplar (3.6 vs. 7.2 vs. 9.9 g/100 mL) although they underwent exactly the same process of extraction. The minimal content in total polyphenols recommended as quality criteria for the poplar and *Baccharis* propolis (21% and 5% minimal in weight of

raw material) is largely reached by our extracts (28 and 20%, respectively). To our knowledge, no specific standardization and quality standard criteria have yet been defined for the propolis from *Dalbergia*. Regarding our data, is it logical to be tempted to compare and to interpret the efficiency of extracts which do not present the same quantity of active compounds?

In our second series of experiments, we diluted our extracts in order to present exactly the same content in active molecules and MIC was determined in the first experience (0.1 and 0.5% v/v on *S. agalactiae*) to verify if the magnitude of efficiency of the extracts would be the same. The extracts of propolis from *Baccharis* and poplar no longer showed an inhibitory effect on the growth of Gram + bacteria contrary to the first experiment whereas they were applied in both series to the same MIC expressed in % of volume. While the increasing order of efficiency observed in the first series was *Baccharis* $<$ poplar $=$ *Dalbergia*, the order of efficiency became *Baccharis* $=$ poplar $<$ *Dalbergia* although they were applied in both experiments in the same percentage. Similar results were obtained with *E. coli*. Thus, the order of efficiency between these three extracts of propolis changed between both experiments whereas it concerned the same botanical extract of propolis, applied to the same MIC expressed in % of volume.

Taken individually, the interpretations of these two experiments on the efficiency of each of these extracts compared with the others are completely different regarding the same samples of propolis. How can this divergence of interpretation be explained? Maybe that the mode of expression of the MIC expressed in % of volume is not the most appropriate method and, even though it is practical, it requires knowing the concentration of active compounds exactly beforehand in each of the tested extracts. We suggest that one of the best ways to allow for a relevant and objective comparison seems to be to express the MIC in concentration of total polyphenols and to standardize samples before testing them according to the recommendations established by Bankova et al. (2016a). Certainly, the various types of propolis do not contain the same active molecules, and to date very few studies were able to demonstrate a correlation between the total polyphenols content and the antibacterial activity (Popova et al., 2017). This standardization seemed to be efficient in the understanding of the antibacterial effect of the various extracts of propolis which could be better related to the presence of some molecules, even in small quantity, rather than to the global content in active compounds. Besides, this method could also allow an objective comparison of the efficiency of the extracts of propolis in relation to antibiotics.

The results obtained in this conceptual work demonstrated that the biological efficiency of the same botanical extracts of propolis can be interpreted completely differently. The standardization of propolis extracts before being tested, whatever the biological activity, has to become an obligation if we want to compare the results of different studies properly and in a relevant manner in the future. A better understanding of the mechanisms of action of each type of propolis is imperative to indicate the best sample to be used in each therapeutic condition.

Disclosure statement

No potential conflict of interest was reported by the authors.

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