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# Correlation analysis between phenolic levels of Brazilian propolis extracts and their antimicrobial and antioxidant activities

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**Abstract**

Propolis extracts are currently used for the treatment of oronasal infections and as antioxidant agents. Ethanolic commercial Brazilian propolis extracts were assayed for their ability to inhibit the growth of *Staphylococcus aureus* and also for their ability to scavenge DPPH radicals. These activities were correlated with their total phenolic and flavonoid levels. In one group of extracts there was a strong linear relationship between total phenol contents and the measured activities, while in the other group this relationship was weaker. It was also found that flavonoid levels had a greater influence on the antioxidant activity of these extracts than on their antimicrobial profiles.


*Keywords:* Propolis; *Staphylococcus aureus*; Flavonoids; Phenolic acids; DPPH

## 1. Introduction

Propolis is a resinous material gathered by *Apis mellifera* from plants, used in the hives to prevent the decomposition of carcasses and as a sealant (Bankova, Castro, & Marcucci, 2000). The use of propolis extracts in folk medicine dates back to 300 BC (Banskota et al., 1998) and its world consumption is estimated to be around 700–800 tons/year (Nothenberg, 1997). Antimicrobial (Krol, Scheller, Shani, Pietsz, & Czuba, 1993), antioxidant (Pascual, Gonzalez, & Torricella, 1994), antiviral (Vynograd, Vynograd, & Sosnowski, 2000) and antineoplasic (Grunberger et al., 1988) activities have been attributed to these extracts. These activities are associated with the phenolic constituents, especially flavonoids and phenolic acids (Marcucci et al., 2000; Tazawa, Warashina, & Noro, 1999), although some terpenoid compounds have also been implied in these pharmacological actions (Bankova et al., 1996; Matsuno, 1995; Pereira, Silva, Kiltzke, Cardoso, & Aquino Neto, 1999). For reviews on the biological and pharmacological activities of propolis, see Tuo and Chan (2003), Castaldo and Capassao (2002), De Castro (2001). These classes of compounds, as well as non-aromatic acids and enzymes (catalase and proteases) also contribute to the antimicrobial activity of honey, another bee product (Mato, Huidobro, Simal-Lozano, & Sancho, 2003; Weston, 2000).

Although many authors describe the antimicrobial activity of propolis extracts, the mechanism of action is still unknown. Takaisikikuni and Schilcher (1994) showed that *Streptococcus agalactiae*, when grown on a nutrient medium containing propolis extract, prevented its cell division, leading to the formation of pseudo-multicellular streptococci. Recently, the antimicrobial activity of propolis from *A. mellifera* and from *Tetragonisca angustula* against *Staphylococcus aureus* was reported (Miorin, Junior, Custodio, Bretz, & Marcucci, 2003).

The DPPH$^{\cdot}$-scavenging activity of flavonoids and phenolic acids has been studied in depth. Some authors, including Hotta et al. (2002), whose electrochemical investigations led to the proposal that this activity is related, not

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only to their H donation ability, but also to the slower subsequent polymerisation reaction. Goupy, Dufour, Loonis, and Dangles (2003) proposed that the kinetics of the H atom transfer can be more important than the stoichiometry of the overall process.

New legislation on propolis extracts was published recently in Brazil, and it delineates that the ethanolic propolis preparations extract must contain at least 0.25% of flavonoids and 0.50% of phenolic components in relation to the dry weight (Brazilian Ministry of Agriculture, 2000). Usually, crude propolis preparations with higher flavonoid contents are sold at higher prices and are also destined for the foreign market.

Since the chemical composition and the quantitative levels of specific components of propolis extracts are dependent on the geographical origin and the season of collection of the resins by the bees, the present study was conducted to evaluate the in vitro antimicrobial activity over *S. aureus* and the antioxidant activity (suppression of DPPH radical) of Brazilian propolis extracts produced in different geographical regions. Therefore, the quantitative levels of phenolic and flavonoid components of these extracts were determined and a search for a correlation between these parameters was performed.

## 2. Materials and methods

### 2.1. Propolis extracts

The propolis extracts were bought in natural products stores or directly from beekeepers. The 49 extracts analysed were named after their city of origin and date of production: Bacaxa (July 1999), Carmo A (February 2000), Carmo B (January 2002), Conservatoria (March 2000), Mage (November 2000), Nova Friburgo A (April 1998), Nova Friburgo B (May 2000), Nova Friburgo C (June 2001), Nova Friburgo D (February 2002), Paracambi (December 2000), Pati do Alferes (June 1999), Petropolis A (November 1999), Petropolis B (June 2000), Petropolis C (January 2001), Petropolis D (October 2001), Petropolis E (March 2002), Rio Bonito (May 1998), Rio de Janeiro A (August 1999), Rio de Janeiro B (February 2001), Rio de Janeiro C (January 2002), Teresopolis A (March 2000) and Teresopolis B (June 2000) (Rio de Janeiro state); Botelhos (May 2000), Carangola A (March 1998), Carangola B (July 1998), Carangola C (April 1999), Carangola D (July 1999), Carangola E (October 1999), Carangola F (March 2000), Carangola G (December 2000), Carangola H (April 2001), Carangola I (December 2001), Divinopolis (November 2000), Itamonte (April 2000), Juiz de Fora (November 1999), Lima Duarte A (September 1998), Lima Duarte B (October 1999), Lima Duarte C (April 2000) and Sao Lourenço (May 2000) (Minas Gerais state); Colombo A (May 1998), Colombo B (May 2000), Curitiba A (June 1998) and Curitiba B (May 2000) (Parana state); Atibaia (October 2000) and Franca (June 1999) (Sao Paulo state); Salvador (October 1999) (Bahia state); Fortaleza (August 1999) (Ceara state); Picos A (March 1999) and Picos B (January 2000) (Piaui state). The flora of Rio de Janeiro, São Paulo, Minas Gerais and Parana states is tropical and sub-tropical (Atlantic Forest) while, in Bahia, Ceara and Piaui states, there is a predominance of semi-arid vegetation.

### 2.2. Determination of phenolics contents

The phenolic components were determined by using the Folin-Ciocalteau reagent solution (Lugasi, Dworschák, Blázovics, & Kéry, 1998). Gallic acid was employed as the standard and the results were obtained as g of gallic acid per 100 ml of propolis extract (%g).

### 2.3. Determination of flavonoid contents

The flavonoid content was determined by the method described by Dowd (1959), employing $AlCl_3$ to form a complex, which was measured spectrophotometrically at 425 nm. Quercetin was employed as the standard and the results were obtained as g of quercetin per 100 ml of propolis extract (%g).

### 2.4. Microbiological assay

The antimicrobial activity of the propolis extracts against *S. aureus* ATCC 29213 was performed as described by the NCCLS, using Muller-Hinton agar as culture medium (National Committee for Clinical Laboratory Standards, 1993). The minimum inhibitory concentration (MIC) was described as ml of propolis extract per 100 ml of culture medium (%v).

### 2.5. Antioxidant assay

This test was performed as described by Davalos, Gomez-Cordoves, and Bartolome (2003). The antioxidant activity was measured as the percentage of DPPH remaining in solution, calculated by the formula

$$\%\mathrm{DPPH}_{\mathrm{rem}} = A_{\text{propolis extract}}/A_{\mathrm{blank}} \times 100.$$

### 2.6. Statistical analysis

All assays were carried out in duplicate. The results were analysed using ANOVA, F distribution and unpaired Student's *t*-test ($p$ value $\leqslant 0.05$).

## 3. Results and discussion

### 3.1. General

The results obtained in the total phenol and flavonoid assays, as well as the MIC and $\%\mathrm{DPPH}_{\mathrm{rem}}$ values for each extract are listed in Table 1. The statistical analyses for the correlation between the parameters evaluated are listed in Table 2.

Table 1
Values of MIC, %$DPPH_{rem}$, total phenol and flavonoid contents of Brazilian commercial propolis extracts

| Extract | MIC (%v) | %$DPPH_{rem}$ | Total phenolics (%g)[a,b] | Flavonoids (%g)[a,c] |
|---|---|---|---|---|
| Atibaia | 0.5 | 37.5 | 1.47 ± 0.40 | 0.26 ± 0.02 |
| Bacaxá | 0.6 | 55.6 | 0.97 ± 0.01 | 0.16 ± 0.01 |
| Botelhos | 0.3 | 34.4 | 1.76 ± 0.05 | 0.54 ± 0.02 |
| Carangola A | 0.4 | 43.8 | 1.10 ± 0.11 | 0.33 ± 0.01 |
| Carangola B | 0.3 | 32.0 | 1.32 ± 0.17 | 0.42 ± 0.03 |
| Carangola C | 0.3 | 17.4 | 1.35 ± 0.17 | 0.47 ± 0.03 |
| Carangola D | 0.2 | 26.6 | 1.35 ± 0.17 | 0.50 ± 0.04 |
| Carangola E | 0.3 | 18.1 | 1.34 ± 0.18 | 0.45 ± 0.03 |
| Carangola F | 0.3 | 26.6 | 1.25 ± 0.11 | 0.43 ± 0.04 |
| Carangola G | 0.2 | 29.9 | 1.53 ± 0.01 | 0.53 ± 0.02 |
| Carangola H | 0.2 | 20.8 | 1.60 ± 0.30 | 0.63 ± 0.03 |
| Carangola I | 0.2 | 12.5 | 1.43 ± 0.16 | 0.44 ± 0.01 |
| Carmo A | 0.5 | 29.9 | 3.69 ± 0.04 | 0.35 ± 0.01 |
| Carmo B | 0.4 | 36.8 | 1.14 ± 0.13 | 0.24 ± 0.01 |
| Colombo A | 0.6 | 51.6 | 1.64 ± 0.04 | 0.15 ± 0.01 |
| Colombo B | 0.5 | 41.7 | 2.01 ± 0.04 | 0.25 ± 0.02 |
| Conservatoria | 0.3 | 34.4 | 2.93 ± 0.03 | 0.44 ± 0.01 |
| Curitiba A | 0.5 | 64.8 | 0.82 ± 0.04 | 0.08 ± 0.01 |
| Curitiba B | 1.2 | 71.9 | 0.57 ± 0.01 | 0.05 ± 0.01 |
| Divinópolis | 0.3 | 24.2 | 1.73 ± 0.08 | 0.45 ± 0.02 |
| Fortaleza | 1.6 | 87.5 | 0.41 ± 0.01 | 0.12 ± 0.01 |
| Franca | 0.3 | 22.2 | 1.71 ± 0.09 | 0.37 ± 0.01 |
| Itamonte | 0.2 | 13.2 | 1.62 ± 0.22 | 0.45 ± 0.01 |
| Juiz de Fora | 0.7 | 51.6 | 1.01 ± 0.03 | 0.21 ± 0.01 |
| Lima Duarte A | 0.4 | 18.1 | 1.55 ± 0.11 | 0.39 ± 0.01 |
| Lima Duarte B | 0.2 | 31.3 | 1.56 ± 0.06 | 0.42 ± 0.01 |
| Lima Duarte C | 0.4 | 35.4 | 1.50 ± 0.25 | 0.30 ± 0.01 |
| Mage | 2.4 | 64.1 | 1.69 ± 0.08 | 0.11 ± 0.01 |
| Nova Friburgo A | 0.3 | 4.3 | 2.74 ± 0.02 | 0.44 ± 0.01 |
| Nova Friburgo B | 0.3 | 26.6 | 2.78 ± 0.08 | 0.46 ± 0.01 |
| Nova Friburgo C | 0.3 | 27.1 | 2.07 ± 0.01 | 0.34 ± 0.01 |
| Nova Friburgo D | 0.3 | 54.7 | 0.82 ± 0.04 | 0.31 ± 0.02 |
| Paracambi | 0.2 | 19.5 | 1.97 ± 0.28 | 0.65 ± 0.01 |
| Pati do Alferes | 0.2 | 4.2 | 3.90 ± 0.02 | 0.65 ± 0.01 |
| Petrópolis A | 0.3 | 20.8 | 3.44 ± 0.04 | 0.51 ± 0.01 |
| Petropolis B | 0.3 | 21.5 | 1.14 ± 0.01 | 0.39 ± 0.01 |
| Petropolis C | 0.2 | 16.7 | 1.50 ± 0.35 | 0.45 ± 0.01 |
| Petropolis D | 0.3 | 18.8 | 1.72 ± 0.45 | 0.56 ± 0.01 |
| Petropolis E | 0.3 | 27.3 | 1.47 ± 0.07 | 0.45 ± 0.03 |
| Picos A | 0.2 | 29.7 | 2.94 ± 0.02 | 0.33 ± 0.01 |
| Picos B | 0.3 | 39.1 | 2.88 ± 0.04 | 0.26 ± 0.01 |
| Rio Bonito | 0.3 | 28.1 | 1.29 ± 0.15 | 0.45 ± 0.04 |
| Rio de Janeiro A | 0.4 | 55.5 | 1.85 ± 0.01 | 0.21 ± 0.01 |
| Rio de Janeiro B | 0.7 | 60.4 | 1.18 ± 0.01 | 0.11 ± 0.01 |
| Rio de Janeiro C | 0.8 | 66.4 | 0.93 ± 0.07 | 0.06 ± 0.01 |
| Salvador | 3.2 | 83.6 | 0.50 ± 0.01 | 0.06 ± 0.01 |
| São Lourenço | 0.4 | 19.5 | 1.84 ± 0.06 | 0.35 ± 0.01 |
| Teresopolis A | 0.3 | 34.0 | 3.33 ± 0.02 | 0.36 ± 0.01 |
| Teresopolis B | 0.4 | 49.3 | 2.08 ± 0.01 | 0.42 ± 0.01 |

[a] Values are means ± SD ($n = 2$).
[b] As gallic acid equivalents.
[c] As quercetin equivalents.

### 3.2. Phenolic and flavonoid contents and antimicrobial activity

All propolis extracts assayed possess antimicrobial activities ranging from 0.2% to 3.2%v, and they show a weak positive correlation with the total phenolic contents. However, when plotting these values, two different groups of extracts, regarding the relationship between MIC values and the levels of these components, were observed. The first group (Group A), formed by the extracts Carmo A, Conservatoria, Mage, Nova Friburgo A, Nova Friburgo B, Pati do Alferes, Petropolis A, Picos A and B, Salvador, and Teresopolis A, showed a strong relationship between MIC values and total phenol contents while, in the second group, which included all other extracts analysed (Group B), a weaker relationship could be found. In the latter group, some highly active extracts could be identified, the most important one being Nova Friburgo D, which showed a MIC value of 0.3%v (67% of the maximum observed), while containing only 21% of the highest phenolic content. The extracts of this group probably contain highly active components, which are currently being isolated and identified, and they are expected to furnish new lead compounds for further development of new antimicrobial agents.

Some authors state that the biological activities of Brazilian propolis are mostly due to the high levels of phenolic acids (Bankova, Christov, Kujumgiev, Marcucci, & Popov, 1995), whilst flavonoids are considered to be responsible for the activity of European propolis extracts (Hegazi, Hady, & Allah, 2000).

The correlation between MIC values and the flavonoid contents was analysed, and a medium positive relationship could be observed. This result may indicate that flavonoids also have an important role in the determination of Brazilian propolis antimicrobial activity, although this relationship coefficient may be suffering interference from the correlation that exists between phenol and flavonoid contents. A higher correlation between these parameters could be identified in Group A extracts. For Group B, a higher correlation factor was found for the relationship between MIC and flavonoid values than for MIC and phenol levels, which may lend support to the hypothesis that flavonoids are also important for the determination of the antimicrobial potency of Brazilian propolis extracts.

### 3.3. Phenolic and flavonoid contents and antioxidant activity

The analysis of the relationship between the antioxidant activity of propolis extracts and their phenolic content showed a medium positive correlation coefficient. Again, two distinct groups could be observed when these values were plotted: a group with a very strong correlation (Group C) and another with a weaker one (Group D). Group C is formed by the extracts that belongs to Group A (with the exception of Nova Friburgo A extract) plus the extracts Colombo A and B, Curitiba A and B, Fortaleza, Rio de Janeiro A, B and C and Teresopolis B, while the second group is formed by all other extracts that are out of the 90% confidence limit of the Group B correlation plot.

For the relationship between antioxidant activity and flavonoid contents, a higher correlation was found than when correlating this activity and phenolic levels if all

Table 2
Statistical analysis for the correlation between the parameters evaluated

| Correlation | $r$[a] | $r^2$[b] | Es[c] | $F_o$[d] | $F_c$[e] | $S_{xy}$[f] |
|---|---|---|---|---|---|---|
| *Antimicrobial activity* | | | | | | |
| MIC × phenolics content | 0.35 | 0.13 | 0.77 | 6.76 | 4.05 | −0.15 |
| MIC × phenolics content (group A) | 0.90 | 0.82 | 0.43 | 40.04 | 5.12 | −0.90 |
| MIC × phenolics content (group B) | 0.62 | 0.37 | 0.32 | 22.93 | 4.11 | −0.07 |
| MIC × flavonoid content | 0.63 | 0.39 | 0.12 | 30.73 | 4.05 | −0.05 |
| MIC × flavonoid content (group A) | 0.81 | 0.66 | 0.11 | 17.16 | 5.12 | −0.81 |
| MIC × flavonoid content (group B) | 0.75 | 0.55 | 0.11 | 45.75 | 4.11 | −0.03 |
| *Antioxidant activity* | | | | | | |
| $\%DPPH_{rem}$ × phenolics content | 0.50 | 0.25 | 16.97 | 16.00 | 4.05 | −7.80 |
| $\%DPPH_{rem}$ × phenolics content (group C) | 0.95 | 0.91 | 6.90 | 167.14 | 4.45 | −24.09 |
| $\%DPPH_{rem}$ × phenolics content (group D) | 0.67 | 0.45 | 9.29 | 22.94 | 4.20 | −3.09 |
| $\%DPPH_{rem}$ × flavonoid content | 0.85 | 0.72 | 0.08 | 120.90 | 4.05 | −2.57 |
| $\%DPPH_{rem}$ × flavonoid content (group C) | 0.90 | 0.82 | 9.69 | 76.59 | 4.45 | −3.56 |
| $\%DPPH_{rem}$ × flavonoid content (group D) | 0.63 | 0.39 | 9.76 | 18.24 | 4.20 | −11.92 |
| Flavonoid content × phenolics content | 0.45 | 0.20 | 0.14 | 11.69 | 4.05 | 0.06 |

[a] $r$ – correlation coefficient.
[b] $r^2$ – determination coefficient.
[c] *Es* – standard error.
[d] $F_o$ – observed $F$.
[e] $F_c$ – critical $F$ (for statistical significance, $F_o \geqslant F_c$).
[f] $S_{xy}$ – covariance coefficient.

extracts were analysed together, suggesting that flavonoids play an important role in the antioxidant activity of Brazilian propolis extracts. When this analysis was performed on Groups C and D, a higher correlation coefficient was obtained for the former and, in both cases, lower than that observed for the correlation with the phenolic levels.

The spectroscopic methods employed in this work, although simple and non-sophisticated, have already been validated during analysis of poplar-type propolis by comparison with results obtained from these methods with those obtained by HPLC analysis (Popova et al., 2004). The authors concluded that the spectrophotometric methods are useful for routine analysis due to their acceptable repeatability and accuracy. Bruschi, Franco, and Gremiao (2003) have also demonstrated the reliability of the spectroscopic quantification of flavonoids in propolis when using an HPLC-UV methodology.

## 4. Conclusions

Our results are in accordance with reports of the major role of phenolics in the determination of the biological activities of Brazilian propolis extracts (Bankova et al., 1995), but they also lend support to the hypothesis that flavonoids may share with phenolic acids an important role in the determination of these activities.

The groups of propolis extracts identified (A/B for the antimicrobial activity and C/D for the antioxidant activity) contain products from very distinct geographical regions, and also extracts from areas near to one another can show different patterns for the measured activities. This fact is a consequence of the different chemical compositions of these propolis extracts, which turn out to be a function of the biogeographical (local flora, climate, seasonal effects) variables of the locality of origin of the crude propolis.

These results also show that the measured levels of these chemical components do not directly reflect the biological activity of these extracts, and MIC and antioxidant activity measurements should be carried out during the quality control of propolis extracts.

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