

Physicochemical properties and antioxidant capacity of propolis of stingless bees (*Meliponinae*) and *Apis* from two regions of Tocantins, Brazil

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ABSTRACT

The composition of propolis depends on time, vegetation and the location of the collection area. The objective of this study was to determine the physicochemical characteristics, the concentration of phenol compounds and the antioxidant capacity of propolis of native stingless bees (*Meliponinae*) and *Apis* from the State of Tocantins. Extraction with 80% ethanol (v/v) was performed in order to obtain the extracts. Parameters examined were: propolis mass loss by desiccation at 105 °C, ashes, wax concentration and pH. Furthermore, the propolis antioxidant activity was measured, as well as the total concentration of phenol compounds. The extracts were also analyzed by high performance liquid chromatography. The total concentration of phenol compounds varied between 121.78 and 631.29 (mg GAE g⁻¹). The antioxidant activity expressed by the value of CE₅₀ varied between 29.81 and 845.38 µg mL⁻¹. High performance liquid chromatography analysis allowed us to infer the existence of phenol compounds. The results indicated that the studied propolis samples constitute good sources of natural antioxidants. The variety of phenol compounds identified in this study, and the diverse biological functions reported in literature for these compounds indicated that this stingless bee propolis (*Meliponinae*) and *Apis* has a high pharmacological potential.

KEYWORDS: Propolis, antioxidant, phenol compounds.

Propriedades físico-químicas e capacidade antioxidante de própolis de abelhas sem ferrão (*Meliponinae*) e *Apis* de duas regiões do Tocantins, Brasil

RESUMO

A composição da própolis depende do tempo, da vegetação e a localização da área de coleta. Este estudo teve como objetivo determinar as características físicas e químicas, o teor de compostos fenólicos e capacidade antioxidante da própolis de abelhas nativas sem ferrão (*Meliponinae*) e *Apis* do Estado do Tocantins. Para a obtenção dos extratos empregou-se extração com etanol 80% (v/v). Os parâmetros analisados foram: perda de peso por dessecação a 105 °C, cinzas, Teor de cera e pH. Além dessas foi mensurada a atividade antioxidante e o teor de compostos fenólicos totais. Adicionalmente os extratos de própolis foram também analisados por cromatografia líquida de alta eficiência. O teor de compostos fenólicos totais variou entre 121,78 e 631,29 (mg GAE g⁻¹). A atividade antioxidante expressa pelo valor de CE₅₀ variou entre 29,81 e 845,38 µg mL⁻¹. As análises por cromatografia líquida de alta eficiência permitiram inferir a existência de compostos fenólicos. Os resultados indicaram que as amostras de própolis estudadas constituem boas fontes de antioxidantes naturais. A variedade de compostos fenólicos identificada neste estudo, e as diversas funções biológicas relatadas na literatura para estes compostos, indicaram que a própolis de abelhas sem ferrão (*Meliponinae*) e *Apis* tem um grande potencial farmacológico.

PALAVRAS-CHAVE: Própolis, antioxidante, compostos fenólicos.

INTRODUCTION

Propolis is a generic designation given to products with viscous consistencies elaborated by *Apis mellifera* bees from plant parts, salivary secretions and wax produced by the bees themselves. In this way, the composition of propolis is a reflex of vegetable flora, which serves bees (Somnez *et al.* 2005; Cabral *et al.* 2012). Geopropolis is produced by *Meliponinae* bees, which collect resinous material from plants and bring to the beehive, mix with wax and clay or soil (Dutra *et al.* 2008).

Among bee products, propolis has been the subject of pharmacological studies due to its antimicrobial (Simões *et al.* 2008), anti-inflammatory (Franchin *et al.* 2013), antioxidant properties (Perchyonok *et al.* 2013), among others. This biological potential is due to a synergy that occurs between the many constituents. Propolis is an important therapeutic alternative, from the economic point of view, and pharmacologically efficient, easily obtained, presenting pharmaceutical properties (Alencar *et al.* 2005).

Recent studies described the benefits of antioxidant substances for health, due to its effects on disease prevention such as cancer, cardiovascular diseases and aging. Phenol compounds are antioxidant substances frequently found on propolis. Among the most studied is gallic acid (Prado 2009). The use of substances with antioxidant capacity can be of great relevance in the prevention and treatment of diseases related to the increase of oxidative stress (Marquele *et al.* 2006; Lima *et al.* 2009; Restivo *et al.* 2014).

Stingless bees, also known as native bees, social and stingless, occupy most tropical regions in the planet, especially South America. In Brazil, there are more than 400 species of native bees, which are responsible for 90% of pollination of native vegetables (Dutra *et al.* 2008).

Due to the socio-economic importance for small farmers and the environmental preservation provided by the creation of native bees, indicate the relevance of this matter and initiate, in a certain way, the study of therapeutic properties of propolis produced by *Melipona scutellaris* L., *Melipona fasciculata* S. e *Apis mellifera* L. bees in the State of Tocantins.

The objective of this study was to determine the physicochemical characteristics, the concentration of phenol compounds and the antioxidant capacity of propolis of stingless native bees (*Meliponinae*) and *Apis* from the regions of Novo Acordo and Santa Maria of Tocantins, North of Brazil (State of Tocantins).

MATERIALS AND METHODS

Sampling

Samples of propolis were collected, produced by *M. scutellaris* bees from the city of Novo Acordo, as well as samples

of propolis produced by *M. fasciculata* bees from the city of Santa Maria of Tocantins, besides the samples of propolis produced by *A. mellifera* bees from both cities, between January and August of 2014. The samples were collected directly from the apiaries, conditioned in sterile bottles, protected from light and forwarded to the Food Security and Nutritional Laboratories, Research on Natural Products Laboratory and Scientific Instrumentation Laboratory from the Federal University of Tocantins – UFT, for the conduction of the analyzes.

Obtaining the extracts

In order to obtain the extracts, the methodology proposed by Park *et al.* (1998) was used with some modifications. Samples of 4 g of propolis were grinded, homogenized and submitted to extraction with 50 mL of 80% ethanol (v/v), at a temperature of 70 °C, in water-bath (CE-160, CIENLAB, Campinas, Brazil), under constant agitation during 30 minutes. The samples were then centrifuged (80-2B, CentriBio, Campinas, Brazil) at 3500 rpm for 10 minutes; the residue was re-extracted twice using the same concentration of ethanol. The supernatants were combined, concentrated in rotary evaporator (Q344B2, QUIMIS, Diadema, Brazil) and dried in a exhaustion hood. The dry extract was stored in a desiccator until the analyzes were performed.

Physicochemical analyzes specifications

For the analysis of loss by desiccation 105 °C, 3 g of gross propolis, previously pulverized, were weighed and taken to the stove (Q317M-12, QUIMIS, Diadema, Brazil) at 105 °C, for two hours, repeating this process until a constant weight was obtained. The result was calculated by the rate between the volatized material mass and the initial propolis mass, expressed in percentage (European Pharmacopoeia, 2002). In the concentration of ashes analysis, the samples previously dried and weighed were heated in a muffle (BT 201/RP, Biothec, Piracicaba, Brazil) at 600 °C, for 7 hours. The concentration of ashes was calculated by the rate between the mass of the ashes and the initial propolis mass, expressed in percentage (European Pharmacopoeia 2002). In order to determine the concentration of wax, 1.0 g of propolis was weighed, placed in glass bottles with lids and submitted to extraction with three fractions of 10 mL of petroleum ether, under heating in boiling water-bath (b.w.b).

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Statistical analysis

All analyzes were conducted in triplicate. The statistical analyzes used comprised average and standard deviation. Afterwards, the results were submitted to ANOVA analysis and *Post-hoc* Fischer ($p < 0.05$). The correlation analysis by Pearson coefficient was performed according to Figueiredo-Filho and Silva-Júnior (2009). The calculations were conducted using the BIOESTAT 5.3^o software.

RESULTS

It was observed that all propolis produced by *M. fasciculata* and *M. scutellaris* bees produced propolis with sandy physical aspects, while propolis produced by *A. mellifera* bees presented a resinous aspect. The physicochemical characteristics of the propolis evaluated are presented in Table 1.

The correlation equation and the linearity coefficient obtained for the calibration curve used to quantify total phenols were, respectively, $y = 0.0946x + 0.0097$ and $r^2 = 0.9613$. As shown in Table 2, the content of total phenol compounds measured by the Folin-Ciocalteu method

varied significantly ($p < 0.05$) between propolis samples of the two locations.

The evaluation of the total antioxidant activity of propolis extracts collected in both locations and the rutin and ascorbic acid positive controls, in the concentrations of 50, 100 and 200 $\mu\text{g mL}^{-1}$, are shown in Figure 1. Both geopropolis extract presented antioxidant activity statistically equal to the positive controls in the 100 $\mu\text{g mL}^{-1}$ and 200 $\mu\text{g mL}^{-1}$ concentrations, achieving a maximum of $95.38 \pm 0.31\%$ for *M. scutellaris* extract. A lower antioxidant activity was observed in the *A. mellifera* propolis extract collected in the city of Novo Acordo.

The CE_{50} DPPH analysis (Table 2) also showed that propolis extracts from both locations presented significant differences in their potential as sources of free radicals sequestrant substances. Values lower than the CE_{50} reflect a higher antioxidant activity, which was detected in both geopropolis extracts, which were very close to the ascorbic acid and rutin standards. This activity turned out to be very well correlated to total phenol concentrations (-0.8042 , $p = 0.1957$) measured by the Pearson coefficient correlation.

Table 1. Physicochemical requirements from propolis samples from Tocantins and limits established by the Ministry of Agriculture, Livestock and Supply (MAPA).

Sample	Loss by desiccation at 105 °C (% m m ⁻¹)*	Ashes (%m m ⁻¹)*	Wax (%m m ⁻¹)*
<i>A. mellifera</i> NA	6.97 ± 0.26	2.92 ± 0.05	25.45 ± 4.16
<i>M. scutellaris</i> NA	2.94 ± 0.30	1.76 ± 0.02	4.33 ± 1.39
<i>A. mellifera</i> SM	3.30 ± 0.08	2.47 ± 0.06	24.09 ± 4.22
<i>M. fasciculata</i> SM	3.11 ± 0.03	1.40 ± 0.04	0.95 ± 0.91
Requirements in Legislation	Maximum 8%	Maximum 5%	Maximum 25%

*Results presented in average ± standard deviation. NA (Novo Acordo); SM (Santa Maria).

Table 2. Average and standard deviation values of antioxidant activity (DPPH CE_{50} in $\mu\text{g mL}^{-1}$ and total phenol concentrations (mg GAE g⁻¹) found in four propolis samples collected in the cities of Novo Acordo (NA) and Santa Maria of Tocantins (SM).

Propolis Sample	DPPH(CE_{50} $\mu\text{g mL}^{-1}$)	Total phenol (mg GAE g ⁻¹)
<i>M. scutellaris</i> NA	50.23 ± 1.60 c	620.01 ± 6.45 a
<i>A. mellifera</i> SM	260.34 ± 12.27 b	150.78 ± 0.610 b
<i>M. fasciculata</i> SM	29.81 ± 2.49 c	631.29 ± 4.22 c
<i>A. mellifera</i> NA	845.38 ± 31.60 a	121.53 ± 3.05 d
Rutin	53.29 ± 4.10 c	-
Ascorbic Acid	19.20 ± 2.53 c	-

Averages followed by the same letter do not differ statistically between each other at the level of 5% significance by Tukey test.

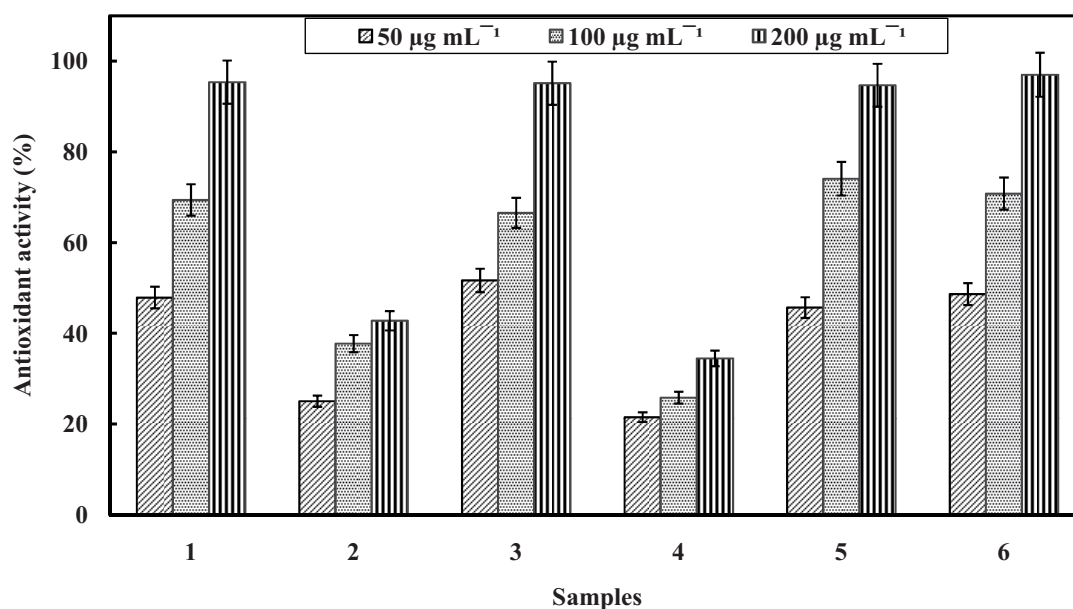


Figure 1. Percentage of antioxidant activity of ethanol extract from propolis samples collected in the cities of Novo Acordo (NA) and Santa Maria de Tocantins (SM) and from positive controls in three concentrations (50, 100 and 200 µg mL⁻¹). 1 – *M. scutellaris* NA, 2 – *A. mellifera* SM, 3 – *M. fasciculata* SM, 4 – *A. mellifera* NA, 5 – rutin positive control and 6 – ascorbic acid positive control. The bars indicate the standard errors.

The evaluation by preparative thin layer chromatography – TLC showed, in all propolis extracts, the existence of phenol compounds, mainly flavonoids, as evidenced by the development of fluorescence in R_f stains, which is characteristic for reference standards.

The *fingerprinting* of propolis samples obtained by HPLC are presented in Figure 2. The comparison of the retention time of samples and the authentic standards allowed the identification of various phenol compounds, such as: ellagic acid (time 29.687 min.), gallic acid (time 8.504 min.), catechin (time 12.596 min.), galocatechin (time 9.774 min.), hesperidin (time 27.873 min.), kaempferol (time 30.393 min.), luteolin (time 31.487 min.), morin (time 28.889 min.), naringin (time 28.419 min.), naringenin (period 30.7652 min.) and rutin (time 28.618 min.). The detection of these substances in extracts are shown in Table 3.

DISCUSSION

The physical aspects of propolis observed in this study, sandy and resinous, are coherent with the Kerr data (1996), where different forms of propolis production were observed between *Meliponinae* and *A. mellifera* bees. Dutra *et al.* (2008) found that *Meliponinae* add clay or soil to the resinous material collected, forming geopropolis, differently from propolis produced by *A. mellifera*. In terms of chemical composition, the products produced by native bee (*Meliponinae*) are less known when compared with the ones produced by *A. mellifera* bees (Evangelista-Rodrigues *et al.* 2005).

Propolis obtained from the *A. mellifera* has a higher concentration of moisture when compared to propolis from *Meliponinae*. The moisture concentrations suggest that propolis produced by *A. mellifera* bees has a chemical composition capable of retaining a higher quantity of water. The moisture concentration results demonstrated that the propolis analyzed are in accordance to the values preconized by current legislation, where it is established that gross propolis must have a maximum moisture concentration of 8% (m m⁻¹) (BRAZIL 2001).

The ash results found are within the maximum limit of 5%, established by specific legislation (BRAZIL 2001). The result was consistent with the data obtained in the ash concentration analyzes conducted by Longhini *et al.* (2007).

The wax concentration is in accordance to the legislation requirements, which is 25% of wax. However, considered high levels when compared to the results obtained by Rodríguez *et al.* (2012). Propolis is a complex mixture containing resin, balm and wax, besides other components. Since phenol compounds are not present in wax, their quantification in the sample becomes an important parameter. In this way, it is desirable to achieve a lower wax concentration in the sample. The results showed that propolis produced by *Meliponinae* bees presented a lower wax concentration (%) due to the presence of clay and soil in their composition.

Castro *et al.* (2007) found total phenol compound values in propolis produced by *A. mellifera* bees similar to the values

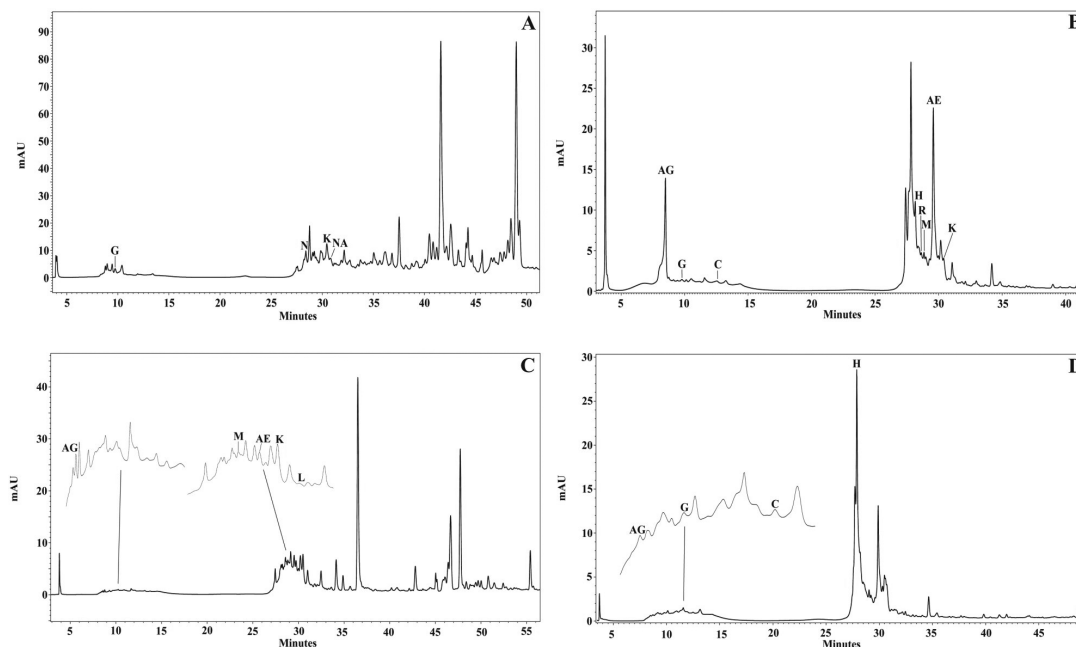


Figure 2. HPLC fingerprinting of ethanol extract from propolis samples collected in the cities of Novo Acordo and Santa Maria of Tocantins. A. *A. mellifera* – City of Novo Acordo; B. *M. scutellaris* – City of Novo Acordo; C. *A. mellifera* – City of Santa Maria; D. *M. fasciculata* – City of Santa Maria. (EA) ellagic acid, (GA) gallic acid, (C) catequin, (G) galocatequin, (H) hesperidin, (K) kaempferol, (L) luteolin, (M) morin, (N) naringin, (NA) naringenin, (R) rutin.

Table 3. Phenol compounds identified by HPLC in the propolis sample extracts produced by native atrophied sting bees and Africanized bees from the region of Novo Acordo and Santa Maria of Tocantins.

Phenol Compounds	Novo Acordo		Santa Maria	
	<i>A. mellifera</i>	<i>M. scutellaris</i>	<i>mellifera</i>	<i>M. fasciculata</i>
	EtOH	EtOH	EtOH	EtOH
Ellagic acid	-	+	+	-
Galic acid	-	+	+	+
Catequin	-	+	-	+
Galocatequin	+	+	-	+
Hesperidin	-	+	-	+
Kaempferol	+	+	+	-
Luteolin	-	-	+	-
Morin	-	+	+	-
Naringin	+	-	-	-
Naringenin	+	-	-	-
Rutin	-	+	-	-

(+) compound detected in the extract; (-) not detected.

found in propolis produced by *A. mellifera* bees from the city of Santa Maria of Tocantins. However, Rodríguez *et al.* (2012) and Cottica *et al.* (2011) found higher values for propolis produced by this species. No studies were found showing total phenol concentrations as high as that from the propolis produced by *M. fasciculata* – city of Santa Maria.

The difference between the total phenol concentrations from these bees constitutes a differential characteristic of this species, which can be associated to the production of geopropolis by *Meliponinae* and propolis with resin by *A. mellifera*.

This difference between total phenol concentrations and antioxidant activity, found in this study, can be related to

the high wax concentration, typical in propolis produced by *A. mellifera*, which presented a lower phenol constitution and free radical sequestrant substances. Because, according to Rodríguez *et al.* (2012), the quantity of wax is a major influencing factor in phenol content of bee products.

Significant amounts of total phenol, obtained in this study, with both *Melliponas* bees corroborate data obtained by Silva *et al.* (2013), indicating that it is characteristic for this type of bee the collection of a major quantity of phenol compounds, since these authors studied two different species for the same genre.

The influence of the extract concentration over the development of antioxidant activity may be related to the high concentrations of phenol compounds present in the propolis samples (Lima *et al.* 2009), since these compounds have a major influence in the biological role. This is confirmed by the high correlation observed between total phenol concentrations and antioxidant activity. Palomino *et al.* (2009) elaborated a correlation and identified that around 60% of the antioxidant activity of evaluated propolis resulted from the contribution of phenol compounds.

The presence of phenol compounds detected in the preparative chromatography analysis TLC and the quantifications in spectrophotometry of propolis samples were confirmed by HPLC analysis, which allowed establishing particular characteristics for each extract. As observed in *fingerprinting* (Figure 2 and Table 3), flavonoid luteolin was restricted to the samples collected in Santa Maria of Tocantins, while, naringerin and rutin, were restricted to the samples collected in Novo Acordo, demonstrating substantial differences between bee pasture of the studied regions. At the same time, it was observed that gallic acid, catequin, galocatequin and kaempferol are fundamental compounds in the formation of propolis of these species, since they were present in most extracts (Cueto *et al.* 2011). It is important to notice that the diversity of compounds observed is consistent with data from literature (Maróstica Jr *et al.* 2008; Cueto *et al.* 2011; Morlock *et al.* 2014). The identified compounds are described as important in the defense to oxidative damage of biological molecules and able to aid in the treatment of various pathologies (Tao *et al.* 2014).

CONCLUSION

Propolis samples collected in the two regions of Tocantins presented concentrations of moisture, ash and wax that fall under Brazilian legislation for propolis quality. They also evidence a high concentration of phenol compounds and good antioxidant capacity. The variety of phenol compounds identified in propolis samples collected for this study, compared to the diverse biological functions described in literature for these compounds, indicates that there is a great pharmacological potential in this raw material.

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