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CHEMICAL CHARACTERIZATION, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF PROPOLIS DRY EXTRACT

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Abstract: The present work identified the bioactive compounds and the antibacterial and antioxidant activity of the dry extract of propolis produced by *Apis mellifera* from the Bay of Iguape, Brazil. Dry extracts of propolis was dissolved in distilled water, ethanol or dimethyl sulfoxide and the bioactive compounds in them were identified. The antibacterial activity against Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and Gram-negative (*Escherichia coli* and *Salmonella* Enteritidis) bacteria, was determined by microdilution, and inhibitory- and bactericidal-concentrations were measured. A major bioactive component was identified to be *p*-coumaric acid (5.178 mg/mL), which has a strong antioxidant effect. Ethanol was the best solvent for extracting antibacterial components, followed by DMSO, while water could not extract such an ingredient. All varieties of bacteria studied were inhibited by the propolis extracts, and Gram-positive bacteria were more sensitive than the Gram-negative bacteria, and the effective concentrations depended on the solvent used.

Keywords: Antimicrobial, *Escherichia coli,* ferulic acid, *p*-coumaric acid, phenolic acids, *Staphylococcus aureus*.

INTRODUCTION

Propolis is an opotherapeutic product, made by bees from plant secretions that undergo enzymatic changes after the addition of the β-glucosidases from the saliva of the bees, wax and pollen (Anvisa, 2003; Anjum et al., 2019). Several studies credit propolis with chemical and biological properties that enables it to be a part of various drugs, cosmetics and food additives, in addition to its proven action as an antibacterial (De Lima et al., 2016), antiviral (Mazia et al., 2016), antioxidant (Olegário et al., 2019), and anticancer (Ebeid et al., 2016) agent, among others.

The chemical composition of propolis is complex and varies according to the place of collection, its source, and the species of bee that produces it. The plant materials available for bees to make propolis (e.g., plant exudates and lipophilic materials from leaves and leaf buds, mucilage, gums, resins and latex) contain a variety of secondary metabolites (Bankova, 2000; Zheng et al., 2017). It enriches the Brazilian propolis in prenylated derivatives of p-cumaric acid (Bankova & Marcucci, 1999), in addition to compounds such as phenolic acids, flavonoids, terpenes, aldehydes, alcohols, fatty acids, stilbenes, amino acids and lignans (Szliszka et al., 2009; Varvara et al., 2017; Anjum et al., 2019), and trace elements (Falcão et al., 2013).

In view of the increasing number of antibiotic-resistant microorganisms, propolis can be an excellent alternative to antibiotics due to its pharmacological properties. The antimicrobial and antioxidant activities, combined with the chromatographic profiles of propolis have been studied, with a view to use propolis in the food industry (Siripatrawan & Vitchayakitti, 2016). These surveys are aimed at ensuring the consistency in chemical composition of propolis and identifying its active principles, to standardize this product (Silva et al., 2012) and direct its use in various commercial products.

Considering the importance of evaluating propolis according to the region of collection, the present work characterized the bioactive compounds and the antibacterial and antioxidant activity of the dry extract of propolis produced by *Apis mellifera* L. from Baía do Iguape, Bahia, Brazil.

MATERIALS AND METHODS

PROPOLIS SAMPLES

Propolis from *Apis mellifera* was collected every month over a year from the apiaries located around the Bay of Iguape, Bahia, Brazil (12° 45' S; 38° 53' W), an area of the second largest coastal bay in Brazil, with diverse vegetation and a low tree cover. Thirty-six samples of propolis were collected from three apiaries. The monthly samples were grouped into four composite samples identified as Sample 1 (for the months from January to April); Sample 2 (May to August); and Sample 3 (September to December).

PROPOLIS DRY EXTRACT

The production of the propolis hydroalcoholic extract followed the methodology of Park et al. (1998). They were weighed, aliquoted and stored in a freezer. For estimating their antibacterial activity, the dry extracts were resuspended in distilled water, 70% ethanol, and dimethyl sulfoxide (DMSO) 1:4 (v/v).

PHYSICOCHEMICAL ANALYSES

Moisture analysis, determination of ash content, and estimation of oxidation activity were performed (AOAC, 2000), followed by determination of waxes, mechanical mass (Brasil, 2001) and total phenolic compounds (Singleton et al., 1999; Park et al., 2004).

ANTIOXIDANT ACTIVITY

The ability to reduce free radicals 2,2 diphenyl-1-picrylhydrazyl (DPPH) was estimated using the method described by Sanchez-Moreno et al. (1999). Antioxidant action of propolis during the peroxidation of linoleic acid, was estimated according to the method used by Ahn, Kumazawa, Hamasaka, Bang, & Nakayama, (2004). As a positive standard synthetic antioxidant was used butilhidroxianisol (BHA) in 0,07 $mg.mL^{-1}$.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY - HPLC

The Shimadzu HPLC system was used, equipped with a prominence pump LC-20AD,

one prominence degasser DGU-20AS, an CTO-10AS VP Column Oven, an Highlighted automatic samplers in the series SIL-20A HT and a photodiode SPD-M20A matrix detector (DAD) (Kyoto, Japão). Phenolic compounds in samples of propolis extracts were identified and quantified by comparing the retention time and UV-vis spectra of pure standards of 4-hydroxybenzoic acid (≥99%), 4-hydroxyphenylacetic acid (98%), chlorogenic acid (> 95%), ferulic acid (≥99%), gallic acid (≥98%), p-cumaric acid (≥98%), protocatechuic acid (99,63%), syringic acid (≥98%), vanillic acid (≥97%); flavonoids: (+) – catechin (≥98%), kaempferol-3-O-glucoside (95%), myricetin (≥96%), naringenin (98%), quercetin (95%), quercetin-3-O-glucopyranoside (≥99%), rutine (≥94%), tiliroside (≥98%); and resveratrol stilbenes (99%). The UV-Visible spectra were recorded in a wavelength range of 190-600 nm and the absorbance was measured at 280, 320 and 360 nm, depending on the maximum absorption of the phenolic compound, as recommended by Moreira et al. (2017). With a gradient program, a Phenomenex Gemini C18 column (250 mm × 4.6 mm, 5 µm) and a protection column maintained at 25°C were used to separate the phenolic compounds. The mobile phase showed 0.1% formic acid in methanol (solvent A) and 0.1% formic acid in water (solvent B), these were degassed and filtered, and later used for elution at a flow rate of 1, 0 ml / min. The following gradient was applied: 0-13 min: 20-26.5% A; 13-18 min: 26.5% A; 18-25 min: 26.5-30% A; 25-50 min: 30-45% A; 50-60 min: 45-50% A; 60-70 min: 50-55% A; 70-90 min: 55-70% A; 90-100 min: 70-100% A, followed by 100% A for 5 min and back to 20% A in 10 minutes and 5 minutes for reconditioning before the next injection. Before injection, the extracts were filtered through a 0.2 µm nylon membrane and 10 µL of each sample was injected. The concentrations of the compounds were calculated in triplicate and expressed in mg/g of sample extract.

MICROORGANISMS AND CULTURE CONDITION

The microorganisms used in the tests were acquired from the Center for Fisheries and Aquaculture Studies (NEPA) at the Federal University of Recôncavo da Bahia, Brazil and from the Escola Superior Agrária (ESA) of the Polytechnic Institute of Bragança, Portugal. These included four Gram-positive strains (*Staphylococcus aureus* ATCC 6538 and *S. aureus* isolated from the environment; *Enterococcus faecalis* ATCC 29212 and *E. faecalis* environmental) and four Gram-negative strains (*Escherichia coli* ATCC 25922 and *E. coli* isolated from the environment; *Salmonella* Enteritidis ATCC 13076 and *Salmonella* Enteritidis isolated from the environment); all of these strains were preserved in tryptone soy agar (TSA) at 10°C.

ANTIBACTERIAL ACTIVITY

The analysis of antibacterial activity were performed following a technique recorded by Morais et al. (2011), using the Mueller-Hinton broth in 96-well microplates. The extracts diluted in the tested solvents were transferred to the first well, with a concentration of 8%, and successive dilutions were performed, obtaining (4; 2; 1; 0.5; 0.25; 0.125; 0 0625%). For procedures where 70% ethanol was used, the microplates were kept open in the laminar flow for 40 minutes for the evaporation of ethanol, to prevent its interference as an antimicrobial agent. The commercial antibiotic (0.1% gentamicin) was used as a control, in addition to the positive and negative controls. After this period, 20 µL of 0.5% TTC (triphenyl tetrazolium chloride) was added to all wells, and incubated at 37°C for 3 hours, before the minimum inhibitory concentration (MIC) was noted for each category. Absence

of color in the wells indicates inhibition of microbial growth.

To determine the minimum bactericidal concentration (MBC), 20 µL of the content of the wells that showed inhibition of microbial growth, were plated on petri dishes containing the Mueller-Hinton agar medium, and incubated at 37ºC for 24 hours before determining the bactericidal effect of the of the extracts at different concentrations. All tests were performed in triplicate.

STATISTICAL ANALYSIS

The results were described as the mean values ± standard deviation. The differences between relevant parameters were analyzed using analysis of variance (ANOVA) followed by the Tukey test. *p* values less than or equal to 0.05 were scored as statistically significant.

RESULTS AND DISCUSSION

The physicochemical parameters of the propolis extract are shown in Table 1 and can be compared with the reference values of Normative Instruction No. 3 of January 19, 2001 (Brasil, 2001).

Among the samples analyzed, statistical differences ($p<0.05$) were observed for ash, mechanical mass, oxidation activity, dry extract, phenols and β-carotene. The differences in the physicochemical composition of propolis are thought to be due to variations between geographic regions, climatic conditions and vegetation that grows around the hives, in addition to the time and conditions of collection (Dias et al., 2012; Andrade, Denadai, Oliveira, Nunes, & Narain, 2017). In this light, it is not surprising that the present study did not show values that were far different from each other, as they were from the close by apiaries that shared the same climate and vegetations.

In all the samples analyzed, the phenol content was more abundant than the flavonoids. Phenolic and flavonoid compounds are the main bioactive components of propolis and are responsible for its functional properties. The present data showed an average 10.01% phenolic content and 0.42% flavonoids. The literature show several reasons for the variation in the contents of these compounds, including the geographical diversity. In addition to their relative contents, the types of constituent compounds are important in determining their biological action, since there are reports of synergistic action among the ingredients (Hochheim et al., 2019). Regarding the flavonoid content, the legislation classifies propolis with values above 2% as high, and those with values up to 1% as low; for phenolic compounds the minimum value established is 5% (Brasil, 2001).

The antioxidant contents showed an average EC50 of 0.37 mg/mL, and there were no significant differences between the different samples evaluated ($p<0.05$). The antioxidant property is important in preservation of food during storage, and for extending its useful life (Seibert et al., 2019). In the pharmaceutical industry, minimization of oxidative stress is essential to control pathological conditions such as diabetes, cancer, cardiovascular diseases, hypertension, atherosclerosis, nephropathy, in addition to cataracts and Alzheimer's disease (Lau et al., 2019). In view of the diversity of molecules found in propolis, it is difficult to determine with precision, which are the main components responsible for the antioxidant activity. According by Banskota et al. (2000), the antioxidant activity of the aqueous and hydroalcoholic extract of propolis is mainly due to derivatives of caffeic acid and cinnamic acid.

All the samples evaluated showed the same bioactive compounds and there was no significant difference ($p <0.05$) between them; p-coumaric acid (5.178 mg/mL) and ferulic acid (1.423 mg/mL), the two

Parameters	Samples			Reference values *
	1	2	3	
Moisture (%)	6.61 ± 0.35 a	5.96 ± 0.56 a	6.84 ± 0.73 a	-
Ash (%)	1.81 ± 0.26 a	1.36 ± 0.15 b	2.00 ± 0.04 a	5 (maximum)
Mechanical mass (%)	38.29 ± 1.85 a	37.41 ± 0.70 ab	34.6 1 ± 0.92 b	40 (maximum)
Insoluble solids %	61.70 ± 1.85 a	62.58 ± 0.70 a	65.38 ± 0.92 a	-
Wax (%)	30.90 ± 3.51 a	26.71 ± 2.28 a	31.82 ± 1.01 a	25 (maximum)
Oxidation activity (“)	21.33 ± 0.40 b	31.53 ± 1.94 a	21.88 ± 0.69 b	22 (maximum)
Dry extract (%)	10.72 ± 0.92 ab	7.34 ± 0.86 b	11.86 ± 2.29 a	-
Fenois (%)	8.48 ± 3.26 b	10.65 ± 5.28 a	10.91 ± 1.41 a	5.0% (minimum)
Flavonoid (%)	0.30 ± 0.60 a	0.36 ± 1.51 a	0.60 ± 0.71 a	0.5% (minimum)
DPPH Ec50	0.39 ± 0.01 a	0.35 ± 0.01 a	0.39 ± 0.05 a	-
β-Carotene (%)	82.27 ± 2.12 a	54.60 ± 3.50 b	83.30 ± 0.98 a	-

* Reference values based on Normative Instruction No. 3 of January 19, 2001. Values followed by the same letter on the line, did not differ statistically ($p < 0.05$).

Table 1. Physical and chemical parameters of the propolis extract from the Bay of Iguape, Bahia, Brazil.

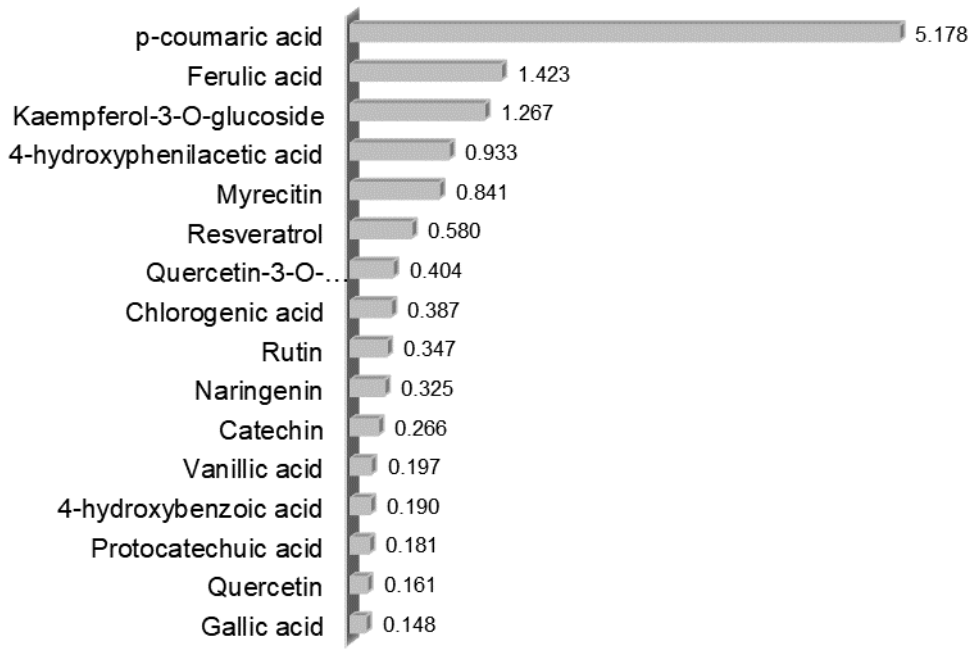

Fig. 1. Compounds identified in the propolis extracts from Baía do Iguape, Bahia, Brazil, using HPLC. Averages of each set of triplicate values are shown as mg/mL.

major components of the propolis samples used in this study, belong to the group of hydroxycinnamic acids, which are a potent antioxidants, and have similar properties (Fig. 1). Chromatographic studies of the different constituents of propolis revealed the presence of these ingredients in relatively high frequency, and these are organic compound that can be used both in the pharmaceutical and food industries (Teixeiraet al., 2013; Keskin et al., 2019). Kaempferol-3-O-glycoside, (1.267 mg/mL), was the third major bioactive constituent found in this study. These compounds confer the propolis samples used in the present study, a significant antimicrobial and antioxidant activity similar to other types of propolis with high commercial value, such as green and red propolis (Andrade et al., 2019).

The differences between the quantities of the phenolics, such as ferulic acid, and flavonoids, like kaempferol-3-O-glucoside, in propolis stem from the different methods of extraction and botanical origins of the propolis used. There are reports of the presence of these substances in important plants, like *Canavalia ensiformis* (L.) DC (Pereira et al., 2018), *Justicia* spp. (Corrêa & Alcântara, 2012), *Acacia nilotica* Lam, *Lycium barbarum* and *Pteridium aquilinum* (Cid-Ortega & Monroy-Rivera, 2018), among others. These authors attribute anti-cancer and anti-diabetic effects of the propolis to these chemical, in addition to the properties studied here.

The chemical profile of propolis must be determined in samples from different regions, as the characteristics vary depending on the climate, vegetation and management, favoring propolis with unique chemical compositions, suitable for specific commercial products and purposes. Utility of propolis depends on its antimicrobial and antioxidant actions, determined mainly by its phenolic contents (Hochheim et al., 2019; Olegário et al., 2019).

Physicochemical and chromatographic analyses indicate the concentrations of the bactericidal and bacteriostatic chemicals in the extract. The MIC was defined as the lowest concentration capable of inhibiting cell growth, while MBC, as its lethal concentration. Table 2 shows the results of the inhibitory effects of different extract of propolis based on the concentration and the type of solvent. The statistical analysis of these results indicated that there was no significant difference between the sample collected from different apiaries or between the different samples analyzed; however there was a significant difference between the extracts using different solvents (ethanol, DMSO and water); and between the different bacterial inoculum employed ($p < 0.001$).

Eethanol was proved to be the best solvent for preparation of the dry extract of the propolis used in this study. Table 2 shows that the inhibition rates on Gram-positive bacteria, where the lowest MIC was seen for the *S. aureus* (1.67 mg/mL), followed by *E. faecalis* (5.0 mg/mL), both in strains isolated from the environment. However, when water was used as a solvent, there was such effects.

All the microorganisms studied showed sensitivity to the propolis extract solubilized in DMSO, even though, at concentrations higher than that dissolved in ethanol. Among them, only the strain *E. coli* ATCC 25922 was resistant to the lethal effects of the extract at the concentrations used in this study. Dimethyl sulfoxide is a better solvent than water for many substances like proteins and steroids (Sturion et al.,1999); in addition, it does not have the disadvantages that ethanol has in terms of altering the sensory characteristics of food products (De Lima et al., 2016) and has less influence on the bactericidal action of the test chemical. Ethanol extracts of propolis are reported to inhibit the growth of Gram-negative bacteria, through the action

Bacteria	MIC*		MBC*	
	Ethanol	DMSO	Ethanol	DMSO
Escherichia coli ATCC 25922	26.67±11.54	80.0±0.0	26.67±11.54	>80.0
Escherichia coli (isolated)	20.0±0.0	40.0±0.0	10.0±0.0	60.0±28.2
Salmonella Enteretidis ATCC 13076	40.0±0.0	66.67±23.1	40.0±0.0	80.0±0.0
Salmonella Enteretidis (isolated)	20.0±0.0	40.0±0.0	10.0±0.0	80.0±0.0
Staphylococcus aureus ATCC 6538	8.33±2.88	20.0±0.0	10.0±0.0	80.0±0.0
Staphylococcus aureus (isolated)	1.67±0.72	26.67±11.54	5.0±0.0	66.67±23.1
Enterococcus faecalis ATCC 29212	10.0±0.0	40.0±0.0	26.67±11.54	80.0±0.0
Enterococcus faecalis (isolated)	5.0±0.0	53.33±23.1	13.33±5.77	60.0±28.3

* mean ± standard deviation

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), expressed as mg/mL, obtained from the dry propolis extracted with ethanol and DMSO.

of aromatic and flavonoid compounds in interrupting metabolism and destruction of the cell wall, only at higher concentrations (Anjum et al., 2019).

The antimicrobial activity of propolis is closely related to the solvent and the extraction method used, as its bioactive components depend on the combined action of the phenolic compounds extracted in the samples. For this reason, although the components responsible for the antimicrobial action are poorly soluble in water, caffeic acid for example, is soluble in water at higher temperature (Jug et al., 2014). However, use of water as a solvent to extract propolis requires specific techniques, and is not as efficient as ethanol (Silva et al., 2012).

Our results also showed that regardless of the solvent used, there was greater resistance from the reference strains in general, requiring larger amounts of the propolis extracts to inhibit growth. This result differs from the study by Silva et al., (2012), which found the inhibition concentrations to be lower for the reference cultures, suggesting the effectiveness of propolis in microorganisms resistant to antibiotics. Hur et al. (2012) demonstrated the resistance of clinical isolates of *Salmonella* sp. to combinations of antimicrobials, and proposed that the bacterial resistance genes act at different sites on the chromosome. However, Barros et al. (2013) suggested that the microbial isolates that were not certified, were likely to be only the colonizers, arguing that these bacteria were not able to express their pathogenicity, but merely survive, under the conditions and techniques used for collection, transport and storage. According to Freires et al. (2016), the mechanism of antimicrobial activity of propolis is complex and can be attributed to the presence of several bioactive compounds, mainly isoflavonoids. Damage to the cytoplasmic membrane, inhibition of nucleic acid synthesis due to topoisomerase inhibition, and reduced energy metabolism are reported.

CONCLUSIONS

Propolis from the Bay of Iguape, Brazil is rich in p-coumaric acid and ferulic acid, and is responsible for its antioxidant effect, in addition to the proven antibacterial action of this product, for use as a potential natural preservative.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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REFERENCES

Andrade, J. K. S., Denadai, M., Oliveira, C. S., Nunes, M. L., & Narain, N. (2017). Evaluation of bioactive compounds potential and antioxidant activity of brown, green and red propolis from Brazilian northeast region, *Food Research International*, *101*, 129-138. https://doi.org/10.1016/j.foodres.2017.08.066

Ahn, M. R., Kumazawa, S., Hamasaka, T., Bang, K. S., & Nakayama, T. (2004). Antioxidant activity and constituents of propolis collected in various areas of Korea. *Journal of Agriculture and Food Chemistry, 52*, 7286-7292. https://doi: 10.1021/jf048726s

Anjum, S. I., Ullah, A., Khan, K. A., Attaullah, M., Khan, H., Ali, H, Bashir, M. A., Tahir, M., Ansari, M. J., Ghramh, H. A., Adgaba, N., & Dash, C. K. (2019). Composition and functional properties of propolis (bee glue): A review, *Saudi Journal of Biological Sciences*, *26* (7). p 1695-1703, https://doi.org/10.1016/j.sjbs.2018.08.013.

AOAC, Association of Official Analytical Chemists. (2000). *Official Methods of Analysis*. AOAC International: Maryland, USA.

Banskota, A.H., Tezuka, T., Adnyana, K., Midorikawa, K., Matsushige, K., D. Message, K., Huertas, A. A., & Kadota, S. (2000). Cytotoxic, hepatoprotective and free radical scavenging effects of propolis from Brazil, Peru, the Netherlands and China, *Journal of Ethnopharmacology*, *72*, pp. 239-246.

Bankova V. S., Castro S. L., & Marcucci M. C. (2000). Propolis recent advances inchemistry and plant origin. *Apidologie*, *31*, 3 -15.

Barros, E., Machado, A., & Sprinz, E. (2013). *Antimicrobianos: consulta rápida*. 5ª Edição – Porto Alegre. Artmed, p. 26-27.

Brasil (2001). Ministério da Agricultura. Instrução Normativa nº 3 – ANEXO VI – *Regulamento técnico para fixação de identidade e qualidade de própolis*. Diário Oficial da República Federativa do Brasil. Brasília, 19 jan. 2001. http://www.apacame.org.br/mensagemdoce/60/normas.htm

Cabral, I. S. R., Oldoni, T. L. C., Prado, A., Bezerra, R. M. N., Alencar, S. M., Ikegaki, M., & Rosalen, P. L. (2009). Composição fenólica, atividade antibacteriana e antioxidante da própolis vermelha brasileira. *Química Nova*, *32,* 1523-1527.

Cid-Ortega, S., Monroy-Rivera, J. A. (2018).Extraction of Kaempferol and Its Glycosides Using Supercritical Fluids from Plant Sources: A Review. *Food technology and biotechnology*, *56*(4), 480–493. https://doi.org/10.17113/ftb.56.04.18.5870

Corrêa, G. M., Alcântara, A. F. C. (2012). Constituintes químicos e atividades biológicas de espécies de Justicia: uma revisão. *Revista Brasileira de Farmacognosia*, *22* (1), 220-238. https://doi.org/10.1590/S0102-695X2011005000196

De-Lima, G. G., Souza R. O., Bozzi, A. D., Poplawska, M. A., Devine, D. M., Nugent, M. J. (2016). Extraction Method Plays Critical Role in Antibacterial Activity of Propolis-Loaded Hydrogels *Journal of Pharmaceutical Sciences*, *105*, 1248 – 1257.

Dias, L. G., Pereira, A. P., Estevinho, L. M. (2012). Comparative study of different Portuguese samples of propolis: Pollinic, sensorial, physicochemical, microbiological characterization and antibacterial activity. *Food Chemistry*, *50*, 4246-4253.

Ebeid, S. A., El Moneim, N. A. A., El-Benhawy, S. A., Hussain, N. G., Hussain, M. I. (2016). Assessment of the radioprotective effect of propolis in breast cancer patients undergoing radiotherapy. New perspective for an old honey bee product, *Journal of Radiation Research and Applied Sciences, 9*, 431-440.

Falcão, S. I., Tomás, A., Vale, N., Gomes, P., Freire, C., Vilas-Boas, M. (2013). Phenolic quantification and botanical origin of Portuguese propolis, *Industrial Crops and Products, 49*, 805-812. https://doi.org/10.1016/j.indcrop.2013.07.021.

Freires, I. A., Alencar, S. M., Rosalen, P. L. (2016). A pharmacological perspective on the use of Brazilian red propolis and its isolated compounds against human diseases. *European Journal of Medicinal Chemistry*, *110*: 267-79. https://doi: 10.1016 / j.ejmech.2016.01.033.

Hochheim, S., Guedes, A., Faccin-Galhardi, L., Rechenchoski, D. Z., Nozawa, C., Linhares, R. E., Silva, H. H., Rau, M., Siebert, D. A., Micke, G., Cordova, C. M. M. (2019). Determination of phenolic profile by HPLC–ESI-MS/MS, antioxidant activity, in vitro cytotoxicity and anti-herpetic activity of propolis from the Brazilian native bee *Melipona quadrifasciata*, *Revista Brasileira de Farmacognosia, 29* (3), 339-350, https://doi.org/10.1016/j.bjp.2018.12.010.

Hur, J., Jawale, C. Lee, J. H. (2012). Antimicrobial resistance of Salmonella isolated from food animals: a review. *Food Research International*, *45* (2), 819-830. https://doi.org/10.1016/j.foodres.2011.05.014

Jug, M., Koncic, M. Z., Kosalec, I. (2014). Modulation of antioxidant, chelating and antimicrobial activity of poplar chemo-type propolis by extraction procures, *LWT - Food Science and Technology, 57* (2), 530-537. https://doi.org/10.1016/j.lwt.2014.02.006

Keskin, M., Keskin, S., Kolayli, S. (2019). Preparation of alcohol free propolis-alginate microcapsules, characterization and release property. *LWT - Food Science and Technology*, *108*, 89-96. https://doi.org/10.1016/j.lwt.2019.03.036

Lau, M., Giri, K., Garcia-Bennett, A. E. (2019). Antioxidant properties of probucol released from mesoporous silica. *European Journal of Pharmaceutical Sciences*, 138. https://doi.org/10.1016/j.ejps.2019.105038

Mazia, R. S., Pereira, R. R. A., Francisco, L. M. B., Natali, M. R. M., Filho, B. P. D., Nakamura, C. V., Bruschi, M. L., Ueda-Nakamura, T. (2016). Formulation and evaluation of a mucoadhesive thermoresponsive system containing Brazilian green propolis for the treatment of lesions caused by herpes simplex Type I. *Journal of Pharmaceutical Sciences*, *105* (1), 113-121. https://doi:10.1016/j.xphs.2015.11.016

Morais, M., Moreira, L., Feás, X., Estevinho, L.M. (2011). Honeybee-collected pollen from five Portuguese Natural Parks: palynological origin, phenolic content, antioxidant properties and antimicrobial activity. *Food and Chemical Toxicology*, *49* (5), 1096-1101. https://doi.org/10.1016/j.fct.2011.01.020

Moreira, M. M., Barroso, M. F., Boeykens, A., Withouck, H., Morais, S., Delerue-Matos, M. C. (2017). Valorization of apple tree wood residues by polyphenols extraction: Comparison between conventional and microwave-assisted extraction. *Industrial Crops and Products*, *104* (1), 210-220. https://doi.org/10.1016/j.indcrop.2017.04.038

Olegário, L. S., Andrade, J. K. S., Andrade, G. R. S., Denadai, M., Cavalcanti, R. L., Da Silva, M. A. A. P., Narain, N. (2019). Chemical characterization of four Brazilian brown propolis: An insight in tracking of its geographical location of production and quality control. *Food Research International*, *123*: 481-502. http://doi:10.1016/j.foodres.2019.04.004

Park, Y. K., Ikegari, M., Abreu, J. A. S., Alcici, N. M. F. (1998). Estudo da preparação dos extratos de própolis e suas aplicações. *Ciência e Tecnologia de Alimentos*, *18* (3), 313-318. http://dx.doi.org/10.1590/S0101-20611998000300011

Park, Y. R., Paredes-Guzman, J. F., Aguiar, C. L., Alencar, C. L., Alencar, S. M., Fujiwara, F. Y. (2004). Chemical constituints in Baccharis dracunculifolia as the main botanical origino f southeastern Brazilian própolis. *Journal of Agricultural and Food Chemistry*, *52* (5). http://doi: 10.1021 / jf021060m

Pereira, J. C., Paulino, C. L. A., Granja, B. S., Santana, A. E. G., Endres, L., Souza, R. C. (2018). Potencial alelopático e identificação dos metabólitos secundários em extratos de *Canavalia ensiformis* L. *Revista Ceres*, 2018, *65*(3), 243-252. https://doi.org/10.1590/0034-737x201865030004

Seibert, J. B., Bautista-Silva, J. P., Amparo, T. R., Petit, A., Pervier, P., Almeida, J. C. S., Azevedo, M. C., Silveira, B. M., Brandão, G. C., Souza, G. H. B., Teixeira, L. F. M., Santos, O. D. H. (2019). Development of propolis nanoemulsion with antioxidant and antimicrobial activity for use as a potential natural preservative. *Food Chemistry*, *287*, 61-67. https://doi.org/10.1016/j.foodchem.2019.02.078.

Sanchez-Moreno, C., Larrauri J. A., Saura-Calixto F. (1999). Free radical scavenging capacity and inhibition of lipid oxidation of wines, grape juices and related polyphenolic constituents. *Food Research International*, *32*, 407-412, 1999. http://dx.doi.org/10.1016/S0963-9969(99)00097-6

Singleton, V. L., Orthofer, R., Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin- Ciocalteau reagent. *Methods of Enzymology*, *299*, 152–178. http://dx.doi.org/10.1016/S0076-6879(99)99017-1

Silva, J. C., Rodrigues, S., Feás, X., Estevinho, L. M. (2012). Antimicrobial activity, phenolic profile and role in the inflammation of propolis. *Food and Chemical Toxicology*, *50* (5), 1790-1795. https://doi.org/10.1016/j.fct.2012.02.097

Siripatrawan, U., Vitchayakitti, W. (2016). Improving functional properties of chitosan films to be used as active food packaging by incorporation with propolis. *Food Hydrocolloids, 61,* 695-702. https://doi.org/10.1016/j.foodhyd.2016.06.001

Slanzon, G. S., Toledo, A. F., Silva, A. P., Coelho, M. G., Silva, M. D., Cezar, A. M., Bittar, C. M. M. (2019) Red propolis as an additive for preweaned dairy calves: Effect on growth performance, health, and selected blood parameters. *Journal of Dairy Science*, *102* (10): 8952-8962. https://doi:10.3168/jds.2019-16646.

Sturion, D. J., Pinheiro, E. R., Pardo, E., Tanaka, N. M. (1999). Efeitos hepatotóxicos e nefrotóxicos do dimetil sulfóxido em aplicações tópicas em cães. UNOPAR Cient., *Ciências Biológicas e da Saúde*, *1*(1), 41-47. https://doi.org/10.17921/2447-8938.1999v1n1p%25p

Szliszka, E., Czuba, Z. P., Domino, M., Mazur, B., Zydowicz, G., Krol, W. (2009). Ethanolic extract of propolis (EEP) enhances the apoptosis – inducing potential of TRAIL in cancer cells. *Molecules*, *14* (2), 738-754. https://doi.org/10.3390/molecules14020738

Teixeira, J., Gaspar, A., Garrido, E. M., Garrido, J., Borges, F. (2013). Hydroxycinnamic Acid Antioxidants: An Electrochemical Overview. *BioMed Research International, 251754*, 1–11. https://doi:10.1155/2013/251754

Varvara, S., Bostan, R., Bobis, O., Gaina, L., Popa, F., Mena, V., Souto, R. M. (2017). Propolis as a green corrosion inhibitor for bronze in weakly acidic solution. *Applied Surface Science, 426*, 1100-1112. https://doi.org/10.1016/j.apsusc.2017.07.230

Zheng, Y. Z., Deng, G., Liang, Q., Chen, D. F., Guo, R., Lai, R. C. (2017). Antioxidant Activity of Quercetin and Its Glucosides from Propolis: A Theoretical Study. *Scientific reports, 7*, 7543. https://doi:10.1038/s41598-017-08024-8