DIFFERENT SOLVENTS FOR EXTRACTION OF BRAZILIAN GREEN PROPOLIS: COMPOSITION AND EXTRACTION YIELD OF PHENOLIC COMPOUNDS

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Abstract. Propolis has been used as a remedy in folk medicine, in apitherapy, as a constituent of biocosmetics, health foods and in numerous other purposes. The various biological activities of propolis have been attributed mainly to the presence of phenolic compounds, especially flavonoids and phenolic acids. Propolis has antioxidant, antibacterial and antifungal properties, combined with the fact that several of its constituents are present in food and/or food additives make it an attractive candidate as a natural preservative in new food applications. Natural compounds with high biological activity can be obtained by conventional techniques, such as soxhlet or by alternative methods such as supercritical fluid extraction (SFE). In this context, the aim of this study was to investigate different extraction techniques, such as extraction with water and ethanol as solvents and supercritical fluid extraction to obtain concentrated flavonoids extracts from green Brazilian propolis. The supercritical assays were carried out using the dynamic method to obtain extraction curves and the global yield (X_0) at temperatures of 40 and 60 °C and pressure of 400 bar, using ethanol as co-solvent. All extracts were analyzed to total phenols and flavonoids. All supercritical extracts presented higher flavonoids concentration to the initial value found in EEP (ethanolic extract of propolis), indicating that SFE has a tendency to focus on flavonoids, which makes supercritical fluid extraction very interesting to fractionate compounds of propolis, which according to several authors, have higher biological activity.

Keywords: Supercritical fluid extraction, Soxhlet extraction, Phenolics compounds, Green brazilian propolis

1. Introduction

Over the last few decades, interest in functional foods has been growing fast, leading to the discovery of new functional components or processes that can improve food processing, as well as products that may help to retard aging or avoid diseases. In this context, bee products have gained the attention of consumers and researchers, due to their chemical compositions and functional properties.

Propolis possesses antibacterial, antifungal and antiviral properties and many other beneficial biological activities: anti-inflammatory, antiulcer, local anaesthetic, hepatoprotective, antitumos, immunostimulating [1], anti-HIV, among others. These biological activities are attributed to compounds such as phenolic acids, flavonoids, terpenes and sesquiterpenes [2]. Due to these characteristics, which can bring health benefits, propolis is considered a functional ingredient and is used in food, beverages, cosmetics and medicine to improve health and prevent diseases [3].

The chemical composition of propolis is complex and variable, being related to the vegetation of the region visited by bees. Propolis generally contains 50 % resin, 30 % wax, 5 % pollen, 10 % aromatic oils and 5 % other organic residues [4].

Propolis cannot be used as raw material, and it must be purified by extraction with solvents. This process should remove the inert material and preserve the polyphenolic fractions. A multi-step extraction with ethanol is particularly suitable to obtain dewaxed propolis extracts rich in polyphenolic components [4].

The extraction technique used to obtain aggregate value compounds from natural raw materials defines the product quality. There are several well-established extraction procedure, called conventional methods, such as hydro-distillation (HD) and organic solvent extraction like Soxhlet (Sox) and maceration (Mac) techniques [5]. The limitations of conventional methods are high energy costs, elevated solvent use, high temperatures, injurious for thermolabile substances, and solvent residue in the solute, reducing the product quality [6].

Supercritical fluid extraction (SFE) is considered a technological innovation, adequate for high valuable products due to the use of low temperatures, efficiency in solvent use, recycling possibility, reduced energy consumption and product quality due to the absence of solvent in solute phase. SFE is a versatile technology, attributed to its selectivity characteristic, acquired by the control of the extracting conditions temperature and pressure. Therefore, SFE is exceedingly considered a viable alternative for pharmaceutical, fine chemistry and food stuff areas [7,8].

Raw propolis was extracted by Stahl et al. [9] using supercritical CO_2 at 600 bar and 40 °C to extract the wax and leave the insoluble flavonoids behind. Catchpole et al. [10] used SC-CO_2 both as an antisolvent to precipitate high-molecular mass components, and also as a solvent to extract the ethanol and soluble components of the EEP (non-dried). Lee et al. [11] extracted DHCA from Brazilian propolis using SC-CO_2 modified with co-solvent, followed by column chromatography to obtain very pure DHCA. Chen et al. [12] using a SC-CO_2 extract containing 41.2 % (wt) DHCA, successfully suppressed growths of human colo-205 cancer cells, although the total yield of the SC-CO_2 extract was relatively low when compared with the extract obtained with ethyl acetate in a Soxhlet apparatus. Paviani et al. [13] studied the SFE of dried ethanolic extract from green Brazilian propolis, investigating the fractionation of components of interest present in the propolis extract and the results indicated higher selectivity at low density.

In this context, the aim of this study was to investigate different extraction techniques, such as extraction with two different solvents (water, ethanol) and supercritical fluid extraction to obtain concentrated flavonoids extracts from red and green Brazilian propolis.

2. Material e Methods

2.1. Sample Preparation

Samples of green propolis, native to the State of Minas Gerais, Brazil, classified as group 12 for Park et al. [14] (Brazil has 13 different groups of propolis, with distinct characteristics), were obtained from Natucentro Ltda. (Bambuí, Minas Gerais, Brazil). The moisture content determined by the Karl Fisher method (Methom 701KF Tritino equipped with 832 KF Thermoprep) was 5.20 ± 0.2% for green propolis, similar results to those obtained in studies of green propolis. The raw materials were packed into plastic bags and stored in a domestic freezer (Consul, model 220, São Paulo, Brazil) at –10 °C.

2.2. Ethanolic Extract of Propolis (EEP)

Ethanolic extract of green and red propolis (EEP) were obtained using the methodology of Paviani et al. [13] where 3 g of crude propolis was mixed with 10 mL of ethanol (Merck, Darmstdadt, Germany) and stirred using a magnet stirrer for 1 day at room temperature. The insoluble portion was then separated by filtration; the filtrates kept in a freezer at –10 °C overnight and then filtered again to reduce the wax content of the extracts. The solvent was evaporated off in a vacuum oven at a temperature of 60 °C to obtain dry ethanolic extract of green and red propolis and the yield results were calculated based on the initial amount of propolis.

2.3 Aqueous Extract of Propolis (EAP)

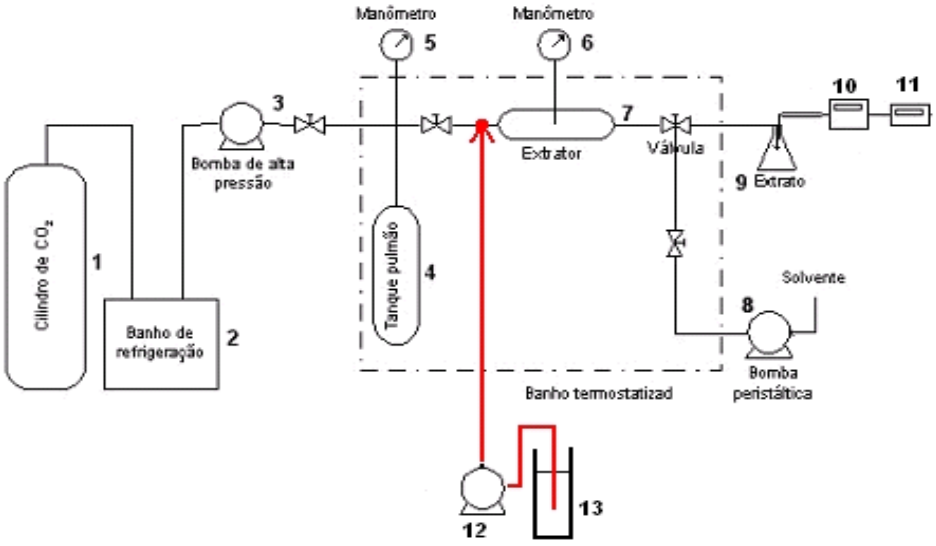
The raw propolis was ground and sieved on a sieve mesh 32 in order to increase the surface area extraction. Then, the weighed raw propolis in a centrifuge tube and added with deionized water in the following proportions: each 0.5 g of propolis using 10 mL of water. In a bath at 80 °C, the sample was allowed to stand for 10 minutes. The tube was led to the centrifuge for 20 minutes at 10.000 rpm. The supernatant was filtered on paper filter and the filtrate was evaporated in an oven with vacuum pump, resulting in a dried aqueous extract of propolis. This extract was stored in a freezer at 10 °C. The yield of aqueous extract of propolis (EAP) was defined as the mass of aqueous extract of propolis obtained by dry mass of raw propolis used in percentage.

2.3. Soxhlet Extraction (Sox)

The Soxhlet extraction (Sox) was performed according to Cunha et al. [15]. Pulverized raw green propolis (5 g) placed inside a paper timber was submitted to 6 h Soxhlet extraction at a maximum temperature of 60 °C, using 150 mL of solvent. Two different solvents were used: ethanol (EtOH) and distilled water (H_2O); with polarities of 5.2 and 9.0 [16], respectively, in order to evaluate the influence of their different polarities on extraction yield and total phenols and flavonoids. The extracts were maintained at freezer overnight and then filtered to remove waxes. The resulting extracts were evaporated in a rotary evaporator (Marconi, MA120, Brazil) with vacuum control and a thermostatic bath to obtain the dry extract and the yield results were calculated based on the initial amount of propolis (w/w). All extractions were performed in triplicate.

2.4. Supercritical Fluid Extract (SFE)

The experimental apparatus used in this work is shown in Fig. 1. It consisted of a 100 mL equilibrium cell (stainless steel AISI 316, Suprilab, Campinas, Brazil) (7) immersed in a water bath controlled by a heater (Suprilab) to within 0.1 °C. The CO_2 from the supply tank (1) was cooled to a liquid state (refrigerated bath model 12101-30, Cole Parmer Instrument Company, Vernon Hills, IL) (2) and compressed into the equilibrium cell by a high-pressure pump (model AA100S, Eldex Laboratories, Inc., Napa, CA) (3). The extracts were obtained in duplicate at a temperature of 40 °C 60 °C and pressure of 400 bar. In a typical experimental run, approximately 5 g of dry EEP was mixed with glass balls and packed inside the extractor. The CO_2 was pumped into the extractor bed, which was supported by two 300-mesh wire disks at both ends. A static period of 30 min was used to allow contact between the samples and the supercritical solvent, so as to guarantee that the operational conditions of temperature and pressure were stabilized. The CO_2 (White-Martins, 99.95%) mass flow rate was 1.0 g/min. The samples were collected and all the tubing in the process line was washed with ethanol to recover the extract deposited in it. The global extraction yields *X*o (%) were calculated as the ratio of the total mass of extract (extraction + cleaning process) to the initial mass of raw material (dry basis). The solute/solvent separation system consisted of a micrometering valve.



1 - CO_2 cylinder
2 - Refrigeration bath
3 - High pressure pump
4 - CO_2 tank
5,6 – Manometers
7 - Extractor
8 – Peristaltic pump
9 – Extraction vessel
10,11 – Flow meters
12 – Co-solvent pump
13 – Co-solvent flask

Figure 1. Schematic diagram of the supercritical carbon dioxide extractor.

2.5. Extraction Composition

Determination of the total polyphenol content (TPC). The total polyphenol content was quantified using the Folin–Ciocalteu reagent, according to the procedure of Singleton et al.[18]. 1mL of diluted extract was transferred to a 25 mL volumetric flask containing 9mL of ultra pure water. The Folin–Ciocalteu reagent (1 mL) was added and mixed. After 5 min, 10mL of sodium carbonate (7 %) were added and the volume completed with ultra pure water. After 90 min of incubation at 23 °C in the dark, the absorbance was measured at 750 nm in a spectrophotometer (UV–VIS lambda 40, Perkin Elmer, USA), and the result calculated using a pre-prepared gallic acid calibration curve (0–100 mg/L). The blank was prepared using the same procedure with 1mL of ultra pure water in the place of the 1mL extract. The results expressed as equivalents of gallic acid (mg GAE/g dry extract).

Determination of total flavonoid contents (TF). The total flavonoids were measured as described by Zhishen et al. [19]. An aliquot (1 mL) of adequately diluted extract or of the aqueous catechin solutions (0–100 mg/L) was added to a 10 mL volumetric flask containing 4mL of ultra pure water, and 0.3mL sodium nitrite (5 %) added. After 5 min, 0.3 mL of aluminum chloride solution (10 %) was added, and after a further 6min, 2mL of sodium hydroxide solution (1M) was added and the volume completed with ultra pure water. The absorbance was measured against ultra pure water as the blank at 510 nm, using a UV/VIS spectrophotometer (UV–VIS lambda 40, Perkin Elmer, USA). The results were expressed in catechin equivalent (mg CE/g dry extract).

3. Results and Discussion

Extraction yield of conventional techniques and supercritical fluid extraction. The results of extraction yield (in %) are shown in Table 1 for low pressure extraction methods: EEP and Soxhlet with different solvents for green propolis: EtOH and H_2O.

Table 1. Global yield (X_0 % w/w) of propolis extract obtained by conventional techniques**.**

Extraction method	Solvent[a]	Solvent polarity index[b]	X_0 (% w/w)
EEP- Green propolis	EtOH	5.2	41.80 ± 2.9
EAP- Green propolis	Water	9.0	21,20 ± 1,30
Sox- Green propolis	EtOH	5.2	49.38 ± 1.37
Sox- Green propolis	Water	9.0	15.75 ± 0.99

[a]EtOH: ethanol; [b]Ref. [16].

The results of EEP yield of green Brazilian propolis (36.78%) was similar to that obtained by Funari et al.[20], Paviani et al. [13] and Biscaia and Ferreira [21] that presented a value of 38.34 ± 2.05%, 39.45 ± 1.20 and 46.00 ± 6.00, respectively. The differences in extraction yield and the concentrations were probably related to characteristics of the raw propolis, such as the harvesting season, bee species (in the case of Brazilian bees, also including the degree of "Africanization" with *Apis mellifera*) and regional flora. The dry aqueous extract of propolis (EAP) was analyzed in terms of average and obtained 21.20 ± 1.30%, This result is consistent with those found in the literature.

Sox-water showed lower extraction yield because water has affinity with polar compounds, the –OH group turns water in to a bad solvent for organic compounds. Although water presents higher polarity index than all other solvents used in sox but the yield was lower, because the polarity is not the only factor affecting the extraction effiency, and it is important to understand the different interactions between solute and solvent [22]. The yield of soxhet extraction with water as solvent was 15.75 ± 0.99 %, similar to that obtained of Biscaia and Ferreira [21] which obtained 14.3 ± 0.5 %.

The largest yield was obtained by Sox-EtOH (49.38 ± 1.37), solvent with intermediate polarity, probably due to high temperature and solvent recycle in Sox method, which contribute to increase the solubilization of components from raw material. Cunha et al [15] observed the same behavior for the extraction of green propolis from Brazilian Southwest region.

Table 2. Global yield (X_0%) of fractionation of EEP-green propolis obtained by supercritical fluid extraction.

Run	T (°C)	P (bar)	ρCO_2 (kg/m^3)[a]	X_o (% w/w)
SFE-1	40	300	840.80	3.56
SFE-2	40	400	910.30	5.00
SFE-3	50	300	870.80	6.57
SFE-4	50	400	923.40	9.74
SFE-5	60	300	830.00	12.86
SFE-6	60	400	890.20	12.26

[a]Ref. [23].

The results of supercritical extraction yields (Table 2) indicate that the solubilization power of supercritical CO_2 depends mainly on the density, which increases with increasing pressure at constant temperature and decreases with decreasing temperature at constant pressure. In general, the extraction yields increase with increasing temperature and pressure. The highest yield obtained by SFE was about 13% at 60°C and 300 bar, which is much lower than X_o by Sox-EtOH (49.38 ± 1.37% w/w). The SFE yield data were similar to the value obtained by Sox-Hex, a low polarity solvent, similar to CO_2. Lower extraction yield represents high selectivity and vice-versa, therefore, the combination of yield and selectivity is fundamental for the definition of the best extraction conditions for substances with biological activity [21].

The supercritical extraction of EEP in the temperature conditions of 60 ºC and pressure of 400 bar was obtained 12.26 % of extraction yield. . In this experiment were spending approximately 250.3 L CO_2, which is equivalent to approximately 0.41 kg of CO_2 (the average pressure = 13.68 psi and average temperature 29.27 °C).

Total polyphenol and flavonoid contents. Some authors state that the biological activities for Brazilian propolis are mostly due to the high levels of phenolic acids, whilst flavonoids are considered to be responsible for the activity of propolis extracts [1,24].

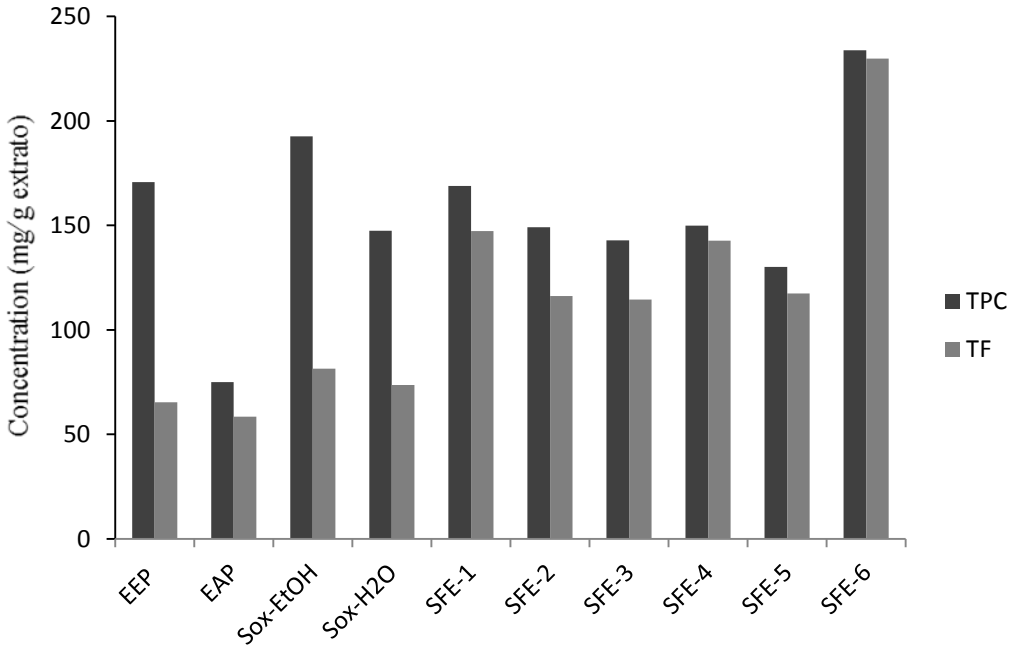


Figure 2. Concentration of polyphenolics and flavonoids compounds.

Extraction of EEP-green propolis (170.74 mg GAE/g) was more efficient when compared with EAP-green propolis (75.04 mg GAE/g), in terms of TPC contents. The TPC contents in the propolis extracts obtained by Soxhlet extraction is higher when using ethanol as solvent, then the soxhlet process is dependent of solvent polarity.

The highest concentration of total phenolics and flavonoids were obtained at 40 °C and pressure of 400 bar (Fig. 2), obtained 184.30 mg GAE / g and 311.2 mg CE / g, respectively. These values were higher than

those obtained by the EEP of red propolis, this is an indication these operational conditions give a extract concentrated in phenolic compounds. At 40 °C and 300 bar, 887 % of total phenols (168.82 mg GAE/g) are flavonoids (147.27 mg CE/ g), this condition the extract showed the highest concentration of flavonoids in the extract.

At 60 °C the amount of total phenols at 400 bar pressures bar was higher those obtained at 40 and 50 ºC. Therefore, the increase in pressure at 60 °C promotes the increase in total phenols and flavonoids. Flavonoids content tends to increase with increasing temperature at a pressure of 400 bar.

The extraction of phenolic compounds from EEP was more efficient than those obtained by supercritical fluid extraction, except for the run SFE-6 (60°C/400 bar). For the flavonoids, the EEP is lower than those found in supercritical extraction in all conditions analyzed.

4. Conclusion

The results of the extraction of raw green propolis different solvents showed high extraction yields, especially when ethanol was used as solvent. The highest supercritical fluid extraction yield was obtained at 60 °C. All supercritical extracts presented higher flavonoids concentration to the initial value found in EEP, indicating that SFE has a tendency to focus on flavonoids, which makes supercritical fluid extraction very interesting to fractionate compounds of propolis, which according to several authors, have higher biological activity.

This work is part of a research, other results will be published in journals indexed.

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References

[1] G. A. Burdock, 1998. Review of the biological properties and toxicity of bee propolis (propolis). Food and Chemical Toxicology 36 (1998) 347–363.

[2] J. Ito, F.-R. Chang, H.-K. Wang, Y. K. Park, M. Ikegaki, N. Kilgore, K-H. Lee, Anti HIV activity of moronic acid derivatives and the new melliferone related triterpenoid isolated from Brazilian propolis. Journal of Natural Products 64 (2001) 1278-1281.

[3] IFIC – International Food Information Council Foundation. 2009. Functional Foods. Available at <http://www.ific.org/nutrition/funtional/index.cfm.

[4] P. G. Pietta, A. M. Gardana, A. M. Pietta, Analytical methods for quality control of propolis. Fitoterapia, 73 Suppl. 1 (2002) S7-S20.

[5] E. Reverchon, I. De Marco, Supercritical fluid extraction and fractionation of natural matter. Journal of Supercritical Fluids 38 (2006) 146-16.

[6] T. S. Weinhold, L. F. V. Bresciani, C. W. Tridapalli, R. A. Yunes, H. Hense, S. R. S. Ferreira, *Polygala cyparissias* oleoresin: Comparing CO_2 and classical organic solvent extractions. Chemical Engineering and Processing 47 (2008) 109-117.

[7] A. Jouyban, H. Chan, N. R. Foster, Mathematical representation of solute solubility in supercritical carbon dioxide using empirical expressions. Journal of Supercritical Fluids 24 (2002) 19–35.

[8] G. Brunner, Gas extraction, Springer. New York, 1994, p. 5-28.

[9] E. Stahl, K. W. Quirin, D. Gerard, Dense Gases for Extraction and Refining (1998), Springer, Berlin.

[10] O. J. Catchpole, J. B. Grey, K. A. Mitchell, J. S. Lan, Supercritical antisolvent fractionation of propolis tincture. Journal of Supercritical Fluids 29 (2004) 97–106.

[11] Lee Y.-N., Chen C.-R., Yang H.-L., Lin C.-C., Chang C.-M.J. 2007. Isolation and purification of 3,5-diprenyl-4-hydroxycinnamic acid (Artepillin C) in Brazilian propolis by supercritical fluid extractions. Separation and Purification Technology 54, 130–138.

[12] C.-R. Chen, C.-T. Shen, J.-J. Wu, H.-L. Yang, S.-L. Hsu, C.-M. J.Chang, Precipitation of sub-micron particles of 3,5-diprenyl-4-hydroxynnamic acid in Brazilian propolis from supercritical carbon dioxide anti-solvent solutions. Journal of Supercritical Fluids 50 (2009) 176-182.

[13] L. C. Paviani, C. Dariva, M. C. Marcucci, F. A. Cabral, Supercritical carbon dioxide selectivity to fractionate phenolic compounds from dry ethanolic extract of propolis. Journal of Food Engineering 33 (2009) 15-27.

[14] Y. K. Park, S. M. Alencar, C. L. Aguiar, Botanical origin and chemical composition of Brazilian propolis, Journal of Agricultural and Food Chemistry 50(2002) 2502-2506.

[15] I. B. S. Cunha, A. C. H. F. Sawaya, F. M. Caetano, M. T. Shimizu, M. C. Marcucci, F. T. Drezza, G. S. Povia, P.

O. Carvalho, Factors that Influence the yield and composition of Brazilian propolis extracts. Journal of Brazilian Chemical Society 15 (2004) 964– 970.

[16] Byers J.A., Catalog Phenomenex. 2009. Available at: http://www.chemicalecology.net/java/solvents.htm.

[17] H. A. Martinez-Correa, P. M. Magalhães, C. L. Queiroga, C. A. Peixoto, A. L. Oliveira, F. A. Cabral, Extracts from pitanga (*Eugenia Uniflora* L.) leaves: Influence of extraction process on antioxidant properties and yield of phenolic compounds. The Journal of Supercritical Fluids 55 (2011) 998-1006.

[18] V. L. Singleton, R. Orthofer, R. M. Lamuela-Raventos, Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. Methods in Enzymology 299 (1999) 152–178.

[19] J. Zhishen, T. Mengcheng, W. Jianming, The determination of flavonoids contents in mulberry and their scavenging effects on superoxide radicals. Food Chemistry 64 (1999) 555–559.

[20] C. S. Funari, V. O. Ferro, M. B. Mathor, Analysis of propolis from *Baccharis dracunculifolia* DC. (Compositae) and effects on mouse fibroblasts. Journal of Ethnopharnacology 111 (2007) 206-212.

[21] D. Biscaia, S. R. S. Ferreira, Propolis extracts obtained by low pressure methods and supercritical fluid extraction. Journal of Supercritical Fluids 51 (2009) 17-23.

[22] P. P. Almeida, N. Mezzomo, S. R. S. Ferreira, Extraction of *Mentha spicata* L. volatile compounds: Evaluation of process parameters and extract composition. Food and Bioprocess Technology. DOI: 10.1007/s11947-010-0356-y.

[23] S. Angus, B. Armstrong, K. M. Reuck, 1976. International Thermodynamic Table of the Fluid State: Carbon Dioxide. 1° Ed. London: Pergamon Press.

[24] V. Bankova, R. Christov, A. kujumgiev, M. C. Marcucci, S. Popov, Chemical composition and antibacterial activity of Brazilian propolis. Zeitschrift fur Naturforschung 50c (1995) 167-172.

[25] S. M. Alencar, T. L. C. Oldoni, M. L. Castro, I. R. S. Cabral, C. M. Costa-Neto, J. A. Cury, P. L. Rosalen, M. Ikegaki, Chemical composition and biological activity of a new type of Brazilian propolis: Red propolis. Journal of Ethnopharmacology 113 (2007) 278-283.