DOI: 10.2478/JAS-2014-0024 J. APIC. SCI. VOL. 58 NO. 2 2014

DE GRUYTER OPEN

Original Article

BOTANICAL ORIGIN OF THE BRAZILIAN RED PROPOLIS: A NEW APPROACH USING DNA ANALYSIS

Sona Jain*
Giulia Marchioro
Lucyana Mendonça
Marcus Batista
Edilson Araujo

Department of Biology, Federal University of Sergipe
Av. Marechal Rondon, s/n Jardim Rosa Elze, 49100-000 São Cristóvão (Sergipe), Brazil

*corresponding author: sonajain24@yahoo.com
Received 19 August 2014; accepted 12 November 2014

Abstract

Propolis is produced by the honeybees by using resin and other plant secretions. Propolis from different geographical regions have different chemical compositions. This is because the chemical constituents of propolis depend on the vegetation surrounding the apiary. In this report we present a new approach using DNA barcoding for the identification of the botanical origin of propolis. Red propolis samples were collected at different times of the year from the state of Sergipe situated in Northeast Brazil. Extraction of the DNA from propolis was made using a CTAB method. Amplification was done using ITS2 universal primers, followed by DNA sequencing. Sequence analysis confirmed the presence of *Dalbergia ecastaphyllum* in the Brazilian red propolis. Formononetin is a chemical marker for the Brazillian red propolis and *D. ecastaphyllum*. Propolis samples analysed by DNA sequencing, were also checked by Ultra-Fast Liquid Chromatography for the presence of formononetin. Peaks corresponding to formononetin were observed in all the analysed propolis samples. This is the first report of the botanical origin of propolis using DNA technology.

Keywords: *Apis mellifera*, Brazilian red propolis, DNA, formononetin, molecular approach, plant source.

INTRODUCTION

Propolis is a complex resinous mixture collected by *Apis mellifera* from various plant sources. It is composed of resin acquired from the bark of the trees and leaf buds. The material is masticated by the honeybees, partially digested with the salivary enzymes and mixed together with beeswax. (Ghisalberti, 1979; Marcucci, 1995; Bankova et al., 2000). Propolis is used to seal the holes in the honeycombs, smooth internal walls and protect the entrance against intruders (Ghisalberti, 1979; Greenway, 1990). Propolis is known for its antimicrobial, anticancer, anti-inflammatory, anti-HIV, antiseptic, and antioxidant properties. The list of its industrial and pharmaceutical uses is nearly endless (Burdock, 1998; Bankova et al., 2000). Propolis has been used in folk medicine since 300 BC and its use recently in food and beverages, cosmetics, formulations for cold syndromes, mouth washes, and toothpastes has increased extensively due to its health-related benefits (Ghisalberti, 1979; Bankova et al., 2000; Banskota et al., 2001; Wagh, 2013).

Medicinal and biological properties of a particular type of propolis are dependent on its chemical composition which in turn is dependent on the chemical constituent of the resin collected by the bees. Hence, propolis from different geographical regions, or propolis collected in different seasons of the year, vary in their chemical content due to changes in the vegetation type surrounding the apiary (Ghisalberti et al., 1978; Greenway et al., 1990; Marcucci, 1995; Simões-Ambrosio et al., 2010). It is, therefore, important to connect a particular propolis to its plant of origin. In this way, a specific propolis from a determined area or a set time of the year, can be linked to its biological significance or medical applications.

Bud exudate of poplar trees are the main source of propolis from Europe and China (Bankova et

al., 2000). The plant origin of Brazilian propolis is more complicated because of Brazil's rich botanical diversity. Twelve different types of Brazilian propolis were reported by Park et al. (2002) based on their chemical composition and geographical location. A new type of propolis called the Brazilian red propolis, was reported by Daugsch et al. (2008) along the sea and river shores of Northeast Brazil.

Chromatographic methods, microbotanical analysis, and direct observation of the collecting behavior of the bees are the most commonly utilised methods for the botanical identification of propolis. (Salatino et al., 2005; Teixeira et al., 2005; Daugsch et al., 2008). The botanical origin of most of the Brazilian green propolis is reported to be alecrim plants (*Baccharis dracunculifolia*). The origin was found by observing the collecting behavior of the bees and analysing the anatomical characteristics of alecrim vestiges in resin and propolis (Marcucci and Bankova, 1999; Kumazawa et al., 2003). The botanical origin of the Brazilian red propolis was found to be *D. ecastaphyllum* in a similar way: by observing the collection behavior of the bees, and by comparison of the phenolic compounds present in the plant exudate and propolis with the use of reverse phase chromatography (Daugsch et al., 2008; Silva et al., 2008). However, the above mentioned conventional methods can be tedious, and require skilled labor and a good state of conservation of the morphological structures. Molecular methods using molecular genetics are emerging as powerful identification tools due to their accuracy and rapidness. Among these, the use of DNA barcoding is of special interest as it does not require any prior knowledge of the DNA under analysis. Also, DNA barcoding primers amplify short DNA sequences and hence tend to work better in the case of slightly fragmented template DNA (Galimberti et al., 2013).

Propolis contains plant resins, pollen, and most importantly, other plant fragments which can be the source of DNA and utilised for the identification of its botanical origin. Extraction of DNA from propolis was reported for the first time in our previous publication (Jain et al., 2014). Now, in our present manuscript, we present a novel approach for the identification of the botanical origin of propolis. Using DNA sequencing, we also confirm the presence of *D. ecastaphyllum* in the red propolis samples collected from the state of Sergipe, Northeast Brazil.

MATERIAL AND METHODS

Sample collection

All the propolis samples were collected from the same apiary located in Sergipe, Brazil (S 10°28'25" and W 36°26'12", over a period of one year. Samples showing the red coloration typical of Brazilian red propolis from the months of February, April, May, and September were selected for analysis.

Ultra-Fast Liquid Chromatography (UFLC)

The sample for UFLC was prepared by extracting 1g of propolis with 12.5 mL of 70% ethanol at room temperature, for 1 hour in an ultrasound bath. After extraction, the mixture was centrifuged, and the supernatant was evaporated under low pressure to produce Hydroalcoholic Propolis Extracts (HPEs) used for UFLC. A reverse-phase column (XP-ODS 50 x 3 mm; particle size, 2.2 µm) with a diode array detector (Shimadzu Co.) was used according to the method described by Alencar et al. (2007) and Cabral et al. (2009), with modifications. Methanol (50 mg/mL) was used to dissolve the HPE. Then the substance was filtered with a 0.45 µm filter (Millipore). Next, 2 µL aliquots of 1% HPE (w/v) were injected into the UFLC system. The column was eluted using a linear gradient of water (solvent A) and methanol (solvent B) with a solvent flow rate of 0.4 mL/min. The gradient was started at 40% B, increased to 60% B (after 22.5 min), held at 90% B (37.3-42.3 min), and then decreased to 30% B (after 42.3 min). Chromatograms were recorded at 260 nm and processed using LC Solutions software. Formononetin was used as a standard.

DNA extraction and PCR

Propolis (5 g) was washed with hexane. Then, 200 mg of each pre-washed sample was used for DNA extraction using a CTAB method as described by Jain et al. (2013, 2014). The extracted DNA was dissolved in 25 µL of TE and stored at -20°C for further use.

Polymerase chain reaction was carried out to amplify the variable ITS2 region which is a part of ITS locus of nuclear rDNA, using primers on the 5.8S and 28S conserved regions. Amplification was carried out using 1µL of 1:10 diluted stock DNA, 0.5 µM primers Bel1, and S3R (Chen et al., 2010; Gao et al., 2010), and 10 µL of 2 X Red PCR mix (Amplicon, Denmark) in a final volume of 20 µL. The following conditions were used to perform PCR: 94°C/5 min, followed by 40 cycles of 94°C/30 seconds, 65°C/30 seconds, 72°C/45 seconds, and a final extension at 72°C for

10 min. After PCR, 5 µL of the reaction mixture was run on 1.5% agarose gel, stained with SYBR Green (SYBR Green I, Biotecnologia LCG) and visualised under UV. Leaf samples of *D. ecastaphyllum* collected from the same region as the propolis were utilised for PCR analysis as a positive control. DNA preparation, PCR and sequencing were repeated 2 – 4 times for each sample in independent experiments with similar results.

DNA sequencing

Polymerase chain reaction-amplified DNA bands were purified using NucleoSpin® Gel and a PCR Clean-up kit (Macherey-Nagel, Germany), quantified and sequenced. Sequencing reactions were carried out with the ABI PRISM BigDye® Terminator Cycle Sequencing V.3.1 kit (Applied Biosystems). The amplified products were sequenced directly using the ABI 3500 DNA sequencer (Applied Biosystems). Sequencing quality and contig assembly were assessed using Pregap4 and Gap4 programs, which are part of the Staden package (Staden, 1996). Only sequences with a Phred value above 30 were considered for the contig assembly. Local sequence alignments were carried out to determine the sequence identity when compared to other sequences from GenBank, using BLAST with default parameters (Altschul et al., 1990). All the samples were sequenced twice from two independent experiments.

RESULTS

The results of the PCR analysis from four different propolis samples (lanes 3 – 6) and two different *D. ecastaphyllum* samples (lanes 1 – 2) are shown in Figure 1. Polymerase chain reaction amplification with the plant specific primers as described in the Material and Methods section, produced a single band of approximately 400 bp in all the samples including the positive controls. Sequence analysis of these PCR amplified bands was carried out to know the source of these DNA bands. Sequencing results confirmed that all the samples and the controls amplified the same ITS2 region. The DNA sequences presented Phred values above 30, which indicate good quality for molecular identification. An online database-comparison of the 400 bp amplified ribosomal fragment with the sequences present in the GeneBank showed 100% identity with ITS2 fragment from *D. ecastaphyllum,* [Accession number EF451072] and *D. monetaria*as [Accession number EF451073]. Ultra-Fast Liquid Chromatography (UFLC) chromatograms of the four propolis samples analysed in this study are shown in Fig.2. Chromatographic profiles of all the samples showed formononetin as one of the major compounds. The amount of formononetin did differ depending on the time of the year.

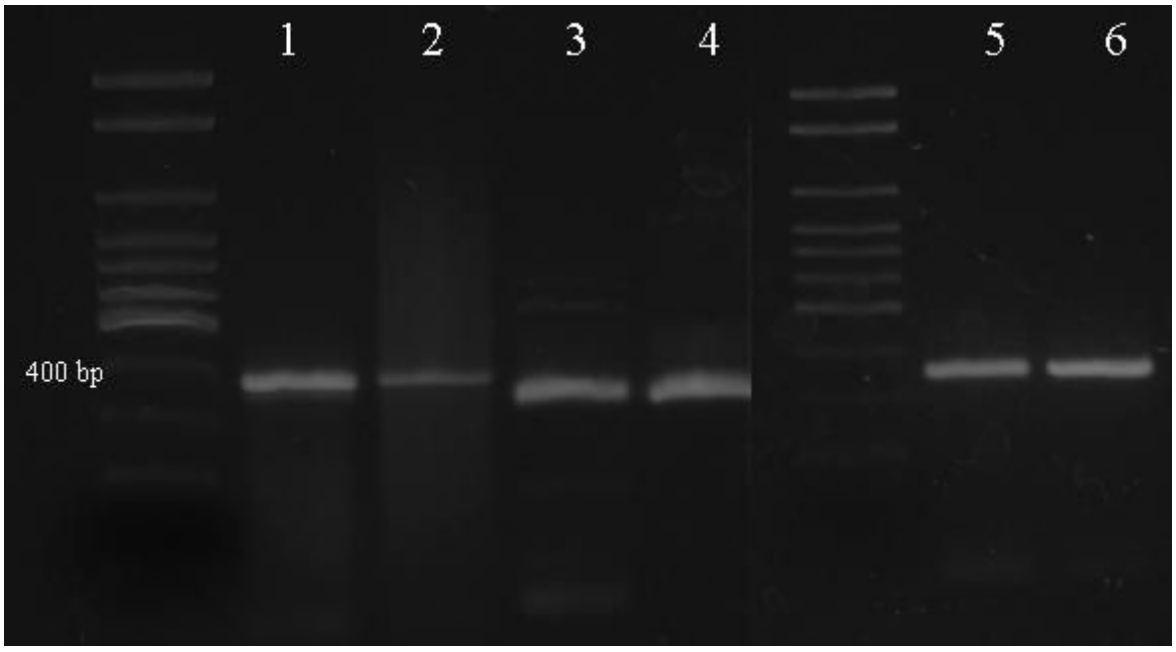

Fig. 1. PCR analysis using primers Bel1 and S3R. Lanes 1 – 2 D. *ecastaphyllum*. Lanes 3 – 6 propolis samples from the months of February, April, May, and September.

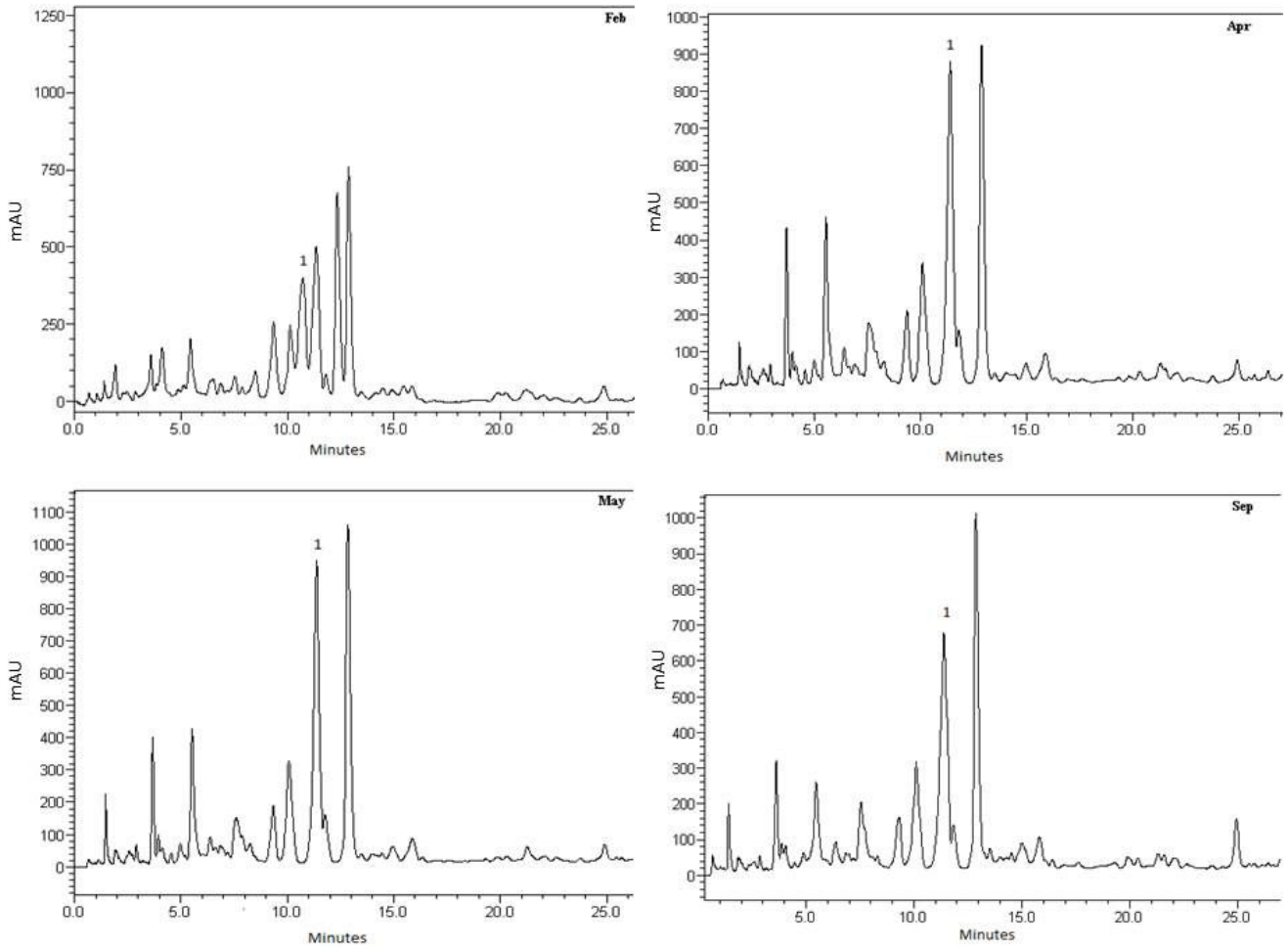

Fig. 2. UFLC chromatograms of the Hydroalcoholic propolis extracts (HPE) collected in the months of February, April, May, and September. Number 1 denotes the peak corresponding to formononetin.

DISCUSSION

A taxonomic identification tool, DNA barcoding, identifies polymorphisms in DNA samples under analysis by PCR and DNA sequencing. It is based on the fact that genome of plants, animals, and microorganisms contain small conserved regions which can be used for their identification. The most commonly utilised barcoding regions in plants are MatK, rbcL, ITS, trnH-psbA (Gao et al., 2010; Crautlein et al., 2011). In this report, ITS2 locus was amplified and sequenced to confirm the presence of *D. ecastaphyllum* in the red propolis samples from Northeast Brazil. To reduce the size of the amplified band, and thus increase the probability of DNA amplification from propolis DNA, the ITS2 region instead of the whole ITS locus was targeted.

Comparison of the PCR amplified bands from the propolis samples and the sequences present in the GenBank, showed 100 % identity with ITS2 fragment from both *D. ecastaphyllum,* and *D. monetaria*. This is probably true because DNA barcodes differ less among individuals of the same species than among species. However, *D. ecastaphyllum* is the only species of *Dalbergia* found in this region (Carvalho, 1997). Moreover, the amplified fragments from *D. ecastaphyllum* used as a control showed 100% identity with the DNA sequences from propolis samples, thus, supporting our results.

Chromatographic analysis confirmed the presence of formononetin in all the propolis samples analysed in this study. Formononetin is reported to be one of the main constituents of *D. ecastaphyllum* and red propolis samples from Northeast Brazil and can be used as a chemical marker for their identification (López et al., 2014). Also, the analysis by Silva et al. (2008), Daugsch et al. (2008) and López et al. (2014) which compare the chemical constituents of propolis from Northeast Brazil and *D. ecastaphyllum by* chromatographic methods, confirmed *D. ecastaphyllum* as one of the main sources of resin for the Brazilian red propolis.

The results of our sequence analysis confirmed the results published by Silva et al. (2008), Daugsch

et al. (2008), and López et al. (2014). In our study, *D. ecastaphyllum* was found to be present in all the propolis samples that we analysed. We are the first to use a DNA based method.

Chemical analysis of the resins present in propolis by: chromatographic methods, micro analysis of the plant fragments present in the propolis as was the case of green propolis from Brazil, and direct observation of bee behavior, are the methods currently utilised for the botanical origin of propolis (Kumazawa et al., 2003; Teixeira et al., 2005; Daugsch et al., 2008). The presence of contaminants and the formation of resin complexes of diverse botanical origins can significantly hamper the sensitivity of the chromatographic methods. Similarly, morphological identification of the plant micro fragments present in the resin can get very difficult and time consuming. The identification of the botanical micro structures require a profound knowledge of the micro-morphology of the plant species, which is an area with a great shortage of skilled professionals. This analysis also requires a good state of conservation of the morphological structures of the plant material, which is generally poor. On the other hand, DNA based methods represent a quick and reliable identification method, less dependent on the state of conservation of the morphological structure of the plant material. In this work, we used the same basic principle used in the identification of green propolis from Brazil. However, we assumed that the presence of micro-botanical fragments in resin could serve as a source of DNA for its amplification by PCR using barcoding primers that amplify small DNA fragments, and seem to work well even in the case of slightly fragmented DNA (Galimberti et al., 2013). This DNA based method represents a new methodological approach completely independent of the most commonly used chemical method for the identification of the botanical origin of propolis, and can be used as a complementary tool for its confirmation. The DNA based method in this study was tested with the red propolis from Brazil and needs to be tested with other types of propolis. Propolis produced from other plant material may contain substances, whose removal using the described procedures, may not be possible. Among these substances, inhibitors of DNA polymerases could occur, inhibiting PCR reaction. Thus, the method might need special adjustments in some cases and might not function with all the types of propolis.

CONCLUSION

The molecular approach presented in this study, was successfully utilised for the identification of the botanical origin of red propolis. The results prove the usefulness of DNA analysis as an important tool for the determination of the botanical origin of propolis. This DNA-based method is a totally new approach for the botanical identification of propolis. The method can be utilised together with conventional methods for the confirmation of the presence or absence of a particular plant species.

ACKNOWLEDGEMENT

The authors would like to thank the Technology Platform of Sequencing of Genomics and Gene Expression Laboratory – LABCEN/CCB/UFPE for the use of its facilities, and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Apoio à Pesquisa e à Inovação Tecnológica do Estado de Sergipe (FAPITEC-SE) for the financial grant that supported this work.

REFERENCES

Alencar S. M., Oldoni T. L. C., Castro M. L., Cabral I. S. R., Costa-Neto C. M., Cury J. A., Rosalen P. L., Ikegakid M. (2007) Chemical composition and biological activity of a new type of brazilian propolis: red propolis. Journal of Ethnopharmacology 113(2): 278-283.

Altschul S. F., Gish W., Miller W., Myers E. W., Lipman D. J. (1990) Basic local alignment search tool. Journal of Molecular Biology 215(3): 403-410. DOI: 10.1016/S0022-2836(05)80360-2

Bankova V. S., Castro S. L., Marcucci M. C. (2000) Propolis: recent advances in chemistry and plant origin. Apidologie 31(1): 3-15. DOI: 10.1051/apido:2000102

Banskota A. H., Tezuka Y., Kadota S. (2001) Recent progress in pharmacological research of Propolis. Phytotherapy Research 15(7): 561-571. DOI: 10.1002/ptr.1029

Burdock G. A. (1998) Review of the biological properties and toxicity of bee propolis. Food and Chemical Toxicology 36(4): 347-363. DOI: 10.1016/S0278-6915(97)00145-2

Cabral I. S. R., Oldoni T. L. C., Prado A., Bezerra R. M. N., Alencar S. M. (2009) Composição fenólica, atividade antibacteriana e antioxidante da própolis vermelha brasileira. Química Nova XY(00): 1-5.

Carvalho A. M. (1997) A Synopsis of the Genus Dalbergia (Fabaceae: Dalbergieae) in Brazil. Brittonia 49(1): 87-109.

Chen S., Yao H., Han J., Liu C., Song J., Shi L., Zhu Y., Ma X., Gao T., Pang X., Luo K., Li Y., Li X., Jia X., Lin Y., Leon C. (2010) Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. PloSONE 5(1): e8613. DOI: 10.1371/journal.pone.0008613

Crautlein M., .Korpelainen H., Pietiläinen M., Rikkinen J. (2011) DNA barcoding: a tool for improved taxon identification and detection of species diversity. Biodiversity and Conservation 20(2): 373-389. DOI: 10.1007/s10531-010-9964-0

Daugsch A., Moraes C. S., Fort P., Park Y. K. (2008) Brazilian red propolis – chemical composition and botanical origin. Evidence-Based Complementary and Alternative Medicine 5(4): 435-441. DOI: 10.1093/ecam/nem057

Galimberti A., Mattia F. D., Losa A., Bruni I., Federici S., Casiraghi M., Martellos S., Labra M. (2013) DNA barcoding as a new tool for food traceability. Food Research International 50(1): 55-63. DOI: 10.1016/j.foodres.2012.09.036

Gao T., Yao H., Song J., Liu C., Zhu Y., Ma X., Pang X., Xu H., Chen S. (2010) Identification of medicinal plants in the family Fabaceae using a potential DNA barcode ITS2. Journal of Ethnopharmacology 130(1): 116-121. DOI: 10.1016/j.jep.2010.04.026

Ghisalberti E. L. (1979) Propolis a review. Bee World 60: 59-84.

Ghisalberti E. L., Jefferies P. R., Lanteri R., Matisons J. (1978) Constituents of Propolis. Experientia 34: 157-158.

Greenway W., Scaysbrook T., Whatley F. R. (1990) The composition and plant origin of propolis: a report of work at Oxford. Bee World 71(3): 107-118.

Jain S., Jesus F. T., Marchioro G. M., Araujo E. D. (2013) Extraction of DNA from honey and its amplification by PCR for botanical identification. Food Science and Technology 33(4): 753-756. DOI: 10.1590/S0101-20612013000400022.

Jain S., Marchioro G. M., Costa A. C., Araujo E. D. (2014) Extraction and amplification of DNA from Brazilian red propolis. Scientia Plena 10(3): 039902.

Kumazawa S. H., Yoned M., Shibata I., Kanaeda J., Hamasaka T., Nakayama T. S. (2003) Direct evidence for the plant origin of Brazilian propolis by the observation of honeybee behavior and phytochemical analysis. Chemical and Pharmaceutical Bulletin 51(6): 740-742. DOI: 10.1248/cpb.51.740

López B. G., Schmidt E. M., Eberlin M. N., Alexandra C. H. F., Sawaya a. c. (2014) Phytochemical markers of different types of red propolis. Food Chemistry 146: 174-180.

Marcucci M. C. (1995) Propolis: Chemical composition, biological properties and therapeutic activity. Apidologie 26(2): 83-99. DOI: 10.1051/apido:19950202

Marcucci M. C., Bankova V. S. (1999) Chemical composition, plant origin and biological activity of Brazilian Propolis. Current Topics in Phytochemistry 2: 115-117.

Park Y. K., Alencar S. M., Aguiar C. L. (2002) Botanical origin and chemical composition of Brazilian Propolis. Journal of Agriculture and Food Chemistry 50(9): 2502-2506.

Salatino A. J., Teixeira E. W., Negri G., Message D. (2005) Origin and Chemical Variation of Brazilian Propolis. Evidence-Based Complementary and Alternative Medicine 2(1): 33-38. DOI: 10.1093/ecam/neh060

Silva B. B., Rosalen P. L., Cury J. A., Masaharu I., Souza V. C., Esteves A., Alencar S. M. (2008) Chemical composition and botanical origin of red propolis, a new type of Brazilian Propolis. Evidence-Based Complementary Alternative Medicine 5(3): 313-316. DOI: 10.1093/ecam/nem059

Simões-Ambrosio L. M., Gregório L. E., Sousa J. P., Figueiredo-Rinhel A. S., Azzolini A. E., Bastos J. K., Lucisano-Valim Y. M. (2010) The role of seasonality on the inhibitory effect of Brazilian green propolis on the oxidative metabolism of neutrophils. Fitoterapia 81(8): 1102-1108. DOI: 10.1016/j.fitote.2010.07.008

Staden R. (1996) The Staden Sequence Analysis Package. Molecular Biotechnology 5(3): 233-241.

Teixeira E. W., Negri G., Meira R., Message D., Salatino A. (2005) Plant Origin of Green Propolis: Bee Behavior, Plant Anatomy and Chemistry. Evidence-Based Complementary Alternative Medicine 2(1): 85-92. DOI: 10.1093/ecam/neh055

Wagh V. D. (2013) Propolis: a wonder bees product and its pharmacological potentials. Advances in Pharmacological Sciences 2013: 308249. DOI: 10.1155/2013/308249