EVALUATION OF PROPOLIS QUALITY FROM TRANSYLVANIA WITH REGARDS TO STANDARDIZATION

(SUMMARY OF PhD THESIS)

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Lately the interest regarding natural alternative of everything that is obtained through synthesis is higher and higher. A large number of studies regarding propolis show that this natural product obtained exclusively by bees and harvested by humans possesses therapeutical effects. Among these effects are: antioxidant capacity, antimicrobial capacity, hepatoprotective effect, antifungal activity, local anesthetic anti-inflammatory effect and others.

Propolis is probably the most complex bee product, having popularity since ancient times. Without a scientific base, our ancestors have used it as a remedy for treatment of different diseases. Much more in our days, when chemical composition of propolis is known and its terapeutical effects, we may trust using it.

Very often natural antioxidants are mentioned. Propolis is one of the most rich natural product in antioxidants (flavonoids, phenolic acids and their esters). Along antioxidant capacity, this product, propolis, shows a great antimicrobial activity, which is supported by recent research studies. Due to these properties, propolis will become a trusted substitute of antibiotics, which nowadays appear to have many side effects.

In the present PhD thesis, there is an important contribution regarding propolis study by modern chemical analysis, with the purpose of investigating Transylvanian propolis quality. Quality control parameters are going to be proposed, which should be checked when this product is used as a remedy. Propolis quality is determined by biological active compounds content (phenolic acids and flavonoids), antioxidant capacity, antimicrobial capacity and also polyphenolic profile. All analysis were conducted in vitro under laboratory controlled conditions. Experimental procedures were realized in the Bee Products Quality Control Laboratory, except microbiological experiments which were conducted in the Microbiology Laboratory, both from University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, during October 2008 – September 2011.

The present PhD thesis “Evaluation of propolis quality from Transylvania with regards to standardization” is structured in two major parts. First one, “Literature review” (Chapters I – IV) present the actual state of knowledge with regards to proposed
Main objectives of the present PhD thesis are:

- Research regarding propolis composition, by determination of total amounts of biological active principles (phenolic acids and flavonoids) by means of classical spectrophotometric methods, and also wax content by authomatic Soxhlet extraction.
- Research regarding antioxidant capacity of propolis from Transilvania (DPPH method and FRAP method) evaluated *in vitro* by means of spectrophotometric methods.
- Research regarding polyphenolic profile of propolis by High Performance Liquid Chromatography (HPLC – PDA) with the purpose of identification and quantification of specific compounds.
- Research regarding correlations between chemical composition and therapeutic effects (antioxidant and antimicrobial capacities) of propolis.
- Indication of quality parameters of propolis which should be included when proposing a standard.
CHAPTER I. PROPOLIS – GENERALITIES

1.1. PROPOLIS IMPORTANCE

All bee products (honey, propolis, polen and royal jelly) show interes within research teams, being products with a composition not much influenced by humans. All these products posses therapeutical effects which are recognized worldwide.

1.2. CHEMICAL COMPOSITION

Propolis chemical composition is very variable, depending greatly of the flora available to bees during harvesting (Marcucci, 1995; Bankova et al., 2000; Ahn and colab et al., 2007). The interest of many research teams was and is to fully elucidate the chemical composition of propolis. Until now more than 300 compounds from propolis composition are known, from different categories: resins, wax, essential oils, minerals and other substances (Burdock, 1998; Kosalec et al., 2003; Cunha et al., 2004; Mărghitaș, 2005).

CHAPTER II. BIOLOGICAL ACTIVE COMPOUNDS FROM PROPOLIS: POLYPHENOLS

Polyphenols are important antioxidants due to their high redox potential. Theirs antioxidant capacity is considered to be much more higher than of essential vitamins, having a significant contribution over therapeutic effects of products which contains them (Tsao și Deng, 2004). Among the role of antioxidants, polyphenols (especially flavonoids) show antimicrobial activity (Boyanova și colab., 2006). Due to therapeutic effects of polyphenols, already proven by scientific studies, the interest for their availability is higher and higher, being preferred natural sources.
CHAPTER V. MATERIAL AND METHOD

5.1. BIOLOGIC MATERIAL

Biologic material subjected to the present study is constituted of 53 propolis samples from Transylvania (figure 1). These samples were provided by beekeepers during March 2009 – March 2010. All samples were kept in the freezer at -20°C until analysis. Samples were harvested by scraping propolis using hive tools according to beekeepers.

Figure 1. propolis samples PS1-PS53 (original photo)

5.2. APPLIED EXPERIMENTAL METHODS

Experimental procedures were conducted using four types of analysis: physico-chemical, spectrophotometric, chromatographic and microbiological.

CHAPTER VI. RESULTS REGARDING PHYSICO–CHEMICAL PARAMETERS OF PROPOLIS

Phisico-chemical analysis were used as a first indicator of propolis quality with regards to water content, wax content and dry residue content.
Water content or humidity of propolis samples was registered around 2.39±0.44%. The lowest value was determined for sample PS 45 (1.46±0.09%), while the highest for sample PS 2 (3.39±0.11%).

Propolis samples distribution according to water content is shown in figure 2. Samples show a normal distribution.

Wax content of propolis samples ranged between wide limits: 13.49±0.50% (PS 46) and 70.89±1.08% (PS 39), with the average of 34.20%. As for the propolis samples distribution according to wax content, could be a normal one with the exception of three samples with showed a very high content (PS 9, PS 30 and PS 39) (figure 3).

Dry residue content of propolis ranged between 37.11±0.35% (PS 30) and 98.06±0.35% (PS 19), with the average of 69.50%. According to this physico-chemical parameter, two samples stand out with unexpected high values: PS 19 (98.06±0.35%) and PS 13 (97.82±1.41).
Dry residue content is a parameter with interest due to the fact that it reflects the amount of extracted active principles from propolis by means of used extraction solvent. Propolis samples distribution according to dry residue content is shown in figure 4.
CHAPTER VII. RESULTS REGARDING SPECTROPHOTOMETRIC DETERMINATIONS OF POLYPHENOLS, FLAVONOIDS AND ANTIOXIDANT CAPACITY OF PROPOLIS

Total polyphenols content of the 53 used propolis samples follows a normal distribution as shown in figure 5.

The range of total polyphenols content was very wide, values were registered between 23.25±3.20% (PS 12) and 63.23±2.44% (PS 41), with the average of 43.01%.

The two methods used for total flavonoid content show very different results as shown in figures 6 and 7.

Antioxidant capacity of propolis was evaluated by means of two different methods. The first one was DPPH method (radical scavenging activity), which offered results in the range of 0.29±0.10 mmol Trolox / g propolis (PS 30) and 1.40±0.10 mmol Trolox / g propolis (PS 19). The second one was FRAP method (Ferric reducing antioxidant potential). “FRAP values” obtained for propolis samples were in the range of
0.57±0.04 mmol FeSO$_4$ / g propolis (PS 17) and 2.55±0.10 mmol FeSO$_4$ / g propolis (PS 29) (figures 8 and 9).

Figure 6. Total flavonoid content determined by the two methods applied (samples PS 1 – PS 25)
Figure 7. Total flavonoid content determined by the two methods applied
(samples PS 26 – PS 53)
Figure 8. Comparative study of the two applied antioxidant methods
(samples PS 1 – PS 25)
Figure 9. Comparative study of the two applied antioxidant methods (samples PS 26 – PS 53)
CHAPTER VIII. RESULTS REGARDING PHENOLIC COMPOUNDS OF PROPOLIS

All propolis samples were subjected to HPLC-PDA analysis, chromatograms were registered at 280 nm and 340 nm, wavelengths specific to phenolic acids and flavonoids. UV-Vis spectra corresponding to each signal from chromatograms were recorded in the range 190-650 nm. Two of the propolis samples outstauded due to identification of 10 reference compounds in their chemical composition (figures 10 and 11).

Figure 10. HPLC – PDA chromatogram of propolis sample PS 22

Both samples, were harvested in Mureș county (PS 22 and PS 24), being the only ones in which t-cinnamic acid was quantified in relatively low concentrations, of 0.64 and 0.71 mg t-cinnamic acid / g propolis. Among reference compounds identified in studied propolis samples, three (caffeic acid, p-coumaric acid and ferrulic acid) were present in all samples. Flavonoids used as reference compounds for spectrophotometric determinations resulted to be appropriate according to HPLC – PDA analysis.
Pinocembrin, the flavanone used for flavanones/dihydroflavonols content determination was identified and quantified in 39 propolis samples, the concentration range being 0.03 ÷ 2.85 mg / g propolis, with an average of 1.05 mg / g propolis. Among identified flavonoids in propolis, chrysin was the flavone most abundant. The concentration range for chrysin was 0.07 ÷ 3.91 mg / g propolis, with an average of 1.59 mg / g propolis. Regarding the flavonols galangin, it was quantified in 34 propolis samples, in the concentration range of 0.31 ÷ 3.20 mg / g propolis, with an average of 1.39 mg / g propolis.

**CAPITOLUL IX. RESULTS REGARDING ANTIMICROBIAL CAPACITY OF PROPOLIS**

Antimicrobial activity was investigated on six different bacterial strains as mentioned in chapter “Material and method”. Propolis samples distribution according to inhibition zone diameter which represent the antimicrobial activity (Staphylococcus...
aureus, Bacillus cereus, Listeria monocitogenes, Escherichia coli and Candida albicans) is shown in figures 12 – 16.

Regarding gram-negative bacteria *Pseudomonas aeruginosa*, samples distribution is not shown due to the resistance it presented to all tested alcoholic propolis extracts.

**Figure 12. Histogram of propolis samples distribution according to antimicrobial activity against Staphylococcus aureus**

**Figure 13. Histogram of propolis samples distribution according to antimicrobial activity against Bacillus cereus**
Figure 14. Histogram of propolis samples distribution according to antimicrobial activity against *Listeria monocitogenes*

Figure 15. Histogram of propolis samples distribution according to antimicrobial activity against *Escherichia coli*
CHAPTER X. GENERAL CONCLUSIONS

According to proposed objectives of the present PhD thesis, the following could be concluded:

1. Chemical composition of propolis from Transylvania was determined, by evaluation of total amounts of biologic active principles (polyphenols and flavonoids), wax content, water content and dry residue content.

2. Antioxidant capacity of propolis was determined by means of two spectrophotometric methods: radical scavenging activity (DPPH method) and total antioxidant potential (FRAP method). Both methods confirm that propolis possesses antioxidant capacity.

showed *in vitro* antimicrobial activity against Gram-positive bacteria and selective activity against Gram-negative bacteria.

4. Polyphenolic profile of propolis was determined by means of HPLC – PDA chromatographic technique, which was as follows: syringic acid, caffeic acid, vanillin, p-coumaric acid, ferrulic acid, t-cinnamic acid, pinocembrin, chrysin, galangin and pinostrobin. The presence of pinocembrin, chrysin, galangin and caffeic acid in the polyphenolic profile of propolis allowed us to classify it as poplar propolis.

5. Correlations between chemical composition and studied therapeutic effects were determined. Flavonoid content correlates in a high manner with antioxidant capacity of propolis. Flavones/flavonols and flavanones/dihydroflavonols are responsible for antimicrobial activity of propolis as shown by Pearson correlation coefficients. Total polyphenols concentration can not be considered a major criteria when estimating antioxidant and antimicrobial capacities, due to weak correlations shown between these parameters. Wax content could be used as a first indicator of propolis quality. A high content of wax correlates with a low content of active principles (flavonoids and phenolic acids).

6. Parameters that should be considered for quality control of propolis are:
   a. Water content – maximum 10%;
   b. Dry residue content – minimum 35%;
   c. Wax content – maximum 35%;
   d. Total flavonoids content – minimum 10%;
   e. Total polyphenols content – minimum 20%;
   f. Antioxidant capacity;
   g. Antimicrobial capacity.
REZUMAT

ORIGINAL ELEMENTS

1. Polyphenolic profile characterization of propolis from Transylvania by means of chromatographic technique (HPLC – PDA) ant its classification as poplar propolis.
2. Evaluation of antimicrobial capacity of propolis against six international bacterial strains.
3. Indication of quality control parameters (wax content, water content, dry residue content, total polyphenols, total flavonoids, antimicrobial and antioxidant activities) necessary to be checked before its use as therapeutical agent.

RECOMMENDATIONS AND PERSPECTIVES

1. Extension of the present study on country level and standard proposal requiring propolis quality parameters.
2. Wax content and total flavonoid content should be checked as first indicators of propolis quality.
3. Use of propolis for therapeutical purposes only after quality control.

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