

**UNIVERSITY OF AGRICULTURAL SCIENCES AND VETERINARY MEDICINE
CLUJ-NAPOCA
ANIMAL BREEDING AND BIOTECHNOLOGIES FACULTY
DOCTORAL SCHOOL**

ING. RODICA BOBIŞ (MĂRGĂOAN)

**SUMMARY
OF PhD THESIS**

**Researches on the nutritional and
biological value of bee pollen**

**SCIENTIFIC COORDINATOR
Prof. Univ. Dr. Ing. LIVIU AL. MĂRGHITAŞ**

**CLUJ-NAPOCA
2014**

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SUMMARY

INTRODUCTION

The relationship between bees, flowers and man is one of the wonders of the universe, being a living proof that the flora, fauna and man were made to live in harmony.

Bees need flowers to feed themselves, plants need bees to be pollinated and to produce seed to ensure the perpetuation of plant species. Although known and used since ancient times, pollen and its nutritional value are still surrounded by mystery and there are questions that have not yet been answered.

However, pollen is called the only perfect and complete food.

Although bee pollen began to be used in human nutrition after World War II, its value has been described much later, when they began to make the first measurements of the chemical composition and associate with some observations of medical practice.

PURPOSE AND OBJECTIVES OF THE THESIS

Given the complexity of this product of the hive, bee pollen, a comparative study is proposed, attention being given to the chemical composition which give nutritional compounds, the biologically active compounds from pollen and biological activity related to their botanical origin.

The objectives of the thesis are:

- To establish the botanical origin of bee pollen samples by palynological method.
- Quantitative and qualitative determination of the parameters giving the nutritive value of bee pollen.
- Determination of biologically active compounds present in pollen by spectrophotometric methods, the high-performance liquid chromatography (HPLC) and gas chromatography (GC).
- Determination of the biological activity of bee pollen: antioxidant, antitumor and antimicrobial activity *in vitro*.

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- Correlation of botanical origin of pollen samples with biologically active compounds and their activity, using chemometrics as statistical tool.

CHAPTER I

POLLEN – GENERAL REMARKS

Pollen is found in the form of a fine powder on the anthers of flowers. It is made up of grains of different shape and color, characteristics of each plant. Pollen grains can be distinguished by the shape of the outer surface, by different content in nutrients, vitamins and biologically active substances.

Pollen grains show various forms: elliptical, spherical, triangular, flattened, rhombic, discoid, square according to botanical origin of pollen. Some pollen grains, out of the pollinic bags, are reunited as four (tetrad) or more (clusters) shapes.

Ornamental elements of the pollen grains are specific to the type of pollination: those originating from the entomophilous plants have surface with lumps, favoring their transport by the pollinating insects, it is stickier, produced in small amounts, and those coming from anemophilous plants generally have smooth and dry surface, is easily carried by wind (Mărghitaș, 2005) and is produced in large amounts (the male inflorescence of corn to produce 50,000 pollen grains).

Bee pollen is a product of the hive, obtained by collecting millions of floral pollen grains, which adds nectar or regurgitated honey and saliva, rich in enzymes, which leads to changes in the composition and improve its therapeutic qualities (Mărghitaș, 2005).

Palynology is the science of studying pollen collected from air, water, sediment deposits and not least the study of bee pollen. Palynology is an interdisciplinary science, a branch of earth science (geology) and biological sciences, particularly botany. A scientist working in this field, have primarily a very good knowledge in botany.

Bee pollen is analyzed in terms of palynology to determine its biological origin. Since bee pollen is present in different colors, first step in palynological analysis should be a separation of pollen grains based on their color.

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CHAPTER II

CHEMICAL COMPOSITION AND BIOLOGICALLY ACTIVE COMPOUNDS FROM BEE POLLEN

Knowing that bee pollen is the main source of protein and other essential components of life (the essential amino acids, organic acids, vitamins), the man thought that pollen should be a valuable product, which together with honey, contains every nutrients needed for life (**Popescu and Meica, 1997**).

Chemical composition of bee pollen, following synthetizing literature data, is presented in table 1.

Table 1

Chemical composition of bee pollen (%), following different authors*

Component	Limit values (g/100g)	Mean values (g/100g)
Water	7-24	11.0
Sugars	15-54	27.0
Proteins	7-35	23.7
Lipids	1-18	4.8
Ash	0.9-7.6	3.1

*by **Mărghitaș, 2005**

According to technical regulations proposed by **Campos et al. (2008)**, bee pollen can be classified according to its water content. Bee pollen is a product of the hive with a water content of between 20-30% (fresh pollen), dehydrated pollen, with a water content of 10-12%, and dry pollen, pollen that has undergone a process of by their drying at a temperature of from 42°C, where water content does not exceed 6%.

Carbohydrate content of the pollen varies within wide limits, depending on the origin and the way of harvesting. Carbohydrates such as lactose, glucose and sucrose make up about 90% of the low molecular weight sugars bee pollen (**Serra-Bonvehi et al., 1986**).

Lipids are important for bees as a source of energy, with some components involved in the synthesis of fat and glycogen reserves, or cell membranes (**Herbert, 1997**).

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Pollen is the food of young bees and its protein composition is about 40% of dry matter. According to numerous research studies, total proteins in pollen amounts to between 3.8 and 40.8%, an average value being set around 25% (**Campos et al, 2008; Mărgăoan et al., 2012**).

It is important to note that pollen contains significant amounts of macro and micro elements with special nutritional value, such as potassium (K), calcium (Ca), magnesium (Mg), zinc (Zn), iron (Fe), manganese (Mn), copper (Cu).

Pollen is an excellent source of hydro and lipo-soluble vitamins. Among the water-soluble vitamins we mention: vitamin C, B vitamins and the fat soluble vitamins are represented by E vitamin and β -carotene.

Bee pollen has gained increased interest in recent years, not only for its nutritional value due to the presence of carbohydrates, proteins or lipids, but also by beneficial effects on human body and its physiological properties (**Kroyer and Hegedus, 2001**).

The color of bee pollen grains is determined by the presence of natural pigments such as flavonoids and /or carotenoids (*Montenegro et al., 1997*).

CHAPTER III

FUNCTIONAL PROPERTIES OF BEE POLLEN. ACTIVITIES AND DETERMINATION METHODS

The functional properties of bee pollen is given by different classes of chemical compounds present in its composition. The major bioactive constituents of pollen are flavonoids. Another class of substances that seem to be involved in pollen antiprostatic activity are phytosterols. Another group of compounds involved in the biological action of pollen is β -carotene. The most important biological activity of bee pollen is the antioxidant activity. This activity was attributed to the polyphenolic compounds in pollen because of their intrinsic capacity to reduce reactive oxygen species.

The antimicrobial activity of pollen is demonstrated by existing studies on monofloral pollen (**Basim et al., 2006; Carpes et al., 2007**), but also on the studies on

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multifloral pollen, which is obtained more easily by beekeepers and also sold in much larger quantities (Morais et al., 2011).

There are many scientific studies showing antitumor action, immunostimulatory or proapoptotic of bee pollen, or various types of bee pollen extracts, but the chemical constituents of pollen that causes these activities have or still are not very clear.

CHAPTER IV MATERIALS AND METHODS

The research was conducted on 16 samples of bee pollen (Fig. 1) harvested from three counties in the northwestern Transylvania. Samples were obtained directly from beekeepers in April - August 2010, were collected using pollen collectors. The samples reached the laboratory received a code in order of arrival, were cleaned of impurities and stored in a freezer at - 18 ° C until the start of testing.

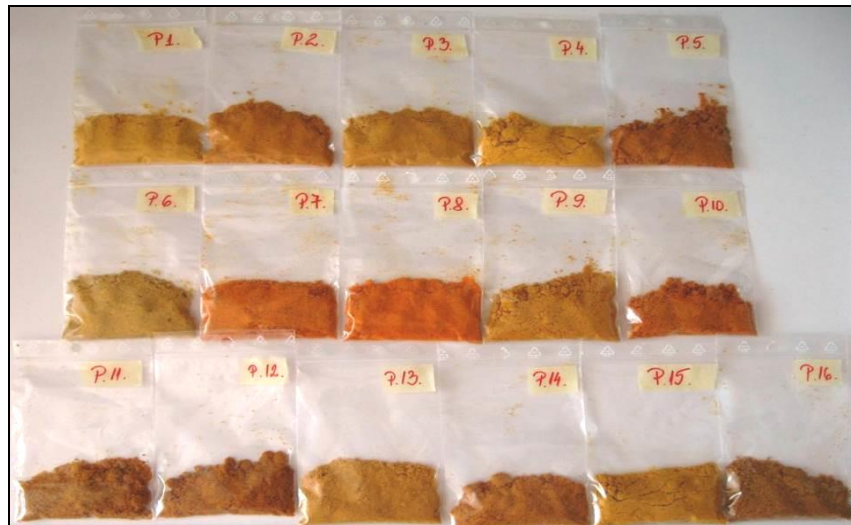


Fig. 1. Pollen samples P1-P16 (original image)

The research was conducted in the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca as follows:

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- Physical and chemical analyzes were performed in the Laboratory of Quality Control for Bee Products (APHIS) of the Technology of bee products and sericulture Department USAMV Cluj-Napoca;
- Determination of vitamin C, carotenoids and fatty acids was performed in the Department of Biochemistry, Veterinary Medicine Faculty Cluj-Napoca;
- Antimicrobial activity was performed in the Microbiology Laboratory of Veterinary Medicine Faculty Cluj-Napoca;
- *In vitro* antitumoral action was performed in the Laboratory of Certification and Crioconservation of Germoplasm, Life Science Institute of USAMV Cluj-Napoca.

CHAPTER V RESULTS AND DISCUSSIONS

To determine the nutritional and biological value of bee pollen, standard techniques were used, found in different standards, using modern spectrophotometry techniques and chromatography.

After determining the botanical origin, the samples were analyzed for their physico-chemical parameters (the water content, the individual sugars, total lipids, proteins), and the content of vitamin C, fatty acids, carotenoids content and the amounts of total polyphenols were flavonoids. Also, the biological activity was determined by determining the antioxidant activity, anti-microbial and anti-cancer activity.

Results and discussions regarding the palinological analysis

Bee pollen samples taken in this study were presented as a mixture of glomerules, of different shapes and colors. Every pellet generally comes from one or more floral species (Mărghițaș, 2005).

Bee pollen samples were subjected to microscopic examination for floral species determination.

Microscopic images of pollen samples are presented in Fig. 2-4.

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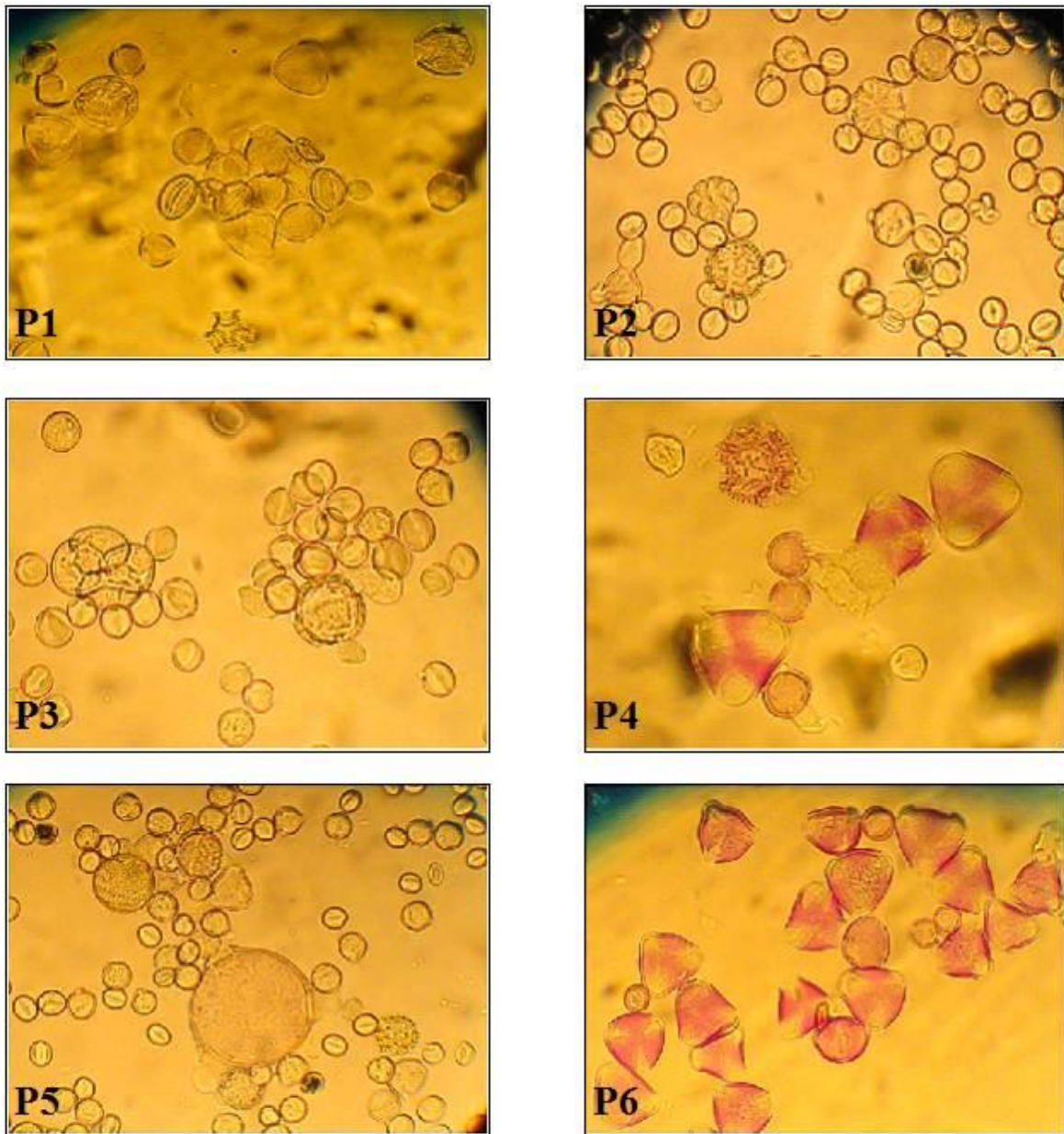


Fig.2. Microscopic images of pollen samples P1-P6 taken in study (original images)

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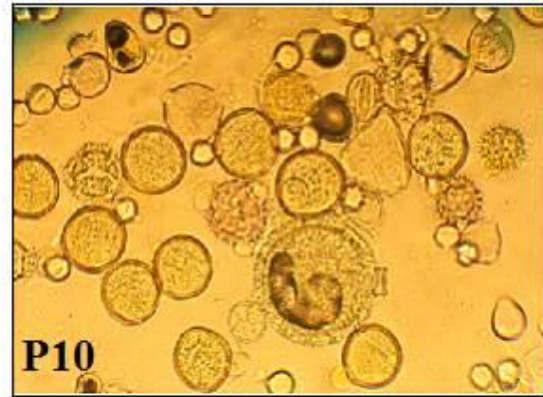
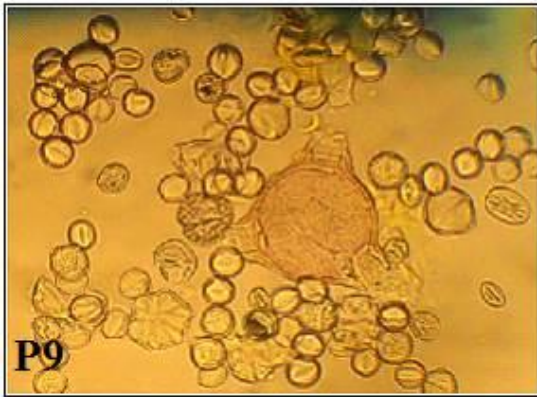
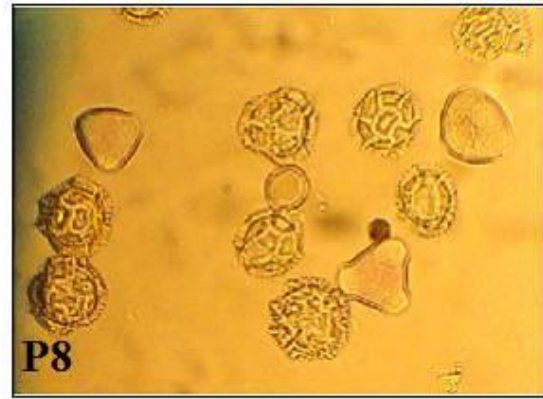
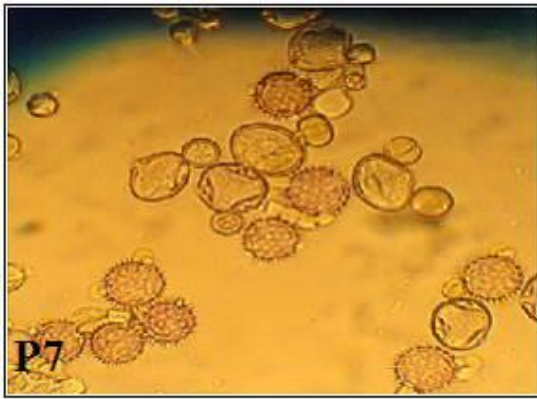


Fig. 3. Microscopic images of pollen samples P7-P12 taken in study (original images)

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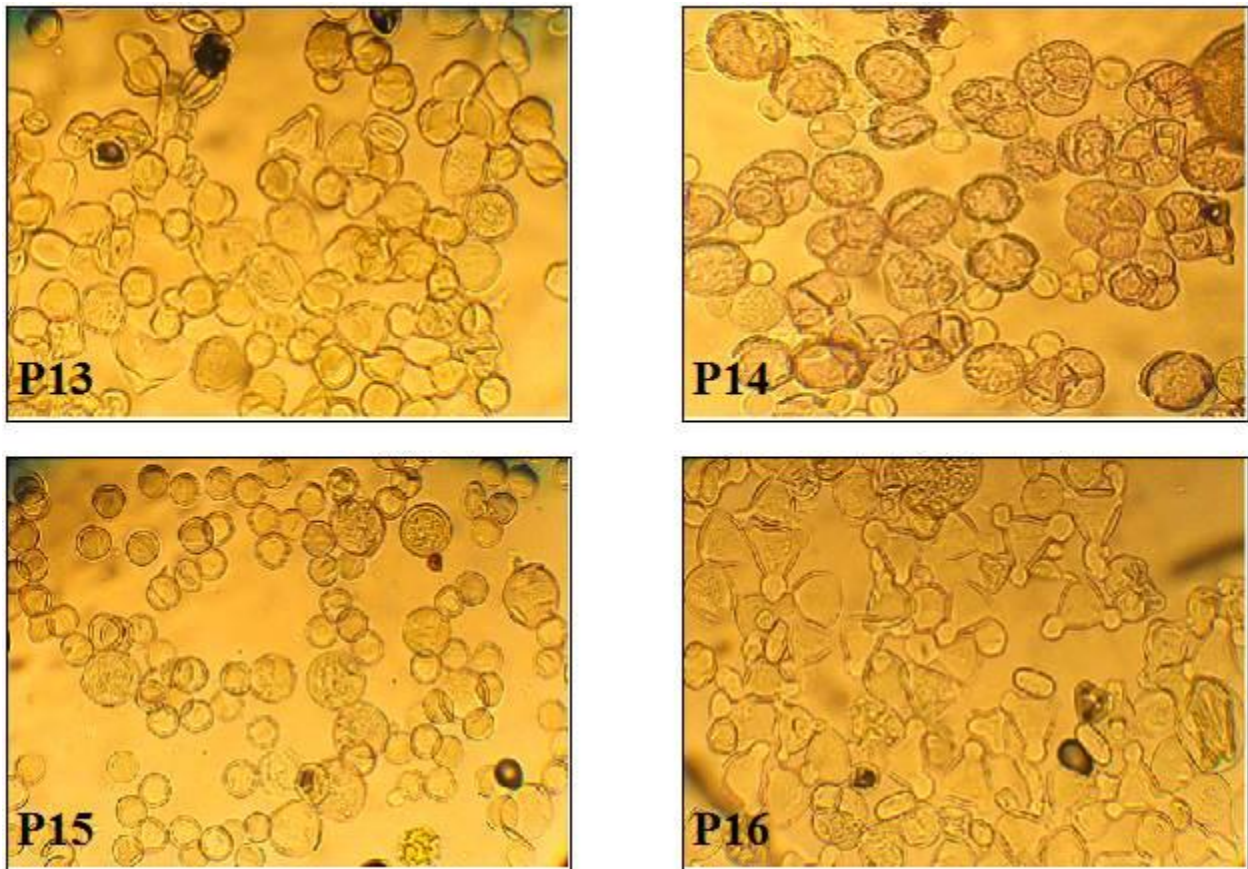


Fig. 4. Microscopic images of pollen samples P13-P16 taken in study (original images)

According to palinological analysis, all 16 pollen samples were multifloral, having different percents and types of pollen.

Six plant families were found in the 16 bee pollen samples as predominant pollen (> 45% from total content): *Rosaceae* (*Crataegus monogyna*, *Filipendula ulmaria*, *Malus domestica*, *Prunus sp.*, *Rosa canina*), *Fabaceae* (*Trifolium repens*, *Anthyllus sp.*, *Robinia pseudoacacia*), *Asteraceae* (*Calendula officinalis*, *Taraxacum officinale*), *Brassicaceae* (*Brassica sp.*), *Ericaceae* (*Calluna vulgaris*), and *Salicaceae* (*Salix sp.*). Secondary pollen of the samples belong to 10 families.

Rosaceae family was dominant (> 45%) in 7 pollen samples (P1, P2, P3, P4, P6, P9, P11) and was present as secondary pollen (16-45%), in other three samples (P8, P13, P14), meanwhile *Fabaceae* family give predominant pollen for 3 samples (P5, P10, P16) and secondary pollen for other three (P7, P10, P12).

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Asteraceae famil, with *Taraxacum officinale*, *Calendula officinalis* and *Carduus sp.* species, were present in 3 different samples (P7 and P8).

Results and discussions regarding nutritive value determination for bee pollen

Based on physico-chemical parameters assessed, the nutritive value was established and a hierarchy of nutritionally pollen samples was made.

The results were compared with the quality standards imposed by Switzerland, Brazil, Argentina, and data from the literature.

Water content of studied bee pollen samples showed a $24.76 \pm 0.15\%$ average. The lowest water content was determined for sample P8 (Fam. *Asteraceae*, *Taraxacum officinale*) of $17.59 \pm 0.41\%$ while the sample P5 (Fam. *Fabaceae*, *Trifolium repens*) showed the highest value of $35.85 \pm 0.04\%$ that's found far beyond the limits of specific legislation. Water content of pollen samples was influenced to a large matter of climatic conditions at the time the pollen was collected from bee entrance and less to botanical origin. However we can mention the fact that from the 16 pollen samples studied, seven showed significant positive differences (P2, P5, P7, P10, P11, P12, P14) of water content toward the average 24.76%, considered as control, while the other 7 samples (P1, P3, P4, P6, P8, P15, P16) were very significantly negative.

Results and discussions regarding ash content of pollen samples

Ash content of the samples had a mean of $2.56 \pm 0.05\%$. The limits of this parameter were $1.75 \pm 0.01\%$ (P8: Fam. *Asteraceae*, *Taraxacum officinale*) and $3.25 \pm 0.07\%$ (P6: *Rosaceae*, *Prunus sp.*). These values lies in the limits of 0.5-3% proposed by **Swiss Food Manual (Bogdanov, 2004)** and those proposed by **Brazilian Standard (MAPA, 2001)** and **Argentinian (Codigo Alimentario Argentino, 1998)** which specify a maximum of 4% for this parameter.

Regarding the influence of botanical origin upon ash content, one can say that bee pollen came from *Rosaceae* family (P2, P3, P4, P6) is very significant compared to the mean which is considered as control. Also a significant difference had samples P5 (Fam. *Fabaceae*) and P13 (Fam. *Brassicaceae*).

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Results and discussions regarding protein content of the samples

Protein content of the bee pollen samples lies between $16.27 \pm 0.13\%$ (P15: Fam. *Salicaceae*) – $26.50 \pm 2.10\%$ (P11: Fam. *Rosaceae*) with a mean of $22.26 \pm 1.29\%$. **Brazilian Standard (MAPA, 2001)** accepts a minimum of 8% for this parameter, and the **Argentinian one (Codigo Alimentario Argentino, 1998)** propose an interval of 15-28%.

Influence of botanical origin upon protein content is obvious from the results found.

Thus a very significant difference presented P2, P5, P6, P9, P11 samples, all having as predominantly pollen (> 45%) *Rosaceae* family pollen, exception P5 which had as predominant pollen the *Fabaceae* family.

Results and discussions regarding lipid content of pollen samples

Lipid content of bee pollen samples, presented values between $2.13 \pm 0.30\%$ for P14 sample (Fam. *Ericaceae*) and $8.93 \pm 0.72\%$ for sample P8 (Fam. *Asteraceae*), with a mean of $4.96 \pm 0.67\%$.

In all 16 samples significant results were obtained, especially sample P8 with *Asteraceae* as predominant pollen. High value of lipids registered samples P13 (7.91%) from *Brassicaceae* family and P1 (7.30%) from *Rosaceae* family.

Results and discussions regarding sugar content , by HPLC-IR

Common carbohydrate compounds of 16 pollen samples were fructose, glucose (monosugars) and turanose, maltose belonging to disugars.

Mean value for fructose was 16.56% with limits between $10.91 \pm 0.13\%$ (P5: Fam. *Fabaceae*) and $19.50 \pm 0.04\%$ (P15: Fam. *Salicaceae*). In terms of glucose content the minimum was 2.94% for P5, and the maximum $10.03 \pm 0.10\%$ for P8 (Fam. *Asteraceae*). For turanose content, same minimum value $0.18 \pm 0.02\%$, was obtained in two samples P6 (Fam. *Rosaceae*) and P13 (Fam. *Brassicaceae*) and the maximum value $1.44 \pm 0.02\%$ for P8 (Fam. *Asteraceae* - *Taraxacum officinale*). The mean value for turanose was $0.38 \pm 0.02\%$. Instead maltose showed $0.15 \pm 0.04\%$ minimum value of the sample P14 (Fam. *Ericaceae*), and the maximum in the sample P8 (Fam. *Asteraceae* - *Taraxacum officinale*) of $0.47 \pm 0.03\%$. Other disugars were identified in some samples, for example trehalose in

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sample P5, isomaltose in 4 probe (P1, P2, P5 and P7), erlose in 3 samples (P3, P8 and P16), and sucrose was identified in 6 samples (P1, P3, P4, P6, P8 and P15).

Results and discussions regarding nutritive value of bee pollen

Energetic values for studied pollen samples lies in the limits of 377.14 P8 (*Taraxacum officinale*) and 274.22 P5 (*Trifolium repens*) kcal/100g pollen.

Analyzing the data presented in Table 2 it can be observed that the first three places regarding energetic values: P8 (*Taraxacum officinale*), P1 (*Crataegus monogyna*) and P16 (*Robinia pseudoacacia*) with energetic values of 377.14, 352.64 and 341.14 kcal/ 100g pollen. The next two places are samples P4 (*Malus domestica*) and P6 (*Prunus sp.*).

Following the determination of nutritional value parameters, and energetic value for all samples, the following conclusions can be drawn:

- Water content of bee pollen samples lies inside the limits of 17.59 - 35.85%, values that depend to some matter by the botanical origin of pollen, but mostly by most climatic conditions on pollen from the bee entrance recoltării .
- Ash content of the studied samples lies between the limits imposed by certain quality standards (Swiss, Argentinean and Brazilian) and in the literature average of 2.56%. Ash represents an important quality index, because a high content (above 4%) of this parameter can give us clues about the impurities that can accidentally get in the sample.
- Protein, an important parameter that characterizes the nutritional value of a food presents an average of 22.25% and is in accordance with literature. The samples are characterized by high values of protein (over 25%) especially *Rosaceae* family (*Filipendula ulmaria*, *Prunus sp.*, *Rosa canina*) and *Fabaceae* (*Trifolium repens*).
- Lipid content with limits of 2.13% (P14: *Calluna vulgaris*) and 8.93% (P8:*Taraxacum officinale*), place our samples among the limits imposed by literature and the three regulations, which are very permissive with this parameter.

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- Sugar content, varies in a broad way, the maximum for fructose belong to *Salix sp.* pollen with the value of 19.50%, meanwhile for glucose, the maximum is present in sample P8: *Taraxacum officinalis*. Minimum for the two compounds belong to sample P5: *Trifolium repens*.
- Energetic value of the pollen samples, depend on the lipid, protein and sugar content, the highest value belong to the pollen having *Taraxacum* as predominant specie, characterized by high lipid and glucose content, meanwhile the lowest value belong to sample P5: *Trifolium repens*, having the lowest amount of glucose, fructose and sugars in general.
- Spring pollen samples (P8, P1, P16, P4, P6) are the most valuable from nutritional point of view, sustaining the revigoration and regeneration process of the bee family, after the winter season.

Results and discussions regarding biologically active compounds from bee pollen

Total polyphenolic content from bee pollen was determined by spectrophotometric method Folin-Ciocalteu and is presented in Table 2.

Table 2

Total polyphenolic content in studied multifloral pollen samples *

Sample	Total polyphenols (mg GAE/g probă)	Sample	Total polyphenols (mg GAE/g probă)
P1	8.80±0.19	P9	7.24±0.23
P2	5.41±0.16	P10	3.50±0.04
P3	4.52±0.14	P11	2.46±0.04
P4	7.74±0.12	P12	5.53±0.12
P5	5.66±0.24	P13	8.24±0.23
P6	8.87±0.03	P14	3.76±0.03
P7	5.62±0.14	P15	7.69±0.07
P8	5.63±0.07	P16	4.05±0.05
Mean		5.92±0.12	

* rezultats represent the mean value of three independent determinations ± standard deviation

Total polyphenols from studied samples presented a mean concentration of 5.92±0.12mg GAE/g sample and varied between a minimum of 2.46±0.04mg GAE/g (sample P11) and a maxim of 8.87±0.03mg GAE/g (sample P6). What can be observed is

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that both samples (P6 and P11) had as predominant pollen *Rosaceae* family. Major difference consist of the specie that give the secondary pollen: P6 - *Prunus sp.* (83%) and P11 - *Rosa canina* (48%).

Total flavonoid content was determined spectrophotometrically, and the results obtained are presented in Table 3.

Table 3

Total flavonoid content in analysed pollen samples *

Sample	Total flavonoids (mg QE/g probă)	Sample	Total flavonoids (mg QE/g probă)
P1	5.93±0.26	P9	5.70±0.22
P2	5.58±0.19	P10	4.05±0.03
P3	6.33±0.16	P11	2.11±0.03
P4	6.30±0.02	P12	2.27±0.06
P5	7.57±0.08	P13	3.17±0.39
P6	5.21±0.28	P14	3.67±0.16
P7	2.56±0.01	P15	5.07±0.06
P8	3.58±0.22	P16	1.39±0.04
Mean	4.40±0.14		

* rezultats represent the mean value of three independent determinations ± standard deviation

Samples having predominant pollen *Rosaceae* family (P1, P2, P3, P4, P6, P9), *Salicaceae* P15 and *Fabaceae* P5 presented a total flavonoid content above the mean value of 4.40±0.14 mg QE/g sample. Mean determined value was 1.39±0.04 mg QE/g sample (P16), respectively a maximum value of 7.57±0.08 mg QE/g sample (P5), aboth samples having *Fabaceae* family, the major difference being the dominant pollen: P16 – *Robinia pseudoacacia* (84%) and P5 – *Trifolium repens* (46%).

Total carotenoid content in saponified samples was determined spectrophotometrically, using UV-VIS spectrometry. Quantitative analysis evidenced broad limits of concentrations: 49.90 – 425.32 µg/g in fresh samples and 63.94–527.27 µg/g when the results were reported to dry weight. (Fig. 5).

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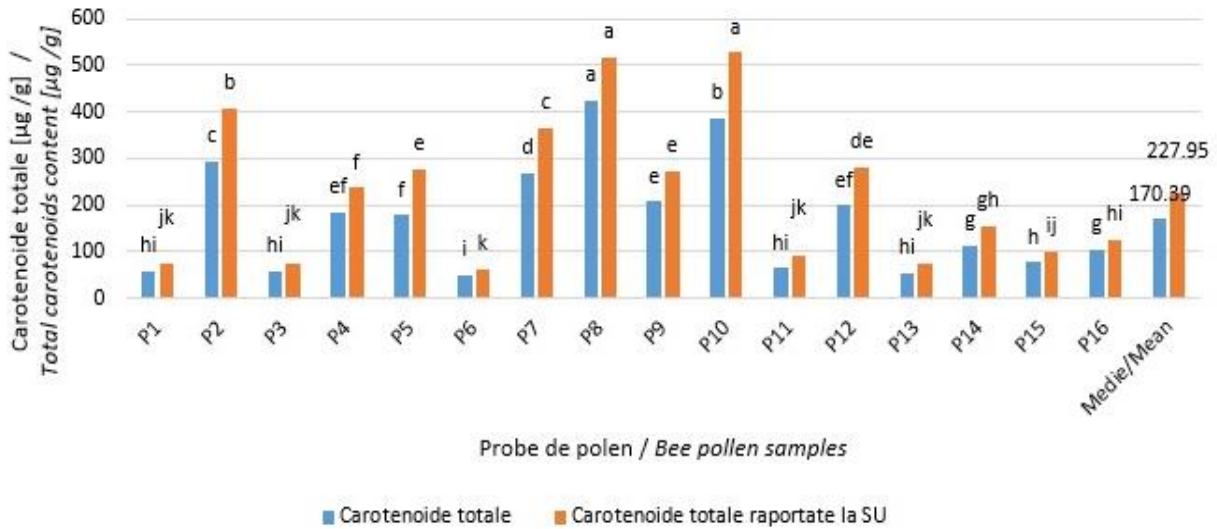


Fig.5. Summary of comparisons by Tukey's test on the content of total carotenoids from bee pollen studied samples

Chromatographic method HPLC-PDA for pollen carotenoids determination permit the separation, identification and quantification of individual carotenoids present in the samples, the three identified carotenoids being: lutein, β -criptoxanthin and β -caroten. Average content of the three individual carotenoids in the samples are presented in Fig. 6.

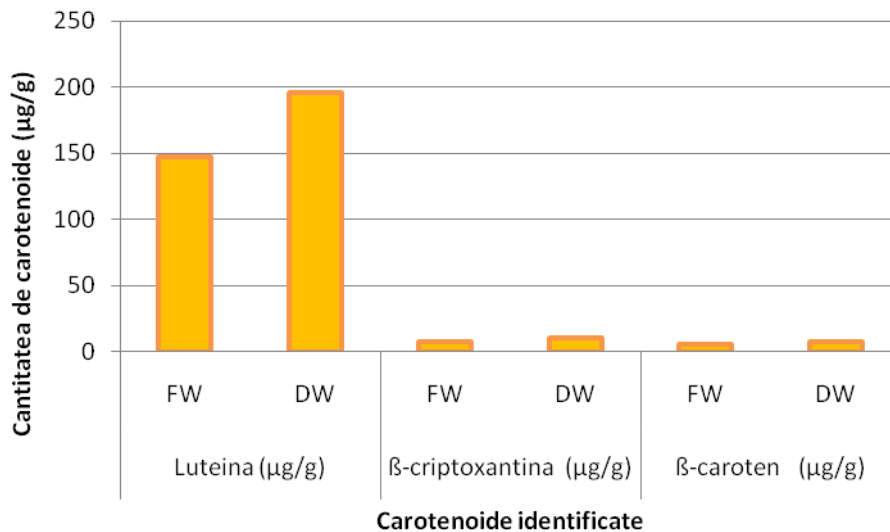


Fig.6. Average quantity of carotenoids found in all pollen samples

Lutein and β -criptoxanthin were identified in all samples. Lutein was the majority carotenoid from all the samples. The concentration varied between 44.52 and 392.52 $\mu\text{g/g}$

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fresh pollen (57.04- 476.30 $\mu\text{g/g}$ dry pollen). The highest concentration was found in sample P8 (392.52 $\mu\text{g/g}$), followed by P10 (317.11 $\mu\text{g/g}$) (Mărgăoan și colab., 2014). The smallest concentration of lutein was found in sample P6 (44.52 $\mu\text{g/g}$).

β -criptoxanthin concentration had limits of 1.02-24.96 $\mu\text{g/g}$ fresh pollen. The highest concentration was found in sample P12, and the lowest in samples P1 and P13.

Regarding β -caroten content, some samples have only traces, while others had high amounts, like samples P2 and P10 (13.20 and 12.56 $\mu\text{g/g}$ fresh pollen) and 18.18 and 17.18 $\mu\text{g/g}$ fresh pollen.

Resultats for vitamin C content, determined by HPLC, lies in the interval of 0.66 ± 0.03 (P8: *Taraxacum officinale*) and 74.65 ± 2.67 mg/100g (P5: *Trifolium repens*), with a mean of 25.15 ± 1.11 mg/100g for the 16 samples of pollen.

In pollen extracts 14 types of fatty acids were identified, the one found in the highest quantity was α – linolenic acid [18:3 (n - 3)], with values of 20.28 % (P1) and 49.37 % (P11), the second being linoleic acid [18:2 (n - 6)] (7.62 % - P11 to 33.21 % - P1), palmitic acid (16:0) (18.39 % - 30.93 % in P8 - P11) and oleic acid [18:1 (n - 9)] (3.86 % - P5 to 15.34 % - P6). Likewise, small and very small quantities (< 3 %) from the following acids were determined: stearic (18:0); miristic (14:0); lauric (12:0); arahidic (20:0), eicosenoic [20:1 (n - 9)]; behenic (22:0); elaidic [18:1(9t) (n - 9)]; caproic (6:0); caprilic (08:00) and capric (10:00). n-6 / n-3 (PUFA-poliunsaturated fatty acids) ratio's varied from 0.15 (P11) to 1.64 (P1) (Table 4). Mean value of this ratio (0.76) is close to that recommended by Simopoulos (2008) (n-6/n-3 = 1-5/1), ratio's which are benefic for human health.

Table 4 present the synthesis in fatty acids of the pollen samples (P1-P16).

Table 4. Fatty acids composition (% from total lipids) existing in total lipids from bee pollen samples

Pollen samples																	
Fatty acids	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	Media
1. 6:0	0.05	0.03	0.08	0.05	0.05	0.04	0.11	0.15	0.03	0.10	0.02	0.08	0.12	0.07	-	0.04	0.06
2. 8:0	-	0.01	0.04	0.08	0.03	0.02	1.74	0.34	0.01	0.29	0.06	0.19	0.12	0.02	-	0.16	0.19
3. 10:0	0.05	0.97	1.68	0.17	0.98	0.11	2.23	0.16	0.87	0.79	0.29	0.20	0.20	0.34	0.04	0.08	0.57
4. 12:0	0.60	1.96	3.65	3.39	2.14	1.53	2.06	8.51	1.98	2.67	1.56	2.30	0.13	3.59	0.49	0.63	2.33
5. 14:0	1.10	2.26	3.62	1.21	2.46	0.77	1.13	0.18	2.08	1.69	0.62	0.83	1.86	0.89	0.69	0.35	1.36
6. 16:0	24.28	28.99	23.30	23.01	25.58	22.29	27.00	18.39	27.92	23.88	30.93	30.00	28.05	27.58	24.46	27.32	25.81
7. 18:0	3.25	1.99	2.21	4.32	2.07	3.24	2.51	8.99	2.17	3.32	1.63	2.67	3.09	2.01	3.50	1.62	3.04
8. 18:1(n-9)	14.41	4.18	8.06	8.51	3.86	15.34	8.78	8.60	4.61	9.52	5.23	6.81	5.15	6.54	14.28	5.55	8.09
9. 18:1(9t)(n-9)	0.99	0.52	0.34	0.42	0.54	0.36	0.56	0.46	0.67	0.33	1.02	0.42	0.60	0.42	0.36	0.88	0.56
10. 18:2(n-6)	33.21	20.86	24.68	26.99	25.31	30.75	13.50	22.06	19.85	20.28	7.62	14.79	11.44	23.63	30.65	29.10	22.17
11. 18:3(n-3)	20.28	35.87	28.44	29.25	33.77	22.48	35.15	29.58	37.04	34.76	49.37	38.43	46.93	29.40	24.26	32.33	32.96
12. (20:0)	0.73	1.23	2.09	0.97	1.34	0.96	0.83	1.30	1.32	0.72	0.58	1.47	0.84	3.18	0.55	0.67	1.17
13. 20:1(n-9)	0.19	0.26	0.25	0.33	0.28	0.45	3.63	0.53	0.33	0.38	0.22	0.35	0.11	0.51	0.23	0.23	0.52
14. (22:0)	0.87	0.88	1.56	1.30	1.58	1.66	0.77	0.74	1.13	1.26	0.85	1.46	1.37	1.83	0.49	1.02	1.17
$\sum_{n-3} PUFAs$	20.28	35.87	28.44	29.25	33.77	22.48	35.15	29.58	37.04	34.76	49.37	38.43	46.93	29.40	24.26	32.33	32.96
$\sum_{n-6} PUFAs$	33.21	20.86	24.68	26.99	25.31	30.75	13.50	22.06	19.85	20.28	7.62	14.79	11.44	23.63	30.65	29.10	22.17
$\sum_{n-9} PUFAs$	15.59	4.96	8.65	9.26	4.69	16.15	12.97	9.59	5.61	10.24	6.47	7.59	5.87	7.46	14.87	6.67	9.16
$n-6/n-3$	1.64	0.58	0.87	0.92	0.75	1.37	0.38	0.75	0.54	0.58	0.15	0.38	0.24	0.80	1.26	0.90	0.76

Values represents the mean of three determinations, analyzed in triplicate (n=3x3); P1- P16-pollen samples; PUFA poly unsaturated fatty acids

Ac. caproic (6:0); Ac. caprilic (8:0); Ac. capric (10:0); Ac. lauric (12:0); Ac. miristic (14:0); Ac. palmitic (16:0); Ac. stearic (18:0); Ac. oleic [18:1 (n-9)]; Ac. elaidic [18:1 (9 t) (n-9)]; Ac. linoleic [18:2 (n-6)]; Ac. α -linolenic [18:3 (n-3)]; Ac. arahidic (20:0); Ac. eicosenoic [20:1(n-9)]; Ac. behenic (22:0)

REZUMAT

Following the results obtained in analyzing bioactive compounds from bee pollen, the following conclusions can be made:

- Total polyphenolic content was situated within the limits reported in literature studies, similar values being determined for some of the pollen types: *Calluna vulgaris*, *Salix sp.*, *Filipendula ulmaria* și *Brassica sp.*
- In literature, total flavonoids are determined in many ways, using different methods and reference compounds, leading to very different results, making difficult the comparison of the results.
- In bee pollen having as predominant specie *Taraxacum officinale*, the highest quantity of total carotenoids was determined.
- The highest quantity of lutein was identified in the pollen having *Asteraceae* family pollen in its composition (P8: *Taraxacum officinale*), β -criptoxanthin was present in the highest amount in sample P12 (*Fabaceae* and *Asteraceae* families) and maximum quantity of β -caroten was identified in the sample with predominant pollen of *Filipendula ulmaria*. An important role in determining this compound, had also the secondary pollen present in the sample (*Hypericum*).
- The content in vitamin C varied greatly, values above average, approx 45mg/100g, being quantified in samples P13 (*Brassica sp.*), P14 (*Calluna vulgaris*) and P16 (*Robinia pseudoacacia*).
- 14 types of fatty acids were identified, the most abundant being linolenic acid [18:3 (n-3)], varying between 20.28 % (P1) and 49.37 % (P11), followed by linoleic acid [18:2 (n-6)] (7.62 % - P11 to 33.21 % - P1).
- Knowing the benefic effect of α -linolenic acid (n-3) in prevention and treatment of cardiovascular diseases and type 2 diabetis, together with a close to 1/1 ratio of n-6/n-3 acids, one can mention that those pollen types are important sources of polyunsaturated fatty acids (P1, P4, P6, P15, P16).

SUMMARY

Results and discussions regarding biologic activity of bee pollen

Biologic activity of methanolic extracts from bee pollen was evidenced by antioxidant methods (radical scavenging activity)(DPPH), ferric reducing antioxidant power (FRAP), antimicrobial activity and antitumour action, all tested *in vitro*.

Radical scavenging capacity of DPPH radical is presented in Table 5.

Table 5

Radical scavenging capacity of pollen, evaluated by DPPH method

Sample	Inhibition percent (%)	Antioxidant capacity (mmoli Trolox/g sample)
P1	84.31±1.13	1.59±0.03
P2	83.38±1.32	1.72±0.03
P3	64.25±1.13	1.15±0.03
P4	83.10±0.98	1.60±0.02
P5	83.10±0.90	1.94±0.03
P6	83.29±0.84	1.59±0.02
P7	71.96±1.43	1.41±0.04
P8	75.39±1.29	1.33±0.03
P9	83.57±1.39	1.65±0.03
P10	81.34±1.95	1.65±0.05
P11	81.62±0.28	1.66±0.01
P12	83.01±1.00	1.76±0.03
P13	84.59±0.90	1.72±0.02
P14	31.38±0.98	0.39±0.02
P15	85.24±0.28	1.66±0.01
P16	84.68±0.48	1.37±0.30
Mean	77.76±1.02	1.51±0.04

Minimal values for this parameter were registered in sample P14 (Fam. *Ericaceae*) 0.39±0.02 mmoli Trolox/g sample and maximal values for sample P5 (Fam. *Fabaceae*) 1.94±0.03 mmoli Trolox/g sample, with a mean of 1.51±0.04 mmoli Trolox/g sample.

Radical scavenging capacity can be expressed as inhibition percent (PI), representing the proportion in which the sample inhibit the discoloration of the radical, by the antioxidants from the chemical composition.

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Antioxidant power (FRAP) of pollen samples is presented in Table 6. What is obvious is that spring pollen, represented by *Crataegus monogyna* (P1), *Malus domestica* (P4), *Brassica sp.* (P13) and *Salix sp.* (P15) present a high antioxidant power, around 2.00 mmoli Fe^{II}/g sample, meanwhile samples having summer plants pollen (P2, P3, P5, P7, P9, P10, P11, P14) had a lower antioxidant capacity, situated between 0.39 - 1.32 mmoli Fe^{II}/g sample.

Table 6

Antioxidant capacity of bee pollen evaluated by FRAP method*

Sample	Antioxidant capacity (mmoli Fe ^{II} /g sample)	Sample	Antioxidant capacity (mmoli Fe ^{II} /g probă)
P1	2.22±0.02	P9	1.16±0.05
P2	1.12±0.02	P10	1.32±0.01
P3	0.60±0.04	P11	1.04±0.02
P4	2.00±0.23	P12	1.32±0.01
P5	1.42±0.00	P13	2.61±0.06
P6	3.17±0.11	P14	0.39±0.03
P7	0.96±0.01	P15	2.47±0.11
P8	1.11±0.05	P16	1.00±0.01
Mean	1.50±0.05		

* results represent mean value of three independent determinations ± standard deviation

Antibacterial activity was determined by measuring the inhibition zone diameter for every pollen extract upon 10 bacterial strains as follows: 5 Gram positive strains: *Staphylococcus aureus* ATCC 6538P, *Bacillus cereus* ATCC 14579, *Bacillus laterosporus* 6932, *Paenibacillus larvae* 9820 and *Paenibacillus alvei* 13253, 4 Gram negative strains *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enteritidis* ATCC 13076, *Salmonella typhi* ATCC 14028 and a levure *Candida albicans* ATCC 90028, and also by determining minimum inhibitory concentration for the same pollen extracts.

Antitumoral activity of methanolic extract of pollen was tested *in vitro*, in tunoral culture cells of mouse colon (cellular line C26). A model was conceived for this experiment, consisting of treatment with three different concentrations (0.25, 0.5 and 1mg/ml pollen

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methanolic extract (sample P9) exposed 6, 12, 24 and 48 hours, following the morphological modifications and antiproliferative and apoptotic effects.

The observations upon the cellular morphology give a precise answer of the action of pollen extract, culture plates for all experiments being made with the same cellular suspension. Due to this fact, covering degree of the monolayer at the analyzing point is different for 6, 12, 24, and 48 hours respectively. Different concentration in analyzing moment may influence the cell morphology, and for this reason this method may be subjective, conferring an orientative value in evaluation of antitumoral effect of bee pollen extract. Nevertheless, important modifications could be seen of shape and cellular aspect meantime and after treatment. Thus, in 6 and 12 hours, in the case of 0,25 and 0,5mg/ml concentrations, the cells did not suffer visible modifications at cellular level, the monolayer being complete and uniformly distributed in the plate. Raising the concentration to 1mg/ml lead to appearance of intracytoplasmic vacuolation and grit. Nevertheless, the most visible morphological modifications could be observed after a longer exposure period of the cells to the treatment (24 - 48 hours).

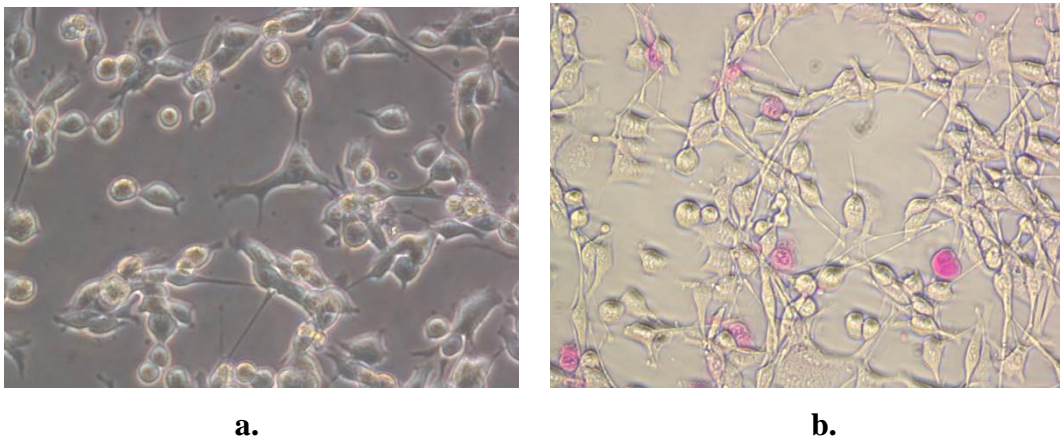


Fig. 7. Morphology and apoptosis in cellular line C26 after 6 hours of treatment with 1 mg/ml pollen extract (a. negative control; b. 1mg/ml; objective 20x)

SUMMARY

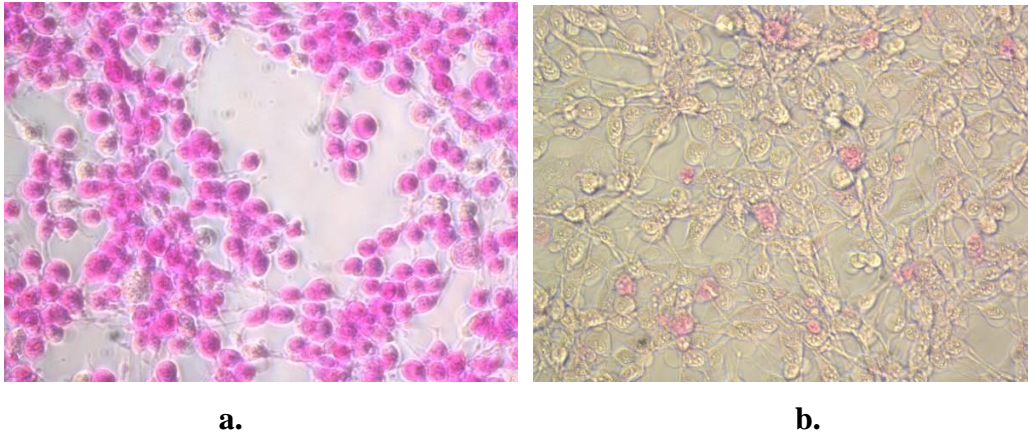


Fig. 8. Morphology and apoptosis in cellular line C26 after 12 hours of treatment with 1 mg/ml pollen extract (a. negative control; b. 1mg/ml; objective 20x)

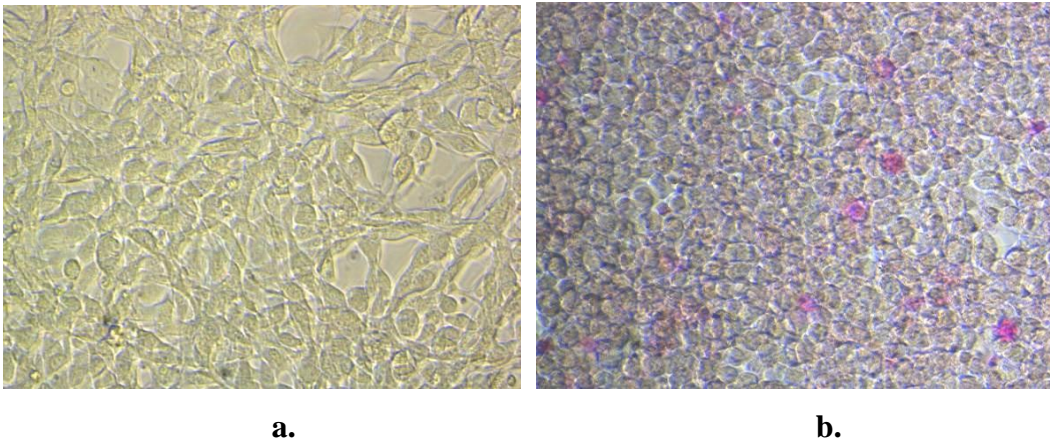


Fig. 9. Morfologia Morphology and apoptosis in cellular line C26 after 24 hours of treatment with 1 mg/ml pollen extract (a. negative control; b. 1mg/ml; objective 20x)

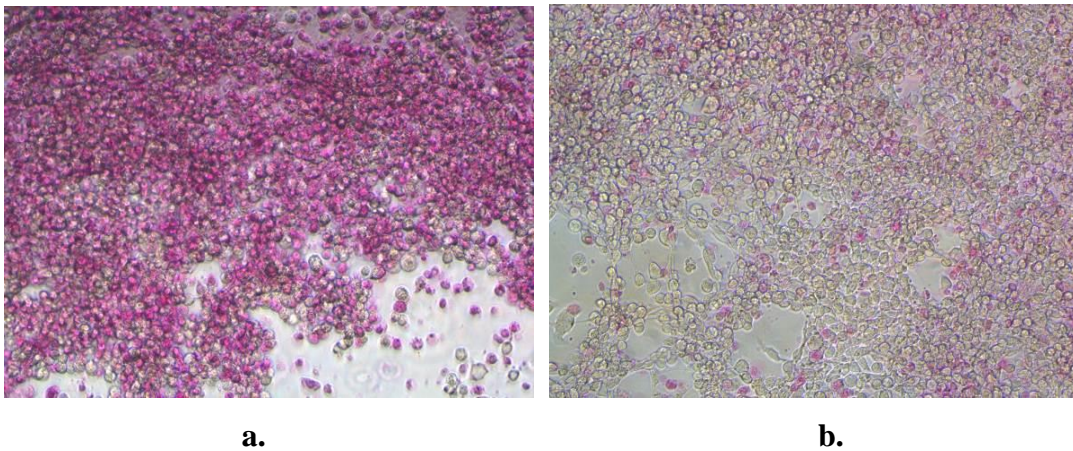


Fig. 10. Morphology and apoptosis in cellular line C26 after 48 hours of treatment with 1 mg/ml pollen extract (a. negative control; b. 1mg/ml; objective 10x)

SUMMARY

Statistical correlations and chemometrics for determined parameters

Statistical correlations in a study are made to describe the relations between many variables and to see if these are connected in between, or are reciprocal influenced.

In laboratory studies, to determine the chemical composition of a matrix and also the biological action of that matrix, statistical correlations are very important to show that the biological activity of studied matrix is influenced by its chemical composition as a whole, or certain parts of it.

Principal Component Analysis (PCA) is a chemometric method used to establish interrelations between different variables and the detection and interpretation of similarities and differences characteristic of the analyzed samples. PCA analysis for experimental data was performed by using the software Unscrambler X, version 10.1 (CAMO Software AS, Oslo, Norway).

Taking into consideration the total composition of pollen samples taken for analysis (individual sugars, fatty acid profile, total lipids, proteins, ash content, polyphenols, flavonoids, carotenoids, antioxidant activity (DPPH), vitamin C, pallinology) good differentiation of the samples were performed using a PCA, the first two components explaining 91% of variance of the data. A similar profile presents evidence P16 and P14, other samples were well differentiated. Parameters that have a role in pollen samples discrimination are: vitamin C, maltose, glucose, lutein, cryptoxanthin, total carotenoids content, AG18, AG18: 1Z and n-9 PUFA (as correlation-loadings).

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CHAPTER VI CONCLUSIONS

The researches carried out in this study have shown some important conclusions in relation to the chemical composition of bee pollen and also with regard to its biological activity, as assessed by several methods.

We have analyzed several samples of pollen collected from the Transylvania beekeeping throughout the year, namely the counties of Cluj, Salaj and Satu Mare, in April, May, June, July and August 2010.

To highlight the botanical origin of the studied samples, the palynological analysis was performed, establishing dominant plant species in the sample, secondary pollen and minor pollen.

According to the established objectives, the conclusions would be as follows:

Objective 1. Researches regarding botanical origin establishing of bee pollen samples by palinological method.

- Pollen samples studied were determined as multifloral with different percentages of specific types pollen .
- Six families of plants were present as predominant pollen (> 45%): *Rosaceae* (*Crataegus monogyna*, *Filipendula ulmaria*, *Malus domestica*, *Prunus sp.*, *Rosa canina*), *Fabaceae* (*Trifolium repens*, *Anthilis sp.*, *Robinia pseudoacacia*), *Asteraceae* (*Calendula officinalis*, *Taraxacum officinale*), *Brassicaceae* (*Brassica sp.*), *Ericaceae* (*Calluna vulgaris*) and *Salicaceae* (*Salix sp.*).
- Secondary pollen samples in the study is determined by 10 families of plants
- *Rosaceae* family was present as the predominant pollen in 7 samples (P1, P2, P3, P4, P6, P9, P11) and secondary pollen in three samples (P8, P13, P14).
- *Fabaceae* family was present as the predominant pollen in 3 samples (P5, P10, P16) and in 3 samples as secondary pollen (P7, P10, P12).
- *Asteraceae* family was present in three different samples (P7 and P8) (as predominant and secondary).

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Objective 2. Researches regarding cantitative and calitative determination of the parameters that characterize the nutritive value of bee pollen

- Water contents of bee pollen samples lies between: 17.59 - 35.85%, values that depend mostly on climatic conditions in the day of harvesting.
- Ash content of the studied samples lies within the limits imposed by certain quality standards (Swiss, Argentinean and Brazilian) and in the literature, having an average of 2.56%.
- The protein content presents an average of 22.25% and is in accordance with our literature. The samples are characterized by high protein values (over 25%) mostly in *Rosaceae* family (*Filipendula ulmaria*, *Prunus sp.*, *Rosa canina*) and *Fabaceae* (*Trifolium repens*).
- Lipid content of 2.13% and 8.93 % are in the limits imposed both in literature and in the 3 existing standards at this time.
- Carbohydrate content varies within very wide limits, and is correlated with the botanical origin of the sample.
- Energetical value of studied pollen samples is dependent on the content of fat, protein and carbohydrates, highest value belonging to *Taraxacum* pollen, characterized by high fat and glucose, meanwhile the species *Trifolium repens* being characterized by smaller amounts of glucose, fructose and carbohydrates in general.
- Pollen samples collected in spring are the most valuable from nutritionally point of view, having great importance for the bee family coming out of hibernation period.

Objective 3. Researches regarding biologically active compounds determination by spectrophotometric, liquid chromatographic (HPLC) and gas chromatographic (GC) medthods.

- Total polyphenolic contnt lies inside the limits reported in the literature, determining similar values for some types of pollen: *Calluna vulgaris*, *Salix sp.*, *Brassica sp* and *Fillipendula ulmaria*.
- For samples having *Taraxacum officinale* as predominant species, the highest value for total carotenoid content was obtained.

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- Three carotenoids were identified and quantified: lutein (maximum quantity of lutein for samples having *Taraxacum officinale* predominant pollen), β -cryptoxanthin (samples having *Fabaceae* and *Asteraceae* families as predominant pollen), and β -carotene (*Filipendula ulmaria* predominantly pollen).
- Vitamin C content varied in large limits, depending on the predominant pollen botanical species.
- 14 types of fatty acids were identified, of which the most abundant was linolenic acid [18: 3 (n-3)], ranging from 20.28% to 49.37%, and linoleic acid [18: 2 (n-6)] (7.62% - 33.21%).

Objective 4. Researches regarding biological activity determination of pollen samples: antioxidant activity, antimicrobial and antitumoral activity *in vitro*.

- Free radicals scavenging activity of bee pollen evaluated by DPPH method was situated between 31.38 and 85.24%, with an average of 77.76%
- Bee pollen from spring floral species: *Crataegus monogyna*, *Malus domestica*, *Brassica* sp. and *Salix* sp. present an antioxidant activity situated above the average of all samples.
- Antimicrobial activity upon Gram-positive bacteria was good, while Gram-negative bacteria have shown resistance to the concentrations used in this study.
- Gram-positive bacteria *Paenibacillus alvei* showed the best survival susceptibility to methanolic extracts used.
- The concentration of phenolic compounds does not completely determine the antibacterial activity of the extract, but mostly the nature of the phenolic is more important.
- Methanolic extract used to determine the anti-tumor effect *in vitro* showed a concentration-dependent cytotoxic effect and also dependent on exposure time.
- Blocking the proliferation of half of the mouse colon tumor cells has been determined by a concentration of 1 mg/ml pollen methanolic extract and an exposure of 24 hours, or a lower dose (0.5 mg / ml) but for a longer period of exposure (48h).

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- APOPercentage test shows a lower activity in the apoptosis direction, compared to antiproliferative activity, as determined by the MTT assay.

Objective 5. Researches regarding the correlations of botanical origin of pollen samples with biologically active compounds and biologic activity (chemometry).

- Chemometric analysis showed that there are correlations between physico-chemical determined parameters
- Botanical origin of the sampls is a decisive factor in classifying pollen from a nutritional perspective
- The type and quantity of predominant pollen from the analyzed samples are decisive factors in determining the biological activity of bee pollen.

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