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**FACULTY OF MEDICINE**
**Department III – Functional Sciences**

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# DOCTORAL THESIS

## CONTRIBUTIONS TO THE EXPERIMENTAL CHARACTERIZATION OF SOME INDIGENOUS PROPOLIS VARIETIES

## ABSTRACT

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**KEYWORDS**: propolis, aqueous extracts, ethanolic extracts, polyphenols, resveratrol, citronellol, antioxidant activity, antimicrobial activity, antitumoral activity, cell lines, malignant melanoma, colon cancer, volatile oil, aromatic water, polyurethane microstructures.

# I. PURPOSE AND OBJECTIVES OF THE RESEARCH

Propolis has been used in empirical medicine since ancient times, and the interest in its use for curative purposes has been considerably increased by the scientific demonstration of its therapeutic actions. In the past decades, research regarding the composition of propolis correlated with its therapeutic actions has seen spectacular progress in line with the sustained interest for apitherapy and phytotherapy as therapeutic approaches adjuvant to classical medicine. Currently, the studies carried out on different experimental models are aimed at identifying the compounds responsible for the therapeutic effects of propolis together with the characterization of their mechanisms of action in wide range of pathologies.

Propolis is primarily an important source of polyphenols, which are considered responsible for its antitumoral/cytotoxic, antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory properties, to name but a few of its therapeutic effects.

The geographical features of the provenance area of propolis do influence its chemical composition and, thus, its biological activities. Today, it is well known that a particular therapeutic effect can be determined by different chemical constituents of propolis. On the other hand, depending on its composition, propolis can display particular therapeutic properties. Similarly to phytocompounds, an increasing number of studies suggest that the effects of propolis are the result of the synergistic and mutually reinforcing actions of its constituents.

The present thesis was purported to systematically characterize the chemical composition in relation with the *antioxidant, antibacterial and antitumoral activities* of a couple of indigenous varieties of propolis harvested from the Western part of Romania. To the best of our knowledge the antitumoral effect of Romanian propolis has been tested so far only in breast cancer cell lines. Therefore, in the present work the anticancer effect of propolis was further investigated in two different human cell lines, malignant melanoma and colon cancer, respectively. In addition, propolis extracts rich in active components that were introduced in in new pharmaceutical formulations with potential therapeutic and/or prophylactic effects were prepared.

**The research objectives** were as follows:

4. Comprehensive chemical characterization of eight propolis samples collected from the Western Romania.
5. The *in vitro* assessement of the antioxidant, antibacterial and antitumoral activity of propolis extracts.
6. Preparation and characterization of novel pharmaceutical formulations based on propolis extracts.

## II. CHARACTERIZATION OF CRUDE PROPOLIS SAMPLES FROM WESTERN ROMANIA

The study material consisted of eight propolis samples (P1-P8) harvested in 2015 (P1→P6) and 2016 (P7, P8) from the Western Romania counties: Bihor, Caraș-Severin, Arad, Satu-Mare and Timiș. The characterization of propolis consisted of: evaluation of its lipid, protein and mineral profile, determination of its water and carbohydrates content, and the calculation of its energy value. The chemical composition was further correlated with the biological activities, and the propolis samples were clustered based on the similarity of the lipid, protein and mineral profile, after principal component analysis.

**II.1. Lipids** were extracted from the propolis using Soxtest equipment, after which the fatty acid profile was determined by gas chromatography coupled with mass spectrometry (GC-MS). The lipid content of the propolis varied between 20.20% and 37.73% (Fig. 1).

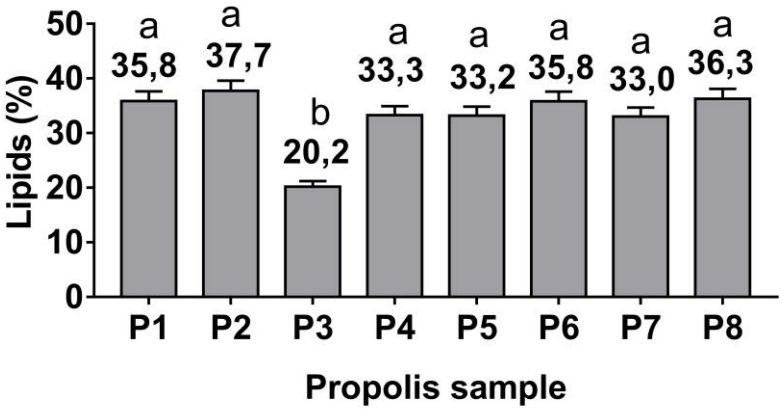


**Figure 1. The total lipid content of the propolis samples.**
*(Significant differences between values are illustrated by distinct letters; p<0.01, Tukey).*

Seventeen fatty acids were identified in the propolis, most of them being saturated; among these, lignoceric, palmitic, behenic, cerotic, arachidic and stearic acid predominated. The unsaturated fatty acids identified were: oleic, *α*-linolenic, *cis*-palmitoleic, 13,16-octadecadienoic acid and hypogeic acid, respectively. All propolis samples contained: palmitic, oleic, behenic, arachidic, stearic, *α*-linolenic, myristic and lauric acid (Fig. 2).

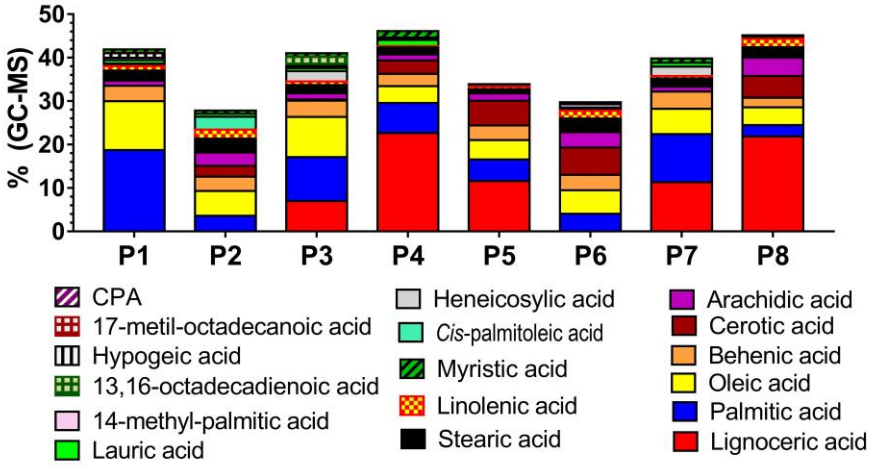


**Figure 2. Distribution of fatty acids identified in the propolis samples.**
*(CPA = 2-undecyl-cyclopropanpentanoic acid).*

**II.2. Proteins** were assayed by the Kjeldahl method and amino acids were assayed by ion exchange chromatography. The protein content varied between 0.80 and 1.90% (Fig. 3).

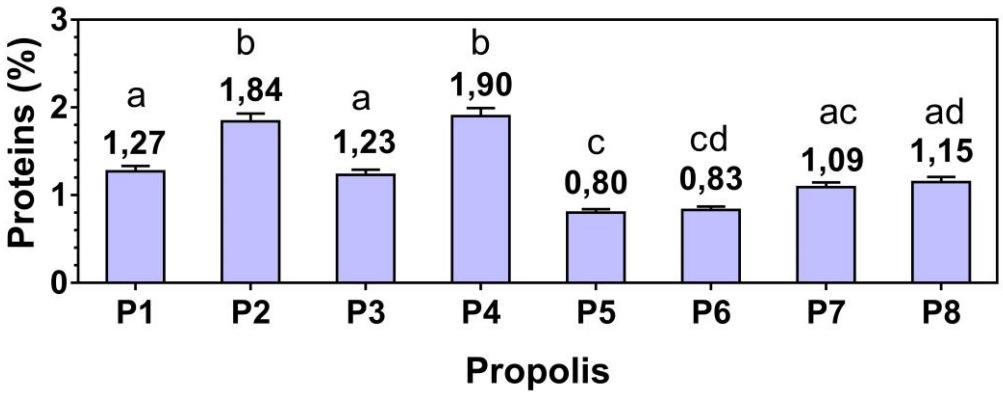


**Figure 3. Protein content of the propolis samples.**
*(Statistically significant differences between values are expressed in distinct letters, p<0.05, Tukey).*

Twelve amino acids were identified in the propolis: valine, alanine, threonine, serine, isoleucine, lysine, arginine, phenylalanine, glycine, tyrosine, leucine and histidine (Fig. 4).

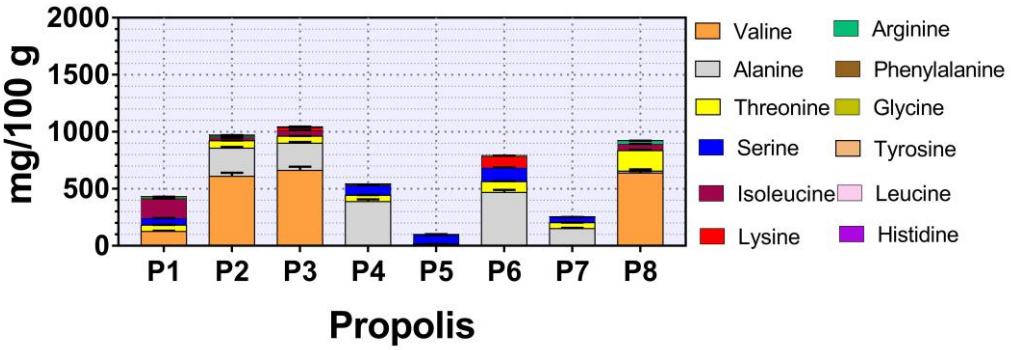


**Figure 4. Amino acids profile of the propolis samples.**

Aliphatic amino acids mono-amino mono-carboxylic, such as valine (essential amino acid) and alanine, were found in high concentration in a few propolis samples. Valine concentration in propolis ranged from 125.99 mg/100 g (P1) to 660.70 mg/100 g (P3).

**II.3.** Total content in **mineral substances** was assessed by calcination and varied between 0.53% and 2.04%. The individual mineral elements were determined by atomic absorption spectroscopy: K> Fe> Ca> P> Mg> Zn> Pb> Mn> Cr> Cu> Ni> Cd (Fig. 5).

Potassium was the main macro element detected in the propolis, followed by calcium and phosphorus. Iron and zinc predominated among the microelements. Regarding the mineral nutritional value of propolis, related to the daily requirements in adults, we noticed a very high content of iron, zinc, chromium and manganese, and a lower content in phosphorus, respectively.

Based on cluster analysis, a distinct mineral profile was observed for samples P2 and P3. Sample P2 was noted with a higher mineral content compared to the other samples (including lead), and sample P3 contained the maximum amount of manganese (22.37 mg/100 g). Toxic elements can be found in propolis as a result of environmental pollution.

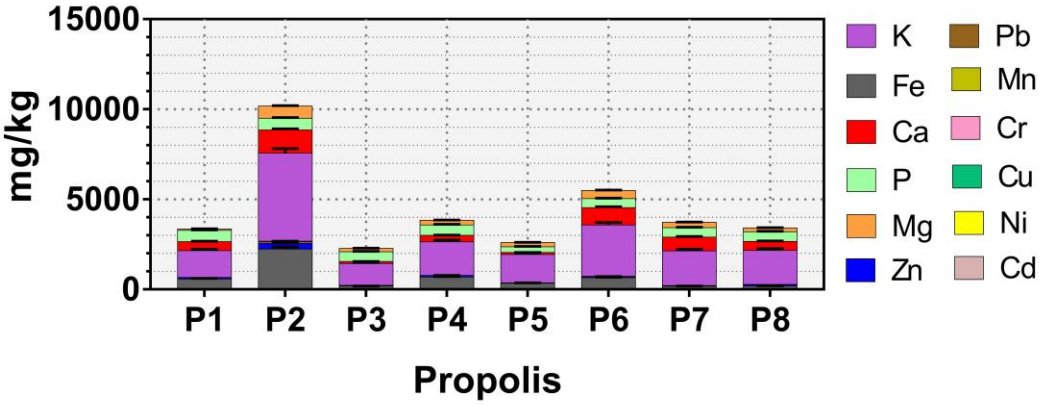


**Figure 5. The content in mineral elements of the propolis samples.**

## III. PREPARATION AND CHEMICAL CHARACTERIZATION OF AQUEOUS AND ETHANOLIC PROPOLIS EXTRACTS

The propolis extracts were prepared using 60% (v/v) ethanol, respectively, distilled water (1:20) as solvents. The extraction yield was 40.26±3.49% for the ethanolic extracts, and 5.18±2.16% for the aqueous ones. Subsequent dilutions were made to characterize the extracts.

The total polyphenols were assessed by means of Folin-Ciocâlteu method (Fig. 6), and the individual ones (gallic acid, protocatechuic acid, caffeic acid, epicatechin, *p*-coumaric acid, ferulic acid, rutin, rosmarinic acid, resveratrol, quercetin and kaempferol) by LC-MS (Fig. 7).

The ethanolic extracts of propolis contained significantly higher amounts of polyphenols (213.92±48.38 mg GAE/g propolis) as compared to the aqueous ones (7.85±3.15 mg GAE/g). Kaempferol, quercetin, resveratrol and rosmarinic acid predominated in the all samples. The aqueous extracts were rich mainly in quercetin, and the ethanolic ones, in kaempferol, respectively.

Resveratrol *was identified in Romanian propolis for the first time*, and the concentration ranged from 4.90 µg/mL (P6) to 188.50 µg/mL (P7) in the ethanolic extracts.

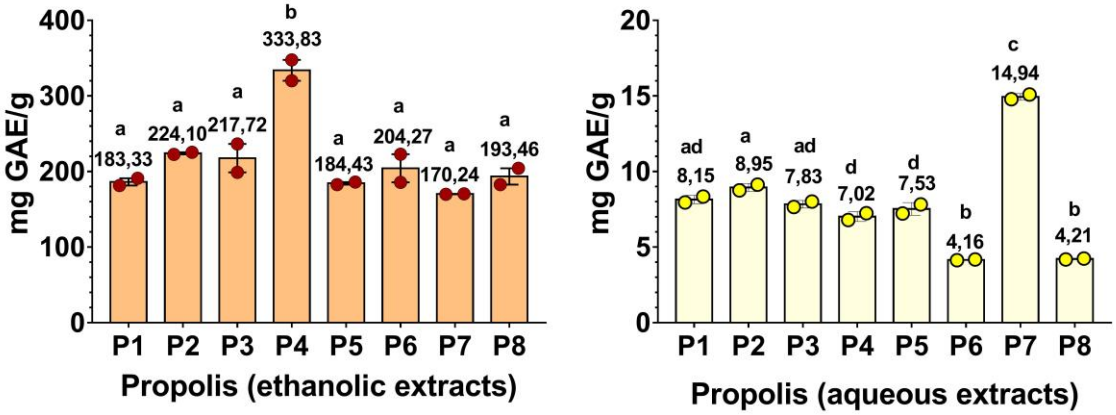


**Figure 6. The total polyphenolic content in the ethanolic & aqueous propolis extracts.**
*(Significant differences among values are expressed by distinct letters, p <0.05, Bonferroni).*

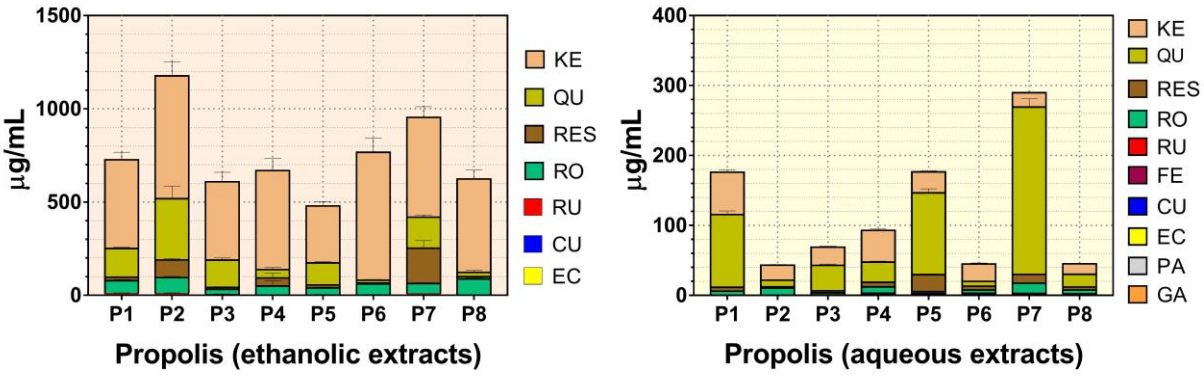


**Figure 7. Quantitative assay of (poly)phenolic compounds in the ethanolic (left) and the aqueous (right) propolis extracts.**

*(KE = kaempferol, QU = quercetin, RES = resveratrol, RO = rosmarinic acid, RU = rutin, FE = ferulic acid, CU = p-coumaric acid, EC = epicatechin, PA = protocatechuic acid, GA = gallic acid).*

## IV. THE BIOLOGICAL CHARACTERIZATION OF THE PROPOLIS EXTRACTS

### IV.1. Assessment of the ANTIOXIDANT ACTIVITY of propolis

The present study assessed the antiradical activity of the ethanolic extracts of propolis view their significantly higher polyphenolic content as compared to the aqueous ones; research was particularly aimed at obtaining extracts rich in active compounds with specific biological activities.

The antioxidant activity of propolis extracts was evaluated by two spectrophotometric methods: DPPH, a method involving the *2,2-diphenyl-1-picrylhydrazyl* radical (Fig. 8) and FOX (*Ferrous Iron Oxidation*) assay, respectively (Fig. 9).

For the first method, the antioxidant activity was determined for concentrations of 10 mg/mL, 5 mg/mL, 3 mg/mL, 1.5 mg/mL, 0.5 mg/mL and, respectively, 0.3 mg/mL, and was monitored for 20 minutes against the activity of ascorbic acid as standard antioxidant. One sample of propolis (P2; $IC_{50}^{DPPH}$=0.0700±0.0132 mg/mL) was noted to have a slightly higher antioxidant capacity than vitamin C.

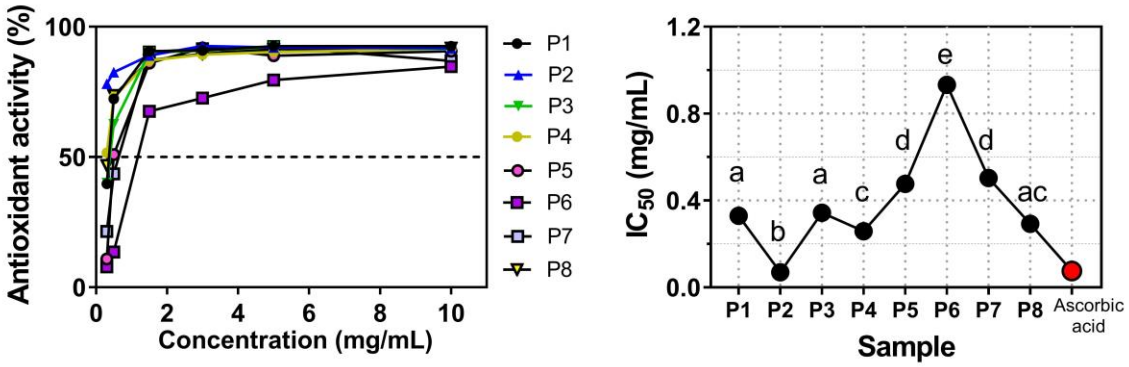


**Figure 8. The dose-dependency of the antioxidant activity and the $IC_{50}^{DPPH}$ variation of the propolis samples assessed by the DPPH method.**

*(Significant differences among the samples are illustrated by distinct letters; p<0.05, Bonferroni. All samples except for P2 significantly differed from ascorbic acid; p<0.0001, Bonferroni).*

Since propolis samples P1, P2, P3, P4, P8 showed significant antiradical capacity (89.91±4.08%, DPPH) at a concentration of 5 mg/mL, which remained high at 0.5 mg/mL

(59.00±20.97%), their antioxidant activity was further evaluated by an additional method, the FOX assay, with catalase (CAT) as standard antioxidant for the $H_2O_2$ scavenger activity(Fig. 9). Samples P5, P6 and P7 showed no antioxidant activity at the FOX assay.

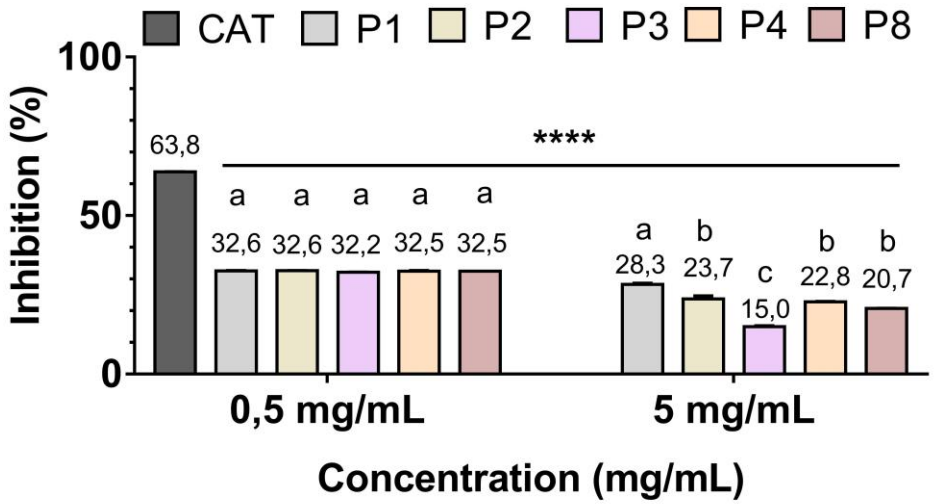


**Figure 9. The antioxidant activity of the propolis samples assessed by the FOX method.**
*(Propolis vs. CAT (catalase), ****p<0.0001, Bonferroni*).

As shown in Fig. 9, all propolis samples displayed an antioxidant activity estimated at ~ 50% of catalase activity when applied in a concentration of 0.5 mg/mL. Paradoxically, when used in a higher concentration (5 mg/mL), a lower antioxidant activity was recorded. This observation suggests the possibility of an *hormetic* effect for propolis, previously reported in the literature for phytochemicals, particularly for resveratrol.

Since polyphenols are the compounds most commonly associated with the antioxidant activity of propolis, the possibility of a correlation between the total polyphenolic content of the ethanolic extracts and their antioxidant activity was investigated. No statistically significant association (p=0.05) was found between the two variables. Regarding the individual polyphenols, significant positive correlations (p<0.05) were found between the antioxidant activity and its content in *p*-coumaric acid (r=0.713); also, the antioxidant effect significantly (p<0.05) and positively correlated with the content in: *cis*-palmitoleic acid (C16:1) fatty acid (r=0.717), zinc (r=0.790), phosphorus (r=0.771) and chromium (r=0.708), respectively.

## IV.2. Assessment of the ANTIBACTERIAL ACTIVITY of propolis

Among the propolis samples, only the aqueous extract of P3 sample showed an anti-bacterial property, especially against *Listeria monocytogenes* species (55.32% inhibition), while the inhibition of *Escherichia coli* and *Staphylococcus aureus* strains was lower (41.89% and 30.78%, respectively) when assessed using the spectrophotometric method. Also, this aqueous extract was efficient against the *Salmonella typhimurium* strain when the agar diffusion assay was employed.

The antimicrobial potential of the ethanolic extract of the P3 propolis sample solubilized in DMSO (dimethylsulfoxide) was further investigated on Gram-positive (*Staphlococcus aureus, Steptococcus pyogenes*), and Gram-negative (*Shigella flexneri, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhimurium, Haemophylus influenzae*) bacteria - Fig. 10. DMSO represented the negative control, and gentamicin (10 mcg) - the positive control, respectively.

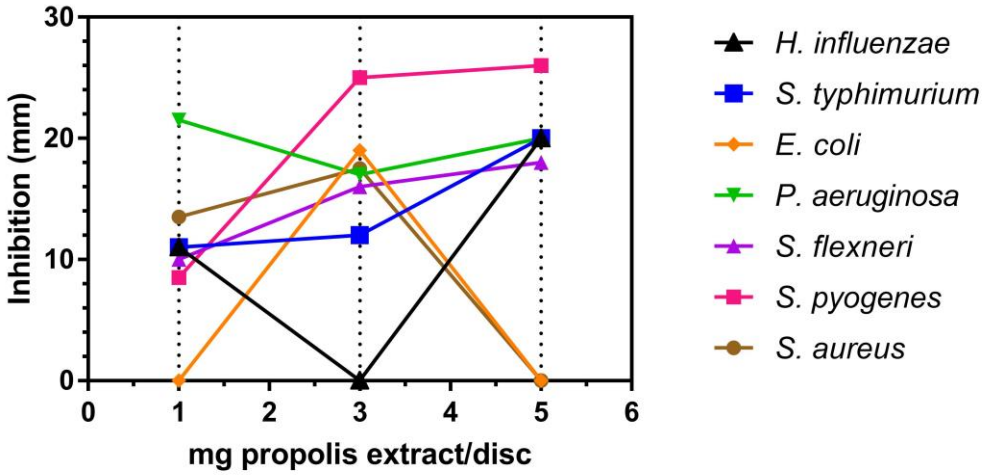


**Figure 10. The dose-dependent antibacterial activity of P3 ethanolic extract.**

P3 dissolved in DMSO elicited a dose-dependent inhibition for *Streptococcus pyogenes* (maximum inhibition at 5 mg propolis extract/disc: 26 mm), that was comparable to the gentamicin effect. A comparable antibacterial effect was recorded at 5 mg for for the Gram-negative bacteria, i.e. *Haemophylus influenzae, Salmonella typhimurium,* and *Shigella flexneri*. Interestingly, for *Staphylococcus aureus* and *Escherichia coli*, the antibacterial effect was present when P3 was applied at 3 mg but was eliminated when applied in the highest dose (Fig. 10). The antibacterial activity of the samples was also presented and discussed in the thesis in relation with the independent effect of the solvent.

## IV.3. Assessment of the ANTITUMORAL ACTIVITY of propolis in human colon cancer (Caco-2) and malignant melanoma (A375) cells

The antiproliferative activity of Western Romanian propolis was firstly assessed in 2 malignant human cell lines using the Alamar-Blue assay, and compared with the effects of equivalent concentrations of quercetin and rosmarinic acid, polyphenolic compounds previously quantified in the propolis extracts. Increasing concentrations ranging between 5 and 250 µg/mL were applied, and the assessment was performed at 48h and 72h, respectively (Figs. 11 and 12).

The propolis extract (P3) showed antitumor action on both tested cell lines (malignant melanoma, colon cancer), being more effective on melanoma cells.

For the **Caco-2 human colon cancer cell line**, the maximal inhibition of cellular proliferation was observed at 48h, and the effect subsequently declined at 72h for all the extracts applied in maximal concentration. The effects of the extracts were compared with the one of a cytostatic, 5-fluorouracil (5-FU) applied in a concentration of 13 µg/mL. Similar to the extracts (at 250 µg/mL), the 5-FU effect was maximal at 48h (450,51±109,96% inhibition) and declined at 72h (118,59±6,02% inhibition). The extracts elicited a dose-dependent increase in the antiproliferative effect after 72 hours of treatment that became evident for all 3 compounds from 100 µg/mL; at 200 µg/mL the inhibition was comparable to the one elicited by 5-FU. Interestingly, at the maximal concentration (250 µg/mL), the inhibition elicited by the extracts was slightly superior (propolis - 141.95±9.52%, quercetin - 140.45±6.44% rosmarinic acid - 157.42±17.77%) to the one induced by 5-FU (118.59±6.02%) (Fig. 11).

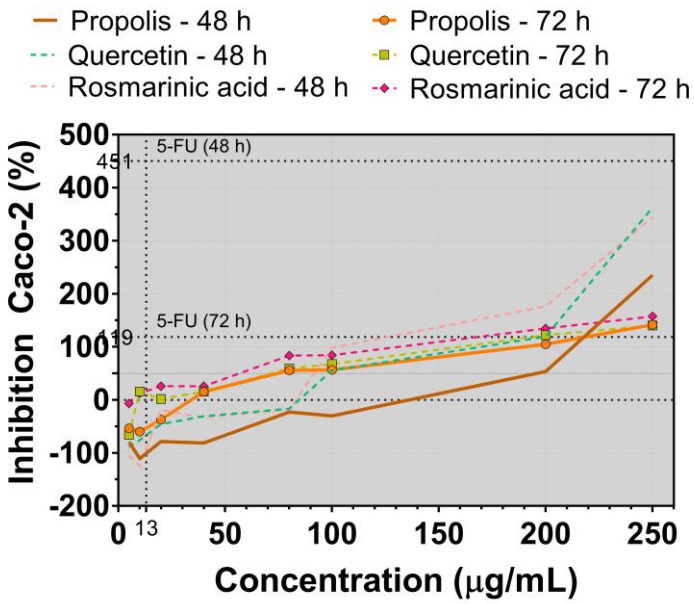


**Figure 11. Evaluation of antiproliferative effect on human colon cancer cell line at 48 and 72 hours of treatment** (5-FU = 5-fluorouracil).

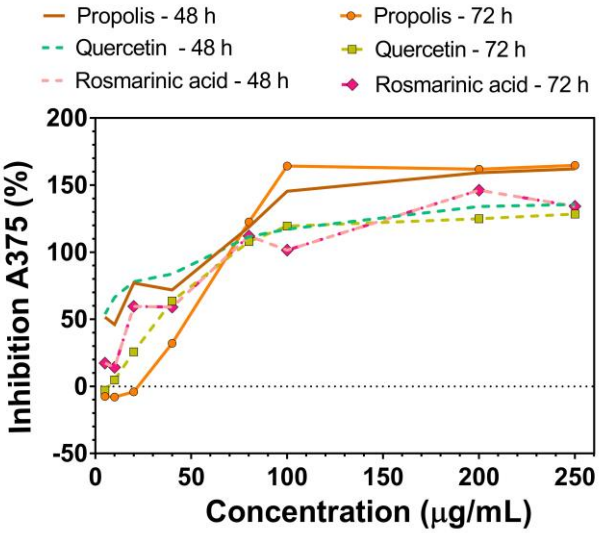


**Figure 12. Evaluation of the antiproliferative effect on human malignant melanoma cell line at 48 and 72 hours of treatment.**

When applied on the **A375 human malignant melanoma cell line**, propolis elicited a superior antiproliferative activity that was present at lower doses (compared to the effects exerted on the Caco-2 cells) and after a short treatment period. Accordingly, propolis applied in the lowest concentration (5 μg/mL) resulted in a significant inhibition of over 50%, after 48 hours of treatment. After 72h, the antiproliferative effects of the propolis were evident starting from the experimental dose of 40 μg/mL (32.17±5.31% inhibition) whereas from 80 μg/mL propolis elicited an inhibition of tumoral cell proliferation that was superior to the one induced by quercetin and rosmarinic acid. When applied in the maximal dose, the inhibitory effect was 164.73±0.75% for propolis, 128.52±2.21% for quercetin, and 149.23±3.29% for rosmarinic acid, respectively. The antiproliferative effect of the propolis at 100 μg/mL was recorded both after 48 h and 72 h of treatment; whether this dose will be also effective on other malignant

cell lines and/or when administered in vivo models of tumorigenesis warrants further investigation (Fig. 12).

## V. PREPARATION AND CHARACTERIZATION OF THE PROPOLIS VOLATILE OIL AND AROMATIC WATER

The volatile oil was extracted from propolis by water vapor entrainment, using a Clevenger device. Its chemical composition was assessed through gas chromatography coupled with mass spectrometry (GC-MS). The compounds that predominated in oil were carvone (43.81%) and anethole (41.20%). *Citronellol* is a component that was *firstly identified* in propolis oil.

The aromatic propolis water resulting from the process of obtaining the volatile oil, was characterized chemically and biologically. The individual polyphenols identified in the propolis water (LC-MS) were: resveratrol, rosmarinic acid, kaempferol, quercetin and *p*-coumaric acid. These were present in small concentrations, ranging from 0.44 to 2.54 µg/mL.

The aromatic propolis water elicited a dose-dependent antimicrobial efficiency against the following strains: *Salmonella typhimurium, Escherichia coli, Candida albicans*, *Haemophylus influenzae,* and *Streptococcus pyogenes*, assessed by the spectrophotometric method. The highest antibacterial efficiency was observed against the *Salmonella typhimurium* strain. Its efficacy against *H. influenzae* was superior to the antifungal effect on *C. albicans.* Interestingly, the antibacterial effect on *E. coli* was inversely related to the dose, the maximal effect being recorded at 25 µL (Fig. 13).

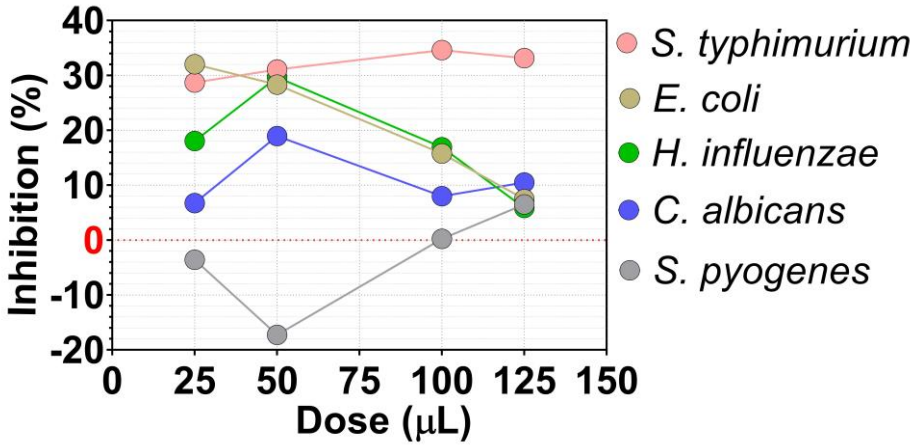


**Figure 13. The antimicrobial activity of the aromatic propolis water.**

## VI. FORMULATION AND CHARACTERIZATION OF PROPOLIS POLYMERIC MICROPARTICLES

Polyurethane microparticles with ethanol propolis extract were prepared and characterized in terms of thermal behavior through differential scanning calorimetry and of physical stability, respectively, by determining their *Zeta* potential. Consequently, the antibacterial activity of the propolis polyurethane microparticles was determined through the agar diffusion method on the following bacterial strains: *S. pyogenes, S. aureus, S. flexneri, P. aeruginosa, E. coli, S. typhimurium* and *H. influenzae*, as compared with the effect of gentamicin (10 mcg). Thermal analysis indicated an efficient inclusion at the particle size of 572.13±35.12 nm; the polydispersion index varied between 0.4 and 0.7, with the *Zeta* potential ranging between 16.3 and 19.8 mV. However, the polyurethane microparticles with ethanol propolis extract did not exhibit antibacterial activity on the tested strains; further studies on their release capacity are warranted.

# VII. CONCLUSIONS

1. The average lipid content of the propolis was 33.17±5.16%, the lipid fractions predominantly identified being saturated fatty acids. In the category of unsaturated fatty acids, oleic and *α*-linolenic acid (an essential fatty acid) predominated.
2. The average total protein content of the propolis was 1.26±0.38%. The following essential amino acids have been identified: valine, threonine, isoleucine, phenylalanine and leucine. Of all the amino acids, valine and alanine predominated.
3. The average total mineral content of propolis was 0.91±0.51%. The propolis was noted for its high potassium content. Regarding the mineral nutritional value of the propolis, considering the daily requirements in adults, a high content of iron, chromium, zinc and manganese was observed.
4. The percentage of reducing sugars in propolis was 0.4±0.1% and the the energy value of the propolis was established at 545.01± 27.30 kcal/100 g.
5. The total amount of polyphenols in the ethanol extracts of propolis was much higher than that in the aqueous extracts. Also, the dry residue of the ethanol extracts was much higher than that corresponding to the aqueous extracts, the ethanol (60% v/v) being a much more efficient solvent for the extraction of polyphenols from the propolis than water.
6. The polyphenolic compounds present in all the propolis samples from the western part of Romania were: kaempferol, quercetin, rosmarinic acid and resveratrol, with these being found in both aqueous and ethanol extracts.
7. Resveratrol was identified *in the Romanian propolis as a national premiere*.
8. The *ethanol* extracts of propolis showed *very good antioxidant activity*, even at low doses: the percentage of DPPH inhibition was 85.97±7.63% for the concentration of 1.5 mg/mL and, respectively, 59.00±22.41% at the concentration of 0.5 mg/mL. The average value of the $IC_{50}^{DPPH}$ for the eight samples was 0.40±0.25 mg/mL.
9. The propolis extracts with an antioxidant activity demonstrated in the DPPH assay were further evaluated through the FOX method; they showed a higher antioxidant activity at a lower concentration, a suggestive aspect for the *hormetic* effect of propolis, described in the literature for other phytocompounds as well.
10. The *ethanol* extracts of propolis were noted for important *antibacterial activity* on *group A beta-hemolytic streptococcus*, including at low doses. Depending on the dose, the propolis showed an antibacterial effect on *Haemophilus influenzae* and *Escherichia coli.* The species *Staphylococcus aureus* and *Shigella flexneri* were less sensitive.
11. The *aqueous* propolis extracts were effective on a smaller number of bacterial strains as compared to the ethanol ones, and were noted for their *efficacy against Salmonella typhimurium*. The species *Listeria monocytogenes, Escherichia coli* and *Staphylococcus aureus* were *sensitive to aqueous extracts.*
12. The aromatic propolis water had its own biological effect being active against the species *Salmonella typhimurium, Escherichia coli, Haemophilus influenzae* and *Candida albicans*.
13. The antitumor activity was first tested nationally on human colon cancer and human malignant melanoma cell lines and showed that the propolis exhibited antitumor effects comparable to quercetin and rosmarinic acid, depending on the applied dose and duration of treatment, respectively.
14. The antiproliferative effect on the melanoma cell line was higher than that obtained on the colon cancer line.
15. Polyurethane microparticles with propolis extract, with a diameter between 0.504 μm and 0.621 μm were synthesized, being physico-chemically characterized and tested for the antibacterial effect, but their reduced capacity of releasing the active principles led to a deficient bacterial inhibition.

## VIII. ORIGINAL CONTRIBUTIONS

1. *First identification of resveratrol* in all ethanolic and aqueous extracts prepared from the propolis samples harvested from Western Romania.
2. *First evidentiation at national level of the antiproliferative effect of Western Romanian propolis on two human malignant cell lines*, melanoma and colon cancer, respectively.
3. *Comprehensive analysis of the lipid fraction in the propolis samples* with the identification of certain components, which albeit present in minute amounts in the composition, have been associated with bioactive potential.
4. Quantification of the mineral content in propolis samples that was compared to the daily dietary requirements, highlighting the presence of essential oligoelements such as: iron, zinc, manganese and chromium.
5. Extraction and characterization of propolis volatile oil and aromatic water and *the first identification of citronellol as an active compound in volatile propolis oil.*
6. *Synthesis of a modern pharmaceutical formulation containing propolis extract* and the preliminary test of its antibacterial property, view potential use in an optimized form as therapeutic adjuvant.

## IX. SCIENTIFIC PUBLICATIONS

4. ***Identification of Resveratrol as Bioactive Compound of Propolis from Western Romania and Characterization of Phenolic Profile and Antioxidant Activity of Ethanolic Extracts.*** Alexandra Duca, Adrian Sturza, Elena-Alina Moacă, Monica Negrea, Virgil-Dacian Lalescu, Diana Lungeanu, Cristina-Adriana Dehelean, Mirela-Danina Muntean, Ersilia Alexa. ***Molecules***, 2019; 24(18), 3368, 19 pag; (ISI journal, IF=3.060).

5. ***Assessment of Lipid Profile of Eight Propolis Samples from Western Romania.*** Alexandra Duca Ersilia Alexa, Cristina Adriana Dehelean,Codruţa Șoica, Corina Danciu, Iuliana Popescu, Ileana Cocan, Dacian Lalescu, Danina Mirela Muntean. ***Farmacia***, 2019; 67(1):126-132. ISSN: 0014-8237; (ISI journal, IF=1.507).

6. ***Formulation and Characterization of Polyurethane Microstructures with Propolis Extract***. Alexandra Duca, Florin Borcan, Danina M. Muntean, Ersilia C. Alexa, Codruța M. Șoica. ***Revista de Materiale Plastice***, 2019; 56(2):321-323. ISSN: 0025-5289; (ISI journal, IF=1.248).