NATIONAL UNIVERSITY OF SCIENCE AND TECHNOLOGY

POLITEHNICA BUCHAREST

DOCTORAL THESIS

Supervisor:

Prof. dr. eng. Vasile Lavric

Ph.D. student:

M.Sc. Mădălina-Maria Nichitoi

Bucharest
2023

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Doctoral School of Chemical Engineering and Biotechnologies

THESIS SUMMARY

SUPERIOR VALORIZATION OF THE APICULTURE PRODUCTS

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Author

Contents

Keywords: propolis, polyphenolic profile, flavonoids, maceration, ultrasound extraction, antioxidant capacity, antimicrobial activity, ABTS, DPPH, UHPLC-MS, multivariate statistics

Introduction

The doctoral thesis is written in "article" format and is structured in 4 main parts.

The first part, represented by **Chapter 1**, presents a literature study regarding the research object, the methods and concepts used to describe the properties of propolis, as well as the mechanisms underlying its antioxidant, antitumor and antimicrobial action, the classification of polyphenols and their mode of action. Polyphenol extraction methods were described, comparing conventional and ultrasound-assisted extraction and highlighting the factors that influence the extraction process. The techniques used to determine propolis composition, antioxidant capacity and antimicrobial activity are also presented. In the end of this chapter are described the mathematical instruments for the advanced processing of the data obtained experimentally during the research activity.

The second part of the paper, the original part, consists of three full articles published as scientific results of the doctoral thesis (Chapter 2, Chapter 3 and Chapter 4).

The aim of the study presented in **Chapter 2** was to evaluate the Romanian propolis composition and to identify whether mild conditions, involving environmentally friendly solvents, lead to significant results. Different operational parameters were applied and their effect on the extract composition was studied. Three equal mass fractions containing fine ($d < 600$ μm), medium (600 μm $< d < 1.25$ mm), and large ($d > 1.25$ mm) size propolis particles were subjected to maceration using demineralized water and 50% ethanol, at 150 rpm and 25°C, ensuring a 10:1 liquid:solid ratio. Extraction duration varied between 1 and 7 days. Extracts were evaluated in terms of polyphenols, flavonoids, and antioxidant capacity. Absorption spectra recorded in the 200 – 500 nm domain were subjected to Principal Component Analysis, Linear Discriminant Analysis, and Partial Least Squares regression. The statistical analysis enabled samples classification, mainly based on the extractant nature, and put into evidence the possibility of linking the main properties in terms of flavonoids and polyphenols content, and the antioxidant capacity to the spectral characteristics.

In the research work presented in **Chapter 3**, propolis extracts, obtained by maceration, were studied to identify the relationship between the polyphenolic derivatives profile and their antioxidant and antimicrobial activity. Extracts were obtained using water and 25%, 50%, and 70% ethanolic solutions (w/w), at 2:1, 4:1, and 6:1 liquid: solid ratios (w/w). 21 polyphenolic derivatives were quantified by UHPLC-MS, proving that the extracts composition strongly depends on the solvent. Antimicrobial efficiency was tested against Gram-positive (*B. subtilis*), Gram-negative bacteria (*E. coli*), and fungi (*C. albicans*) by disc-diffusion method.

Chemometric methods (partial least squares) and a saturation-type model were used to evaluate the contribution of various bioactive principles for antioxidant capacity of extracts.

The **Chapter 4** aims to find optimal conditions for polyphenolic compounds extraction from propolis, using sonication as intensifying technique, under isothermal conditions. The operating parameters were the US exposure time, liquid:solid ratio, and ethanolic solvent concentration. Another goal was to identify if the ultrasonic field and exposure time influence the standard profile of the polyphenolic derivatives (quantified by UHPLC-HRMS method). Data of the study presented in Chapter 3 for the polyphenolic profile of the extracts obtained by maceration are compared with the data of the polyphenolic profiles obtained by sonication.

The contribution of ultrasound to the intensification of the process, the way in which the new profiles influence antioxidant and antimicrobial activities and the quality of propolis extracts were also investigated.

The third and fourth parts of the thesis contain the general conclusions of the doctoral thesis, respectively the elements of originality of this thesis.

Chapter 1 – Presentation of the research object, methods, and concepts used

1.1. Propolis – main apicultural product

Propolis is a dense and sticky substance produced by bees. They collect the resins of various trees such as poplars, conifers, plums, process them by mixing with wax, pollen and their saliva, that contains specific enzymes, resulting in the final, a product rich in biological properties. Propolis is used by bees to cover the cracks in the hive, to cover the bodies of intruders and provide an aseptic environment in the hive [10].

For this research work, we used raw propolis (produced by Apis Meliffera Carpatica), donated by dr. Roxana Spulber (Institute for Research and Development for Beekeeping, Bucharest, Romania), harvested by the beekeepers in March-November 2016 season. It was stored in laboratory at − 20 °C until processing and analysis.

1.1.1. Propolis composition and its effects on human health

Propolis is a valuable product for human health thanks to the synergistic effect of all its constituents, but many studies pay special attention to polyphenols [14, 15].

Polyphenolic compounds are a large family of naturally organic compounds with a great importance in human health [16]. They are divided into four main classes: phenolic acids (divided further in hydroxycinnamic acids and hydroxybenzoic acids), flavonoids, stilbenes and lignans.

The most common polyphenols found in Romanian propolis, but also in European propolis, are: ***flavonoids***, like quercetin, caffeic acid phenethyl ester (CAPE), pinocembrin, apigenin, chrysin, galangin, acacetin, pinostrobin, myricetin, luteolin, kaempferol, naringenin, rutin, catechin, epicatechin and ***phenolic acids*** like caffeic, p-coumaric, gallic, ellagic, syringic, ferulic, cinnamic, benzoic, salicylic, or vanillic [20-27].

1.2. Methods for extracting valuable compounds from propolis

1.2.1. Classical extraction

The extraction methods used to obtain propolis extracts, the factors influencing the extraction process and the techniques used to determine propolis composition, antioxidant capacity and antimicrobial activity were also described in this chapter.

Thus it is described the maceration technique that is a classic extraction method, cheap and simple that can be performed at room temperature. Compounds with bioactive properties are extracted from various plants, plant derivatives and propolis using different solvents and different extraction times.

The most important factors influencing propolis maceration extractions as well as other types of extractions are: the nature of the solvent, contact time between the plant and the solvent, temperature, the material and solvent ratio, particle size of the material subjected to extraction [56, 57].

1.2.3. Ultrasounds assisted extraction

Ultrasound assisted extraction is a simple-to-use technique that does not require much extraction time or hight investments for a good efficiency. Also, ultrasounds facilitate the diffusion of the solvent in the sample and increases the migration of the compounds in the liquid, by dilating the pores of the material subjected to extraction or by cracking or breaking the walls of it [73, 74].

The most important factors influencing ultrasonication are: power, amplitude, frequency and intensity.

1.3. Analytical methods for quantification of polyphenolic acids and flavonoids in propolis extracts

1.3.1. Spectrophotometric methods

The Folin-Ciocalteu (F-C) test is a colorimetric measurement widely used in analyz-es which aims to estimate the total content of phenolic compounds in plant extracts, foods, and other types of samples [102].

Total phenolic content

The total polyphenols content of the propolis extracts obtained in this research work was determined by reaction with the Folin- Ciocalteu reagent in basic medium, using gallic acid as model compound. After proper dilutions, 1 mL diluted extract was mixed with 5 mL Folin-Ciocalteu reagent, 10 % solution. The mixture was made up to 10 mL with Na_2CO_3, 7.5 % solution, after 5 min. The absorbance at 765 nm was measured, after 60 min rest in the dark, in 1 cm quartz cell, against water [115,148].

Total flavonoid content

The total flavonoid content was determined by reaction with $AlCl_3$, after 30 min reaction time. Quercetin was used as model compound. Typically, 0.5 mL standard, and 1.5 mL $AlCl_3$ 2 % solution in ethanol, were made up to 5 mL with ethanol. Absorbance at 452 nm was measured in a 10 mm quartz cell, against ethanol [115, 148].

Chemicals and equipment used in present research

Ethanol (99,8 %), Folin-Ciocalteu reagent (2 M, 1,27 g/mL), 2,2-diphenyl- 1-picryl-hydrazyl (DPPH), gallic acid (ACS standard, 95,5%), $AlCl_3$ (99,99%), quercetin (95%), Trolox (95%), 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid – ABTS – (98%), $K_2S_2O_8$ (99%), Na_2CO_3 x 10 H_2O (99,8%) were purchased from Sigma-Aldrich (Germany). The extractions were carried out in an ES 80- Grant Instruments orbital shaker**.**

A Kern 770 Analytical Balance (Germany), having a weighing accuracy of 0.0001 g was used for weighing purposes. All solutions were prepared in class A laboratory glassware. Water was purified using a TKA demineralization system (Germany), reaching a conductivity of 18.2 MΩ × cm. A Varian Cary 50 *UV-Vis* (USA) monofascicle spectrophotometer was used to measure sample absorbances [87, 115, 148].

1.3.2. Chromatographic techniques

UHPLC system coupled with mass spectrometer (UHPLC-MS) is the most sensitive and efficient equipment, used in specialized laboratories for the separation and identification of new compounds, especially organic ones, offering much more structural information than conventional analysis methods [109].

In the present study [87, 148], polyphenolic derivatives were quantification by UHPLC-MS. The phenolic acids and flavonoids were quantified with an UltiMate 3000 UHPLC System (Thermo Fisher Scientific), coupled with a Q Exactive Focus Hybrid Quadrupole-Orbitrap mass spectrometer equipped with Heated Electrospray Ionisation (HESI) probe (Thermo Fisher Scientific). The Kinetex C18 column (Fusion-RP, 100 Å, LC Column 100 × 2.1 mm, particle diameter 1.7 μm) was operated at 30 °C. The injection volume was 10 μL, each injection being repeated three times. Mobile phase A contained formic acid, 0.1%

aqueous solution, while solution B was a methanolic 0.1% formic acid solution. The gradient elution program started with 100% A: 0–2 min, from 100% A to 98% A, 2% B at 400 µL/min; 2–5 min, from 98% A, 2% B to 50% A, 50% B at 300 µL/min; 5–17 min from 50% A, 50% B to 2% A, 98% B at a 300 µL/ min; 17–18 min, from 2% A, 98% B to 98% A, 2% B at 400 µL/min; 18–20 min to 100% A. Mass spectra were recorded in the negative ionization mode and nitrogen was used as collision, sheath, and auxiliary gas at 11–48 arbitrary units flow rates. The spray voltage was 2.5 kV and the capillary temperature 320 °C. Data were acquired and analysed with the Thermo Xcalibur software package (Version 4.1). Calibrations were carried out in the 50–1750 µg/L concentration range, by serial dilution of the 10 mg/L methanolic standard mix. Propolis extracts were filtered through a 0.45 µm polytetrafluoroethylene membrane and diluted before injection into the UHPLC-MS system [87, 148].

Analytical standards for flavonoids (apigenin, (+)–catechin, chrysin, (–)–epi–catechin, galangin, hesperidin, isorhamnetin, kaempferol, pinocembrin, quercetin, rutin) and phenolic acids (caffeic, 3,4- dihydroxybenzoic, t-ferulic, gallic, 4-hydroxybenzoic acid, p-coumaric, syringic, and vanillic acids), phenolic acids derivatives (CAPE, ellagic, and chlorogenic acids), stilbenes (t-resveratrol) acid, vanillic acid) and terpenes (abscisic acid) from Sigma-Aldrich (Steinheim, Germany) were used to prepare 500 mg/L methanolic stocks [87, 148]. .

1.4. Methods for evaluation of antioxidant and antimicrobial activity

The spectrophotometric method for determining the antioxidant activity by the cati-on-radical ABTS method is based on the reaction between the dianion 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) and potassium persulfate, leading to the formation of the stable blue-green chromophore of ABTS•, which acquires a positive charge and becomes a cation [118].

DPPH is a simple, inexpensive, and widely used method to evaluate the ability of compounds to donate H atoms or scavenge free radicals [128].

Antioxidant capacity

Extracts were tested for antioxidant capacity by scavenging the long-lived free radicals ABTS+ and DPPH, Trolox being model compound. ABTS assay was carried out 734 nm, against a reagent blank in water, while DPPH assay was performed at 515 nm.

Propolis extracts were diluted prior reacting with the free radicals' solution. The antioxidant capacity was calculated, irrespective of the method at hand, as equivalent concentration of Trolox from the calibration curves, namely Trolox Equivalent Antioxidant Capacity (TEAC), and reported as µg TEAC/mL [87,115, 148].

1.5. Mathematical instruments for advanced data processing

1.5.1. Descriptive statistics instruments for primary treatment of experimental Data

1.5.2. Multivariate statistics instruments

Descriptive statistics were used to evaluate the experimental errors for chemical and micro-biological measurements. The graphical representations of components concentration ob-tained in propolis extracts at various liquid: solid ratios, both in maceration and ultrasound field exposure gave a good insight for process evaluation and estimation of experimental errors. The experimental errors were clearly represented in data tables and graphs in all published papers.

Multivariate analysis (MA) can extract as much useful information from data as possible, considering all variables at the same time. MA uses statistical-mathematical methods that can simultaneously investigate relationships between three or more variables [152-155].

1.5.2.1. Principal component analysis

PCA (Principal Component Analysis) is the oldest chemometric method in multivariate analysis that uses mathematical statistical calculations to interpret data. This method is based on extracting the lowest number of components that contains as much information as possible from the original data of the studied system.

A data matrix {n × m}, X, consists of n samples or objects in the space of m variables measured. The main idea of PCA is to find m PCs (PC_1, PC_2,…, PC_m) which are obtained by linear combinations of the original variables describing each sample (X_1, X_2,…, X_m) [152].

So:

$$PC_1 = a_{11}X_1 + a_{12}X_2 + a_{13}X_3 + \ldots + a_{1m}X_m$$

$$PC_2 = a_{21}X_1 + a_{22}X_2 + a_{23}X_3 + \ldots + a_{2m}X_m \quad (4)$$

$$\ldots$$

$$PC_m = a_{m1}X_1 + a_{m2}X_2 + a_{m3}X_3 + \ldots + a_{mm}X_m$$

1.5.2.2. Linear discriminant analysis

LDA, like PCA, is a statistical method of selecting features and reducing the size of initial data, while keeping a large part of the discriminant information.

The purpose of LDA is to classify objects into two or more groups based on a set of n characteristics that describe the objects, trying to find the best direction for data projec-tion in which the vectors of the different classes are best separated.

1.5.2.3. Partial least squares regression

The PLS method aims to analyze the relationships between an array of independent variables (X) and an array (Y) or a vector (y) of dependent variables, called the response. It practically is a combination of PCA and regression and is applied when the number of independent variables is higher than number of data sets.

PLS establishes a new set of variables, called latent variables (LVs), with a role in maximizing the covariance between the X and Y matrices, thus, PLS extracts those varia-bles that separate the information from the matrix X from the noise, and a calibration model is obtained, described by the equation:

$$Y = XB + E \quad (15)$$

- B is the matrix of PLS regression coefficients.

Chapter 2 - Romanian propolis extracts: characterization and statistical analysis and modeling [115]

2.2. Propolis samples

The frozen propolis was weighed and grinded, and the particle size distribution was measured using a Retsch AS 200 set of sieves. All resulting fractions were clustered according to the particle size into fine (d < 600 μm), medium (600 μm < d < 1.25 mm) and large (d > 1.25 mm) particles fractions.

2.8 Statistical analysis and modelling

Multivariate statistical analysis of the spectral data was performed using principal component analysis (PCA) and linear discriminant analysis (LDA) for the analysis of 300 data sets (spectra in the 200–500 nm range).

3. Results and Discussions

The effects of changing operational parameters were monitored in terms of total content of polyphenols and flavonoids, antioxidant capacity in the presence of ABTS, and spectral characteristics in the 200 – 900 nm domain.

Larger levels of polyphenolic compounds and flavonoids, together with higher antioxidant capacities were recorded for ethanolic extracts than the corresponding levels in aqueous extracts.

Extraction of polyphenolics and flavonoids is expected to tend towards saturation concentrations in time, therefore experimental data for all three granulometric classes at varying extraction durations were correlated using the general accepted saturation model:

$$C(\tau) = C_{\max} \cdot \frac{\tau}{K + \tau} \qquad (4)$$

where C(τ) is the concentration in the extract at time τ, C_{max} the maximum concentration reachable in the given experimental conditions, and K the extraction kinetic constant.

In Table 1 are presented results, with Err standing for the absolute relative error between experimental and computed values.

Table 1

Solid – liquid extraction parameters

Parameter	Polyphenols					
	Ethanolic extracts			Water extracts		
	Small particles	Medium particles	Large particles	Small particles	Medium particles	Large particles
C_{max}, mg/g	92.7	90.1	89.7	15.1	6.80	Inadequate model
K, d	0.25	0.11	0.32	1.68	0.68	
Err, %	5.1	5.4	2.8	27	17	
Parameter	**Flavonoids**					
	Ethanolic extracts			Water extracts		
	Small particles	Medium particles	Large particles	Small particles	Medium particles	Large particles
C_{max}, mg/g	3.09	4.099	4.156	0.317	0.33	0.167
K, d	0.049	0.32	1.105	14.77	14.75	14.98
Err, %	3.00	12.6	17.5	26.2	27.6	37.13

Fig. 1 gives the estimated model predictions for ethanolic extracts as regards the total extracted polyphenols and flavonoids.

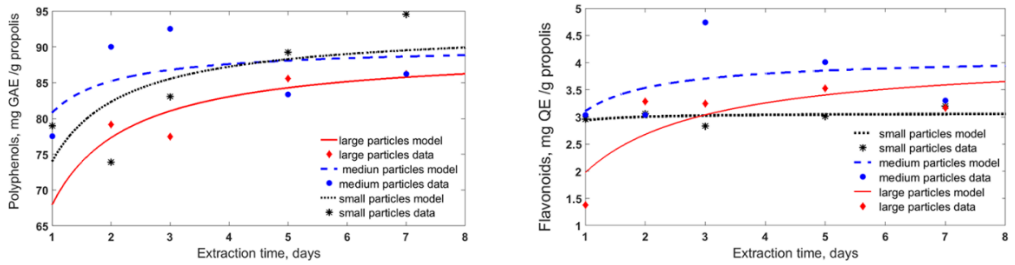


Fig. 1. Polyphenols and flavonoids in ethanolic extracts according to the saturation kinetic model

As expected, the increase in antioxidant capacity for samples corresponding to higher extraction duration follows a time limitation tendency, as this property is mainly due to polyphenols and flavonoids present in the extract (Fig. 2).

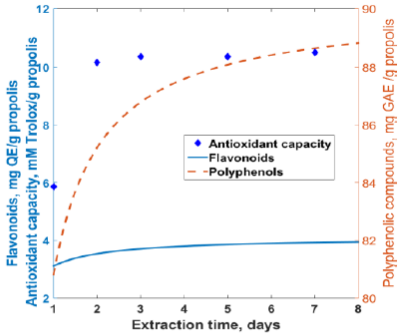
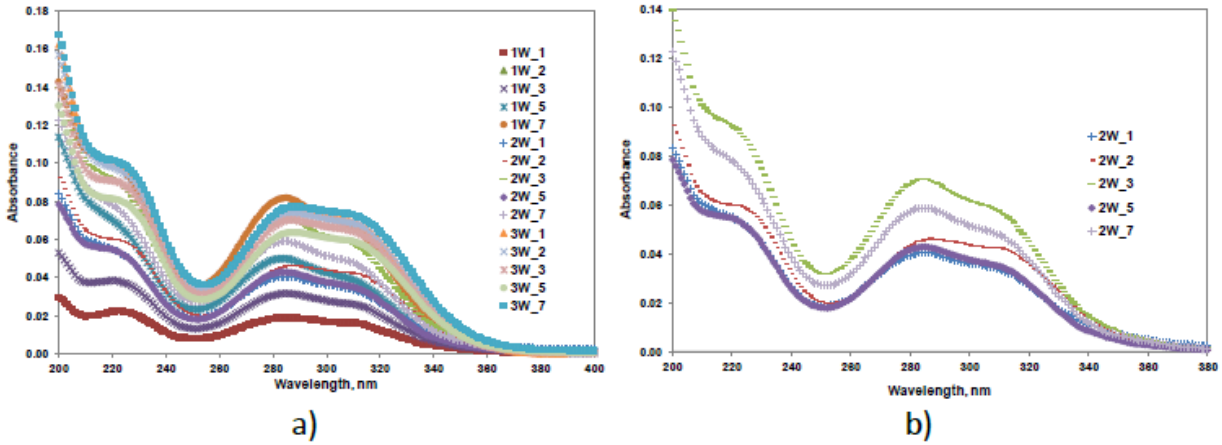
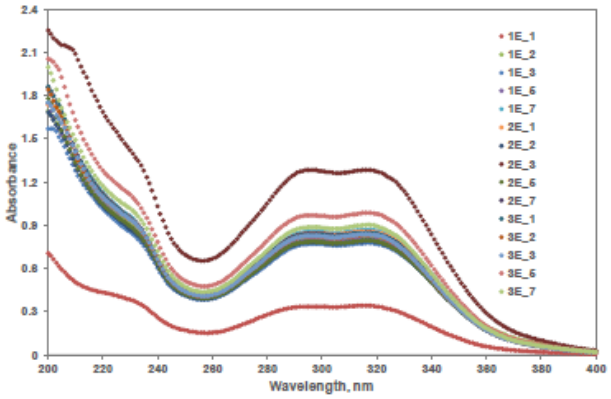


Fig. 2. Time evolution of the antioxidant capacity in ethanolic extracts of medium size propolis particles

The spectral study concentrated in the 200 − 500 nm range is represented in (Fig. 3). Differences in time and with particles size can be observed. Aqueous extracts presented two overlapping bands of different intensities, centred at 320 and 280 nm, together with a shoulder in the 220 − 230 nm region. The extracts yielded by the large and small particles showed only hyperchromic changes in time (Fig. 3a).

Medium size particles extracts display unexpected spectral changes in the 280 − 300 nm domain, with at least two visible isosbestic points (Fig. 3b). The 1:1 water:ethanol extraction medium lead to different spectral features (Fig. 3c).





c)

Fig. 3. Spectral details for extracts obtained processing different size propolis particles in time (1 – 7 days): a) aqueous extracts from large (1W), medium (2W) and small (3W) particles; b) extracts in 1:1 ethanol:water mixture from large (1W), medium (2W) and small (3W) particles; c) medium particles in aqueous phase

PCA applied to this absorption spectra of all extracts in the 200 - 500 nm domain (300 wavelengths) revealed that the first 7 PCs reflect over 99.9 % of data variability. In the PC1 - PC2 space (Fig. 4) aqueous and ethanolic extracts form two different classes, but aqueous samples are less differentiated by thegranulometric characteristics than the ethanol-water extracts, where the dimension of propolis particle seems to influence the extract properties reflected by the absorption spectra.

LDA was carried out using the first 7 PCs for samples characterization. It gave clearer samples separation in terms of extraction solvent and granulometric fraction used (Fig. 5). The main differentiation is on LDA1, proving that the use of aqueous *versus* ethanolic solutions can lead to different extract properties. The three granulometric classes used for ethanolic extraction appear as separate groups, mainly on the direction of LDA2 function, proving that propolis granulation is a factor likely to influence the extract content, as also shown in Figs. 1-2.

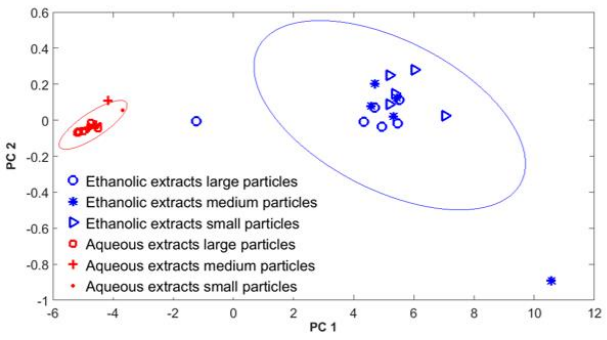


Fig. 4. Water and ethanolic extracts represented in PC1-PC2 coordinates

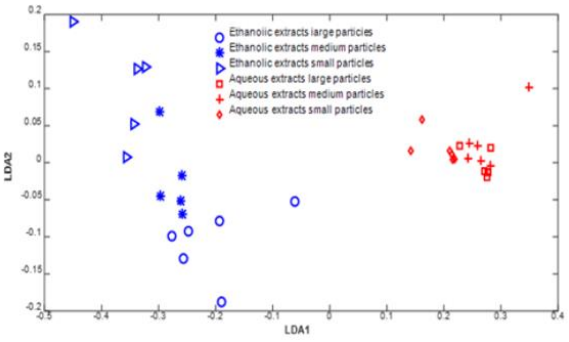


Fig. 5. Samples representation in LDA functions coordinates

Regression analysis by PLS was carried out separately for ethanolic and aqueous extracts for the main properties measured: polyphenols, flavonoids, and antioxidant capacity. The minimum number of PCs, reflecting over 95% of data variability, was used.

Table 2

Results of the PLS regression analysis

Property	Ethanolic extracts				Aqueous extracts			
	PCs	R^2	Training error, %	Testing error, %	PCs	R^2	Training error, %	Testing error, %
Polyphenols	6	0.992	1.53	6.50	4	0.995	3.17	15.8
Flavonoids	6	0.993	1.48	8.62	5	0.993	5.08	14.2
Antioxidant capacity	7	0.978	2.00	6.64	6	0.997	0.99	12.0

Correlation results and relative importance of original variables (the wavelengths) in the build-up of the regression model are presented in Figs. 6 and 7.

Figs. 6b and 7b show that the main contribution for establishing a correlation between the spectral data and polyphenols content are represented by wavelength around 220 nm, 250 nm, and 320 nm.

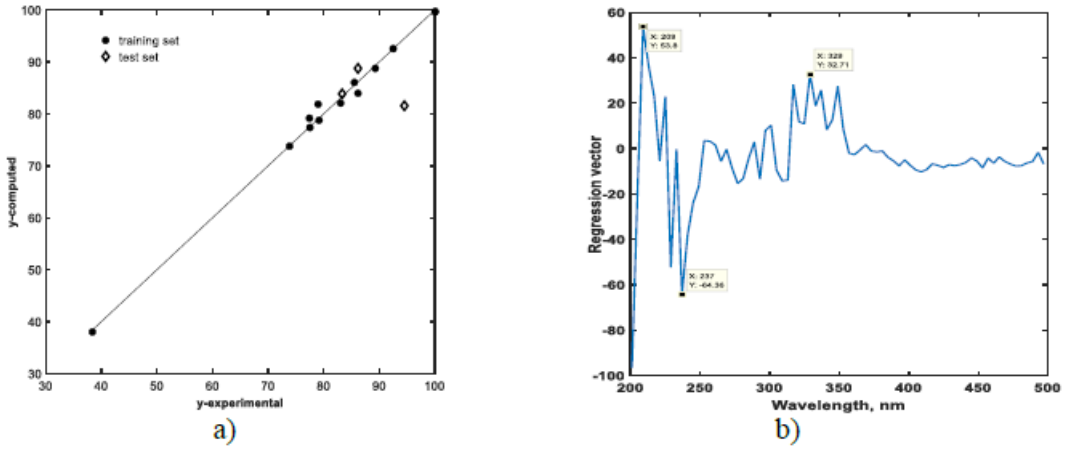


Fig. 6. Polyphenols PLS correlation results for ethanolic extracts a) correlation data, b) relative importance of wavelengths in the correlation model

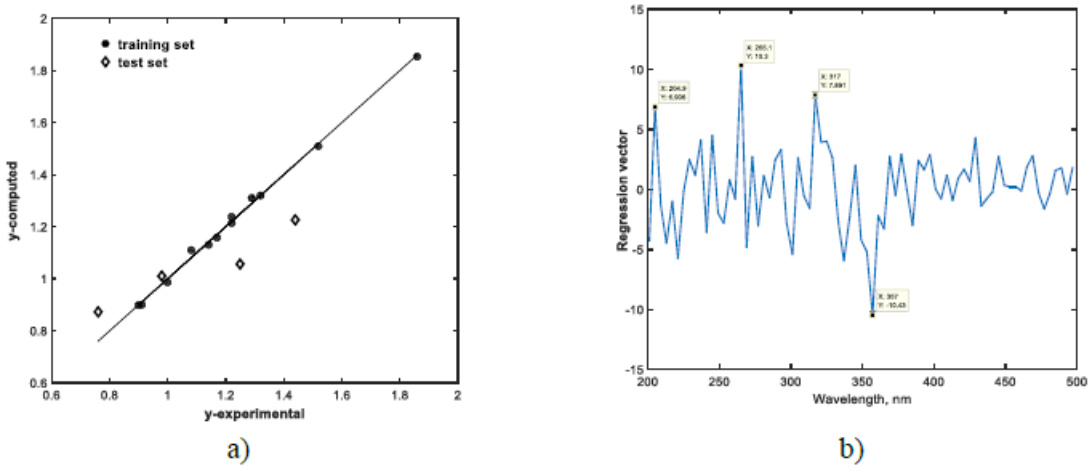


Fig. 7. Polyphenols PLS correlation results for aqueous extracts a) correlation data, b) relative importance of wavelengths in polyphenols correlation model

Chapter 3 – Polyphenolic profile effects upon the antioxidant effects upon the antioxidant and antimicrobial activity of propolis extracts [148]

Extraction procedures

Water and ethanolic solutions (25%, 50%, 70% w/w) were used for extraction. The liquid:solid ratios (w/w) were 2:1, 4:1, and 6:1. Phases were contacted at room temperature, 150 rpm for 1, 3, and 5 days, in an ES 80-Grant Instruments orbital shaker (UK). Extracts were separated from waxes with Filtrak No 389, Ø 12.5 cm filter paper, and stored at − 20 °C until analysis. The extracts coding system is an alpha-numerical combination with the letter representing the solvent (A— water, E—25%, EE—50%, EEE—70% ethanol), the first digit, the liquid:solid ratio (**2**:1, **4**:1 and **6**:1), while the second digit stands for the contact duration (**1** for 24 h, **3** for 72 h, **5** for 120 h).

Results and discussion

In the 36 propolis extracts prepared and analysed, 21 polyphenolics derivatives were quantified.

Data collected, with relative standard deviations below 5%, identified bioactive principles with average content lower than 100 μg/g, below 1 mg/g, and a major group exceeding 1 mg/g.

Phenolic acids representing on average 98.5% of the extracted compounds in water and are accompanied by 1.45% flavonoids, and 0.05% abscisic acid, the only terpenoid identified in this study. In 25% hydroalcoholic solutions, phenolic acids exceed 86%. In 50% ethanol, they represent less than 44% of the total, dropping to 40% in 70% ethanol.

The best extracted compound in water is p-coumaric acid (1.73 mg/g), while in 25% ethanol is ferulic acid (1.5 mg/g) (Fig. S3 and S4). The trend is less clear cut for 50% ethanol, while 70% ethanol extracts chrysin most in all tests (Fig. 2. and Fig. S5).

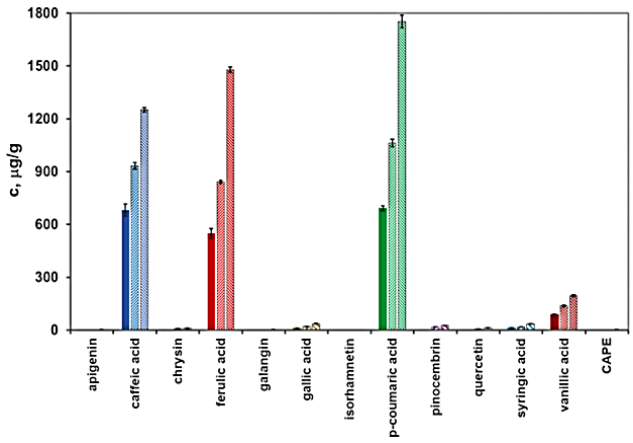


Figure S3. Polyphenolics composition pattern at different liquid : solid ratios in water (A25 – full colour, A45 – diagonal stripes upward, and A65 – diagonal stripes downward).

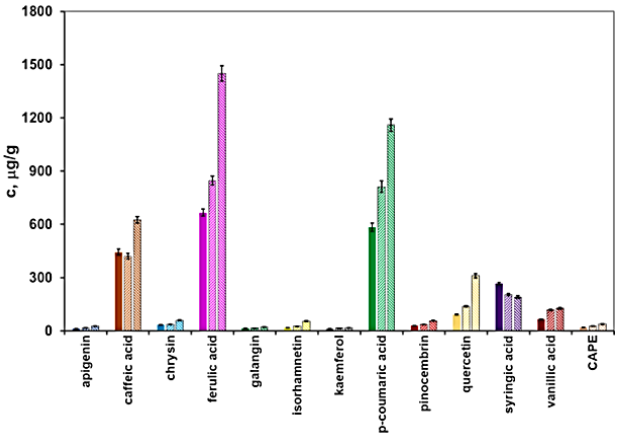


Figure S4. Polyphenolics composition pattern at different liquid : solid ratios in 25 % ethanol (E25 – full colour, E45 – diagonal stripes upward, and E65 – diagonal stripes downward).

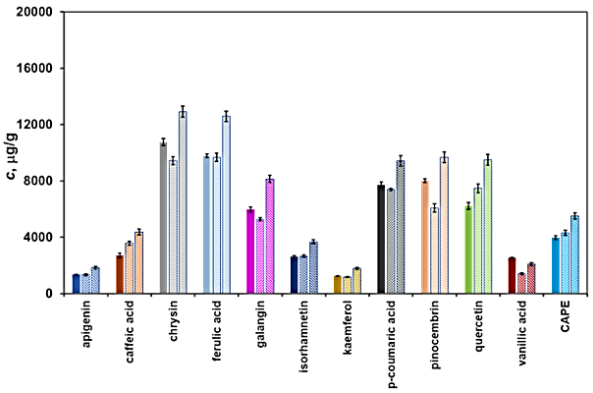


Figure 2. Polyphenolic derivatives composition pattern at different liquid : solid ratios in 50% ethanol (EE25– full colour, EE45–diagonal stripes upward, and EE65–diagonal stripes downward).

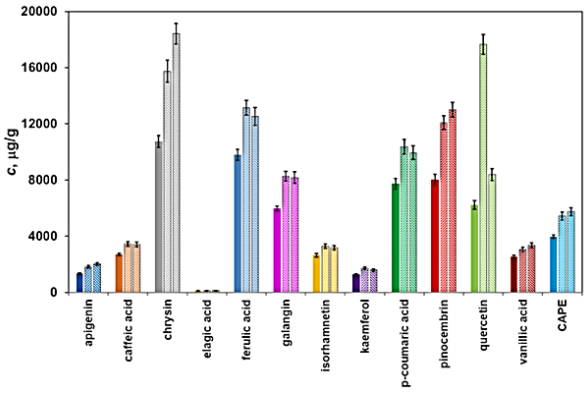


Figure S5. Polyphenolics composition pattern at different liquid : solid ratios in 70 % ethanol (EEE25 – full colour, EEE45 – diagonal stripes upward, and EEE65 – diagonal stripes downward).

Antioxidant effects variation

The registered antioxidant effects in aqueous type solvents, determined by ABTS quenching, are induced by acids and their esters, as flavonoids are present in negligible amounts (Fig. 3).

Larger volumes of alcohol offer the premises of extracting more flavonoids, along larger amounts of some phenolic acids. The large increase in the antioxidant effects is attributable to the flavonoids (Fig. 3b).

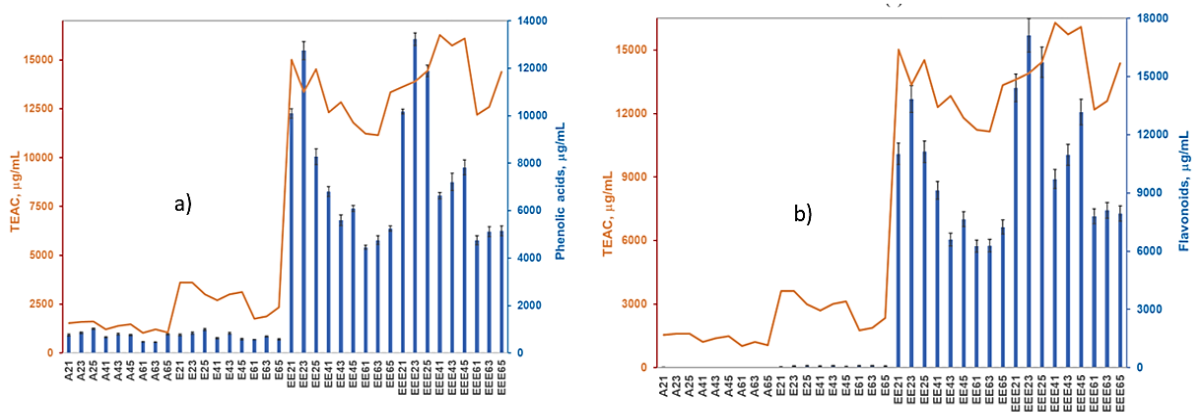


Figure 3. Antioxidant effects variation (ABTS assay) with phenolic acids (a) and flavonoids (b) distribution.

Statistical modelling was applied to investigate the correlation between the extracts' polyphenolics content and the antioxidant capacity. A saturation type model (Eq. 1) was used, to find out if there is a limiting polyphenolic acids and flavonoids concentration, cp, for which the antioxidant capacity, Qa, attains saturation:

$$Q_a = \frac{K_{\max} \cdot c_p}{K_c + c_p} \qquad (1)$$

where Q_a is the antioxidant capacity (TEAC, µg/mL), cp is the polyphenolic derivatives concentration (µg/mL).

The model parameters are K_{max}, (µg Trolox/mL), the extract antioxidant potential at theoretically very high cp values ($cp \rightarrow \infty$), and Kc (µg/mL), the critical concentration (c_p for

which Q_a is half K_{max}). The model parameters were identified minimizing the objective function (2):

$$F = \sum_{i=1}^{n} (Q_{a,\text{exp}} - Q_{a,\text{model}})^2 \quad (2)$$

where n being the number of samples considered.

The saturation model regression against the experimental data was performed for correlating the concentration of chemical compounds identified in each extraction solvent with the corresponding antioxidant capacity of the extract (Fig. 6).

The coefficient of determination, R^2, is 0.96 for K_{max} = 18,216 µg TEAC/mL extract, and K_c = 5632 µg polyphenolic derivatives/ mL extract. As the maximum antioxidant capacity registered experimentally is 16,287 µg TEAC/mL extract, the K_{max} value shows a possible increase of antioxidant capacity by increasing the polyphenolic derivatives content in the extracts.

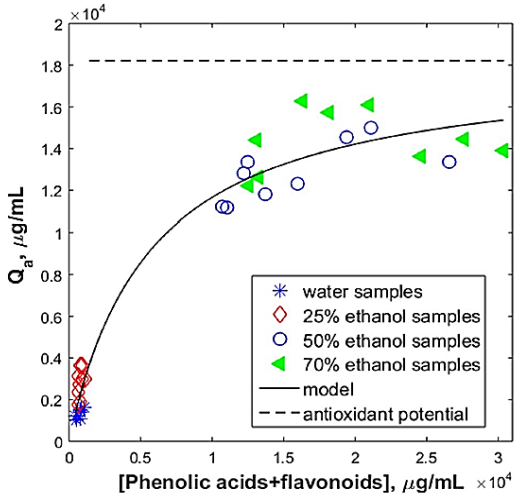


Figure 6. Antioxidant capacity as function of samples total polyphenolic derivatives content.

The antioxidant capacity increases with the growth of total phenolics concentration, with a steep increase in the 25–50% ethanol region.

The model slope decreases drastically for 50–70% ethanol, reflecting the experimental (values oscillating around 15,000 µg TEAC/mL). According to this model, the increase above 50% ethanol content in the extractant does not bring important changes in the antioxidant capacity.

Statistical analysis also correlated the individual concentration of polyphenols in the extracts with the corresponding antioxidant capacity. Therefore, the link between antioxidant capacity and polyphenolic composition, identifies chemical compounds synergistically involved in antioxidant capacity (Figures S6-S9).

Data correlation was performed by partial least squares (PLS), a combination of Principal Component Analysis (PCA) and regression.

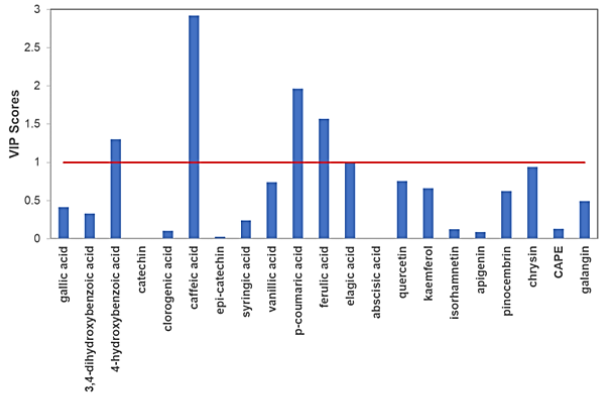


Figure S6. Chemical components with important contribution in the PLS model (VIP scores >1) for water extracts.

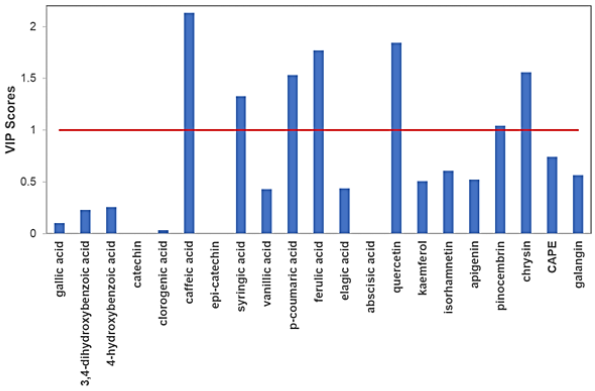


Figure S7. Chemical components with important contribution in the PLS model (VIP scores >1) for 25% ethanolic extracts

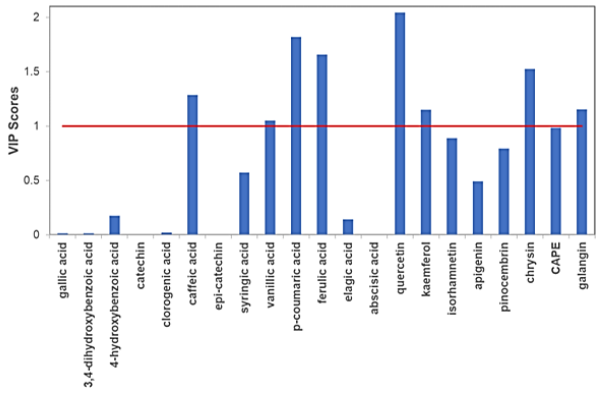


Figure S8. Chemical components with important contribution in the PLS model (VIP scores >1) for 50% ethanolic extracts

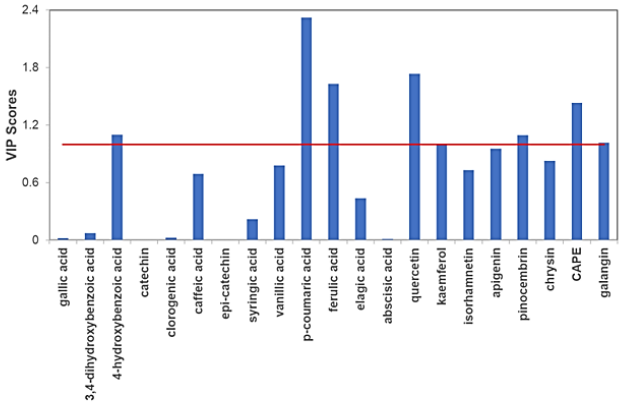


Figure S9. Chemical components with important contribution in the PLS model (VIP scores >1) 70% ethanolic extracts.

These components included, in decreasing order, the variability between samples in terms of polyphenolics composition and antioxidant capacity. The "variable influence on projection" known as "VIP" was used to identify the bioactive principles significantly influencing the antioxidant capacity variation.

A VIP score summarizes the contribution of each variable to the regression model. Variables with VIP values larger than 1 are generally considered relevant. The VIP score, defined for each variable (extracted bioactive principle), was calculated as a sum over its PLS components' loadings weighted by the percentage of explained Y variance by each PLS component.

Antimicrobial activity quantification

Table 2 presents the antimicrobial activity of the studied propolis extracts. Aqueous propolis extracts show a constant and substantial bactericidal activity against G −, but not so high against G +.

Aqueous extracts, containing mainly phenolic acids, proved to act on E. coli and C. Albicans. The extract with best antibacterial effect on E. coli is A65, with higher liquid:solid ratio during extraction.

The 50% ethanolic extracts contain, in addition to phenolic acids, large quantities of chrysin and galangin, flavonoids with already proven high antioxidant contribution.

The high flavonoids levels seem responsible for the better reaction towards B. subtilis. 50% ethanolic extracts showed the lowest inhibitory concentration for G − after 5 days, 768.5 µg/mL.

More ethanol in the extracting solvent (70%) does not bring further increase, neither in the antimicrobial, nor in the anti-fungal activity.

Extract	*E. coli*		*B. subtilis*		*C. albicans*	
	Inhibition zone, mm	MIC, µg/mL	Inhibition zone, mm	MIC, µg/mL	Inhibition zone, mm	MIC, µg/mL
A21	0.50[a]		1.00[a]		1.33 ± 0.47	
A41	1.00[a]	< 1663	*na*	4990	2.33 ± 0.94	< 832
A61	2.00[a]		*na*		2.67 ± 0.47	
A23	0.50[a]		0.67 ± 0.24		1.00 ± 0.82	
A43	1.00[a]	< 832	0.50[a]	2495	2.00 ± 0.82	< 832
A63	2.33 ± 0.47		*na*		1.33 ± 0.47	
A25	*na*		0.50[a]		2.01	
A45	1.00[a]	< 832	0.50[a]	< 1633	2.00 ± 1.00	< 832
A65	2.67 ± 0.47		0.508		2.67 ± 0.47	
E21	*na*		1.67 ± 0.47		*na*	
E41	*na*	1610	2.00[a]	< 805	*na*	1610
E61	1.33 ± 0.7		2.00[a]		1.67 ± 0.47	
E23	*na*		1.33 ± 0.47		na	
E43	*na*	1610	2.00[a]	< 805	0.33 ± 0.12	2415
E63	1.67 ± 0.47		2.67 ± 0.47		*na*	
E25	*na*		*na*		1.00 ± 0.94	
E45	*na*	1610	2.67 ± 0.47	1208	na	805
E65	1.00[a]		*na*		0.83 ± 0.5	
EE21	1.00[a]		2.00		*na*	
EE41	1.00[a]	< 1537	4.67 ± 0.47	< 768.5	1.00[a]	2305
EE61	1.33 ± 0.47		4.33 ± 0.47		1.00[a]	
EE23	1.00 ± 0.41		3.67 ± 1.25		0.67 ± 0.14	
EE43	1.00[a]	< 1537	1.67 ± 0.47	< 769	1.33 ± 0.47	769
EE63	1.67 ± 0.47		2.33 ± 1.25		1.33 ± 0.47	
EE25	*na*		2.33 ± 1.25		*na*	
EE45	2.00[a]	< 769	4.00[a]	< 76	1.67 ± 1.25	1153
EE65	1.33 ± 0.47		3.00 ± 0.82		*na*	
EEE21	0.06[a]		0.33 ± 0.27		*na*	
EEE41	1.94 ± 0.63	< 1440	2.78 ± 1.16	< 720	*na*	1440
EEE61	1.61 ± 0.68		3.22 ± 0.16		0.33 ± 0.02	
EEE23	*na*		2.00 ± 0.47		*na*	
EEE43	1.72 ± 0.72	< 720	4.67 ± 0.47	< 720	1.00 ± 0.16	1080
EEE63	3.39 ± 0.47		3.78 ± 0.42		1.22 ± 0.31	
EEE25	*na*		1.56 ± 0.31		*na*	
EEE45	1.50 ± 0.54	1080	3.33 ± 0.98	< 720	0.89 ± 0.16	< 2160
EEE65	1.83 ± 0.57		2.22 ± 0.16		0.78 ± 0.16	

Table 2. The antimicrobial activity of propolis extracts. *na* non active (no areas of inhibition reported), [a] identical replicates. *E. coli* Escherichia coli. *B. subtilis* Bacillus subtilis spizizenii nakamura. *C. albicans* Candida albicans.

Chapter 4 – Do ultrasonic field effects upon the polyphenolics profile of propolis extracts improve their antioxidant and antimicrobial activity? [87]

UAE procedure

Three types of solvents (water, 25 % (w/w) ethanol, and 50 % (w/w) ethanol), three liquid:solid ratios (2:1; 4:1; 6:1, w:w) and five extractions times (10, 20, 30, 40 and 100 min) were envisaged. 5 g of propolis were added to 43 glass jars over which 10 g solvent were added for the ratio of 2:1, 20 g of solvent for the ratio of 4:1, and 30 g for the last ratio. The jars were closed tightly with lids and all samples were placed in pairs in the ultrasonic bath for extraction, always at the same height above the transducers. The jars for 100 min and 10 min exposure times were placed first for each liquid: solid ratio, the last being replaced, in due time, with the jars for 20, 30 and 40 minutes extraction time. Samples were filtered through filter paper in sterile plastic containers with lids and stored in the freezer (-20◦C) until the analyzes were performed. The ultrasonic bath frequency was 40 kHz (Elma Transsonic, Germany), ultrasonic power was set to 110 W (100 %) and the water temperature (the coupling fluid) was maintained around the room ambient temperature, replacing it every other 10 min of sonication. The volume of water in the ultrasonic bath was always the same, 1300 mL.

Data processing

The PCA, PLS, and nonlinear regression over the experimental data to evaluate the saturation model parameters were carried out using an inhouse software written in Matlab® R2022a (MathWorks, Natick, MA,USA) programming environment. US field topology was computed
Acoustic physics from COMSOL Multiphysics® 5.2a (COMSOL, Inc., Burlington, MA, USA).

Results and discussion
Composition of extracts

Data in Table 1 demonstrate that even the longest extraction time (100 min) spent in an ultrasonic bath has not brought around the much-sought enhancement of aqueous extractions compared to maceration. The main gain, apart from reducing the operating time, was the presence of around 10 % flavonoids in a solvent which, normally, solubilizes very slowly such structures.

The 50% ethanol solution improved the extraction yield in the ultrasonic field compared to maceration.

Table 1
Process efficiency for different extraction techniques applied to propolis.

Solvent	Extraction yield, %					
	5 days maceration [38]			US 100 min		
	2:1	4:1	6:1	2:1	4:1	6:1
water	0.206	0.306	0.485	0.010	0.076	0.117
25 % ethanol	0.228	0.273	0.416	0.382	0.362	0.487
50 % ethanol	4.25	6.01	8.20	12.38	24.67	23.20

Figure S2 show that caffeic, ferulic, and p-coumaric acids as best extracted compounds in water, at room temperature (Fig. S2).

US treatment allowed flavonoids to solubilize even in aqueous extracts. Pinocembrin was leading, at a 14.6 µg/g average, accompanied by isorhamnetin, 9.6 µg/g, and small amounts of chrysin, 4.6 µg/g in 6UA10 sample.

Ellagic acid was the top acid extracted in 50 % ethanol, 38.6 mg/g. Resveratrol and rutin were extracted at approximately 18 mg/g. Caffeic acid did not exceed the values extracted in 25 % ethanol (0.8 mg/g), but its phenyl-ester derivative, CAPE, increased significantly (24 mg/g in 6UEE100).

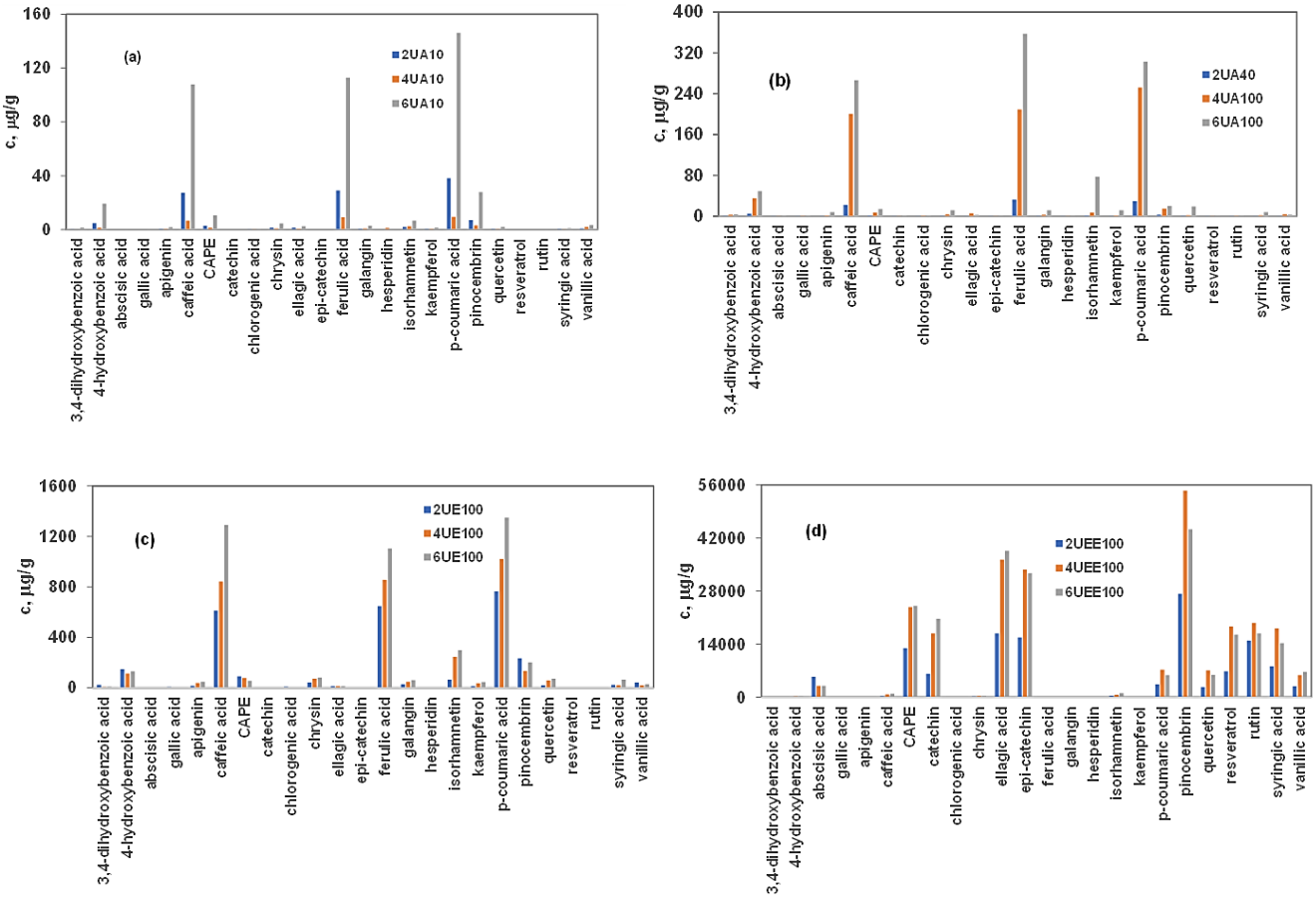


Figure S2. Polyphenolics profiles of (a - b) aqueous, (c) 25%, and (d) 50% ethanolic extracts, in US field, and different exposure times.

The US field topology (Fig. 2.) was heavily influenced by both the physical properties of the fluid in which the former developed and its level in the vessel and that was a contribution to getting different polyphenolics profiles for different concentrations of hydroalcoholic solution.

For the same characteristics of the US parameters (amplitude of the transducer and the input power), the knots and venters distribution will be different for different fluids subjected to the US in the same ultrasonic bath, keeping the coupling liquid the same (Fig. 2).

The density (for the energy needed to periodically move the liquid mass), the viscosity (for the internal heat dissipation of the mechanical energy and the cavitation phenomena) and

the interfacial tension (for the cavitation phenomena) are the main properties which will dictate the topology of knots and venters, together with the field distribution in between.

Two fluids were used to illustrate this, 50 % ethanol (density 900 kg/m^3, viscosity 1.05 mPa·s, interfacial tension 21.8 mN/m, Fig. 2a, b, and e) and water (density 1000 kg/m^3, viscosity 1 mPa·s, interfacial tension 72 mN/m, Fig. 2c, d, and f), while water was chosen as coupling liquid in the ultrasonic bath. The US field distribution was computed for the aforementioned intensity and frequency, the beakers being filled with the amounts corresponding to the highest ratio, 6:1, and to the lowest one, 2:1. The computations were done using Acoustic physics from COMSOL Multiphysics® 5.2a (the chosen fluid model was linear elastic), the ultrasonic bath geometry being implemented using COMSOL geometry primitives.

Fig. 2a-d clearly shows that the computed distribution field is completely different for the two fluids present in the beaker, which must reflect in the interactions between the US field and the solid phase – therefore, when explaining the differences between the performance of different solvents subjected to an US field, keeping the operating conditions the same, the field distribution should be accounted for, as a hidden, but powerful cause. The US field topology will change, also, when the level of the extractants in the beaker (or any other kind of vessels) changes, even if the height above the transducer is kept the same (Fig. 2e and f, against Fig. 2a and d).

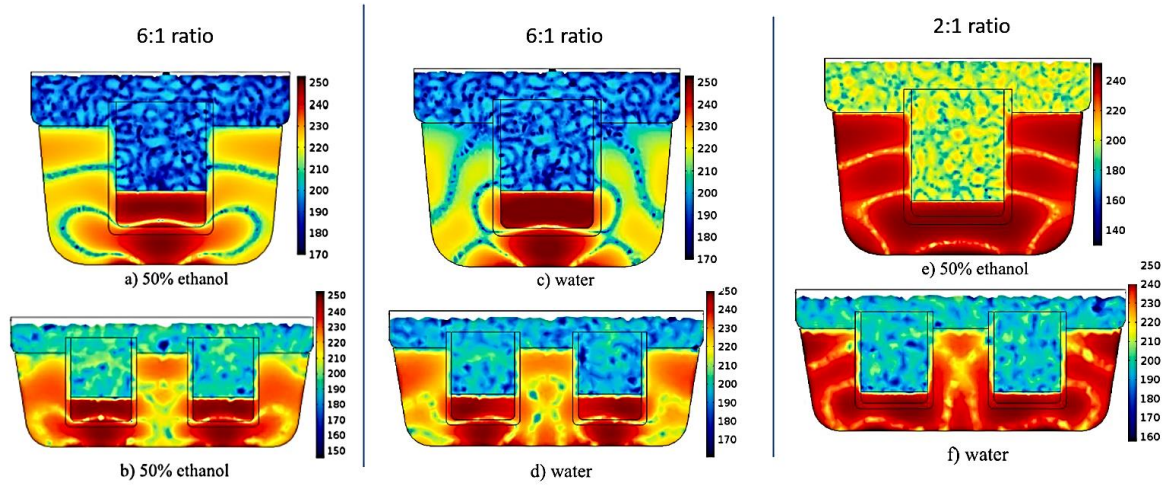


Fig. 2. US field distribution in cross sections YZ, passing through the center of one of the transducers (**a**, **c,** and **e**) and XZ, passing through the center of the US bath (**b**, **d,** and **f**). 50% ethanol (**a** and **b**) and water (**c** and **d**) are in beakers, for the 6:1 ratio case, while 50 % ethanol (**e**) and water (**f**) are in beakers, for the 2:1 ratio case, water being the coupling liquid. The

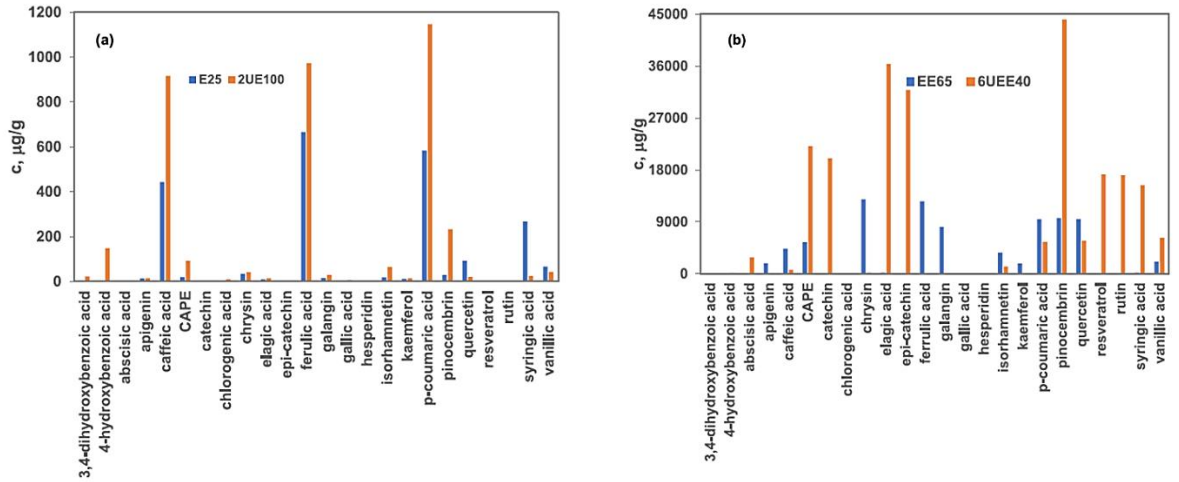


Fig. 3. Polyphenolics profile extracts variation with ethanol level and liquid:solid ratio for: (a) 25 % ethanol, 2:1 liquid:solid ratio, 5 day-maceration (E25) and 100 min in ultrasonic bath (2UE100); (b) 50 % ethanol extracts, 6:1 liquid:solid ratio 5 day-maceration (EE65) and 40 min in ultrasonic bath (6UEE40).

Antioxidant capacity of aqueous US extracts represented approximately 30 % of the values obtained after 5-day maceration at any liquid:solid ratio. 25 % ethanolic extracts presented rather similar antioxidant capacities, either by maceration or by 100 min US field exposure (Fig. 4). The extraction time reduction was the major plus. The three-times increase in the extracted antioxidants was closely followed by the TEAC values of the 50 % ethanolic extracts.

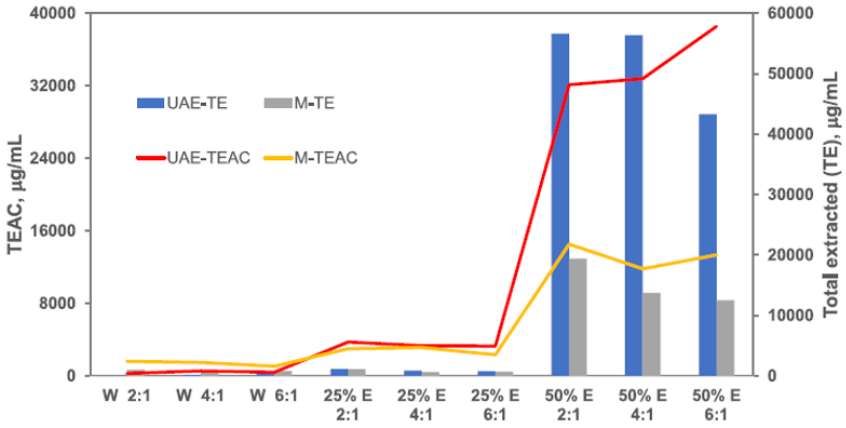


Fig. 4. Variation of antioxidant capacity and total extracted compounds with the experimental conditions in UAE for 100 min and 5-day maceration at similar liquid:solid ratios (2:1, 4:1, 6:1, w:w) in water (W), 25 % and 50 % ethanol as solvents. Maceration data were previously reported [38]

The UAE samples representation in PC1-PC2 coordinates revealed a clear grouping tendency according to the solvent used (Fig. 5). Samples did not differentiate by liquid:solid ratio and UAE time for water extracts. Separation is slightly higher for 25 % ethanolic extractant, but, still, all samples part the same ellipsis. Water extract obtained at 6:1 liquid:solid ratio and US maximum time (6UA100) overlaps with the 25 % ethanol samples, mainly with 2:1 liquid:solid ratio (2UE100), suggesting a similar polyphenolics profile. This might stamp 6UA100 sample as outlier.

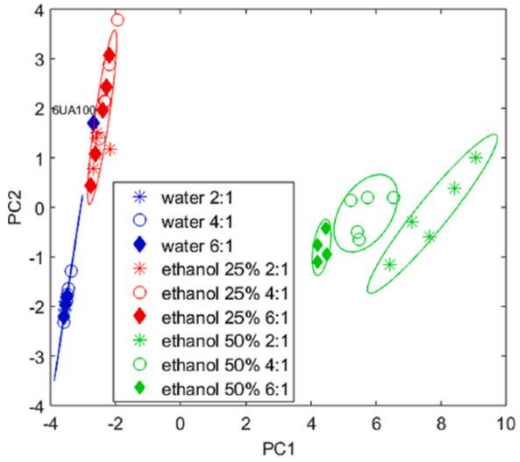


Fig. 5. Projections in PCA coordinates of samples obtained in UAE.

PCA applied for both maceration and UAE samples (PC1 59.2 %, PC2 33.2 %) lead to a clear separation of 50 % ethanolic samples obtained in US field and maceration (Fig. 7a).

Water and 25 % ethanol extracts practically overlap (Fig. 7b), proving that US field did not essentially change the polyphenolics profile.

PCA demonstrates that the solvent nature is the major factor differentiating the polyphenolic profile, while the US field and liquid:solid ratio play an important role for 50 % ethanol, where polyphenolic derivatives with complex structure (mainly flavonoids) are extracted.

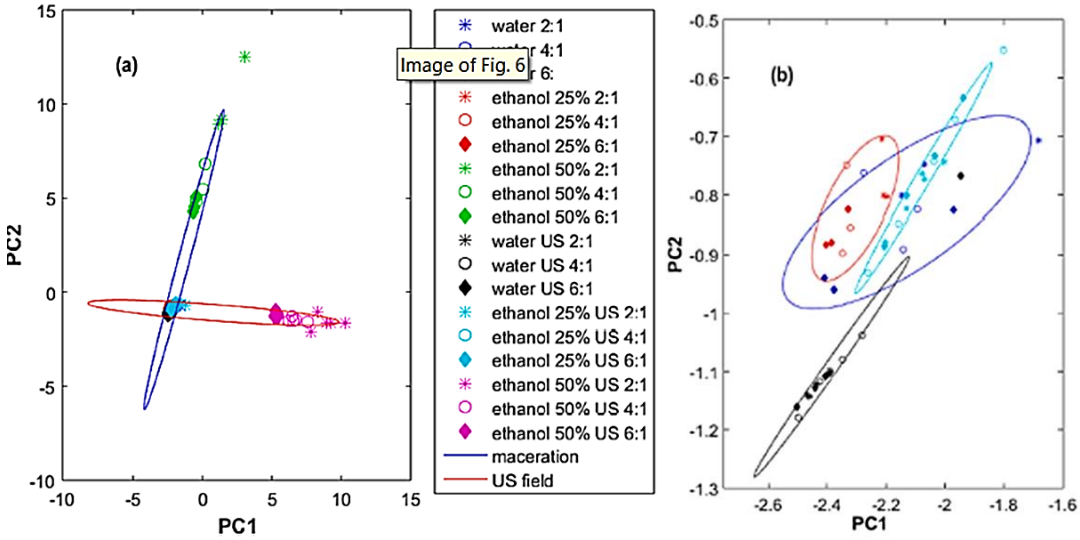


Fig. 7. Projections in PC1-PC2 coordinates of samples obtained using both maceration [38] and US extraction results: (a) grouping along maceration vs US field; (b) close-up for the projections in PC1-PC2 coordinates of water and 25 % ethanol samples.

The maximum antioxidant capacity predicted by the saturation model (Fig. 8.) is quite close to the experimental data obtained for 50 % ethanolic extracts. Generally, data fit well the proposed model, but there is an unexpected pattern, better noticed for 50 % ethanol samples, at 2:1 and 4:1 liquid: solid ratios (Fig. 8): there are samples with higher polyphenolics content having similar or lower antioxidant capacity. At 6:1 liquid:solid ratio the data follow the general rule of increasing antioxidant capacity with increased polyphenolics concentration.

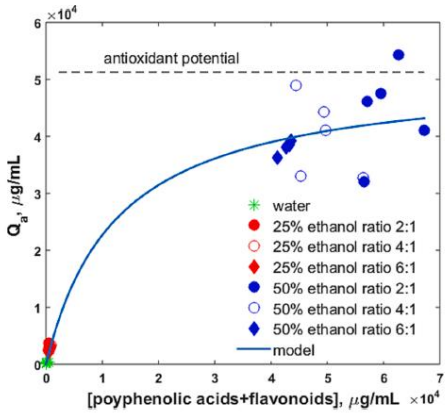


Fig. 8. Antioxidant capacity variation with total polyphenolics concentration.

The 2:1 and 4:1 liquid:solid ratios profiles vary with the US field duration, showing that the concentration of certain polyphenolics is not steadily increasing, possibly due to chemical

reactions caused by free radicals. The 6:1 liquid:solid ratio concentrations profile in Fig. 9 is almost constant in time.

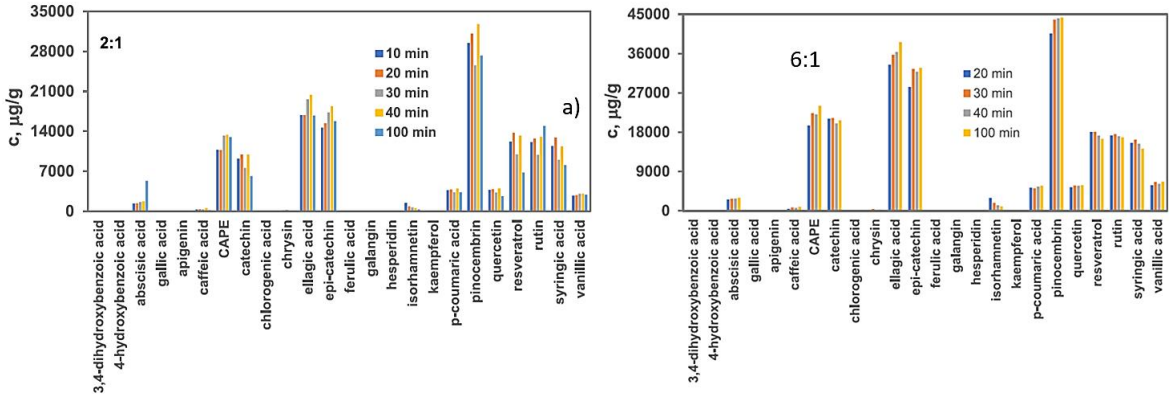


Fig. 9. Polyphenolic derivatives profiles for 2:1 and 6:1 liquid:solid ratio in 50 % ethanol.

Antimicrobial activity

The US exposure time influence upon the antibacterial activity against E. coli increased for 2:1 ratio, while for 4:1 and 6:1, higher value for inhibition zones were recorded for 10 and 40 min, respectively.

Gram-positive bacteria (*B. subtilis*) were most inhibited by the 2:1, liquid:solid ratio and moderate exposure time to US extract (2UA20). The second-best inhibitory effects had 6UA100 extract, thus showing that the change in the US field topology was beneficial for the longest extraction time. Aqueous extract 2UA100 had the maximum inhibitory effect upon Gram-negative bacteria *E. coli*.

The antimicrobial activity for propolis extracts with 25 % ethanol is moderate. The most efficient extract for *E. coli* is 4UE10.

Propolis extracts in 50 % ethanol in the presence of US are the most effective in terms of antimicrobial activity, maximum inhibition being provided by 2UEE20 extract against *B. subtilis* and by 2UEE100 against *E. coli.*

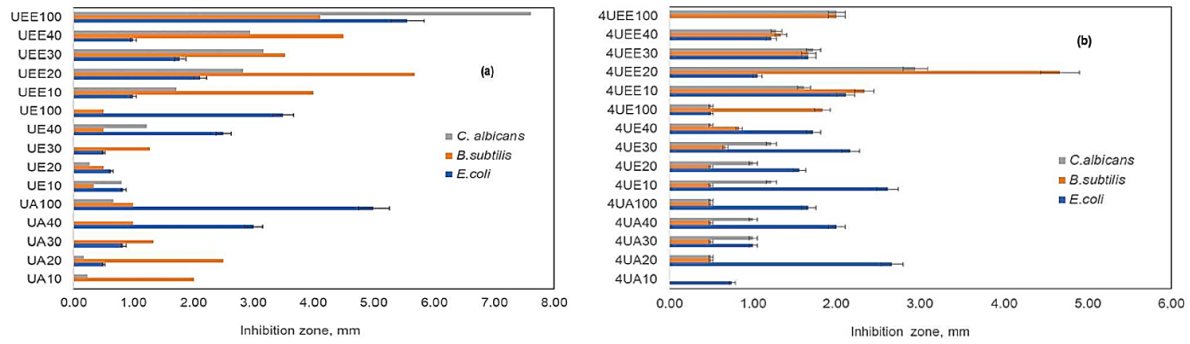


Fig. 10. The antimicrobial activity recorded after 24 h in UAE extracts obtained at different liquid:solid ratios: (a) 2:1 and (b) 4:1.

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General conclusions

1. Experiments and data processing lead to the conclusion that the extracts polyphenolic profile is important for determining antioxidant and antimicrobial capacity.

2. The profile variation is given, first of all, by the solvent nature (50% ethanol has proven to be the best option), then by the solid/liquid ratio, and, to a very small extent, by the particle size.

3. Smaller liquid/solid ratios than those mentioned in literature proved to be efficient in terms of extraction yield and extraction time, thus ensuring lower extractant consumption and avoiding further concentration steps of the final product.

4. Water, as extraction solvent, did not prove to be favorable for the extraction of polyphenols with low solubility in water, and likewise 25% ethanol, whose composition does not favor the extraction of all compounds.

5. The use of the ultrasound field leads to the increase of the extraction efficiency, but also determines a significant polyphenolic profile modification, leading to changes in the antioxidant and antimicrobial capacity.

6. The solid/liquid ratio influences the polyphenolic profile in the case of ultrasonic field extraction. For low solid/liquid ratios, the cavitation process occurring at the liquid surface produces changes in the polyphenolic profile due to the appearance of hydroxyl radicals, HO•, which are more likely to be generated when small amounts of solvent are used.

7. The HPLC-MS analysis proved to be effective in quantifing the extracts composition, allowing, through appropriate statistical methods the analysis of the extracts differentiation and the profile influence on the investigated properties (antioxidant capacity and antimicrobial activity).

8. The of UV-VIS spectra analysis, a cheap and straightforward approach compared to chromatographic and more sophisticated techniques, allows prediction of the antioxidant capacity, considering that the spectrum varies according to the composition of the extract obtained under various conditions (different types of solvent and granulometry).

9. The best operating conditions in terms of producing extracts with a high antioxidant and antimicrobial activity required ultrasounds as intesifying technique and 50% ethanol as solvent, proving that a further increase in the ethnol content does not bring significant improvements in the properties of the extracts.

Original contributions

1. The main original contribution of this thesis is highlighting the influence of *polyphenolic profiles* obtained in various operating conditions upon the *antioxidant capacity and antimicrobial activity* of extracts.The influence of the profile on the properties of the extracts could be attributed to synergistic actions of the polyphenols extracted differently depending on the processing ways.

2. The choice of operating conditions for extraction by maceration had in view the practical fesability of the proces and economically resonable conditions. A temperature of 25°C was chosen and durations up to 5 days. The study proved that more than 50% ethanolic concentration in the solvent and duration over 3 days bring no significant improvements.

3. Isothermic condition, arround 25 °C, were used in US field extraction. Thus the effect of temperature increase due to US was avoided. In an isothermal process, it was possible to emphasize the role of US field on the modification of the polyphenolic profile along with the considerable increase in the antioxidant and antimicrobial capacity.

4. Concerning the most efficient propolis particle sizes for the efficiency of extraction, in terms of total polyphenolic compounds and antioxidant capacity, a range of 200-600 μm was identified which can be easely obtained.

5. The use of low liquid/solid ratios aiming to ensure low extractant consumption and avoid further concentration of extracts.

6. The influence of solvent type and liquid/solid ration upon polyphenolic profile in ultrasound filed extraction was put into evidence by modelling the US field topology. The fluid physical properties, but also the liquid level in the vessel, proved to modify the knots and venters distributions. The US field topology proved to influence the polyphenols profile. At low liquid level (small liquid/solid ratios) the cavitation phenomenon and free radicals that are formed at solid-liquid interface may produce modifications in the concentration distribution of polyphenolic componds.

7. The investigation of polyphenolic profile during US extraction put into evidence the different evolution of each component concentration. Some compounds increase in concentartion while others decrease, which may be explained by the formation of free radicals induced by the US field that interact with some compounds present in the extract modifying the polyphenolic profile. The influence of polyphenolic profile upon the antioxidant capacity is clearly evidentiated for the extraction in US field at 2/1 solid liquid ratio using 50% ethanolic solution where, with increased extraction duration the total polyphenolic compounds concentration increases slightly, but the antioxidant capacity varies strongly, and has higher values for lower total concentrations.

8. The modeling methods proposed and validated, based on multivariate statistics (PCA and PLS) and optimization were capable to correlate the antioxidant capacity with total polyphenol content and with the polyphenolic profile.

9. Using PCA the clear separation of extracts according to solvent used was put into evidence; a better separation is revealed in US filed than in maceration profing that the solvent and US contributes to the modification of polyphenolic profiles. The PCA study also schowed distinct classes according to liquid/solid ratio for extracts obtained in US field in 5% ethanolic solvent as a consequance of the modification of the US fiels topology.

10. The original saturation type model proposed to correlate the antioxidant capacity with total polyphenolic acids and floavonois content put into evidence the steep increase of antioxidant capacity for ethanolic content increases from 25% to 50%. A further increase in ethanol concentration does not lead to a significant increase in the antioxidant capacity.

11. The correlation between the composition of extracts and antioxidant capacity, performed by PLS allowed to evidence the realtive importance of each compound in the buid up of antioxidant capacity. Some compounds have greater influence in predicting the antioxidant capacity. For 50% ethanolic extracts the greater contribution have some poliphenolic acids (ferulic, cafeic and p-cumaric) and flavonoids, such as chrysine, kaempferol, galangin and quercetine for maceration extraction and syringic acid, p-coumaric acid, ellagic acid, catehin, epicathehin, isorhamnetin and resveratrol in US field.

12. Antimicrobial activity of extracts is influenced by polyphenolic profile. The 50% ethanolic extract obtained in US field prooved to have the highest antimicrobial activity explened by the contribution of pinocembrin, epicatechin, resveratrol and syringic acid that are in hight concentration.

13. The change in the topology of the ultrasound field have an important influence on the profile of polyphenolic compounds of the aqueous extracts which, compared to that obtained by maceration, had an increased antimicrobial activity against *E. coli* and *B. subtilis bacteria*. Some of the aqueous samples have inhibitory activity close to that of the ethanolic samples (25% and 50%) obtained by US, and others even higher. This is mainly attributed to the differences in the concentration profile of antimicrobial compounds in all samples subjected to ultasound extraction which vary according to the type of solvent and the its volum in the baker.

14. A rapid method for estimating polyphenol content and antioxidant capacity using easily available experimentally UV spectra of extracts was developped.

Dissemination of results

Published works

1) Nichitoi, M. M.; Josceanu, A. M.; Isopescu, R. D.; Isopencu, G.; Lavric V. "Romanian propolis extracts: characterization and statistical analysis and modelling", U.P.B. Sci. Bull., Series B, **2019**, 81(4), 149-162, WOS:000501994100014
2) Nichitoi, M. M.; Costache, T. A.; Josceanu, A. M.; Isopescu, R. D.; Isopencu, G.; Lavric, V. "Development and Application of an LC-MS/MS Method for Identification of Polyphenols in Propolis Extract", *Proceedings*, vol. 55, no. 10, pp. 1-5, 2020, doi: 10.3390/proceedings2020055010.
3) Nichitoi, M.M; Josceanu, A. M.; Isopescu, R. D.; Geană, E. I.; Ciucure, C.T.; Lavric, V. "Polyphenolics profile effects upon the antioxidant and antimicrobial activity of propolis extracts", Sci. Rep. vol. 11, no. 20113, 2021, https://doi.org/10.1038/s41598-021-97130-9, WOS:000706395800043, Impact Factor: 4.996
4) Nichitoi, M. M.; Josceanu, A.M.; Isopescu, R.D.; Isopencu, G.O.; Geană, E.I.; Ciucure, C. T.; Lavric, V., "Do ultrasonic field effects upon the polyphenolics profile of propolis extracts improve their antioxidant and antimicrobial activity?", Ultrason. Sonochem., vol. 92, 2023, doi: 10.1016/j.ultsonch.2022.106274, WOS: 000911315500001, Impact Factor: 9.336

Conferences

1) Nichitoi, M. M.; Josceanu, A. M.; Isopescu, R. D.; Isopencu, G.; Lavric V. "Romanian Propolis Evaluation", 4th International Conference on Analytical Chemistry 1-3 September 2018, Bucharest, Romania.
2) Nichitoi, M. M.; Josceanu, A. M.; Isopescu, R. D.; Isopencu, G. O.; Lavric V. "Romanian propolis extracts - Characterization and statistical analysis and modelling",

21st Romanian International Conference on Chemistry and Chemical Engineering 4-7 September 2019, Mamaia-Constanta, Romania.

3) Nichitoi, M. M.; Costache, T. A.; Josceanu, A. M.; Isopescu, R. D.; Isopencu, G.; Lavric V. "Development and application of a LC-MS/MS method for identification of polyphenols in propolis extract", Eurachem Online Workshop - Quality Assurance Elements for Analytical Laboratories in the University Curriculum, 14-15 July 2020, Bucharest, Romania.
4) Nichitoi, M. M.; Josceanu, A. M.; Isopescu, R. D.; Costache, T. A.; Lavric V. "Advanced analysis in the extraction of polyphenolic compounds from Romanian propolis", 22nd Romanian International Conference on chemistry and Chemical Engeneering, 7-8 Sept., 2022, Sinaia, Romania.

The present summary includes in a concise form the content of chapters 1-4.
The numbering of chapters, subchapters and tables corresponds to that in the thesis.
The significant bibliographic references used in the work are presented.

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