BIOPRODUSE INGERABILE PE BAZĂ DE COLAGEN HIDROLIZAT PENTRU TRATAREA AFECȚIUNILOR GASTRICE
COLLAGEN HYDROLYSATE-BASED INGESTIBLE BIOPRODUCTS FOR THE TREATMENT OF GASTRIC DISORDERS

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Gastric ulcer disease is a common problem of the gastro-intestinal tract with its increasing incidence and prevalence attributed to the loss of balance between aggressive and protective factors. The purpose of this study was to obtain and characterize biomaterials for the regeneration of the soft tissue of the digestive system. Powders with different concentrations of collagen hydrolysate, zeolite and propolis were obtained by lyophilization. The type I collagen hydrolysate was obtained by acidic hydrolysis. A stable DPPH radical was used for determination of the free radical scavenging activity of the propolis. The morphology (Scanning Electron Microscopy – SEM), the spectral characteristics (Fourier Transform Infrared Spectroscopy – FT-IR, X ray diffraction – XRD), the goniometry (contact angle), in vitro assessment to simulated gastric acid and the biocompatibility (XTT assay) of the designed powders were studied. The collagen hydrolysate, zeolite and propolis biomaterials exhibited characteristics that make them potential candidates for use in the treatment of gastric ulcer.

Ulcerul gastric este o afecțiune comună a tractului gastro-intestinal, ale cărei incidență și prevalență ridicate sunt atribuite dezechilibrului dintre factorii agresivi și defensivi. Scopul acestei lucrări a fost obținerea și caracterizarea unor biomateriale pentru promovarea regenerării țesutului moale al sistemului digestiv. Pulberi cu diferite concentrații de colagen hidrolizat, zeolit și propolis au fost obținute prin procesul de liofilizare. Colagenul hidrolizat de tip I folosit a fost obținut prin hidroliză acidă. Pentru determinarea activității antioxidante a propolisului a fost utilizat radicalul stabil DPPH. Probelor obținute le-au fost analizate morfologia (Microscopie Electronică de Baleiaj – SEM), caracteristicile spectrale (Spectroscopie în Infraroșu cu Transformată Fourier – FT-IR, difracție de raze X – XRD), goniometria (unghi de contact), stabilitatea in vitro sub acțiunea sucului gastric simulat și biocompatibilitatea (Test – XTT). Biomaterialele pe bază de colagen hidrolizat, zeolit și propolis studiate prezintă caracteristici care le recomandă ca potențiali candidați în tratamentul ulcerului gastric.

Keywords: *Collagen hydrolysate, zeolite, propolis, gastric ulcer*

1. Introduction

Gastric ulcer disease is a common problem of the gastro-intestinal tract, with its increasing incidence and prevalence attributed to the loss of balance between aggressive factors (pepsin, hydrochloric acid and bile acids) and defensive factors (prostaglandin, mucus, bicarbonate and blood flow) [1]. The most common cause of the gastric ulcer is *Helicobacter pylori* infection [2]. Approximately 10% of the adult population suffers or has suffered from gastric ulcer caused by infection with this germ [3]. Infection with *Helicobacter pylori* occurs via fecal - oral or oral – oral route, especially in poorly developed countries (at the age of 20 approximately 70% of people are infected) and later in developed countries (at the same age only 15-20 % are infected) [4].

An alternative to treating this disease, to the detriment of current drugs, is the use of collagen-based biomaterials. Throughout history, biomaterials have played an important role in the treatment of diseases and the improvement of health care [5]. Natural biomaterials have been successfully used in the past for the treatment of gastric ulcer [6].

Collagen is a basic protein from conjunctive tissues: skin, bone, tendons, ligaments, basement membranes etc [7]. It is one of the most used biomaterial due to its excellent biocompatibility,

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biodegradability and weak antigenicity, well established structure, biologic characteristics and to the way it interacts with the body, being known by the body as one of its constituents and not as an unknown material [8].

The denatured collagen – collagen hydrolysate – plays an important role in the management of gastric ulcer, bone, joint disorders and osteoarthritis, having properties and characteristics such as very low viscosity in aqueous solutions, neutral odor, solubility, dispersibility, low allergenicity, being a bioavailable source of peptides and aminoacids [9].

On the last decade, zeolites have enjoyed considerable attention, due to the good performance of this material in ion exchange, adsorptive and biocatalytic processes, together with their high chemical stability. Zeolites are aluminosilicates with microporous structures of Si- and Al-tetrahedrons (SiO_4, AlO_4), linked through the common oxygen atoms to form an open crystal structure [10, 11]. Zeolites are materials often used in the pharmaceutical industry as antiacids due to the high content of metallic oxides such as Aluminum and Magnesium [12]. Other authors have shown zeolites are non-toxic materials, controlled release systems and adjuvants for drug, thus showing their potential as biomaterials [11].

Propolis is a resinous material collected by bees from exudates and buds of plants and mixed with wax and bee enzymes [13]. In general, it is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various others substances [14]. Several authors reported its biological activities such as antiulcer, anti-inflammatory, antimutagenic and antibacterial properties [15-19].

For the treatment of gastric ulcer, drugs are preferred in the form of capsules, powders and solutions. Treatments are focused on recovering damaged tissues and restoring lost functions [20]. Formulas in the form of capsules and powders are indicated for the treatment of gastric ulcer because they are easy to administer and are quickly assimilated by the body. These products may contain antacids, antisecretory, protective gastric mucosa and antibiotics [21].

Thus, the purpose of this study was to obtain and characterize some powder biomaterials based on collagen hydrolysate, natural zeolite and propolis for the regeneration of the soft tissue of the digestive system.

2. Experimental part

2.1. Materials

Type I collagen hydrolysate with an average molecular weight of about 6,000 Da was obtained by acidic hydrolysis as we previously described [9,22]. Propolis-having a specific gravity of 1.112-1.136 g/cm^3 and a melting point of between 70-120 °C - was a Romanian commercial product and zeolite - having the following constituents: SiO_2 α quartz, SiO_2 α cristobalite, $3Al_2O_32SiO_2$ mullite and Ca, Fe, K, Mg-type chlorite aluminosilicates- was provided by a commercial company from Turkey.

2.2. Powders synthesis

The powder bioproducts for the regeneration of the soft tissue of the digestive system were prepared with active ingredients such as collagen hydrolysate, zeolite and propolis in water (up to 100 %) forming suspensions according to the composition shown in Table 1. In order to obtain stable bioproducts, the suspensions were lyophilized: the water of suspensions was frozen and then with drawn under vacuum and dried powders were obtained.

Table 1

Composition of samples/*Compoziția probelor*

Samples	Collagen hydrolysate %	Zeolite %	Propolis %
P 1	5	5	0.5
P 2	5	5	1
P 3	5	10	0.5
P 4	5	10	1
P 5	10	5	0.5
P 6	10	5	1

The powders obtained by the lyophilization of resulting suspensions were then characterized.

2.3. Characterization methods

Antioxidant activity of propolis. The antioxidant activity of propolis was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent assay. Absorbance measurement was performed using the Jasco UV-VIS ISV-469 Spectrophotometer. Seven samples were prepared in brown vials to determine the ability to inhibit free radicals (Table 2).

Table 2

Samples composition of propolis for DPPH test/ *Compoziția soluțiilor de propolis pentru testul DPPH.*

Sample	Oil vol., mL	Alcohol vol., mL	DPPH vol., mL
Blank	0	1.9	0.1
a	0.1	1.8	0.1
b	0.3	1.6	0.1
c	0.5	1.4	0.1
d	0.7	1.2	0.1
e	0.9	1	0.1
f	1	0.9	0.1

Immediately after the addition of the DPPH reagent (0.05%), the vials were closed, homogenized and kept in the dark for 30 minutes, after which the absorbance at 518 nm was measured against ethyl alcohol as a control. Quartz cuvettes with a 1 cm layer thickness were used. All samples had the same volume of 2 mL.

The A_{518} = f (concentration of propolis) function is constructed as shown in Figure 1. The EC50 parameter representing the analyte concentration that reduces the absorbance of the DPPH reagent by 50% is plotted. The lower EC50 a compound has, the greater the inhibitory capacity against free radicals.

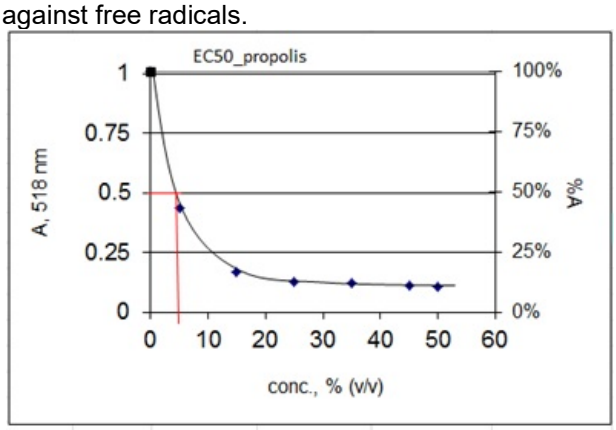

Fig. 1 - Graph to determine EC50/*Grafic pentru determinarea parametrului EC50.*

X-Ray Diffraction. XRD data were acquired using a PANalytical X'Pert PRO MRD instrument (PANalytical, Almelo, the Netherlands) working with Cu*K*α radiation (λ = 1.5418 Å) within the 2Theta range of 5 – 60°.

SEM Characterization. SEM analyses were performed on a FEI, Inspect F50 electron microscope equipped with a Schottky Field Emission source and an EDS detector, in primary electron beam, on samples covered with a thin gold layer.

FTIR-ATR Analysis. FT-IR spectral measurements were recorded by a Jasco FT/IR - 4200spectrophotometer. All the spectra were recorded at the following parameters: spectral range 4000 - 600 cm^{-1}, resolution 4 cm^{-1} with 30 acquisitions per each sample.

Evaluation of powders wettability (contact angle). The wetting behaviour of dried powders was determined by contact angle measurement at room temperature, using a KSV CAM 101 Scientific Instrument, as previously described [23]. Briefly, the measurement of the contact angle was carried out by placing the tested powder on a glass microscope slide covered by a double sided adhesive tape and the distilled water was dispensed with a Hamilton syringe. The pendant drop dynamic method was used and the Young-Laplace equation was applied to mathematically describe the drop shape. For each sample at least six contact angle measurements were performed, and the average value was considered.

***In vitro* assessments.** The samples were exposed to simulated gastric acid (HCl, pH=1.5) as well as in phosphate buffer saline solution (PBS, pH=7.2) for 3 days and the solutions were analysed each day by ICPMS (Agilent, 8800 Triple Quadrupole) in order to evaluate the released ions. For this reason, 0.5 g of each powder was dispersed in 50 mL simulated fluid and each day 0.5mL solution was removed from the solution after 5 min centrifugation at 5000 rpm to avoid powder removal. The obtained samples were diluted 12 times and injected into the ICPMS system.

Cell Viability. HaCaT cells (human keratinocytes line) were seeded on 96-well plates, with a density of 20000/cm^2 in DMEM with 10% fetal bovine serum. After 24h, they were incubated with collagen, zeolite and propolis hydrolysate bioproducts suspended in complete growth medium. Each dilution was performed in triplicate. Three days post-incubation, mitochondrial enzymatic activity was evaluated as an indicator of cell viability using the XTT assay (Thermo Scientific) according to the manufacturer's specifications.

3. Results and discussion

DPPH reagent assay for propolis exhibited a EC50 of 4.80% thus suggesting a high free radical inhibitory capacity.

XRD patterns (Figure 2) highlight the main crystalline phases of the zeolite. Based on these data it can conclude that SiO_2 α quartz (reference code 01-085-0930), SiO_2 α cristobalite (reference code 04-008-7642) and $3Al_2O_3.2SiO_2$ mullite (reference code 00-083-1881) are the main crystalline phases but also amorphous phase is presented as revealed by the diffractogram, especially between 5 and 30°.

Figure 3 shows the SEM images for the obtained powders. By scanning electron microscopy specific morphology can be observed. Collagen and zeolite interact and agglomerates are formed, the size of the agglomerates reaching tens of micrometers. The roughness of the surface reveal the presence of the zeolites but, there are also some limited lamellar structures with smooth surface (at 10 000x magnification) which is most probably because of the lack of zeolite onto the surface (especially visible for the sample P4). Based on the morphology, we can conclude that all the samples exhibit a good homogeneity. The powder morphology was not influenced by the concentrations of the active substances.

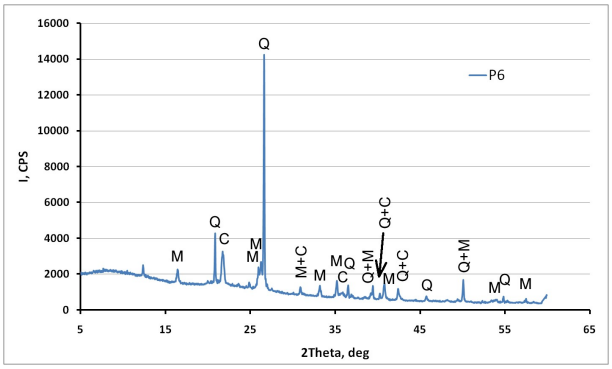

Fig. 2 - Representative XRD pattern for the obtained powders (P6) /*Difractograma de raze X caracteristică pentru pulberile obținute (P6).*

Figure 4 shows the FT-IR images for the obtained powders.

FT-IR spectra revealed peaks at the wavelengths corresponding to the Amide A groups (3300 cm^{-1}), Amide B (3070 cm^{-1}), Amide I (1630 cm^{-1}), Amide II (1530 cm^{-1}) and Amide III (1235 cm^{-1}) characteristic of collagen hydrolysate. Also, peaks between 1110 – 920 cm^{-1} and 690 – 710 cm^{-1} corresponding to Al-O-Si and Al-O-Mg systems indicate the presence of the zeolite. The peaks corresponding to the aromatic rings (630 – 450 cm^{-1}) and the nitro group (1404 – 1450 cm^{-1}) indicate the presence of constituent compounds of propolis. The peaks observed further for sample P2 can be attributed to propolis containing compounds, this sample having the highest propolis content of 1% and this component having a complex composition.

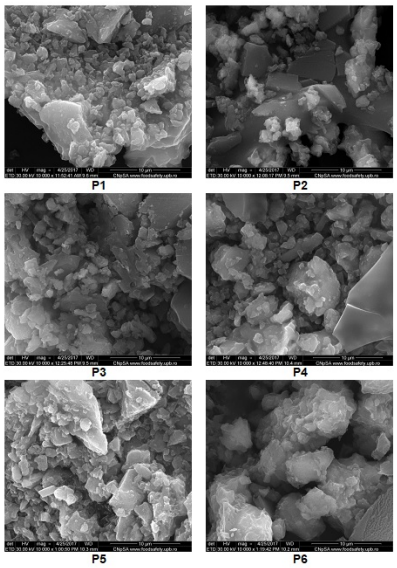

Fig. 3 - SEM images for the obtained powders/*Imagini SEM pentru pulberile obținute.*

The values recorded for the contact angles of obtained materials are given in Table 3.

In the case of the powders, the contact angle (CA) does not have a unique value, the dynamic contact angle measurements showing the drop shape variation in time [20].

The images of the drop shape obtained at different periods of time for the tested materials P1-P6 are presented for exemplification in Figure 5.

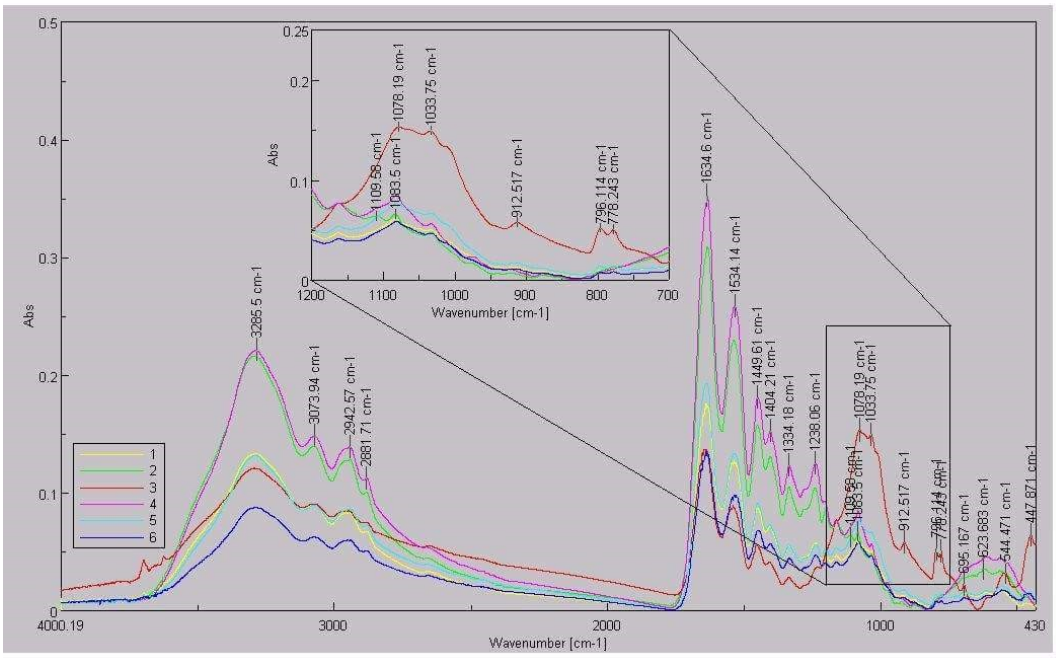

Fig. 4 - FT-IR spectra for the obtained powders/*Spectre FT-IR pentru pulberile obținute.*

Table 3

Contact angles of powders/*Unghiuri de contact ale pulberilor.*

Samples	Contact angle (°)
P 1	25.36
P 2	37.65
P 3	20.30
P 4	25.60
P 5	23.09
P6	33.68

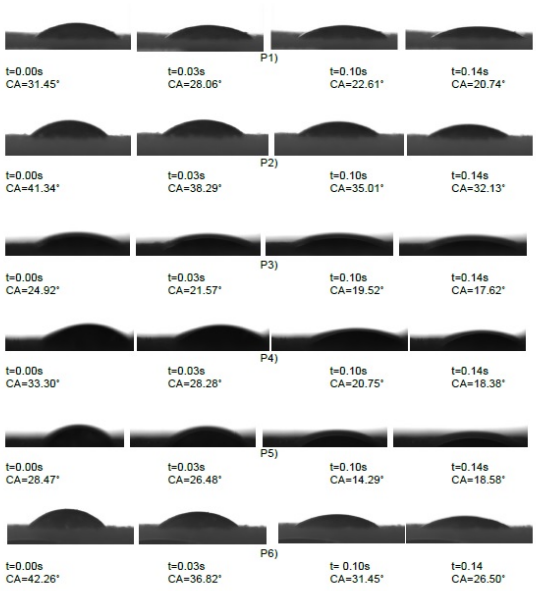

Fig. 5 - Images of the drop shape at different time intervals for the tested powders/ *Imagini cu forma picăturii la diferite intervale de timp*.

As Figure 5 shows, a slightly contact angle hysteresis (the difference between extreme values of contact angle) is noticed.

The KSV CAM 101 device allowed the determination of an average contact angle which indicates the wetting properties of the powder surface. The values obtained for the contact angle (Table 3) indicate a high surface hydrophilicity for all the samples, ranging between 20.30° (P3) and 37.65° (P2), which favours the wetting of the powders by the hydrophilic liquids. Thus, powders derived from collagen hydrolysate, zeolite and propolis exhibit good wetting capacity, a property necessary for ingestible substances. The lowest hydrophilicity is found in samples P2 and P6 containing the maximum concentrations of propolis. This characteristic is due to the content of waxes and hydrophobic compounds in the propolis composition. The good wetting capacity is found in sample P3 (20.30°), sample with low propolis content and high zeolite content. Increased concentration of hydrolysed collagen in samples leads to an increase in hydrophilicity (P1 / P5, P2 / P6), and high propolis content to less hydrophilic characteristics (P1 / P2; P3 / P4, and P5 / P6).

The release profiles of the samples were recorded for all the samples over 3 days of exposure in simulated gastric acid at pH 1.5 respectively in PBS at pH 7.2 (Figure 6). The release profiles of the main cations of the samples, in acidic conditions are presented in Figure 5. It can be concluded that Fe, Al, Ca and Mg are released with different rate from the samples because of the different solubility. Along with these ions, Na is released assuring a high ionic strength because of the up to 12 mg Na / ml SGF for the samples P4, 5 and 6. Aluminum and iron seems to be released increasingly over the 3days of evaluation in acidic conditions but the release of Ca and Mg is slightly different, their release being more complex, most probably because of the higher influence of the ionic strength assured by the release of sodium. In PBS, the release rate of sodium is 30-40% lower while the release of the Fe and Al is very low, usually 50-100 times lower comparing with the release in acidic solution.

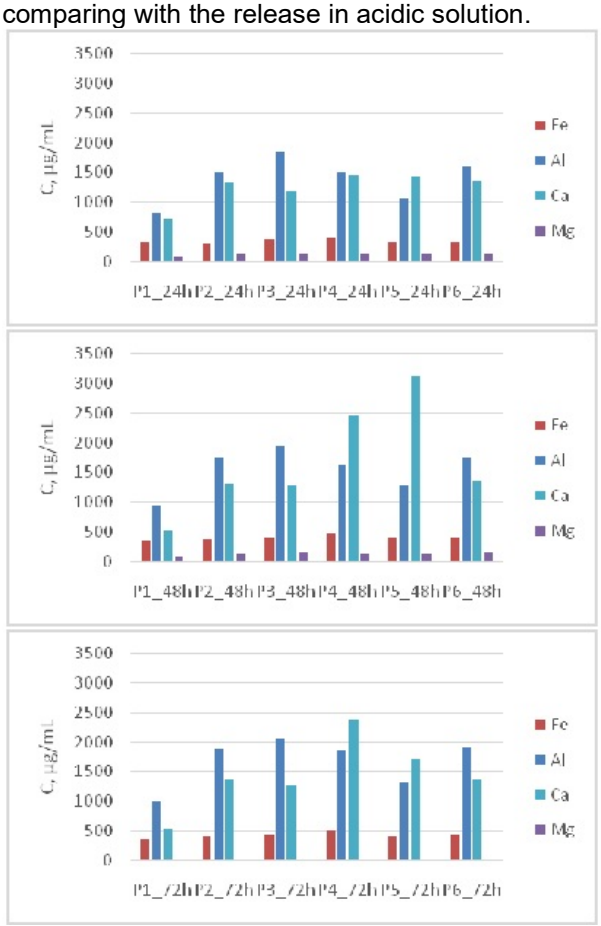

Fig. 6 - Fe, Al, Ca, Mg release over 3 days of immersion in simulated gastric acid/*Eliberarea de Fe, Al, Ca, Mg după 3 zile de imersare în acid gastric simulat.*

Further studied will be necessary to evaluate the long-term activity of these materials, especially in similar conditions as in gastric tract, mainly exposed to acidic solutions for ~200 min followed by exposure to neutral/slightly basic conditions for ~200 min [24].

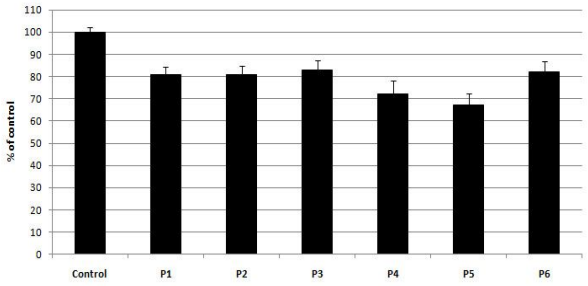

Fig. 7 - Viability of HaCaT cells grown in the presence of powders/*Viabilitatea celulelor HaCaT în prezența pulberilor.*

Figure 7 presents the results of the XTT test for collagen, zeolite and propolis-based biomaterials.

In comparison with the control cell set (HaCaT cells grown in DMEM with 10% fetal bovine serum), it can be observed that the collagen hydrolysate, zeolite and propolis bioproducts do not present a cytotoxic effect on human keratinocytes that were cultivated for 72 hours in the presence of the powders (Figure 7). One of the highest cell viability was recorded for sample P6 with high content of hydrolysed collagen. Comparing with P5 which has the same collagen hydrolysate content, the P6 has better biocompatibility, having double amount of propolis.

4.Conclusions

Powder biomaterials based on collagen hydrolysate, natural zeolite and propolis have been obtained using a freeze-drying process, and characterized after synthesis.

FT-IR analyses confirmed the presence of active principles in the powders obtained by lyophilization while the analysis of the contact angle revealed that the powders are hydrophilic, which is essential for ingestible products. A pronounced hydrophilic character is obtained with the use of low propolis concentrations, its hydrophobic components strongly influencing the hydrophilicity of the powders. Cell viability tests (XTT) performed on HaCaT cells also established a high biocompatibility; in this case the values of this parameter are influenced by high concentrations of collagen hydrolysate and propolis.

The best results for gastric mucosal regeneration were presented by the P6 sample containing high concentrations of collagen hydrolysate and propolis. Further studies are necessary to clearly and completely establish the physico-chemical changes of the zeolites after ingestions and to assess the biological influence.

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