Novel Formulation Based on Chitosan-Arabic Gum Nanoparticles Entrapping Propolis Extract

Production, physico-chemical and structural characterization

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The aim of our study was to prepare and characterize chitosan-based nanoparticles encapsulating propolis extract by ionotropic gelation and glutaraldehyde cross-linking technique. Both spectroscopic (UV-Vis, FTIR) and microscopic techniques (AFM) were applied for structural characterization of nanoparticles, along with entrapment and release study of propolis extract. The physico-chemical properties and morphological features of the obtained nanoparticles demonstrated a good correlation between all the investigated methods. Moreover, the bioactive compounds were stable upon the encapsulation procedure. Propolis release from the polymeric matrix was monitored in both simulated gastric acid and simulated intestinal fluids, concluding that our proposed formulation is suitable for controlled release. These results may provide a novel design, with improved bioavailability, stability and nutritional value of propolis bioactive compounds during processing and storage, with possible applications in food and nutraceutical industries.

Keywords: propolis, nanoparticles, chitosan, Arabic gum, DLS, AFM, FTIR.

Since ancient times, propolis has been extensively employed due to multiple pharmacological properties including antioxidant, antibacterial, antiviral, antifungal, anti-inflammatory, immunomodulatory and antitumor activities [1-5]. Previous studies on chemical composition of propolis demonstrated that the quality of propolis is influenced mainly by the plant source, as different plant species are characterized by its genome, determining secondary metabolites synthesized by the plant enzymes, being responsible for its biological activities [3-6]. The potential of propolis to enhance antibiotics and especially antifungal action for topical application was demonstrated by several studies [7-9]. Moreover, a synergic action of propolis extract and anticancer drugs was demonstrated when propolis was used as dietary supplement in patients during chemotherapy, resulting in reduction of cancer cell growth due to proliferation arrest and apoptosis [10, 11]. It seems that naturally bioactive compounds may have a co-operative action with different types of chemotherapeutics. Particularly, Romanian propolis possessed high amounts of biologically active compounds such as phenolic acids, flavonoids (flavonols, flavanones, flavanols), aminoacids and minerals [12, 13]. So, the composition of propolis varies according to the species of bee, geographic origin, month of collection, as well as to the solvent used in extraction process –higher content of active substances was obtained when ethanol was used [14]. On the other hand, the administration of phenolic compounds requires the formulation of a protecting system, able to maintain the structural integrity of the polyphenol until the consumption or the administration towards a physiological target. From this point of view, micro and nano-encapsulation has been documented to be an interesting approach, the use of encapsulated polyphenols instead of free compounds being the source of numerous works [15-20]. Chitosan is a good candidate for encapsulation, being widely investigated as a drug carrier for many possible routes of administration as chitosan has favorable biological properties, such as non-toxicity, biocompatibility, biodegradability, and antibacterial activity [16, 17]. At acid *p*H, its amino groups are ionized, making it hydrosoluble and positively charged; these properties enable it to interact with oppositely charged polymers by intermolecular electrostatic interaction, forming polyelectrolyte complexation. Chitosan-based nano-formulations can be produced by various techniques including solvent emulsification-evaporation, high pressure homogenization, high-speed stirring, ultrasonication, microemulsion using spray-drying, nanoprecipitation and ionotropic gelation [18]. In order to achieve a desired controlled release of therapeutic agents and a good bioavailability, cross-linking method is usually applied for chitosan-nanoformulations. Examples of cross-linking agents used in fabrication of chitosan nano/micro-particles are: glutaraldehyde, citric acid, calcium chloride, tripolyphosphate, vanillin, sodium sulphate, epichlorohydrin [19, 20].

The aim of our study was to prepare and characterize chitosan-based nanoparticles encapsulating propolis extract by ionotropic gelation and glutaraldehyde cross-linking technique. Both spectroscopic (UV-Visible, Fourier Transformed Infrared- FTIR) and microscopic techniques (AFM-Atomic Force Microscopy) were applied for structural characterization of nanoparticles, along with entrapment and release study of propolis extract. The results may provide a novel design, with improved bioavailability, stability and nutritional value of propolis bioactive compounds during processing and storage, with possible applications in food and nutraceutical industries.

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Experimental part

Chemicals

Chitosan (medium molecular weight, deacetylation degree >75%), Arabic gum (from Acacia tree) and glutaraldehyde solution (50% w/w) were purchased from Sigma-Aldrich, while propolis ethanolic extract (30%) was a gift from SC. PHENALEX SRL (Romanian private company).

Production of chitosan-based nanoparticles encapsulating propolis extract

100 mL chitosan solution 0.7% was prepared by dissolving the chitosan powder in acetic acid (1%) under continuous stirring, during 60 min. 50 mL Arabic gum solution 0.3% was prepared in distilled water at room temperature, to which 10 mL propolis extract (30%) was added gradually, by vigorous stirring, until homogenous mixture was achieved. The mixture Arabic gum/propolis extract was added to chitosan solution and then, 50 mL glutaraldehyde (1%) as cross-linking agent was injected by syringe, under continuous stirring, during 2 hours, allowing nanoparticles formation. The nanoparticles were separated from the suspension by centrifugation in vortex at 4000 rpm, for 60 min, filtrated and washed with distilled water (three times) and re-suspended in PBS solution. The resulted colloidal sol was used for UV-Vis spectroscopy, DLS and Zeta potential measurements. For ATR-FTIR and AFM measurements, the nanoparticles were freeze-dried using Martin Christ Alpha 1-2 LD equipment and stored at -20°C until the investigation.

UV-visible spectroscopy and DLS analysis

After an appropriate dilution of colloidal sol, UV-Vis spectrum was recorded in the range 200-800 nm using Shimadzu UV-Vis 1700 Pharma Spec (Shimadzu Corp. Kyoto) spectrophotometer. The propolis extract spectrum was also recorded for comparison. DLS (Dynamic Light Scattering) was applied using ZEN 3690 (Malvern Instruments) in order to determine the average particle size, size distribution and Zeta potential.

Morphological and structural characterization

Powder nanoparticles were investigated by FTIR (Fourier Transform Infrared Spectroscopy) in the range 400-4000 cm^{-1}, using Spectrum BXII spectrophotometer (Perkin Elmer), equipped with MIRacle ATR accessory (ZnSe crystal), at scanning speed of 32cm^{-1} and spectral width 2.0 cm^{-1}. AFM microscopy (SPM/AFM 5500 Keysight Technologies) was applied in order to observe the surface topography of the drop-coated film of nanoparticles, using tapping mode with RTESP tip.

Encapsulation efficiency, loading capacity and propolis controlled release

The entrapment efficiency of chitosan/Arabic gum nanoparticles was evaluated upon the filtering procedure, as follows: the nanoparticles were separated from free propolis in the mixture using a membrane filter (PVDF Millex filter unit), and the amount of free propolis in the filtrate was measured spectrophotometrically at 450 nm. Entrapment efficiency (EE) and loading capacity (LC) of propolis extract in chitosan/Arabic gum nanoparticles was determined from the equations:

$$EE\ (\%) = \frac{A_t - A_f}{A_t} \times 100 \quad (1)$$

$$LC\ (\%) = \frac{A_t - A_f}{A_d} \times 100 \quad (2)$$

where A_t is the total amount of propolis used in loading procedure, A_f is the amount of free propolis in the filtrate and A_d represent the amount of propolis in nanoparticles after freeze-drying procedure.

Cumulative propolis release in simulated gastrointestinal solutions with *p*H=1.8 and *p*H= 7.4 was also investigated. Simulated fluids were prepared according to literature [21, 22] as follows:

a) Simulated gastric fluid (SGF- an artificial dissolution medium that is intended to represent stomach acid) was prepared by dissolving 2.0 g of sodium chloride and 3.2 g of purified pepsin (derived from porcine stomach mucosa) in 7.0 mL of hydrochloric acid and water up to 1000 mL. The final solution was adjusted to *p*H=1.8.

b) Simulated intestinal fluid (SIF) was prepared by dissolving 6.8 g of monobasic potassium phosphate in 250 mL of water and then adding 77 mL of 0.2N sodium hydroxide and 500 mL of water. Then, 10 g of pancreatine was added and the resulting solution was adjusted with 0.2N hydrochloric acid to a pH of 7.4 and finally diluted to 1000 mL. All the reagents were purchase from Fluka.

A known amount of lyophilized polymeric nanoparticles encapsulating propolis was dispersed in both simulated fluids and the rate of release was divided into a stomach phase (0-180 min) and respectively, a small intestine phase (180-400 min). As free propolis is insoluble in water, the solution containing released propolis was centrifuged (after determined time intervals) at 3000 rpm for 10 minutes to separate the released propolis from simulated fluids. Then, the pelleted propolis was re-disolved in ethanol and measured spectrophotometrically at 300 nm. The concentration of released propolis was calculated from standard absorption curve, while the percentage of released propolis was calculated from the equation:

$$R(\%) = \frac{P_r}{P_t} \times 100 \quad (3)$$

where P_r is the concentration of propolis released after specific time interval and P_t is the total amount of propolis encapsulated in nanoparticles.

Results and discussions

Production of chitosan-based nanoparticles encapsulating propolis extract

Due to the limitation of chitosan in drug delivery systems, because of its hydrophilicity and solubility, chemical modification was performed in our study by combining with a second natural polymer, Arabic gum, in order to improve the stability of nanoparticles. Arabic gum is a negatively charged polyelectrolyte, and thus, the combined use of gum Arabic with chitosan could provide an inter-biopolymer electrostatic complex by forming strong viscoelastic films around propolis nano- droplets and provide them with good barrier properties against oxidation. In the same time, glutaraldehyde cross-linking was performed in order to produce permanent networks by covalent binding between polymer chains, as the aldehyde groups are able to form covalent imine bonds with the amino groups of chitosan. So, the preparation of propolis-loaded chitosan/Arabic gum nanoparticles was based on the electrostatic interaction between positive charge of amino groups in chitosan and negative charge of carboxyl group in Arabic gum, stabilized by the chemical cross-linking to achieve a desired controlled release of propolis extract [20].

UV-visible spectroscopy and DLS analysis

Figure 1 present the UV-VIS spectra of propolis extract and nano-colloidal sol containing chitosan/Arabic gum particles encapsulating propolis, showing absorption maxima at 290 nm both in starting extract and nanoparticles suspension. As there was no change in the absorption maxima, it indicates that the prepared nanoparticles still contains the same bioactive compounds [23, 24]. The formation of nanoparticles was further confirmed by laser diffraction, revealing that particle size obtained from highly dispersed mixture was in the range of 50-400 nm, with large Gaussian distribution, the maximum percentage of size distribution being at around 120 nm (fig. 2a). The zeta potential measurement showed negative charge -28.2 mV (fig. 2b), which indicates a stability of the nanoparticles in suspension.

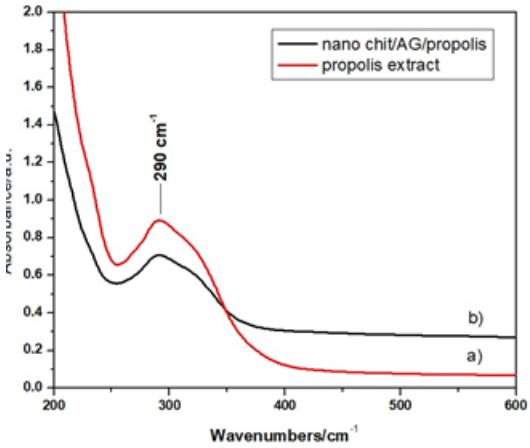

Fig.1 UV-Vis spectra of propolis extract and chitosan/Arabic gum nanoparticles loaded with propolis

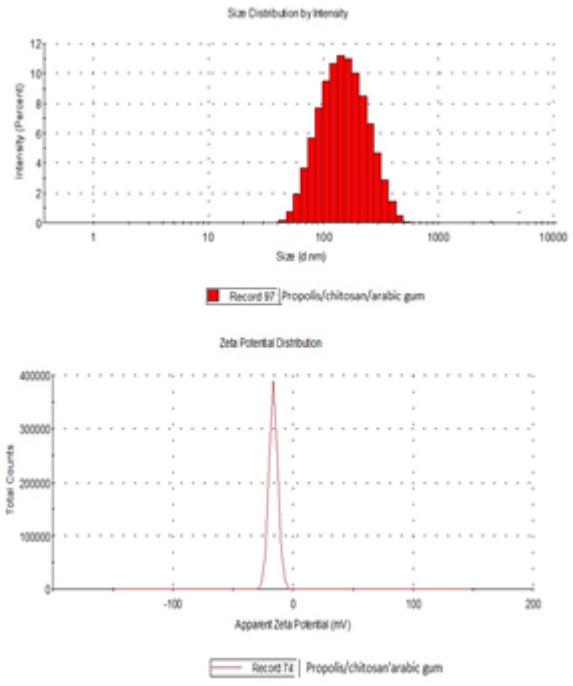

Fig.2 DLS analyses of colloidal chitosan/Arabic gum/propolis mixture: a) Particle size distribution; b) Zeta potential

Morphological and structural characterization

2D and 3D topography of chitosan/Arabic gum nanoparticles entrapping propolis extract is presented in fig. 3(a-c) using AFM, operating in tapping mode, along with the surface profile. Although the lateral dimensions are influenced by the shape of the probe, the height measurements can provide the height of nanoparticles with a high degree of accuracy and precision. In this case, one can observe that the highest value on the Z-axis is 86 nm. However, larger particles are formed due to the aggregation during storage time [25] The results are similar with previous reported data [24] in which particle size was found to be approximately 150 nm and was observed to decrease with increasing dilution as the zeta potential of the particles became more negative, stabilizing the dispersion. But this previous study doesn't use polymers for encapsulation; propolis formulations were gargles, in which propolis tinctures were diluted with water, the dilution process being accompanied by nanoprecipitation. Another similar study employed polycaprolactone in order to prepare nanoparticles loaded with propolis extract [18], but the values of particle size in this case was varying in the range between 208.5 and 280.2 nm, while the zeta potential varied from -18.6 to -26.8 mV. Recently, chitosan and gum Arabic polyelectrolyte complexation was developed as carriers for curcumin [26] and presents the influence of mixing ratio of polysaccharides on the nanoparticles size distribution, zeta potential and interior micropolarity. Size distribution in this case was in the range of 100- 500 nm (with the average diameter in the range of 250–290 nm), while the zeta potential values of nanoparticles were higher than +30 mV.

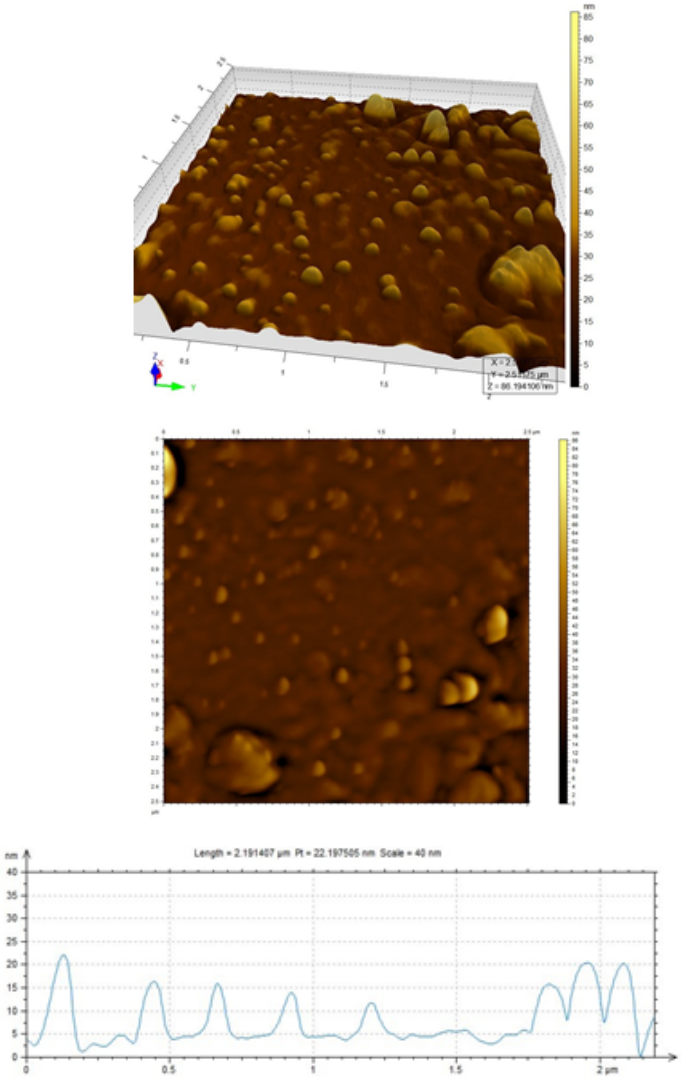

Fig.3 AFM images of chitosan/Arabic gum nanoparticles entrapping propolis extract; a) 2D view; b) 3D topography; c) Surface profile

Structural characterization of polymeric powder nanoparticles entrapping propolis was performed by ATR FTIR spectroscopy, and compared with recorded spectrum

of raw propolis, chitosan powder and Arabic gum as reference. A comparison of ATR FTIR spectra is presented in figure 4 (a, b). The marker bands of propolis can be observed in the high wavenumber region at about 3340 cm^{-1} as a large band due to O-H vibration groups and a doublet at 2840/2930 cm^{-1} due to stretching vibrations of C-H bonds in CH_2 and CH_3 groups. In the lower wavenumber region, a strong band at 1638/1602 cm^{-1} (doublet) is due to C=O stretching vibrations in flavonoids and lipids; 1510 cm^{-1} and 1450 cm^{-1} are assigned to aromatic ring deformations and C-H vibrations; 1260 cm^{-1} is assigned to C-O groups in polyols (such as hydroxyflavonoids); the very strong band at 1155 cm^{-1} is assigned to C-O and C-OH vibrations of polyols, while the week bands below 1100 cm^{-1} are attributed to secondary alcohols and C-O-vibrations [27, 28]. The ATR FTIR spectrum of chitosan showed a large band at 3370 cm^{-1} concerning with OH groups, being broadened because it overlaps the stretching band of –NH, and a week band at 2890 cm^{-1} corresponding to stretching vibration of C-H bond. In the lower wavenumber region, at 1653 cm^{-1}, a characteristic amide I band attributed to C-O vibration of the acetylated units (- $CONH_2$ groups), and amide II band at 1580 cm^{-1} (- NH_3^+ groups). The peaks at around 1375 cm^{-1} are the joint contribution of the vibration of -OH and -CH. The shoulder-peak at 1155 cm^{-1} corresponds to the symmetric stretching of C-O-C. The very strong peaks 1066 and 1030 cm^{-1} are associated to the C-O stretching vibration. The characteristic bands of Arabic gum are: typical bands of the OH bond at 3350 cm^{-1}; stretching vibration of C-H bond at 2920 cm^{-1}; two medium bands at 1600 and 1411 cm^{-1} are due to asymmetric and symmetric stretching vibration of the carboxylic acid salt; the very strong band at 1030 cm^{-1} due to the stretching of the C-O bond [26-29]. In the spectrum of mixture polysaccharides/propolis, these characteristic peaks also existed. As a result of the interaction of the biopolymers, the FT-IR spectrum of the polymeric mixture changed significantly in the carbonyl-amide region; the NH groups and asymmetric/symmetric COO stretching vibration in the range 1640-1260 cm^{-1} almost disappeared, concomitant with appearance of a new band at 1448 cm^{-1}, indicating the electrostatic interaction between the amine groups of chitosan and carboxyl groups of Arabic gum. In the same time, the marker bands of propolis are well preserved in the polymeric mixture, indicating that the bioactive compounds are stable upon the encapsulation procedure. Moreover, the large band at about 3300 cm^{-1} in the mixture become more intense and stiffened, suggesting the hydrogen bonding between chitosan and propolis [28].

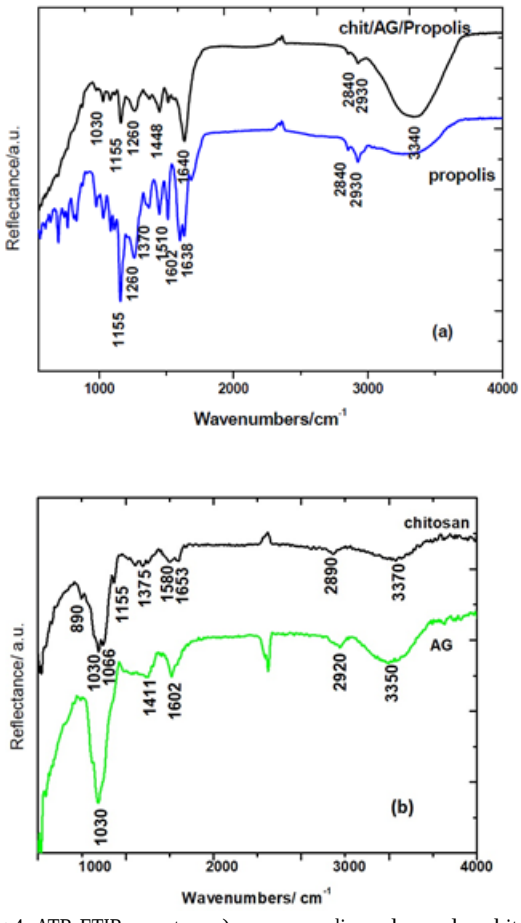

Fig.4. ATR FTIR spectra: a) raw propolis and powder chitosan/Arabic gum/propolis nanoparticles; b) powder chitosan and Arabic gum

Encapsulation efficiency, loading capacity and propolis controlled release

The selected composition presented in our study has emerged from an optimization study regarding the volume ratio of chitosan/Arabic gum and glutaraldehyde concentration as well, and also based on literature documentation [15-19, 26, 30]. The encapsulation efficiency and loading capacity of propolis extract in chitosan/Arabic gum matrix crosslinked by glutaraldehyde were calculated according to relations (1) and (2) described in Materials and Methods section resulting the mean values EE (%) = 71.22± 2.98 % and LC (%) = 23.15±1.72 % (mean values refer to the mean of three determinations ± standard deviation). According to literature, the higher glutaraldehyde cross-linker concentration, the lower EE and LC values, due to the formation of imine covalent bonding between glutaraldehyde and chitosan, resulting in more rigid particles, lowering the free volume space within the nanoparticles [31]. On the other hand, the concentration of Arabic gum also influences these parameters because higher concentration could make the cross-linking less effective. In our formulation, we consider that a balanced crosslinking toward electrostatic interaction was established.

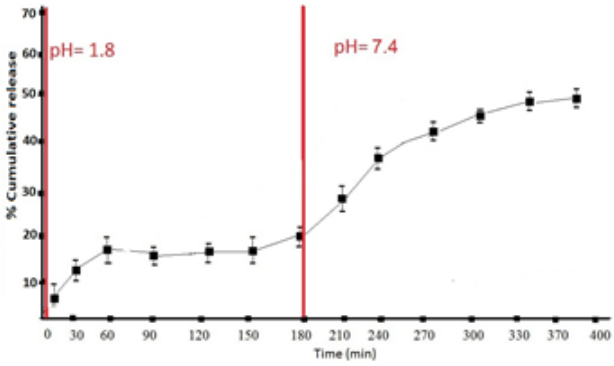

Fig.5 Release profile of propolis from chitosan/Arabic gum nanoparticles

Figure 5 illustrates propolis release profiles plotted as a function of incubating time in SGF (*p*H=1.8) and SIF (*p*H=7.4). One can observe that chitosan/Arabic gum nanoparticles strongly retain the loaded propolis in gastric environment. After the first 180 min in acid medium, the release profile of propolis indicates only 18% release from the chitosan/Arabic gum matrix. In the next 3 hours, after transferring the particles in basic environment, 33% propolis was released from the matrix. We consider that the strong biopolymeric network suppressed the leakage of propolis and such slow release in the stomach is desirable for an oral carrier [21]. This release mechanism

can be explained by the fact that the polymer matrix could swell in acidic environment due to protonation of amine group of chitosan [22].

Conclusions

In this study we succeeded to prepare and characterize natural polymeric nanoparticles based on chitosan/Arabic gum, entrapping propolis extract. The physico-chemical properties of nanoparticles were assessed by UV-visible and FTIR spectroscopy, along with Dynamic Light Scattering, revealing that particle size obtained from highly dispersed mixture was in the range of 50-400 nm, with large Gaussian distribution, the maximum percentage of size distribution being at around 120 nm. In the same time, an efficient encapsulation procedure was described using glutaraldehyde as cross-linking agent. The morphological features of nanoparticles were emphasized by AFM microscopy, demonstrating a good correlation between the results obtained by DLS technique. The FTIR analysis demonstrated that the marker bands of propolis are well preserved in the polymeric mixture, indicating that the bioactive compounds are stable upon the encapsulation procedure. In our formulation, we consider that a balanced crosslinking toward electrostatic interaction was established. Propolis release from polymeric matrix was monitored in both simulated gastric acid and simulated intestinal fluids, concluding that our proposed formulation is suitable for controlled release and pharmaceutical applications. Our results may provide a novel drug design, with improved bioavailability, stability and nutritional value of propolis bioactive compounds during processing and storage, with possible applications in food and nutraceutical industries.

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References

1. SFORCIN, J. M., BANKOVA, V., J. Ethnopharmacol., 133, 2011, 253-260.
2. De MENDONCA, I.C., PORTO, I.C., DO NASCIMENTO, T.G., De SOUZA, N.S., OLIVEIRA, J.M., ARRUDA, R.E., MOUSINHO, K.C., DOS SANTOS, A.F., BASÍLIO-JÚNIOR, I.D., PAROLIA, A., BARRETO, F.S., BMC Complement. Altern. Med., 15, 2015, p.357.
3. FERREIRA, F.B., TORRES, S.A., ROSA, O.P., FERREIRA, C.M., GARCIA, R.B., MARCUCCI, M.C., GOMES, B.P., Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod., 104, nr. 5, 2007, p.709-16.
4. STEPANOVIC, S., ANTIC, N., ADAKIC, I., SVABIC-VLAHOVIC, M., Microbiol. Res., 158, nr. 4, 2003, p. 353-357.
5. BOSIO, K., AVANZINI, C., D'AVOLIO, A., OZINO, O., SAVOIA, D., Lett. Appl. Microbiol., 31, 2000, p.174–177.
6. BUFALO, M.C., FIGUEIREDO, A.S., SOUSA, J.P.B., CANDEIAS, J.M.G., BASTOS, J.K., SFORCIN, J.M., Appl. Microbiol., 107, 2009, p.1669–1680.
7. OTA, C., UNTERKIRCHER, C., FANTINATO, V., SHIMIZU, M. T., Mycoses, 44, 2001, p. 375–378.
8. KUJUMGIEV, A., TSVETKOVA, I., SERKEDJIEVA, Y., BANKOVA, V., CHRISTOV, R., POPOV, S., J. Ethnopharmacol., 64, 1999, p. 235–240.
9. MISSIMA, F., PAGLIARONE, A.C., ORSATTI, C. L., SFORCIN, J.M., Journal of ApiProduct and ApiMedical Science, 1, 2009, p. 11–15.
10.MARKIEWICZ-¯UKOWSKA, R., BORAWSKA, M. H., FIEDOROWICZ, A., NALIWAJKO, SAWICKA, S.K.D., CAR, H., BMC Complement. Altern. Med., 13, 2013, p. 50.
11. WATANABE, M.A., AMARANTE, M.K., CONTI, B.J., SFORCIN, J. M., J. Pharm. Pharmacol, 63, 2011, p.1378–1386.
12. MARGHITAS, L. Al., DEZMIREAN, D.S., BOBIS, O., Evid. Based Complement. Alternat. Med., 2013, doi:10.1155/2013/159392.
13. GATEA, F., HANGANU, A., TEODOR, E. D., RADU, G. L., GILLE, E., Rev. Chim. (Bucharest), **66**, no. 12, 2015, p. 1938-1942.
14. RAMANAUSKIENE, K., INKENIENE A. M., PETRIKAITE V., BRIEDIS, V., Evid. Based Complement. Alternat. Med., 2013, doi:10.1155/2013/842985.
15. MUNIN, A., EDWARDS-LEVY, F., Pharmaceutics, 3, 2011, p. 793-829.
16. MITRA, A., DEY, B., Indian J. Pharm. Sci., 73, nr. 4, 2011, p. 355–366.
17. TAN, C., XIE, J., ZHANG, X., CAI, J., XIA, S., Food Hydrocolloids, 57, 2016, p. 236-245.
18. DO NASCIMENTO,T. G., DA SILVA, P.F., AZEVEDO, L.F., DA ROCHA, L.G., DE MORAES PORTO, I.C.C., LIMA E MOURA,T.F.A., BASILIO-JUNIOR, I.D., GRILLO, L.A.M., DORNELAS, C.B., DA SILVA FONSECA, E.J., DE JESUS OLIVEIRA, E., ZHANG, A.T., WATSON, D.G., Nanoscale Res. Lett., 11, 2016, p. 301.
19. SHWETA, A., SONIA, P., Int. Research J. Pharmacy, 4, nr. 2, 2013, p. 45-51.
20. ESPINOSA-ANDREWS, H., BAEZ-GONZALEZ, J.G., CRUZ-SOSA, F., VERNON-CARTER, E. J., Biomacromolecules, 8, 2007, p. 1313-1318.
21. CAVALU, S., PROKISCH, J., LASLO, V., VICAS, S., IET Nanobiotechnology, 11, nr. 14, 2017, p. 426 – 432.
22. JAYAKUMAR, R., REIS, R., MANO, J., Drug Delivery, 14, nr.1, 2007, p. 9-17.
23. JAYKUMAR, R., RAMYA, C., SUDHEESH KUMAR, P. T., SNIMA, K. S., LAKSHMANAN, V. K., NAIR, S. V., J. Nanopharm. Drug deliv., 1, 2012, p.1-7.
24. TROUSIL, J., PANEK, J., HRUBY, M., MATEJKOVA, J., KUCKA, J., PETR STEPANEK, P., J. Pharm. Investig., 44, nr.1, 2014, p. 15–22.
25.CAVALU S., KAMEL, E., LASLO, V., FRITEA, L., COSTEA, T., ANTONIAC, I. V.,
VASILE, E., ANTONIAC, A., SEMENESCU, A., MOHAN, A., SACELEANU, V., VICAS, S., Rev. Chim.(Bucharest), **68**, no. 12, 2017, p. 2963-2966.
26. TAN, C., XIE, J., ZHANG, X., CAI, J., XIA, S., Food Hydrocolloids, 57, 2016, p. 236-245.
27.OLIVEIRA, R.N., MANCINI, M.C., DE OLIVEIRA, F.C.S., PASSOS, T.M., QUILTY, B., DA SILVA MOREIRA THIRE, R. S., MCGUINNESS, G.B., Revista Materia, 21, nr. 3, 2016, p. 767-779.
28. FRANCA, J. R., DE LUCA, M. P., TRIBEIRO, T. G., CASTILHO, R.O., MOREIRA, A. N., SANTOS, V.R., FARACO, A.G., BMC Complement. Altern. Med., 14, 2014, p. 478.
29.ESPINOSA-ANDREWS, H., SANDOVAL-CASTILLA, O., VÁZQUEZ-TORRES, H., VERNON-CARTER, E.J., LOBATO-CALLEROS, C., Carbohydrate Polymers, 79, nr. 3, 2010, p. 541-546.
30. GHADI, A., MAHJOUB, S., TABANDEH, F., TALEBNIA, F., Iran. Caspian J. Intern. Med., 5, nr. 3, 2014, p.156-161.
31.TAHTAT, D., MAHLOUS, M., BENAMER, S., KHODJAA, A.N., OUSSEDIK-OUMEHDI H., LARABA-DJEBARIB, F., In. J. Biol. Macromol., 58, 2013, p. 160-168.

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