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ANTIOXIDANT BIOHYBRIDS WITH POTENTIAL APPLICATION IN PHARMACEUTICAL AREA

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Selfassembling by sonochemical processing of the flavonoid extracts on chitosan was studied related to the capacity of free radicals scavenging. The assembling of the flavonoid extract on chitosan, activated in ultrasonic field in DMSO solution, rise to a synergic activity with improved scavenging rate of free radicals. The flavonoidic extract was obtained from *Crataegus monogyna* Jacq species by solvo-thermal extraction at 150KPa in a supercritical high pressure reactor. The antioxidant properties measured by chemiluminescence technique and correlated with AFM topography and FT-IR spectroscopy showed a specific interaction between hydroxyl groups of flavonoids with amide I groups from chitosan leading to new potential applications in pharmaceutical area of the biohybrids made of biopolymer and natural extracts.

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1. Introduction

The biohybrid materials are a particular class of advanced functional materials with applications in nutraceutical and pharmaceutical industries, nanocosmetics and nanomedicine [1]. In particular specific biohybrids for drugs delivery are in continuous designing and they now are a part of "nanopharmaceutical discoveries". The new challenges in therapeutic require efficient and cost-effective tools to transport and recognize targets. The drug transport supported on different biocompatible carrier either by biomimicking or borrowing from nature of different active principles proves a potential impact for health benefits to humans. Biohybrids are made of a supporting biomaterial (usually a biopolymer) and an active principle such as antioxidants or other synthetics in a biological-physical-chemical compatibility. The principles which stand in designing of biohybrids are similar with the functional materials: multiple functions in biocompatibility with the body fluids or skin, high uptake rate, drug transport with molecular recognition to be released to a specific target, to name few. Until now a large class of biohybrids materials have been devised targeting a specific application but they still remain tributary as blind healing drug-biomaterial because no one has function with the molecular recognition although they are selfassembled nanostructures [2]. The nature employs extensively selfassembling using both phase segregation and "lock and key" specific interactions to generate the diverse range of highly ordered systems observed in living organisms. A particular class of biohybrid materials is that enriched with functions of free radicals scavenging from the body fluids, i.e biohybrids with antioxidant properties and the polyphenols are representatives in field. Research and the application of polyphenols, have

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recently attracted great interest in the functional foods and pharmaceutical industries, due to their potential health benefits. However, the effectiveness of polyphenols depends on preserving the stability, bioactivity and bioavailability of the active ingredients. The unpleasant taste of most phenolic compounds also limits their application and they are usually supported on different biopolymers [3]. The flavonoids, the most common group of polyphenolic compounds, have been large studied for different therapeutic applications either as single use or in combination with other active principles. Flavonoid extracts obtained from *Crataegus monogyna* Jacq. have many biological properties especially in the management and prevention of cardiovascular diseases like atherosclerosis, arthritis, and hypertension, and various digestive aliments, good vitamin supplier, as well in geriatric obesity and menopause disturbances [4] . In his context flavonoids need a better protection by increasing their bioavailability and therapeutic activity by selfassembing on a biopolymer such as chitosan.

Usually the standard methods (soxhlet, distillation, reflux) to obtain plant extracts have several drawbacks especially long extraction time and dependent of temperature, which can affect the quality of plant extracts because in the course of the technological process can be destroyed active ingredients by oxidation or denaturation. In addition the natural extracts from plants with high content in flavonoids are quite different than flavonoids complex which lose their properties in subsequent processing. Various novel techniques including ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, and fast solvent extraction have been developed to obtain nutraceuticals from plants targeting short time, low solvent consumption, high yield, enhancing the extract quality [5]. In this study we develop a single step of extraction of flavonoids in systems solid-liquids using solvo-thermal extraction at moderate pressures in sealed reactor used for supercritical fluid reactions based on assumptions: non-contact with environment and the pressure assures a better preservation and less degradation of the active principles. A second approach was to design a biohybrid flavonoids supported on chitosan aiming to the increasing bioavailability of the flavonoids and improving the chitosan functionality. The incorporation of the active principles of the natural extracts from plants by selfassembling on chitosan lead to an increased antioxidant activity. The proposal method consists of high activation of chitosan by sono-chemistry methods with subsequent coprecipitation and selfassembling of flavonoids extracts in biocompatible solvent (DMSO).

2. Experimental details

2.1. Materials

Vegetal materials: *Crataegus monogyna* Jacq. from Romanian areas (source, Fitoterapia SA Romania); *Biopolymer*: Chitosan - Poly-(1-4)-2-Amino-2-deoxy-ß-D-Glucan with the deacetylation degree (DD) of 95% and the molecular weight (Mw) of 360 kDa (Sigma-Aldrich); *Reagents for quantitative determination*: by spectrophotometric methods of flavonoids (aluminium chloride, sodium acetate), polyphenols (Folin-Ciocalteu), polyphenol-carboxylic acids (Arnow); *Reagents for chemiluminescence*: luminol – H_2O_2 in buffer TRIS-HCl, at pH 8.6; *Solvents*: Ethanol, Methanol, DMSO (Sigma-Aldrich, analytical grade); *Standard reagents*: rutin, caffeic acid, gallic acid, chitosan (Sigma-Aldrich);

2.2 Equipments

Spectrophotometer UV-Vis, Jasco, Japan V-570: quantitative determination of flavonoids, polyphenols and polyphenol-carboxylic acids [6,7].

Chemiluminometer (Sirius Luminometer Berthelot - GmbH Germany): for *antioxidant activity* measurements by chemiluminescence tehnique (CL).

Solvo-thermal extraction: High Pressure reactor (HPR-500, Supercriticalfluids USA); High pressure sealed 500 ml-chamber with stirrer (range of pressure: 0-600bar); temperature: RT-500^{0}C (temperature controller and programming); vacuum system for degassing.

Ultrasound system: Ultrasonic processor, UIP 1000W (Hielscher - Ultrasound Technology) 20kHz, sonotrode amplitudes up to 170 micron, liquid pressures up to 10 bars.

FT-IR spectroscopy: FT-IR JASCO 6300, wavelength range 400-12000cm^{-1}, rezolution 0.1 cm^{-1}. IR spectral studies were performed under dry air at ambient temperature using KBr pel-

lets. Samples in quantities of 4 mg each were mixed thoroughly with 200 mg of KBr, and 40 mg of the mixture were pelletized.

Atomic Force Microscopy: NTEGRA PRIMA Platform (NT-MDT) for study of topography at molecular scale.

Rotovapor: B-480, Buchi type, for the extract concentration.

2.3 Methods

a) The Flavonoids extraction.

The flavonoids extraction from Crataegus *monogyna* Jacq., was performed with HPR-500 at 80^0C at 150kPa in 300ml ethanol-water system (70/30,v/v). For each 300 ml ethanol-water the optimum quantity of dried vegetal materials (*Crataegus monogyna* Jacq.) was established at 15 grams. The optimum extraction time: 45 min at an average stirring of 120 rpm. The vegetal material was removed by filtration and the extract was concentrated by vacuum rotoevaporation. In the final stage the flavonoid extract was dried under vacuum up to constant weight (EF-HPR). The content of flavonoids, total polyphenols and polyphenolcarboxilic acids was measured by spectrophotometric technique (JASCO UV-Vis V-570) using methods developed in [6, 7], (table 1).

b) Biohybride (BHS): chitosan- flavonoid extract

In Fig. 1 is shown the preparation method by selfassembling of flavonoidic extract on chitosan activated in high ultrasound field. First chitosan, biopolymeric matrix (BMC) was solubilised in DMSO (5 g/l) for 20 min in high ultrasound field (1000 W sonotrode).

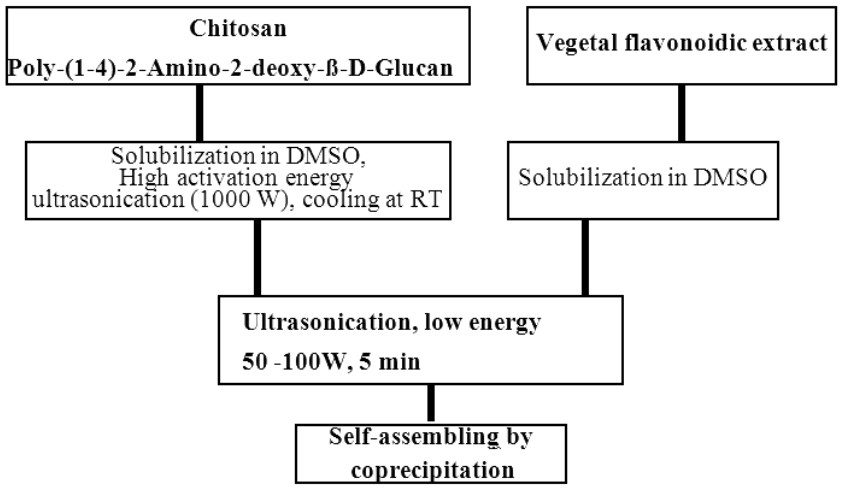

Fig. 1:Self assembling of the flavonoid extracts on chitosan in high energy ultrasound field.

In the next stage the chitosan solution has been mixed in appropriate volumes with flavonoid extract (12 % dry powder of EF-HPR in DMSO) and ultasonicated for 5 min in ultrasound bath. In final stage the solution has been stirred over night at RT (Room Temperature). The precipitate has been collected by filtering under vacuum and dried at 50^0 C to get a constant weight. In such way a biohybrid powder with 24% flavonoids coprecipitated and selfassembled on chitosan has been obtained. DMSO, aprotic polar solvent, was selected because of its ability to dissolve many kinds of biocompounds, plays a role in sample management and high-throughput screening operations in drug design and is compatible with the body fluids. In medicine, DMSO is predominantly used as a topical analgesic, a vehicle for topical application of pharmaceuticals, as anti-inflammatory, and antioxidant. On the other hand the amino group in chitosan has a pKa value of ~6.5, which in high energy of ultrasonic field leads to a protonation in polar aprotic solvent (DMSO) inducing a charge density that make chitosan readily binds to negatively charged surfaces such as mucosal membranes or flavonids, rich in hydroxyl groups [8].

c) The scavenging rate

The antioxidant activity counted by the scavenging rate (SR%) of the flavonoid extract (EF-HRP), chitosan (BMC) and biohybrides (BHS) have been evaluated by chemiluminescence [CL] using a modified and adapted method based on references [9,10] . The chemiluminescent system use dianaions generated by luminol and hydrogen peroxide at pH = 8.6 in presence of TRIS + HCl. The chemiluminiscent quenching induced by compounds are measured by the scavenging rate-SR%

$$\%SR = \frac{I_0 - I_s}{I_0} \cdot 100$$

where: I_0 = CL intensity in the absence of samples at t = 5s; I_s = CL for sample at t = 5s. In case of the biohybrids systems the active principles can be activated or inhibited depending of the biopolymer support. Based on this assumption was defined the specific scavenging rate SR_S (%):

$$\%SR_s = \%SR / m$$

where *m* is the weight (mg) of the flavonoid extract adsorbed / selfassembled on BMC. The rational for definition as percentage of SR% per mass of the natural extract is to evidence the relationship between matrix and selfassembled principles which can amplify or reduce their activity. The chemiluminescence of the reactive oxygen species is quenched due to antioxidant activity of the plant extracts [11,12] . There are lots of generating of free oxygen radicals but the system luminol-H_2O_2 in alkaline solution lead to high reactive oxygen species to be neutralized by the flavonoids and polyphenols at a given concentration. The rate of the chemiluminescence quenching is direct related with the flavonoid structure and its capacity of scavenging being a measure of the capacity to reduce free radicals. The antioxidant activities of the compositions evaluated by their scavenging rates (%)/m, will be a good marker for free radicals scavenging [13,14].

3. Results and discussions

3.1. Antioxidant and physical-chemical characteristics

The flavonoidic from *Crataegus monogyna* Jacq species (EF-HPR), the biopolymeric matrix – chitosan (BMC) and the biohybride system (BHS) were characterized by chemiluminescence. The specific physical-chemical indicators and quantitative determination of the flavonoids, total polyphenols, content of polyphenolcarboxylic acids for EF-HPR were measured according to methods described elsewhere [4a, 6,7] (table 1).

EF-HPR:The antioxidant activity of vegetal extract EF-HPR was evaluated by comparison to flavonoids and polyphenols compounds (Table1) which reach 8.316% total active polyphenolic principles. The antioxidant activity counted by the scavenging rate reaches to 77.17%. The kinetic parameters k_i, respective v_i are very close to the weighted average counted based on the reference date for GA, R and CA (table 2). The specific scavenging rate SR_s counted as weighted average shows a value of 27.52%/mg which is very close with data measured for EF-HPR (25.7%/mg)

Chitosan (BMC): The antioxidant activity for the biopolymer chitosan (BMC) has value of 30.99% (SR_s= 10.3% mg^{-1}) quite low compared to extract flavonoids EF-HPR (77.17%) although has been strongly activated in ultrasound field. Nevertheless the activity is high by comparison with reports [15] which claim neutral antioxidant activity. Other reports assign the antioxidant activity for chitosan and its derivatives to the active hydroxyl and amino groups. The above results with high ultrasonicated chitosan are assigned for amino and OH activated groups.

Biohibrid (BHS)

Although SR% for BHS reach to 73.96% slightly low than EF-HPR when is counted on specific weighted mass SR_s = 38.6 %/mg (table 1) which is high than all standards indexed in table 2. In addition can be observed the kinetic parameters are quite different than standards and EF-HPR k_i-ratio decreases with 22.4% and the ratio of reaction (v_i) decrease with ~17%.

This shows that the BHS has a long lasting scavenging activity with high capacity of free radical retention. These experimental data support the hypothesis that the flavonoids are chemical grafted to the chitosan via amide I groups when are subjected to excitation in high density ultrasonic field. The data are consistent with other reports related to the chitosan grafting [16]. Our re-

port is in agreement with the basic formulation for inducing of high antioxidant activity: 1) to improve the solubility of chitosan in neutral aqueous solution [17] 2) to introduce the H-atom donor group if we are to consider the development of a chitosan-based antioxidant. In addition, owing to the nitrogen atom, chitosan is a potential preventive antioxidant based on its metal ion deactivation. Ultrasonication in high energy field remove some limitation for chitosan which make it intractabil as antioxidant; especially, (i) its poor solubility due to its inter- and intramolecular hydrogen bond network, and (ii) the lack of on H-atom donor to serve as a good chain breaking antioxidant, (iii) the availability of net cationic amino groups in the molecule, which impart intermolecular electrostatic repulsive forces leading to increase in the hydrodynamic volume of the extended chain conformation [18, 19, 20].

Table 1. Physical-chemical characteristics, kinetics parameters, antioxidant activity

Specific physical-chemical indicators				Quantitative determination of the flavonoids, polyphenols, polyfenolcarboxilic acids		
Sample code	Evaporation residuum, %	Relative density at 20ºC	pH	Polyphenols, mass % (vs gallic acid)	Polyfenolcarboxilic acids, mass % (vs caffeic acid)	Flavonoids, mass % (vs rutin)
EF-HPR	17.28	0.9942	6.3	3.384	2.722	2.210

Chemiluminescence and ***kinetic*** characteristics *				
Sample code	k_i (s^{-1})	v_i (s^{-1})	SR (%)	Specific activity (SRs= SR%mg^{-1})
EF-HPR	0.089	283.50	77.17	25.7
BMC	0.125	741.00	30.99	10.3
BHS	0.065	618.00	73.96	38.9

*k: ratio constant; v_i: ratio of reaction, indexed from the chemiluminescence curves [9]

Table 2. Reference data for SR indexed on standards

Sample code	k_i (s^{-1})	v_i (s^{-1})	SR(%)	SR_s (% mg^{-1}) (this work)	Reference for SR
R [a]	0.093	291.40	77.40	25.8	[10]
Q [b]	0.100	118.84	92.80	30.9	[14]
K [c]	0.105	124.80	92.49	30.8	[14]
CA [d]	0.070	318.00	82.90	27.6	[14]
GA [e]	0.112	166.46	85.70	28.6	[21]
CLA [f]	0,096	100.00	87.20	29.1	[14]

a:Rutin, b:Quercetin, c:Kaemferol, d:Caffeic Acid, e:Gallic Acid, f:Chlorogenic Acid

3.2 Atomic Force Microscopy

The topography images of the sample surface were measured with AFM-NT-MDT NTEGRA Prima. The measurements were performed in tapping mode using a scanning probe NSG10. for noncontact/ semicontact modes, resonant frequency 140-390 kHz, force constant 3.1-37.6N/m. Figure 2 shows the surface topography and specific features for chitosan BMC (fig.2a) and chitosan-flavonoid composite BHS (fig. 2b).

By comparison with other reports [22] where large grain size and rough spherical particles are observed in figure 1a chitosan processed in high ultrasound field are stacked in small grains (< 200nm) with a specific topography. In eye of figure 1a are shown the topographic arrangement of the chitosan nanoparticles in spheroid aggregates.

In figure 1b are shown chitosan particles coated with EF-HPR after sonication and free precipitation in the cooling time. The grains are reduced in size and topography of stacking is quite different (eye in fig.2b).

It is clear evidenced the presence of EF-HPR film which surround the chitosan particles. The most interesting feature is that flavonoid extract is stacked in the same topography as chitosan in parallel planes. This led to the suggestion that flavonoid extract bond to the most reactive group in chitosan which is amide I. The results are congruent with the observation in FT-IR spectrum (fig. 3).

3.3 FTIR spectroscopy

The FTIR spectra of chitosan (BMC) and chitosan-vegetal extract (BHS) chitosan are shown in Fig. 3. Chitosan (BMC) has characteristic bands in range 3350-3371 cm^{-1} assigned to $-NH_2$ and –OH groups stretching vibration and the band for amide I at 1651 cm^{-1} is seen in the infrared spectrum. Whereas in the FTIR spectra of high activated chitosan in high density ultrasonic field selfassembeld with EF-HPR the peak of 1651 cm^{-1} disappears and 2 new peaks at 1630 cm^{-1} and 1613 cm^{-1} appears being a consequence of crosslinking and specific interactions with amide I-groups.

In the spectra IR (fig. 3), a peak at 1321 cm^{-1} corresponds to acetyl groups [23,24] . The region between 1419- 1435 cm^{-1}, is considered to be conformation-sensitive for polysaccharides (BMC), dependent of the orientation of the primary hydroxy groups and interactions with the flavonoid indicates a change in the secondary structural environment. As result of the interactions with BMC and EF-HPR a strong deacreasing of their intensities with appearance of characteristic band for GA and CA respective R (dominant compounds in EF-HPR).

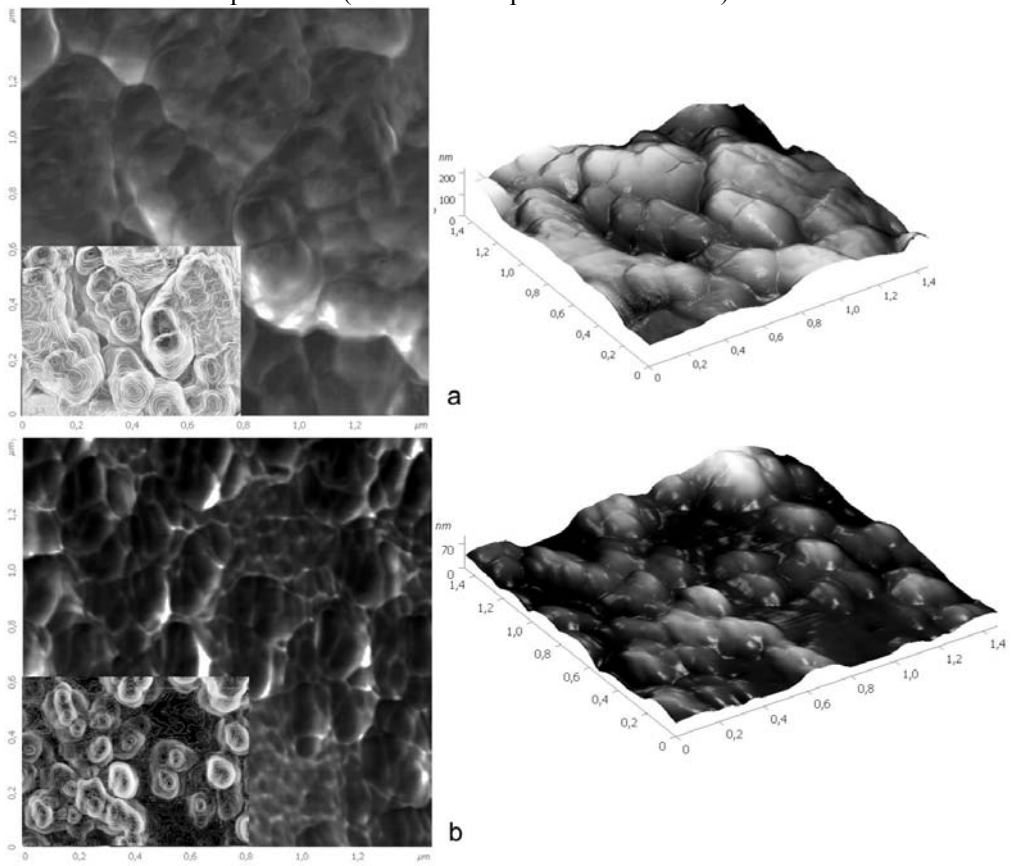

Fig. 2 (a) AFM images of chitosan(BMC), (b) AFM images of Biohybride Chitosan- EF-HPR(BHS)(see comments in text)

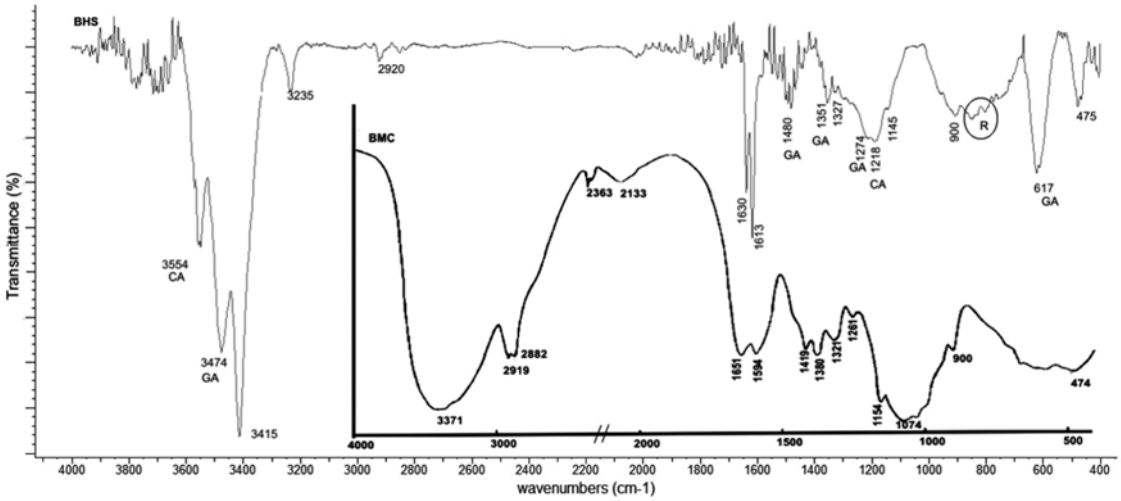

Fig.3 IR spectra for BMC and BHS (see comments in text)

4. Conclusions

It was demonstrated that biohybride chitosan- flavonoid extracts can provide an improved antioxidant activity. That is a consequence of the synergistic effect induced by the content of the active principles on chitosan: polyphenols, polyphenolcarboxilic acids and flavonoids. The constituents in the sample extract (EF-HPR) were quantified in the phenolic, polyphenolcarboxilic acids and flavonoidic, confirmed by spectral analysis. The specific scavenging rate is more effective to evaluate the antioxidant activity by chemiluminescence.

Chitosan activated in high energized ultrasonic field is more appropriate to design biohybrides basd on following assumptions: 1) The amino group in chitosan (pKa value of ~6.5) [25] in high energy of ultrasonic field leads to protonation in polar aprotic solvent (DMSO) that make it readily to bind to the negatively charged surfaces such as mucosal membranes or flavonoids, rich in hydroxyl groups 2) The synergic activity come from chitosan which enhances the transport of polar drugs across epithelial surfaces, and from flavonoids which have high rate of free radical scavenging. Atomic force microscopy reveals the interaction of high activated chitosan with the flavonoid extracts via grafting on amino groups and confirmed by FT-IR spectroscopy. In consequence the benefits of including flavonoidic extract in a biopolymer matrix lead to increased the antioxidant potential and thus leads to new design of drugs for therapeutic applications.

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