New Biopolymeric Membranes with Vegetal Plants Extracts and Potential Anti-inflammatory Effect for Use in Tissue Therapy

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The paper presents a method for elaboration and characterization some chitosan-gelatine membranes with different collagen-gelatine mass ratio, cross-linked with glutaraldehyde (GA) and coupled with extract plants. The aqueous extracts obtained from these plants were analyzed in terms of biologically active substances content (amino acids, polyphenols and phytosterols). The investigation for all obtained membranes involves: SEM microscopy, porosity, degradation test, contact angles measurement, hemolisys and antibacterian index determination.

Keywords: chitosan, gelatine, plants, hemolisys, antibacterian activity

Natural polymers are useful as biomaterials, being also biodegradable polymers. The chitosan polymer, has been intensively used as a topical dressing in wound management, owing to its non-toxic, antimicrobial, biocompatible and biodegradable properties [1]. Gelatine (Gel) is the product of thermal denaturation or desintegration of insoluble collagen [2] with various molecular weights and isoionic points depending on the source of collagen and the method of its manufacturing process of recovery. Collagen exists in many different forms, but gelatine is only derived from source rich in Tipe I collagen. [3]. It has been reported that chitosan-based cross linked matrices using glutaraldehyde(GA) or genipin, have better stability in aqueous medium and better mechanical properties as compared to non-modiûed materials [4]. The evelopment of chitosan membranes with desirable properties sometimes requires modiûcation at surface or bulk level by chemical and physical means [5, 6] or incorporation of various bioactive compounds [7]. Plants have been used to treat infectious diseases due to their antimicrobial properties. [8].The main constituent of birch (Betula verrucosa) named betulin, has antiseptic, anti-inflammatory, antiviral, antibacterial, antifungal and antitumour activities [9-14]. The aqueous extract of M. officinalis demonstrated a high antiviral activity against herpes simplex virus type 1 (HSV-1) in vitro [15] and antimicrobial action against the growth of *P. aeruginosa, K. pneumonia, S. aureus and C. Koseri*[16-18]. The antibacterial effect of *Viola tricolor* as methanolic extract showed a low to moderate antifungal activity against *C .albicans* [19]. Therefore, the purpose of this study was to develop and characterize the chitosan-gelatin membranes with incorporation of vegetal extracts by the emulsion or adsorption method.

Romanian resources in the field of medicinal and aromatic plants are very diversified; in spontaneous flora there are about 3700 species of plants, of which 800 species are medicinal plants and of these, 370 have proven therapeutic properties [20].

In this study the analised plants were: pansy (*Viola tricolor*), melilot (*Melilotus officinalis*), birch (*Betula verrucosa*), lady's mantle (*Alchemilla vulgaris*) and lemon balm (*Mellissa officinalis*).

Experimental part

Elaboration of biopolymeric membranes

Materials: Chitosan (CHI) obtained from crab shells, molecular weight M = 150,000, and 84.5% degree of di-acetylation DD (Fluka BioChemika), high viscosity, <12% loss on drying (Sigma Aldrich). GEL from the dermis and tendons of swine, (Fluka BioChemika). (GA) solution 25% (Merck).

Elaboration of the biopolymeric membranes was the coupling of colloidal dispersions (CHI and GEL) followed by crosslinking of free amino groups of these biopolymers with glutaraldehyde.Initially, colloidal suspensions of the two biopolymers of chitosan gel 1%, w / v were prepared in the acetic solution (2 M acetic acid-sodium acetate 1 M) by dissolving on a water-bath until a final *p*H 5.4 was achieved, respectively gelatin, 1%, w / v, dissolved in distilled water at a temperature of 60°C.

The two components, were mixed in molar ratios 1:1 treated with GA bifunctional agent (concentration 0.02%), considering that it is sufficient for the crosslinking 20% of free amine groups.

The resulting mixture, after stirring 30 min, was left at rest to remove the air bubbles formed and was poured into petri dishes, dried in an oven at 37°C for 48 h. Finally were obtained some elastic and translucent membrane: **M1**: CHI:GEL (1:1), GA; **M2**: M1 in which the plant extracts are embedded by adsorption and **M3**: M1 in which the plant extracts are embedded by including.

The aqueous extracts were obtained by maceration of dried herbs (pansy, melilot, birch, lady's mantle and lemon balm) in bidistilled water during 10 days with periodic agitation at room temperature. Aqueous extracts (extraction ratio 1: 13.2 g/v),were analyzed in terms of content biologically active.

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To achieve membranes, gelatin-chitosan-vegetal extracts having anti-inflammatory effect, the chitosan-gelatin ratio was 1: 1, and GA crosslinking was carried out in the final concentration of 0.02%. Biopolymeric membranes complexed with the plants extracts mentioned above were obtained by the adsorption and inclusion in gel methods.Adsorption was carried out by immerssion the crosslinked membrane of chitosan-gelatin (1: 1) for 24 h in a mixture of 5 extracts of plants, each one taken in the same amount. The extract was added gradually until saturated, the excess was removed. The membrane with the adsorbed extract was dried at 37^{0}C in the oven, weighed and analyzed.

The inclusion in gel method involves addition of chitosan and gelatin (molar ratio 1:1) in the mixture of the five plants extracts composed from equal proportions from each one. It was added 0.02% glutaraldehyde in order to crosslink. The gel after being well mixed, was poured in the Petri dish and left in an oven at 37^{0}C for membrane formatting. Elastic and semitransparent membranes were obtained.

Membranes characterization

For the obtained aqueous extracts were carried out a series of organoleptic tests in order to determine their quality and stability, according to Romanian Pharmacopoeia [21]. Organoleptic properties consist of preliminary characterization of the extracts of plants such as: odor, color, taste, appearance, adhesion.

Is worth to mention that these properties were pursued over a period of 60 days. The extracts were kept at room temperature in the dark and without using any preservatives [22].

The content of amino acids present in the obtained extracts was determined by the TLC (thin layer chromatography), according to Romanian Pharmacopoeia [23]. As materials were used silica gel plates type ALUGRAM SIL (Macherey-NAGEL), G / UV 254, 20x20cm. Mobile phase was: butanol: acetic acid: water (4: 1: 1); revelation was performed using 0.3% ninhydrin solution dissolved in acetone.

The presence of polyphenols in the aqueous extracts was based on the color reaction of these compounds with phosphotungstic acid in alkaline medium, where it forms a blue colored complex. The working method used was described by Singleton et al [24] .

Electron microscopy studies (SEM)

SEM tests were conducted in order to characterize their structure. From the asymmetry of structure and extra micrographs it can be estimated the porosity and also the pore size distribution. Membrane fragments were viewed under a scanning electron microscope, SEM Hitachi SU 1510.

Porosity measurements

A liquid displacement method was used to measure the porosity (P) of scaffolds [25]. Ethanol was used as a liquid medium. The porosity was calculated using the following formula:

$$P(\%) = \frac{m_1 - m_3}{m_2 - m_3} \times 100 \quad (1)$$

where: m_1 is the weight of sample in air, m_2 the weight of sample with liquid in pores, and m_3 the weight of sample suspended in ethanol.

The degradation test was performed in phosphate buffer solution (PBS) *p*H = 7.4, at 37 ± 0.5°C with a continual agitation speed of 120 rpm according to ISO 10993-14. At the end of each immerssion time, distilled water was used to wash the sample surface, and then samples were dried until a constant weight. Weight loss (Dm) for each sample during degradation was measured by equation as follow:

$$\Delta m = \frac{m_0 - m_t}{m_o} \times 100 \quad (2)$$

where: m_0 and m_t stand for initial weight and weight after a specific immersion time, respectively.

Bioactivity studies of membranes

The bioactivity studies of membranes were carried out by biomimetic method with 1.5× simulated body solution (SBF) with the the following composition (mM): Ca^{2+}2.5, HPO_4^{2-} 1, Na^{2+}142, Cl^-147.8, Mg^{2+}1.5, K^+ 5, SO_4^{2-} 0.5, HCO_3^- 4.2. All the membranes were previously subjected to $CaCl_2$ treatment for 3 days at 37°C in order to enhance the bioactivity properties. After this treatment each of them were vertically suspended in 50 ml of 1.5× SBF solutions. Samples were retrieved after 7 days of soaking at 37°C and then they were rinsed with distilled water and dried. Calcein titration method was used to measure the Ca^{2+} concentration change in SBF before and after soaking the membranes [26].

Contact angle measurements

The contact angle (CA) of a drop of water with the membranes surface was measured as previously described [27] with a contact Angle Meter, KSV instruments. Each contact angle value is the mean value of 3 measurements.

Antibacterial activity and Hemolytic study

The occurrence of new infectious diseases and the increase in bacterial resistance have created the necessity for studies for the identification of antimicrobials from natural products, especially from plants as source of new bioactive molecules [28]. Antimicrobial activities have been evaluated with the turbidimetric method. The antibacterial activity of natural products was appraised against two bacteria species: *Escherichia coli* ATCC 25922 (American Type Culture Collection) [29] and *Streprococcus pyogenes* ATCC 19615.

Both bacteria were cultivated overnight on Columbia Agar-5% sheep blood for 24 h at 37°C, then a bacterial suspension (inoculum) was prepared with sterile physiological solution, to 10^8 CFU/mL. The test materials were placed in sterile glass tubes and the inoculum was added. One glass tube was filled with sterile saline as negative control, and another with the inoculum witouht materials as positive control. All recipients were incubated at at 37°C for 24 h, then the absorbance was read at 600 nm with an automated analyzer Chemwell 6010. The antibacterial rates were determined by calculating the bacterial growth inhibition index using the formula:

$$I\% = \frac{(C_{18} - C_0) - (T_{18} - T_0)}{(C_{18} - C_0)} \times 100 \quad (3)$$

where: I is the growth inhibition index, C_{18} and C_0 are the blank-corrected optical densities at 600 nm of the positive control at time 0 and after 18 h, T_{18} and T_0 are the blank–corrected optical densities of cultured media in the presence of test samples at 18 h and respectively at 0 h.

The hemolytic activity was performed according to a wel –established method [30]]

Results and discussions

The results of the organoleptic tests are presented in table 1.

Samples	Apparence	Color	Odor	Taste
Pansy (Viola tricolor),	slightly opalescent	Maron-brown	Characteristic (specific to the plant, but is perceived another smell too}	Pleasant
Melilot (Melilotus officinalis)	Slightly opalescent	Greenish yellow	Characteristic (specific plant)	Aromatic, sweet, Analgesic
Birch (Betula verrucosa)	Slightly opalescent	Yellow orange	Characteristic (specific plant)	Aromatic, sweet, pleasant taste buds.
Lady's Mantle (Alchemilla vulgaris).	Clear	Yellowish brown	Characteristic (pleasantly fragrant).	A little bitter, a sallow hue.
Lemon balm (Mellissa officinalis)	Clear	Yellow green	Characteristic (pleasant aroma).	Aromatic, analgesic

Table 1
ORHGANOLEPTIC PROPERTIES OF PLANTS

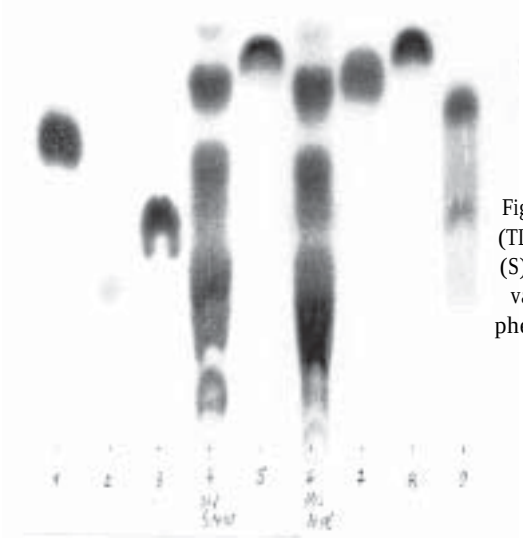

Fig. 1. Chromatographic analysis (TLC), aqueous extracts, melilot (S), pansy (TFP), and standards: valine, proline, glutamic acid, phenylalanine, tyrosine, leucine, methionine

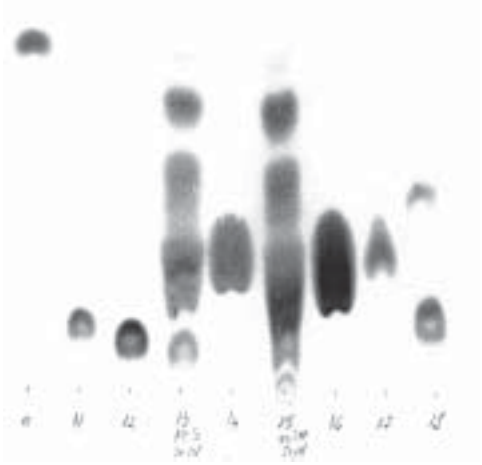

Fig. 2. Chromatographic analysis (TLC), aqueous extracts, melilot (S), pansy (TFP), and standards: tryptophan, histidine, lysine, glycine, serine, aspartic acid, asparagine

The content of amino acids in aqueous extracts of the plants studied was determined by thin layer chromatography (TLC).

In figures 1 and 2 are shown the chromatograms of the standard amino acids (valine, proline, glutamic acid, phenylalanine, tyrosine, leucine, methionine, tryptophan, histidine, lysine, glycine, serine, aspartic acid, asparagine, hydroxyproline, arginine, and alanine) samples and aqueous extracts of the studied plants.

Identification of amino acids in aqueous extracts of plants TLC:

The results regarding the highlighted amino acid content in the aqueous extracts studied in chromatograms are shown in the table 2.

It can be concluded that aqueous extracts of the studied plants contain a significant number of amino acids. In aqueous extract of pansy were identified 11 amino acids, of which essential amino acids; melilot shows eighth amino acids, of which five essential; Lady's mantle contains seven amino acids, the essential balm contains nine of which two essential and birch contains the lowest number of amino acids, five amino acids of which three are essential amino acids. Regarding the TLC analysis it can be seen that the extract of birch, lady's mantle and lemon balm is possible to contain another essential amino

No. crt	Aminoacids (AA)	Rf AA	Aqueous extract Melilot	Aqueous extract Birch	Aqueous extract Pansy	Aqueous extract Lemon balm	Aqueous extract Lady's Mantle
1.	L-hydroxyproline	0.32	+		+	+	
2.	L- arginine	0.17		+	+	+	+
3.	L-histidine	0.17	+	+	+	+	+
4.	L-alanine	0.49			+	+	+
5.	Valina	0.60	+		+	+	+
6.	L-proline	0.32	+		+	+	
7.	Glutamic acid	0.42	+	traces	+	+	traces
8.	Tryptophan	0.71	+		+		
9.	L-lysine	0.14	+		+		
10.	L-asparagine	0.31					
11.	L-serine	0.36		+		+	
12.	Aspartic acid	0.35		+		+	+
13.	L-phenylalanine	0.68					
14.	L-tyrosine	0.68	+		+		
15.	L-leucine	0.70					
16.	L-methionine	0.56			+		+
17.	L-glycine	0.34		+			+

Tabel 2
THE AMINOACIDS PRESENT IN AQUEOUS EXTRACTS

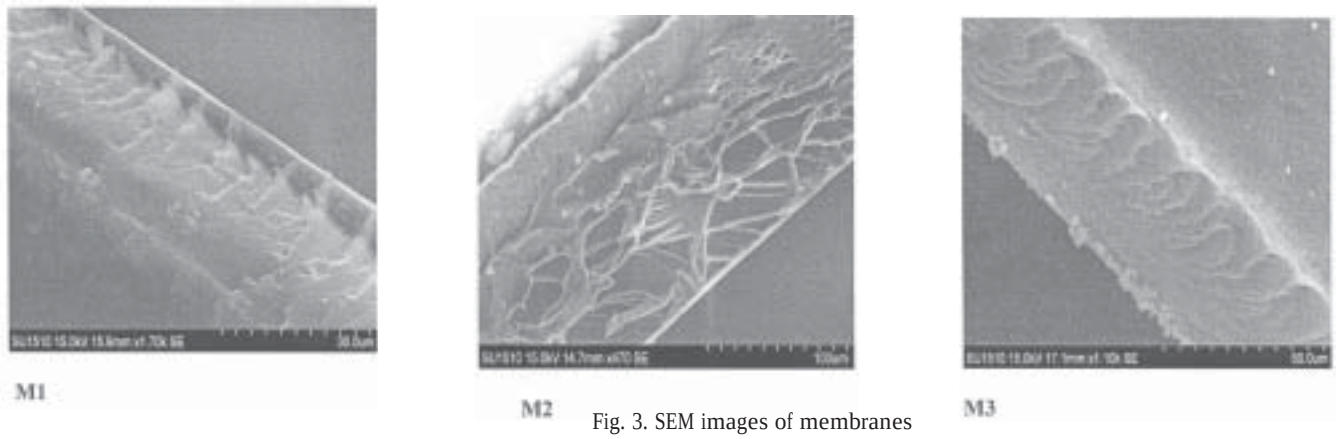

Fig. 3. SEM images of membranes

acid, namely threonine, that have been identified with Rf=0.35.

As regarding polyphenols, substances with proven antioxidant, antibacterial and anti-inflammatory properties, they were identified in the extracts of pansy, birch and melilot. Extracts of lemon balm and lady's mantle shows negative reaction, so they do not contain polyphenols.

SEM and porosity analisys

They were viewed in SEM, three variants of membranes: **M1**; **M2** and **M3.**

The SEM images (fig. 3) shown that the membrane **M2** has a high degree of porosity. The membrane **M3** presents a more compact structure, with low degree of porosity, similar to the membrane structure without extracts **M1.** Porosity of materials plays a critical role in bone formation *in vitro* and *in vivo*. Because of their higher specific surface area, porous structures are able to offer good conditions for cell growth and flow transport of nutrients and metabolic waste. The optimal pore size required for bone in growth has been suggested in the range of 100–800 μm [31]. It was proved that pores above 100 μm (big pores) permit colonization by osteoblasts, while the micro- pores (≤ 50 μm) promote materials vascularization (nutrients supplying for cells, waste products removal) [32]. Structure with pore size of 75±15 μm is observed in the case of **M2,** whereas for sample **M3** pore size decreased to approximately 38±12 μm.

It is known that porous material used for repair of dermal lesions should submit a porous structure with a porosity greater than 70% and interconnected pores that allow cell growth and proliferation [33]. In our cases the porosity determined by liquid displacement method of **M1** was about 72.9%, whereas a bigger porosity (with a value of 92.2%) was obtained for **M2** indicating that the effect of the fabrication methods. The porosity for **M3** was similar with **M1** (around 79.1%).

Degradation test

When one porous material was put into the degradation solution, the liquid contacted with the sample surface first, forming the material-liquid interface. Then the fluid went up through the interconnection pore by capillarity, the pore walls became new material-liquid interfaces. Modifications of the textural porosity of the membranes had an effect on the dissolution behavior (fig. 4) due to changes in the specific surface area of sample exposed to the degradation media.

The increased porosity eventually caused the higher dissolution rate. The highest weight loss was observed for membrane **M2** which had the highest initial porosity. There are no significant differences between the other two membranes, although **M3** has a higher dissolution rate than **M1**.

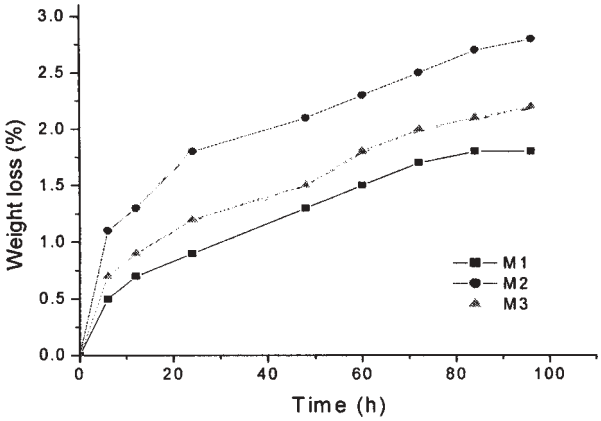

Fig. 4. Weight loss of membranes in PBS solution

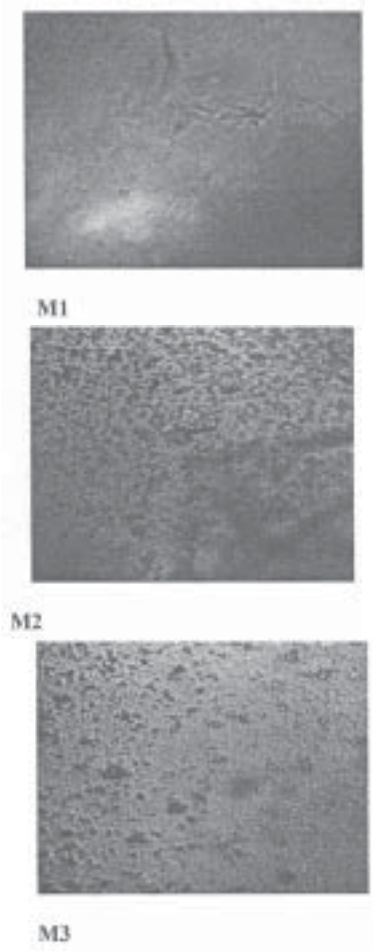

Fig. 5. Optical image of membranes after 7 days of immersion

Bioactivity studies of membranes

Figure 5 shows the optic image (Zeiss Scope A1 microscope) of membranes after 7 days of immersion in 2SBF solution. The surface morphology of **M1** membranes

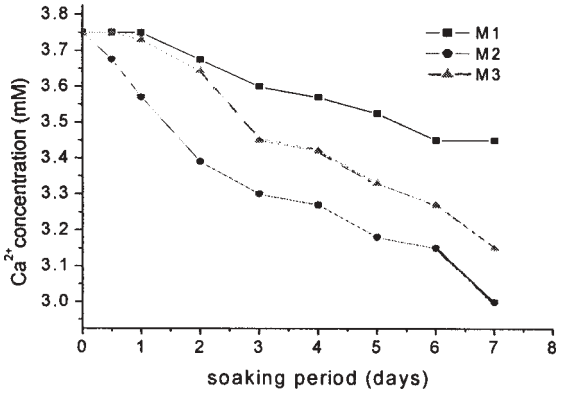

Fig 6. Evolution of Ca^{2+} in 2SBF solution versus soaking times

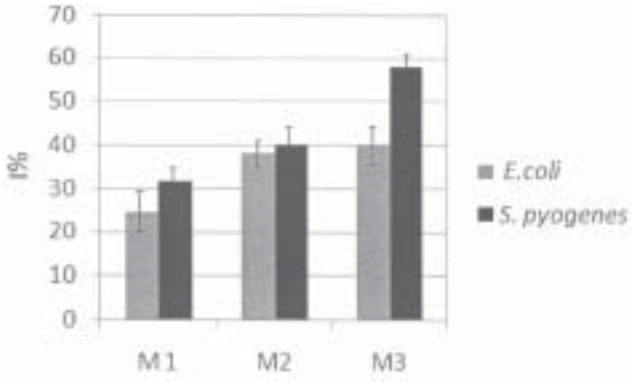

Fig. 7. The index of bacteria inhibition growth

has some small precipitate areas on the surface. The membranes without plant extract were soaked in SBF showed some deposition on their surface. **M2** presents a surface which is more homogeneous with small crystals than **M1**. Based on these results, it can be concluded that the membranes induce the growth of calcium crystals on their surface.

Figure 6 shows the evolution of Ca^{2+} concentration in 2SBF after various periods of immersion.

The calcium ions concentration decreases till the last day of immersion (day 7). This decrease corresponds to the precipitation of calcium on the surface of bioactive membranes to form the apatite layer**.** The slight decrease in Ca^{2+} for membranes **M1** was explained in terms of adsorption of calcium ions on chitosan, forming complexes with the amino groups [34]. In the case of **M2,** after this membrane was mineralized in SBF for 1 day some Ca precipitate was formed.

Contact angle

The contact angle of membranes without extract plant was 52.64 ± 5^0 which showed good hydrophilicity although it was slightly larger than that of the membranes in which the plant extracts are embedded by adsorption (22.54 ± 2^0) and the membrane in which the plant extracts are embedded by including (38 ± 4^0).

Antimicrobial and hemolityc studyes

The results of groowth inhibition of tested bacteria are presented in figure 7. The best inhibition against both bacteria is shown for the M3 with an index as 58. The lowest inhibition factor is 32 for sample M1. The bacteria growth inhibition is higher in the case of Streprococcus pyogenes.

Regarding the hemolysis studies, the membranes showed the following indexes: 1.83 for **M3**, 1.62 for **M2** and 1.36 for **M1**, meaning that all membranes are non-hemolytic considering the recomandations of ASTM F 756-00 [35], where a hemolysis index over 5% means that a material is hemolytic, between 2 and 5% is slightly hemolytic and under 2% is non hemolytic.

Conclusions

The paper presented a method for elaboration and characterization collagen-gelatine membranes with and without extracts plants (adsorbed and including). SEM results indicate that inclusion in the gel method provides more homogeneous membranes. All membranes are hydorphilic, porous and present antibacterial and bioactive properties. The best inhibition against both bacteria is shown for the M3 with an index as 58. The lowest inhibition factor is 32 for sample M1. Their hemolytic activity is low, so these membranes do not act as cytotoxic materials to the red blood cells.

Considering the characteristics which have been studied in this paper, the experimental biomaterials appear to be suitable for medical applications.

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