Spanish Broom (*Spartium junceum* L.) fibers impregnated with vancomycin-loaded chitosan nanoparticles as new antibacterial wound dressing: preparation, characterization and antibacterial activity.

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1. Introduction

Wound dressing as well as devices play an important role in the medical and pharmaceutical wound care market worldwide. Cotton gauze is one of the most successful wound dressings due to the intrinsic properties of cotton fibers. In fact, cotton fibers are highly hydrophilic, absorbent and inexpensive (Edwards et al., 2006). Cotton farming involves environmental risks due to intensive use of pesticides that pollute rivers and groundwater. Moreover, if cotton is cultivated intensively, it requires large amounts of water for irrigation causing soil desalinization and hence a degradation of soil fertility. Taking into account these disadvantages, we explored the potential use of Spanish Broom fibers for wound care. The choice of Spanish Broom fibers as a wound dressing depends on many factors including low cost, availability and hydrophilic character. In fact, Spanish Broom fibers as well as cotton fibers are composed of cellulose and can be extracted by an easy, efficient, convenient and fast physical-chemical process, increasing the possibility of extensive application of these fibers in various fields including pharmaceutical (Cerchiara et al., 2010). Spanish Broom is a small shrub available in Mediterranean countries, where it grows spontaneously. In comparison with flax and hemp, Spanish Broom grows in the most unfavorable limestone soil and once planted it can be used during a period of up to twenty years, whilst hemp and flax demand high quality soil each year.

Cellulose based dressings are prepared in different forms, but they do not possess antibacterial activity. In order to give cotton or other fibers some healing activity, antibiotics such as neomycin, bacitracin, streptomycin, gentamycin and polymixin and/or combinations are used to treat chronic wounds (Boateng et al., 2013). Among the different antibiotics, we used vancomycin (VM) as a model drug for a new modern antibacterial dressing based Spanish broom fibers. VM is a water soluble glycopeptides drug, active against gram-positive bacteria. In literature, therapeutic success of topical application of VM on conjunctivitis, chronic suppurative otitis media and osteomyelitis caused by meticillin-resistant *S. aureus* (MRSA) are reported (Ozcan et al., 2006). Moreover, local delivery of antibiotics is attractive for wound infection prophylaxis because high concentrations are achieved directly at the wound site and systemic toxicity is limited (Yan et al., 2014). However, the slow release of antimicrobial agent from wound dressing has the advantage of treating infected wounds in a mild way (Gomes et al., 2015). So, nanoparticles impregnated into fibers offer great opportunities for improving wounds treatments due to the easier applicability and more uniform dispersion over the wound surface (Aramwit et al., 2016; Romano et al., 2015).

The biomaterial chosen for the preparation of nanoparticles was chitosan (CH), known as biodegradable, nontoxic and biocompatible polymer. CH is widely used as wound dressings and has been shown to have mucoadhesive properties, cationic nature, stimulation of healing, anti-bacterial

and haemostatic properties (Alves et al., 2009; Jayakumar et al., 2010; Dai et al., 2011; Harkins et al, 2014; Romano et al., 2015). Chemically, CH is a natural linear polycationic polymer obtained by partial N-deacetylation of chitin.

In this work, a new antibacterial wound dressing based on Spanish Broom fibers impregnated with CH nanoparticles was developed as drug delivery system for the treatment of infected wounds. Firstly, chemical composition, morphology and tensile properties of Spanish Broom extracted by DiCoDe process varying the experimental conditions were investigated. Then, CH nanoparticles containing VM were prepared using ionic gelation method and different weight ratios of CH and tripolyphosphate (TPP). Nanoparticles were characterized in terms of size, zeta potential, yield, encapsulation efficiency, stability and VM release. Finally, the antibacterial activity against *Staphylococcus aureus* as well as *in vitro* cytotoxicity on HaCaT cells were evaluated.

2. Materials and methods

2.1 Materials

Vancomycin was kindly delivered from Hikma Italia (Pavia, Italy). Low molecular weight chitosan (CH, Mw ≈ 150 kDa, viscosity 20-300 cP, T=20°C, 1 % in 1 % acetic acid; deacetylation degree DD 95 %), penta-sodium tripolyphosphate (TPP), all other chemicals and solvents (HPLC grade) were purchased from Sigma-Aldrich (Milan, Italy).

Phosphate buffer at pH 7.4 and acetate buffer at pH 4.5 were prepared with the following compositions per liter: Na_2HPO_4 x12 H_2O 2.38 g; K_2PO_4 0.19 g; NaCl 8.00 g and $NaCH_3COO$ 8.20 g, CH_3COOH 5.71g, NaCl 0.58 g, respectively.

Spanish Broom fibers were collected from plants cultivated in the Orto Botanico of Calabria University (Italy).

2.2. Extraction of cellulose fibers from Spanish Broom

Spanish Broom fibers were extracted according to the physical-chemical process reported in a previous article, by varying the experimental conditions (NaOH concentration and time of compression-decompression in autoclave) (Cerchiara et al., 2016a). Usually acronym DiCoDe (Digestion-Compression-Decompression) was used to indicate it. Briefly, we prepared two samples: Sample C (control): rough Spanish Broom fibers were extracted by treating the vegetable branches with a 5% (w/w) sodium hydroxide solution at 100 °C for 30 min. The hot sprigs were washed in water to obtain rough fibers that were finally washed in water and dried.

Sample A: prepared as sample C, but fibers were further cleaned from pectin and lignin residues that are decomposed by direct air-oxidation into an autoclave at 120°C and 10 bar pressure for two cycles of 30 minutes. The fibers were finally washed and dried.

2.3. Chemical composition of Spanish Broom fibers

Chemical composition of sample A and C was determined (Cerchiara et al., 2016a). Briefly, the amount of cellulose in the broom fibers was determined using a colorimetric method with the anthrone reagent (Updegraff, 1969). Lignin was determined according to the TAPPI T222 om 02 (2002) method. The ash content of the fiber was determined by weighting the residue remaining after ignition at 575°C for 3 h (Han and Rowell, 1997).

2.4. Mechanical characterization of Spanish Broom fibers

The mechanical properties of Spanish Broom single fibers (samples A and C) were measured by uniaxial tension tests on a Instron 3365 dual column universal testing machine. At least 10 fibers were measured per each extraction process.

Single fibers were carefully extracted from the bundles. Preference was given to thin fibers, as identified with the aid of an optical microscope. In order to facilitate the clamping procedure, each fiber was first mounted on a paper frame composed by two detachable halves with a useful window length of 25 mm, then clamped on the testing machine. Displacement was applied with the constant rate of 2 mm/min and test were carried on until failure of each specimen. Exact fiber size was measured post mortem by optical analysis. This was possible because the fracture behavior of the fibers is brittle, with negligible plastic deformation and low elongation at break, therefore no changes in the section during testing are expected.

From the stress strain curves, the following mechanical parameters were extracted: Young's Modulus E is the slope of the linear region of the stress-strain curve; the strength is the maximum load reached before break; for ease of comparison, data are presented both in MPa and in cN/tex, a common unit for textile materials; elongation at break is the strain corresponding to maximum load.

2.5. Morphological structure

A LEO 420 scanning electron microscope (SEM) was used to observe the morphological features of Spanish Broom fibers. The specimens to be observed were mounted on conductive adhesive tape, sputter coated with gold-palladium and observed in the microscope using an accelerating voltage of 15 kV.

2.6. X- ray diffraction pattern

X-ray powder diffraction (XRPD) was performed to characterize the physical forms (crystalline or amorphous) of the fibers extracted with different experimental conditions. X-ray powder diffractograms were collected on a Panalytical X'Pert Pro automated diffractometer (Almelo, The Netherlands) equipped with X'Celerator, CuKα, using glass sample holder. Tube voltage and amperage were set at 40 kV and 40 mA, respectively. The program used for data collection was set to record only the data points within the range 3–40° 2θ (Hall et al., 2010).

2.7. Preparation of chitosan nanoparticles

CH nanoparticles (NPs) were prepared by ionic gelation method (Abruzzo et al., 2016). Cationic and anionic phase were prepared dissolving CH in acetate buffer (pH 4.5) at concentration range of 2.5-10 mg/mL and TPP in phosphate buffer (pH 7.4), respectively. Chitosan NPs were spontaneously formed by adding dropwise the anionic phase (9 mL) into the cationic phase (1 mL) under constant agitation at room temperature for 15 min. The NP suspensions were centrifuged (10000 rpm, 30 min, T= 25 °C). Supernatants were removed and the NPs re-suspended in deionized water (0.25 mL).
For the preparation of VM-loaded NPs, 50 mg of VM were dissolved in the cationic phase (1 mL) and the final NP suspension was prepared as described before. Unloaded and loaded formulations with different CH/TPP weight ratios and CH concentrations were prepared as shown in Table 2 and 3.

2.7.1. Determination of particle size, polydispersity index and zeta potential

Particle sizes and polydispersities of NPs were measured by photon correlation spectroscopy (PCS) using a Brookhaven 90-PLUS (New-York, USA) with a He-Ne laser beam at a wavelength of 532 nm (scattering of angle 90°) after a sample dilution of 1:10 v/v in ultrapure water. Zeta-potential measurements were carried out at 25 °C using a Malvern Zetasizer 3000 HS (Malvern Instruments ltd., Malvern, UK), after similar dilution. Both the particle size and the zeta-potential measurements were run in triplicate.

2.7.2. Determination of yield, encapsulation efficiency and loading capacity

For the calculation of process yield, the NP suspensions were centrifuged (10,000 rpm, 30 min, T= 25 °C) and the supernatants were discarded. The pellets were dried at 50°C until constant weight, and the actual solid weights were obtained. The yield of the process was calculated as follows (eq. 1):
Eq. 1) % Yield = actual solid weight x 100 / theoretical solid weight

For the calculation of the encapsulation efficiency of VM, loaded NPs were isolated by centrifugation (10,000 rpm, 30 min, T= 25 °C) and the amount of non-entrapped VM was determined in the supernatant (Calderòn et al., 2013) by HPLC method as described in section 2.9.

The particles' drug loading (DL) and the encapsulation efficiency (EE) were calculated using the following equations (eq. 2 and 3, respectively):

Eq. 2) % DL = (Total amount of drug added − Amount of non-entrapped drug) x 100/ NP weight

Eq. 3) % EE = (Total amount of drug added − Amount of non-entrapped drug) x 100 / Total amount of drug.

2.7.3. NP Physical stability

The stability of NPs is one of the most critical issues, as their general tendency is to aggregate upon storage as a suspension (Abruzzo et al., 2016). NPs were tested for their stability in water for 6 hours in order to verify that water can be used as a suitable medium for NP dispersion. Aliquots of fresh NP suspensions were diluted in this media reaching a concentration of 1 mg/mL and the change of NP size and PDI index was measured using PCS at 25°C (n = 3).

2.8. Impregnation of chitosan NPs into Spanish Broom fibers

Before use, Spanish Broom fibers were dried in oven for 24 h; then 20 mg of Spanish Broom fibers were impregnated for 1 h with 0.25 mL of chitosan NP suspension, obtained as described in section 2.6.

2.9. *In vitro* release studies

VM availability from VM solution, chitosan NPs and impregnated Spanish Broom fibers was determined by Franz-type static glass diffusion cell (15 mm jacketed cell with a flatground joint and clear glass with a 12 mL receptor volume, diffusion surface area: 1.77 cm^2), equipped with a V6A Stirrer (PermeGearInc., Hellertown, PA, USA). Spanish Broom fibers (20 mg) were impregnated in 0.25 mL chitosan NPs suspension and then were introduced in the donor compartment of Franz-type cell divided from a receptor compartment by means of cellulose filter (MF-Millipore Membrane, mixed cellulose esters, pore size = 0.45 μm). The receptor compartment was filled with phosphate buffer at pH 7.4 (PBS). The system was thermostated at 37 °C and, at appropriate time intervals, 200 μl aliquots were taken and replaced with the same volume of the fresh buffer (Bigucci et al., 2015). Drug concentration was quantified in the receptor phase by high performance liquid chromatography (HPLC) following the method previously described in Bigucci et al. (2008). Briefly, chromatographic separations were performed using a Shimadzu (model LC-10ATvp) liquid chromatograph connected

to a UV-VIS detector (model SP-10Avp) and to a ChromatoPlus computerized integration system (Shimadzu Corporation, Kyoto, Japan). Manual injections of samples were performed using a Rheodyne 7125 injector with a 20 µl sample loop. A Synergy 4 µm Hydro-RP80A (Phenomenex, Torrance, USA) column was employed and an acetonitrile/sodium phosphate buffer (Na_2HPO_4 x12 H_2O 9.15 g/l adjusted at pH 7.0 with phosphoric acid) 10:90 v/v was used as mobile phase (flow rate of 0.4 ml min^{-1}). Ultraviolet detection was set at 229 nm and the elution time was 15 min. Cotton fibers impregnated with NPs were used as control.

2.10. Antibacterial activity assays

The antibacterial activity of chitosan NP loaded Spanish Broom fibers was evaluated against the Gram-positive bacterium, *Staphylococcus aureus* ATCC 29213. *S. aureus* was cultured in Luria-Bertani broth (LB; Oxoid, UK), aerobically for 24 h at 37 °C and shaking at 130 rpm.

Agar plate diffusion method (Bondock et al., 2013) and overlay method of plating bacteria (Schillinger and Lücke, 1989) were used to determine the antibacterial activity. Briefly, in the agar diffusion method, 100 µL of bacterial suspension, corresponding to 1×10^4 CFU/mL, were inoculated on LB agar plates. Spanish Broom fibers (2 mg) impregnated with chitosan NPs loaded/non loaded with VM were placed on agar plates and left at 4 °C for 45 minutes to permit the diffusion of compounds. In the overlay method, 1×10^8 CFU of bacteria were inoculated in 10 mL of melted LB soft agar (0.7 %), which were poured onto the surface of agar plates containing 2 mg of Spanish Broom fibers impregnated with chitosan NPs loaded/non loaded with VM. LB plates were incubated for 24 h at 37 °C. A solution of VM (20 µL, 3.012 mg/mL) was tested on sterile Whatman filter paper discs (6 mm), as a control. The antibacterial activity was expressed as the diameter of inhibition zone (mm) ± standard deviation. All experiments were performed in triplicate.

2.11. *In vitro* cytotoxic test

2.11.1 Preparation of extracts

For extract preparation, 1 mg of sample (Spanish Broom fibers, Spanish Broom fibers impregnated with VM, Spanish Broom fibers impregnated with unloaded and loaded NPs) was incubated in 12 ml of Dulbecco's modified Eagle's medium (DMEM) for 24 hours so that released substances can be tested (Moritz et al., 2014). Extracts of each dressing were filtrated with 0.45 µm filter and were tested *in vitro* by exposing cultured human keratinocytes to the extracts.

2.11.2 Cell Culture

HaCaT, an immortalized line of human keratinocytes (Boukamp et al., 1988), were grown in DMEM supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin and 10% fecal bovine serum

(FBS). Cells were subcultured in 6 well plates at a density of $1x10^5$ cells/well in the culture medium. After 24 hours and during the exponential phase of cell growth, culture medium was replaced by the extracts of each dressing and cells were cultured for 24, 48 and 72 hours. The controls were cells cultured in medium that were incubated at the same conditions as those used for the extracts.

2.11.3. Cell viability assay (MTT)

Cell survival was determined by estimation of mitochondrial competence in living cells to reduce the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl 2h-tetrazolium bromide), also known as MTT (Mosmann, 1983; Stockert et al., 2012). A stock MTT solution (5 mg/ml in distilled water) was prepared and filtered through a 0.22 µm Millipore filter. After 24, 48 and 72 hours of incubation with original extract of each dressing, the culture medium in well was replaced by fresh medium DMEM containing MTT (solution stock 5 mg/ml) at final concentration of 0.45 mg/ml and incubated for a further 3 hours at 37°C in the dark. Subsequently, the medium was gentle removed and the formazan product was dissolved with dimethylsulfoxide (DMSO). After 1 hour of extraction with shaking, the absorbance of solutions was measured at 540 nm. The MTT assay was performed three times.

2.12. Statistical analysis

All the experiments were done in triplicate. Results are expressed as mean ± SD. t- test was used to determine statistical significance of results. Differences were considered significant for values of $p < 0.05$.

3. Results and Discussion

3.1. Chemical composition of Spanish Broom fibers

The advantages of textiles for wound care applications are due to their excellent qualities, such as strength, extensibility, flexibility, air and moisture permeability, availability in 3-dimensional structures, variety in fibers' length, fineness, cross-sectional shape and geometry, mechanical properties (Petrulyte, 2008). Natural fibers generally used in wound care are cotton, silk and linen. In addition to these natural cellulose based substances (Gupta, 2010), we explored the potential use of Spanish Broom fibers as wound dressing due to advantages such as availability, biodegradability, biocompatibility, non-toxicity and high absorbent properties. Until today, its use in biomedical field was limited by the difficulty to obtain cellulose in sufficiently pure form that is free from lignin and pectin. Consequently, this work concerns the potential use of Spanish Broom fibers for the first time as wound dressing, however traditional applications as textiles and cordage are reported (Gleba, 2008). Moreover, DiCoDe process offers important advantages for the production of Spanish Broom

fibers such as rapid production times and chemical composition comparable to cotton. In fact as cotton fibers, Spanish Broom fibers are predominantly composed of cellulose, as reported in Table 1. The chemical composition of Spanish Broom fibers extracted by DiCoDe process by varying the experimental conditions are similar, suggesting that fibers with good physical-chemical properties are obtained using this mild conditions.

Cellulose content of fibers A increased slightly compared to fibers C, suggesting that these conditions (compression–decompression at 120°C for 30 min) firstly causes the small break of the reticulations inside the fibers influencing their morphology and then determined the partial decomposition by oxidation of the pectin residues. For such reason, the content of components such as pectin in fibers A is lower than in fibers C, while lignin content is similar for both fibers.

3.2. Mechanical characterization of Spanish Broom fibers

Generally, alkali treatments have a lasting effect on the mechanical behavior of fibers, especially on fiber strength and stiffness. This is because mechanical strength and elasticity of the cellulosic fibers depend on different properties such as crystallinity and orientation (Peetla et al., 2006). Spanish Broom fibers extracted by varying the experimental conditions, as reported in section 2.2, showed similar X-ray diffraction pattern (data not shown).

The two extraction methods present similar values of all mechanical parameters, as shown in Figure 1, meaning that differences are not statistically significant ($p > 0.25$). This is attributed to the fact that fibers have similar chemical composition and the autoclave step at these experimental conditions did not deeply influenced the organization of fiber bundles.

Comparison with works from literature gives excellent agreement in terms of strength (Cerchiara et al., 2014) and good agreement in terms of Young's modulus (Angelini et al., 2000).

3.3. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) showed the influence of the two different experimental conditions on fiber morphology. Spanish Broom fibers treated with low concentration of NaOH (5%) for 30 min appeared as bundles of fibers bound by lignin, hemicelluloses, and pectins and a bit rough to the touch (data not shown). Conversely, fibers extracted by DiCoDe at 120°C for 30 min were softer to the touch and were separated (Figure 2) (Cerchiara et al., 2016a) suggesting the potential use of these fibers as wound dressing.

3.4. Preparation of chitosan NPs

Chitosan NPs were prepared through simple, convenient, controllable process adding drop wise TPP solution to the CH cationic phase under gentle magnetic stirring at room temperature. When the anionic phase (based on PBS at pH 7.4 containing TPP) was added to the cationic phase (consisting of CH solubilized in acetate buffer at pH 4.5), CH amino groups (pKa = 6.3) were ionically crosslinked through TPP ions (pH of the final suspension was around 5.5). The ionic gelation process is influenced by different conditions, such as CH and TPP concentrations and the pH of the final suspension (Cerchiara et al., 2016b). For this reason, we prepared many batches to optimize CH and TPP concentrations and CH/TPP weight ratios. Particles' characteristics and the appearance of the suspensions of unloaded and loaded NPs were reported in Table 2 and 3, respectively. With 2.5 mg/mL CH concentration, a lower crosslinking density occurred as suggested by the lower turbidity observed. On the other side, in the presence of CH concentration of 10 mg/ml, NP precipitation was obtained for CH/TPP weight ratios 4:2, 4:3 and 4:4. Instead, the appearance of opalescence was attributed to nanoparticle formation (Cerchiara et al., 2015). The preliminary screening of physicochemical parameters revealed that three CH/TPP weight ratios (4:0.5, 4:1 and 4:2) with 5 mg/mL CH concentration were able to form NPs. Moreover, in this case, the size of NPs increased with the increase of TPP amount, due to TPP crosslinking ability, that leads to the formation of larger structures. Furthermore, unloaded NPs showed lower sizes with respect to loaded NPs, thus demonstrating that VM was included inside NP structure. The three CH/TPP weight ratios (4:0.5, 4:1 and 4:2) with 5 mg/mL CH concentration, were selected for further investigation and named in this study as CH/TPP 4:0.5, CH/TPP 4:1 and CH/TPP 4:2 respectively. Finally, for these formulations, the polydispersity index was lower than 0.3 (0.22-0.21, 0.19-0.23 and 0.18-.24 for CH/TPP 4:0.5, CH/TPP 4:1 and CH/TPP 4:2, respectively) indicating a good particle size distribution, while the zeta potential was positive due to the presence of CH on NP surface. The zeta potential for CH/TPP 4:0.5, CH/TPP 4:1 and CH/TPP 4:2 was +21.7 mV, +21.0 mV and +19.7 mV, respectively (no significant difference was observed between the three formulations, $p > 0.05$).

3.4.1 Determination of yield, encapsulation efficiency and loading capacity

Table 4 reports the yield, encapsulation efficiency and loading capacity of three selected formulations. All formulations showed low encapsulation efficiency due to the small size of the prepared nanoparticles. So, we suggest that the obtained encapsulation efficiency was the maximum capacity for these nanoparticles. As no significant differences can be observed between the three formulations, in order to select the best formulation for wound care, we also performed stability studies.

3.4.2 NP stability

As reported in Figure 3, the formulation based on CH/TPP weight ratio 4:1 showed the best stability among the all NPs. Differently from CH/TPP 4:0.5 and CH/TPP 4:2, CH/TPP 4:1 was probably characterized by a good interaction between all the components, resulting in a better stability over the time and for this reason CH/TPP 4:1 was selected for the next studies.

3.5. In vitro release studies

An ideal wound dressing with effective antibacterial properties should sustain drug release to avoid the frequent changing of the dressing and accelerating the healing process (Zhang et al., 2015).

Figure 4 shows VM availability during time from NPs and fibers (cotton and Spanish Broom) impregnated with loaded NPs with respect to control samples (VM solution, cotton fibers impregnated with VM solution, Spanish Broom fibers impregnated with VM solution). NPs provided lower release (about 30 % of released drug) than all the control samples ($p < 0.05$) due to ability of NPs to interact with drug and control its release. No significant difference ($p > 0.05$) was observed between drug release profile obtained from NPs and Spanish Broom fibers impregnated with loaded NPs, thus indicating that the presence of the fibers does not limit VM release. In addition, when comparing the data of the Spanish Broom fibers impregnated with NPs with those of the cotton fibers, significant differences in VM availability were not observed ($p > 0.05$) indicating that Spanish Broom fibers can successfully replace the cotton fibers.

3.6. Antibacterial activity of chitosan NP loaded Spanish Broom fibers.

The antibacterial activity of Spanish Broom fibers impregnated with chitosan NPs was tested against *S. aureus*, which represent the principal etiologic agent of acute bacterial skin and soft tissue infections. Two different microbiological methods were used in order to have a high reliability of the results. These methods are based on different inocula of *S. aureus*, in terms of CFUs, that reflect the two different operating procedures: inoculum on the surface (agar diffusion method) or submerged inoculum (overlay method). In the second case, the CFUs are higher as the test microorganism is inoculated into a larger volume. Results of the antibacterial activity tests are presented in Table 5. VM (control) and Spanish Broom fibers containing VM showed similar inhibition zones in both agar diffusion method (inhibition zones: 17.98 mm/17.96 mm) and overlay method (inhibition zones: 14.35 mm/14.63 mm). Spanish Broom fibers containing chitosan NPs loaded with VM showed an increased antibacterial activity against *S. aureus* compared to VM and Spanish Broom fibers containing VM, as evaluated by both methods (inhibition zones 22.57 mm/17.80 mm). Meanwhile, Spanish Broom fibers without VM or containing unloaded chitosan NPs did not exert any antibacterial activity. In conclusion, Spanish Broom fibers containing chitosan NPs loaded with VM

have been shown to be promising for wound dressing application for the prevention of skin infections in replacement to common cotton fibers.

3.7. *In vitro* cytotoxic test

To complement antibacterial studies, we performed a simple set of experiments to evaluate the cytotoxicity of Spanish Broom fibers impregnated with loaded NPs against HaCaT cells. In fact, the determination of cytotoxicity is important and necessary since Spanish Broom fibers impregnated with loaded NPs are likely to come into contact with the skin. HaCaT cells, a human keratinocyte cell line, were selected as they constitute a major cellular component of the human skin (Moritz et al., 2014). Figure 5 shows the levels of cytotoxicity of Spanish Broom fibers, Spanish Broom fibers impregnated with VM solution, Spanish Broom fibers impregnated with unloaded and loaded NPs. According to the guideline for determination of *in vitro* cytotoxicity of medical devices (DIN EN ISO 10993-5), materials can be described as non-cytotoxic when the viability of cells is ≥70% after exposure (Moritz et al., 2014). Taking into account this consideration, all tested samples were non-toxic indicating that Spanish Broom fibers and NPs did not influence the proliferation of HaCaT cells. In conclusion, Spanish Broom fibers and NPs are safe and can be applied as wound dressing on the skin without causing any cutaneous adverse effects (Gomes et al., 2015).

4. Conclusions

A new application for Spanish Broom fibers impregnated with antibacterial NPs as a wound dressing is proposed. Spanish Broom fibers, extracted by patented method DiCoDe by varying the experimental conditions, have good physical–chemical properties, such as high mechanical resistance and high elasticity, and rapid production times. Moreover, Spanish Broom fibers impregnated with NPs showed a good antibacterial activity against *S. aureus* and were not toxic to HaCaT keratinocytes cells. In conclusion, Spanish Broom fibers can successfully replace the cotton in wound care and used as medicated dressing for potential active wound healing in infected wounds.

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Conflict of interest

The Authors declare that they have no conflicts of interest to disclose.

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Tables

Table 1. Chemical composition of Spanish Broom fibers extracted by DiCoDe method with different experimental conditions (mean ± SD, *n* = 3).

Table 2. Size (nm) and appearance of unloaded NPs prepared with different CH concentrations and CH/TPP weight ratios (mean ± SD, *n* = 3).

Table 3. Size (nm) and appearance of loaded NPs prepared with different CH concentrations and CH/TPP weight ratios (mean ± SD, *n* = 3).

Table 4. Yield, Encapsulation efficiency (EE) and Drug loading (DL) of loaded NPs obtained with 5 mg/mL CH concentration (mean ± SD, *n* = 3).

Table 5. Zones of inhibition (mm) of Spanish Broom fibers and vancomycin (VM) evaluated by agar plate diffusion method and overlay method. (mean ± SD, *n* = 3)..

Figure captions

Figure 1. Mechanical characterization of single fibers, prepared with both A and C extraction methods: a) Young's modulus, b) elongation at break, c) tensile strength. The differences are not significant: for Young's modulus p=0.591, for elongation p = 0.294, for tensile strength p = 0.250

Figure 2. (A) Photographic image and (B) SEM of Spanish Broom sample A (1000X)

Figure 3. Stability of CH/TPP 4:0.5, CH/TPP 4:1 and CH/TPP 4:2 nanoparticles (5 mg/mL CH concentration) in water over the time (mean ± SD, $n = 3$).

Figure 4. *In vitro* VM release from NPs (CH/TPP 4:1, 5mg/mL CH concentration), cotton fibers impreagnated with VM solution (Cotton+ VM) or loaded NPs (Cotton + NPs), Spanish Broom impregnated with VM solution (SB + VM) or NPs (SB + NPs) and the dissolution profile of VM (used as control). Mt = drug amount released over time, M0 = drug amount at time zero (mean ± SD, $n = 3$).

Figure 5. Viability of HaCaT human keratinocytes cells cultured with different extracts (mean ± SD, $n = 3$).

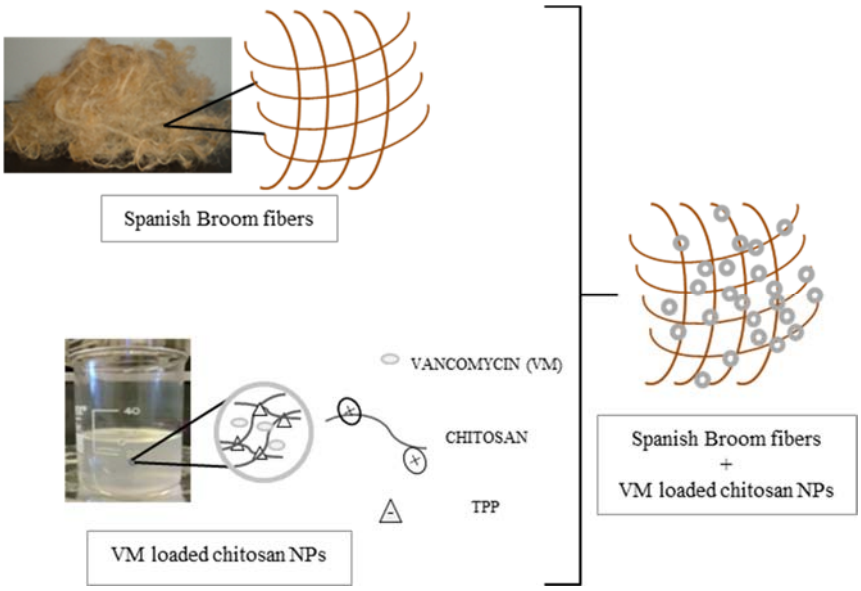

Spanish Broom fibers
VANCOMYCIN (VM)
CHITOSAN
TPP
VM loaded chitosan NPs
Spanish Broom fibers
+
VM loaded chitosan NPs

Table 1. Chemical composition of Spanish Broom fibers extracted by DiCoDe method with different experimental conditions (mean ± SD, $n = 3$).

	Sample C	Sample A
Cellulose (%)	70.20±0.03	72.40±0.05
Lignin (%)	5.84±0.06	5.63±0.09
Other components (hemicellulose, pectins) (%)	23.35±0.07	21.34±0.02
Ash (%)	0.42±0.05	0.41±0.06

Table 2. Size (nm) and appearance of unloaded NPs prepared with different CH concentrations and CH/TPP weight ratios (mean ± SD, *n* = 3).

CH concentration (mg/mL)	CH/TPP weight ratios				
	4/0.5	4/1	4/2	4/3	4/4
10	160 ± 5[a]	180 ± 9[a]	[b]	[b]	[b]
5	159 ± 6[a]	162 ± 4[a]	197 ± 5[a]	[b]	[b]
2.5	[c]	[c]	[c]	285 ± 7[d]	393 ± 10[a]

[a]= opalescent

[b]= precipitation occurred

[c]= clear

[d]= weakly opalescent

Table 3. Size (nm) and appearance of loaded NPs prepared with different CH concentrations and CH/TPP weight ratios (mean ± SD, *n* = 3).

CH concentration (mg/mL)	CH/TPP weight ratios				
	4/0.5	4/1	4/2	4/3	4/4
10	256 ± 3[a]	313 ± 5[a]	[b]	[b]	[b]
5	239 ± 6[a]	247 ± 4[a]	287 ± 5[a]	[b]	[b]
2.5	[c]	[c]	[c]	335 ± 8[d]	453 ± 12[a]

[a]= opalescent

[b]= precipitation occurred

[c]= clear

[d]= weakly opalescent

Table 4. Yield, Encapsulation efficiency (EE) and Drug loading (DL) of loaded NPs obtained with 5 mg/mL CH concentration (mean ± SD, *n* = 3).

Weight ratios CH/TPP	Yield %	EE%	DL %
4/0.5	41.45 ± 4.88	24.13 ± 5.90	52.32 ±12.28
4/1	34.04 ± 5.71	22.86 ± 7.69	59.69 ± 5.48
4/2	35.35 ± 3.88	22.74 ± 7.61	55.92 ± 5.52

Table 5. Zones of inhibition (mm) of Spanish Broom fibers and vancomycin (VM) evaluated by agar plate diffusion method and overlay method. Results are expressed as mean ± standard deviation (mean ± SD, n = 3).

Treatments	Agar plate diffusion method	Overlay method
Spanish broom fibers	0.00 ± 0.00	0.00 ± 0.00
VM	17.98 ± 0.21	14.35 ± 0.43
Spanish broom fibers + VM	17.96 ± 0.37	14.63 ± 0.62
Spanish broom fibers + unloaded chitosan NPs	0.00 ± 0.00	0.00 ± 0.00
Spanish broom fibers + VM loaded chitosan NPs	22.57 ± 0.23	17.80 ± 0.48

Figure 1

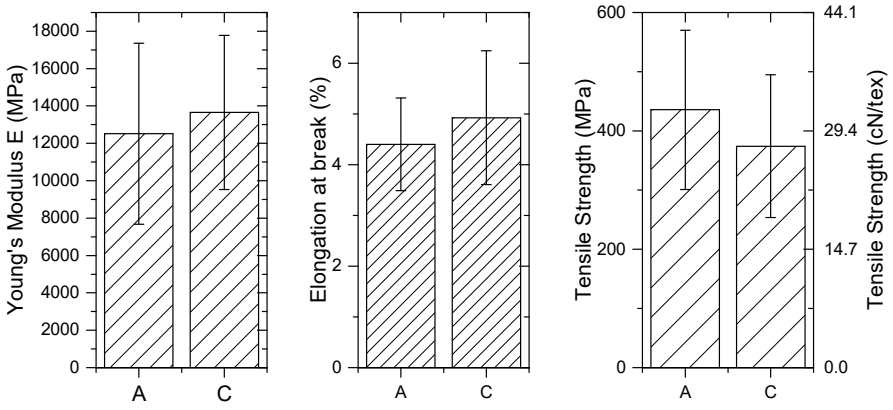

Young's Modulus E (MPa)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
A
C
Elongation at break (%)
6
4
2
0
A
C
Tensile Strength (MPa)
600
400
200
0
A
C
Tensile Strength (cN/tex)
44.1
29.4
14.7
0.0

Figure 2

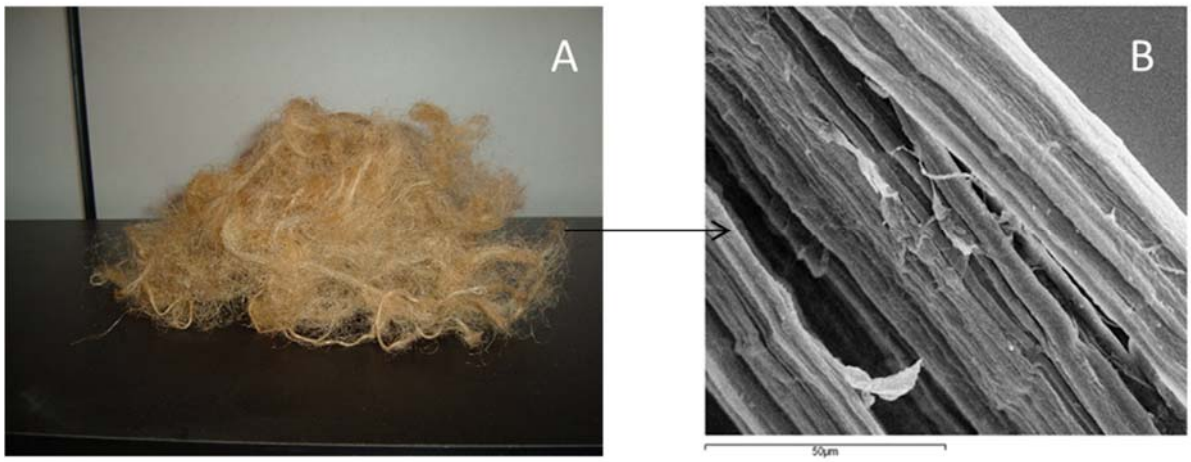


Figure 3.

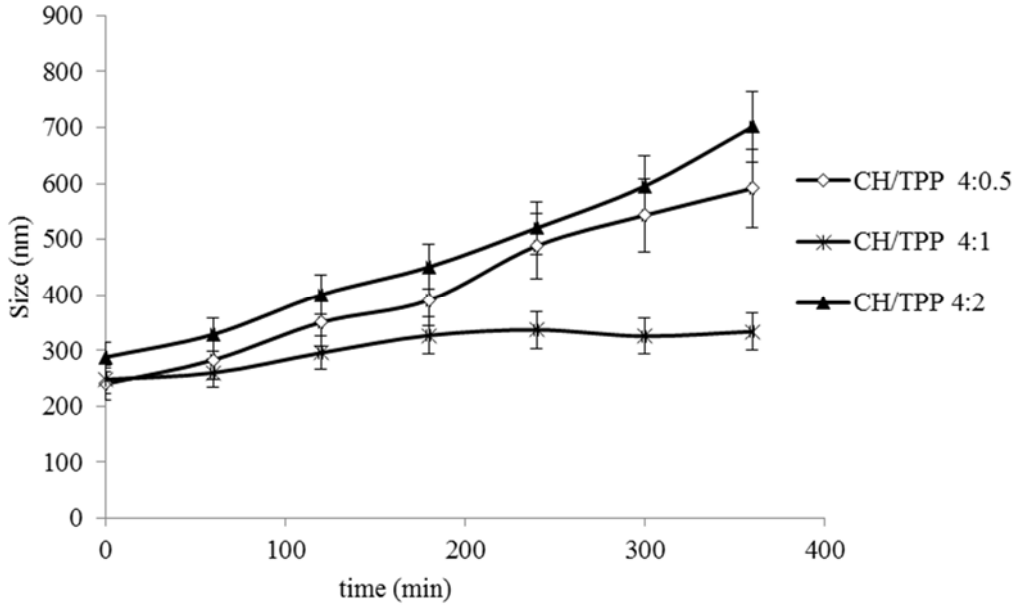

900
800
700
600
500
400
300
200
100
0
Size (nm)
0
100
200
300
400
time (min)
CH/TPP 4:0.5
CH/TPP 4:1
CH/TPP 4:2

Figure 4.

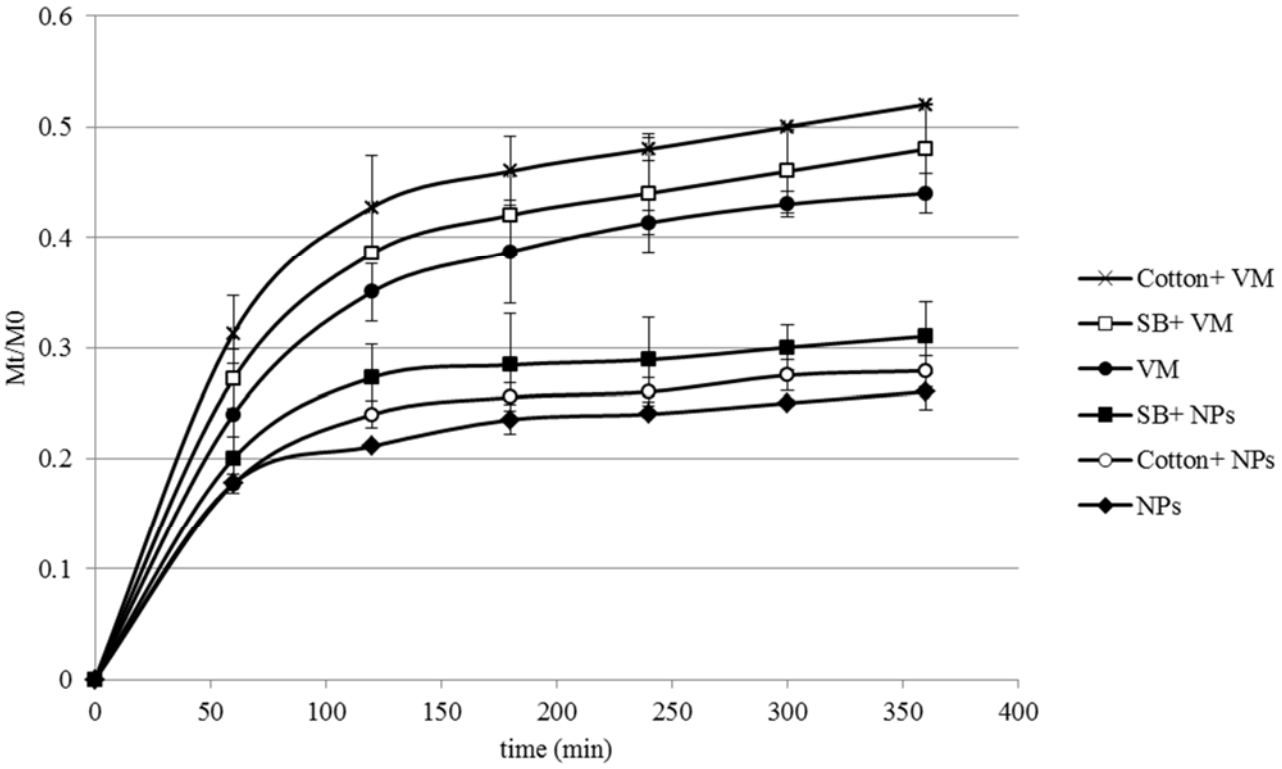
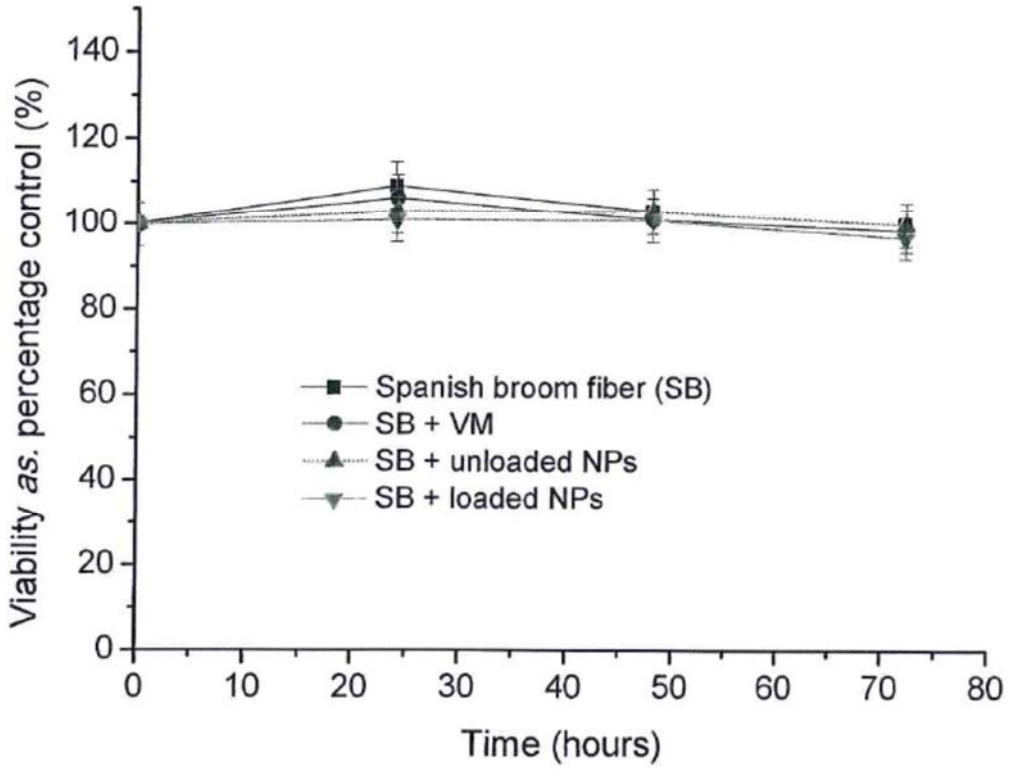

0.6
0.5
0.4
0.3
0.2
0.1
0
Mt/M0
0
50
100
150
200
250
300
350
400
time (min)
Cotton+ VM
SB+ VM
VM
SB+ NPs
Cotton+ NPs
NPs

Figure 5.


Viability as. percentage control (%)
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
Time (hours)
Spanish broom fiber (SB)
SB + VM
SB + unloaded NPs
SB + loaded NPs