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

4  
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## 1 **1. Introduction**

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

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

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

1 and haemostatic properties (Alves et al., 2009; Jayakumar et al., 2010; Dai et al., 2011; Harkins et al,  
2 2014; Romano et al., 2015). Chemically, CH is a natural linear polycationic polymer obtained by  
3 partial N-deacetylation of chitin.

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

12

## 13 **2. Materials and methods**

14

### 15 **2.1 Materials**

16 Vancomycin was kindly delivered from Hikma Italia (Pavia, Italy). Low molecular weight chitosan  
17 (CH, Mw  $\approx$  150 kDa, viscosity 20-300 cP, T=20°C, 1 % in 1 % acetic acid; deacetylation degree DD  
18 95 %), penta-sodium tripolyphosphate (TPP), all other chemicals and solvents (HPLC grade) were  
19 purchased from Sigma-Aldrich (Milan, Italy).

20 Phosphate buffer at pH 7.4 and acetate buffer at pH 4.5 were prepared with the following  
21 compositions per liter: Na<sub>2</sub>HPO<sub>4</sub> x12 H<sub>2</sub>O 2.38 g; K<sub>2</sub>PO<sub>4</sub> 0.19 g; NaCl 8.00 g and NaCH<sub>3</sub>COO 8.20  
22 g, CH<sub>3</sub>COOH 5.71g, NaCl 0.58 g, respectively.

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

25

### 26 **2.2. Extraction of cellulose fibers from Spanish Broom**

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

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

### 4 5 **2.3. Chemical composition of Spanish Broom fibers**

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

### 11 12 **2.4. Mechanical characterization of Spanish Broom fibers**

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

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

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

### 28 29 **2.5. Morphological structure**

30 A LEO 420 scanning electron microscope (SEM) was used to observe the morphological features of  
31 Spanish Broom fibers. The specimens to be observed were mounted on conductive adhesive tape,  
32 sputter coated with gold-palladium and observed in the microscope using an accelerating voltage of  
33 15 kV.

34

## 1 **2.6. X- ray diffraction pattern**

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

## 9 **2.7. Preparation of chitosan nanoparticles**

10 CH nanoparticles (NPs) were prepared by ionic gelation method (Abruzzo et al., 2016). Cationic and  
11 anionic phase were prepared dissolving CH in acetate buffer (pH 4.5) at concentration range of 2.5-  
12 10 mg/mL and TPP in phosphate buffer (pH 7.4), respectively. Chitosan NPs were spontaneously  
13 formed by adding dropwise the anionic phase (9 mL) into the cationic phase (1 mL) under constant  
14 agitation at room temperature for 15 min. The NP suspensions were centrifuged (10000 rpm, 30 min,  
15 T= 25 °C). Supernatants were removed and the NPs re-suspended in deionized water (0.25 mL).

16 For the preparation of VM-loaded NPs, 50 mg of VM were dissolved in the cationic phase (1 mL)  
17 and the final NP suspension was prepared as described before. Unloaded and loaded formulations  
18 with different CH/TPP weight ratios and CH concentrations were prepared as shown in Table 2 and  
19 3.

### 21 **2.7.1. Determination of particle size, polydispersity index and zeta potential**

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

### 29 **2.7.2. Determination of yield, encapsulation efficiency and loading capacity**

30 For the calculation of process yield, the NP suspensions were centrifuged (10,000 rpm, 30 min, T=  
31 25 °C) and the supernatants were discarded. The pellets were dried at 50°C until constant weight, and  
32 the actual solid weights were obtained. The yield of the process was calculated as follows (eq. 1):

33 Eq. 1) % Yield = actual solid weight x 100 / theoretical solid weight

1 For the calculation of the encapsulation efficiency of VM, loaded NPs were isolated by centrifugation  
2 (10,000 rpm, 30 min, T= 25 °C) and the amount of non-entrapped VM was determined in the  
3 supernatant (Calderòn et al., 2013) by HPLC method as described in section 2.9.

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

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

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

9

### 10 **2.7.3. NP Physical stability**

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

16

### 17 **2.8. Impregnation of chitosan NPs into Spanish Broom fibers**

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

21

### 22 **2.9. *In vitro* release studies**

23 VM availability from VM solution, chitosan NPs and impregnated Spanish Broom fibers was  
24 determined by Franz-type static glass diffusion cell (15 mm jacketed cell with a flatground joint and  
25 clear glass with a 12 mL receptor volume, diffusion surface area: 1.77 cm<sup>2</sup>), equipped with a V6A  
26 Stirrer (PermeGearInc., Hellertown, PA, USA). Spanish Broom fibers (20 mg) were impregnated in  
27 0.25 mL chitosan NPs suspension and then were introduced in the donor compartment of Franz-type  
28 cell divided from a receptor compartment by means of cellulose filter (MF-Millipore Membrane,  
29 mixed cellulose esters, pore size = 0.45 µm). The receptor compartment was filled with phosphate  
30 buffer at pH 7.4 (PBS). The system was thermostated at 37 °C and, at appropriate time intervals, 200  
31 µl aliquots were taken and replaced with the same volume of the fresh buffer (Bigucci et al., 2015).  
32 Drug concentration was quantified in the receptor phase by high performance liquid chromatography  
33 (HPLC) following the method previously described in Bigucci et al. (2008). Briefly, chromatographic  
34 separations were performed using a Shimadzu (model LC-10ATvp) liquid chromatograph connected



1 to a UV-VIS detector (model SP-10Avp) and to a ChromatoPlus computerized integration system  
2 (Shimadzu Corporation, Kyoto, Japan). Manual injections of samples were performed using a  
3 Rheodyne 7125 injector with a 20  $\mu$ l sample loop. A Synergy 4  $\mu$ m Hydro-RP80A (Phenomenex,  
4 Torrance, USA) column was employed and an acetonitrile/sodium phosphate buffer ( $\text{Na}_2\text{HPO}_4 \times 12$   
5  $\text{H}_2\text{O}$  9.15 g/l adjusted at pH 7.0 with phosphoric acid) 10:90 v/v was used as mobile phase (flow rate  
6 of 0.4 ml  $\text{min}^{-1}$ ). Ultraviolet detection was set at 229 nm and the elution time was 15 min. Cotton  
7 fibers impregnated with NPs were used as control.

8

## 9 **2.10. Antibacterial activity assays**

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

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

24

## 25 **2.11. *In vitro* cytotoxic test**

### 26 2.11.1 Preparation of extracts

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

### 32 2.11.2 Cell Culture

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

1 (FBS). Cells were subcultured in 6 well plates at a density of  $1 \times 10^5$  cells/well in the culture medium.  
2 After 24 hours and during the exponential phase of cell growth, culture medium was replaced by the  
3 extracts of each dressing and cells were cultured for 24, 48 and 72 hours. The controls were cells  
4 cultured in medium that were incubated at the same conditions as those used for the extracts.

### 5 2.11.3. Cell viability assay (MTT)

6 Cell survival was determined by estimation of mitochondrial competence in living cells to reduce the  
7 (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl 2h-tetrazolium bromide), also known as MTT (Mosmann,  
8 1983; Stockert et al., 2012). A stock MTT solution (5 mg/ml in distilled water) was prepared and  
9 filtered through a 0.22  $\mu\text{m}$  Millipore filter. After 24, 48 and 72 hours of incubation with original  
10 extract of each dressing, the culture medium in well was replaced by fresh medium DMEM containing  
11 MTT (solution stock 5 mg/ml) at final concentration of 0.45 mg/ml and incubated for a further 3  
12 hours at 37°C in the dark. Subsequently, the medium was gently removed and the formazan product  
13 was dissolved with dimethylsulfoxide (DMSO). After 1 hour of extraction with shaking, the  
14 absorbance of solutions was measured at 540 nm. The MTT assay was performed three times.

15

## 16 2.12. Statistical analysis

17 All the experiments were done in triplicate. Results are expressed as mean  $\pm$  SD. t- test was used to  
18 determine statistical significance of results. Differences were considered significant for values of  $p <$   
19 0.05.

20

## 21 3. Results and Discussion

22

### 23 3.1. Chemical composition of Spanish Broom fibers

24 The advantages of textiles for wound care applications are due to their excellent qualities, such as  
25 strength, extensibility, flexibility, air and moisture permeability, availability in 3-dimensional  
26 structures, variety in fibers' length, fineness, cross-sectional shape and geometry, mechanical  
27 properties (Petrulyte, 2008). Natural fibers generally used in wound care are cotton, silk and linen. In  
28 addition to these natural cellulose based substances (Gupta, 2010), we explored the potential use of  
29 Spanish Broom fibers as wound dressing due to advantages such as availability, biodegradability,  
30 biocompatibility, non-toxicity and high absorbent properties. Until today, its use in biomedical field  
31 was limited by the difficulty to obtain cellulose in sufficiently pure form that is free from lignin and  
32 pectin. Consequently, this work concerns the potential use of Spanish Broom fibers for the first time  
33 as wound dressing, however traditional applications as textiles and cordage are reported (Gleba,  
34 2008). Moreover, DiCoDe process offers important advantages for the production of Spanish Broom

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

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

11

### 12 **3.2. Mechanical characterization of Spanish Broom fibers**

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

18 The two extraction methods present similar values of all mechanical parameters, as shown in Figure  
19 1, meaning that differences are not statistically significant ( $p > 0.25$ ). This is attributed to the fact that  
20 fibers have similar chemical composition and the autoclave step at these experimental conditions did  
21 not deeply influenced the organization of fiber bundles.

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

24

### 25 **3.3. Scanning Electron Microscopy (SEM)**

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

32

### 33 **3.4. Preparation of chitosan NPs**

1 Chitosan NPs were prepared through simple, convenient, controllable process adding drop wise TPP  
2 solution to the CH cationic phase under gentle magnetic stirring at room temperature. When the  
3 anionic phase (based on PBS at pH 7.4 containing TPP) was added to the cationic phase (consisting  
4 of CH solubilized in acetate buffer at pH 4.5), CH amino groups ( $pK_a = 6.3$ ) were ionically  
5 crosslinked through TPP ions (pH of the final suspension was around 5.5). The ionic gelation process  
6 is influenced by different conditions, such as CH and TPP concentrations and the pH of the final  
7 suspension (Cerchiara et al., 2016b). For this reason, we prepared many batches to optimize CH and  
8 TPP concentrations and CH/TPP weight ratios. Particles' characteristics and the appearance of the  
9 suspensions of unloaded and loaded NPs were reported in Table 2 and 3, respectively. With 2.5  
10 mg/mL CH concentration, a lower crosslinking density occurred as suggested by the lower turbidity  
11 observed. On the other side, in the presence of CH concentration of 10 mg/ml, NP precipitation was  
12 obtained for CH/TPP weight ratios 4:2, 4:3 and 4:4. Instead, the appearance of opalescence was  
13 attributed to nanoparticle formation (Cerchiara et al., 2015). The preliminary screening of  
14 physicochemical parameters revealed that three CH/TPP weight ratios (4:0.5, 4:1 and 4:2) with 5  
15 mg/mL CH concentration were able to form NPs. Moreover, in this case, the size of NPs increased  
16 with the increase of TPP amount, due to TPP crosslinking ability, that leads to the formation of larger  
17 structures. Furthermore, unloaded NPs showed lower sizes with respect to loaded NPs, thus  
18 demonstrating that VM was included inside NP structure. The three CH/TPP weight ratios (4:0.5, 4:1  
19 and 4:2) with 5 mg/mL CH concentration, were selected for further investigation and named in this  
20 study as CH/TPP 4:0.5, CH/TPP 4:1 and CH/TPP 4:2 respectively. Finally, for these formulations,  
21 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,  
22 CH/TPP 4:1 and CH/TPP 4:2, respectively) indicating a good particle size distribution, while the zeta  
23 potential was positive due to the presence of CH on NP surface. The zeta potential for CH/TPP 4:0.5,  
24 CH/TPP 4:1 and CH/TPP 4:2 was +21.7 mV, +21.0 mV and +19.7 mV, respectively (no significant  
25 difference was observed between the three formulations,  $p > 0.05$ ).

26

### 27 **3.4.1 Determination of yield, encapsulation efficiency and loading capacity**

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

33

### 34 **3.4.2 NP stability**

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

5

### 6 **3.5. *In vitro* release studies**

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

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

19

### 20 **3.6. Antibacterial activity of chitosan NP loaded Spanish Broom fibers.**

21 The antibacterial activity of Spanish Broom fibers impregnated with chitosan NPs was tested against  
22 *S. aureus*, which represent the principal etiologic agent of acute bacterial skin and soft tissue  
23 infections. Two different microbiological methods were used in order to have a high reliability of the  
24 results. These methods are based on different inocula of *S. aureus*, in terms of CFUs, that reflect the  
25 two different operating procedures: inoculum on the surface (agar diffusion method) or submerged  
26 inoculum (overlay method). In the second case, the CFUs are higher as the test microorganism is  
27 inoculated into a larger volume. Results of the antibacterial activity tests are presented in Table 5.  
28 VM (control) and Spanish Broom fibers containing VM showed similar inhibition zones in both agar  
29 diffusion method (inhibition zones: 17.98 mm/17.96 mm) and overlay method (inhibition zones:  
30 14.35 mm/14.63 mm). Spanish Broom fibers containing chitosan NPs loaded with VM showed an  
31 increased antibacterial activity against *S. aureus* compared to VM and Spanish Broom fibers  
32 containing VM, as evaluated by both methods (inhibition zones 22.57 mm/17.80 mm). Meanwhile,  
33 Spanish Broom fibers without VM or containing unloaded chitosan NPs did not exert any  
34 antibacterial activity. In conclusion, Spanish Broom fibers containing chitosan NPs loaded with VM

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

3

### 4 **3.7. *In vitro* cytotoxic test**

5 To complement antibacterial studies, we performed a simple set of experiments to evaluate the  
6 cytotoxicity of Spanish Broom fibers impregnated with loaded NPs against HaCaT cells. In fact, the  
7 determination of cytotoxicity is important and necessary since Spanish Broom fibers impregnated  
8 with loaded NPs are likely to come into contact with the skin. HaCaT cells, a human keratinocyte cell  
9 line, were selected as they constitute a major cellular component of the human skin (Moritz et al.,  
10 2014). Figure 5 shows the levels of cytotoxicity of Spanish Broom fibers, Spanish Broom fibers  
11 impregnated with VM solution, Spanish Broom fibers impregnated with unloaded and loaded NPs.  
12 According to the guideline for determination of *in vitro* cytotoxicity of medical devices (DIN EN ISO  
13 10993-5), materials can be described as non-cytotoxic when the viability of cells is  $\geq 70\%$  after  
14 exposure (Moritz et al., 2014). Taking into account this consideration, all tested samples were non-  
15 toxic indicating that Spanish Broom fibers and NPs did not influence the proliferation of HaCaT cells.  
16 In conclusion, Spanish Broom fibers and NPs are safe and can be applied as wound dressing on the  
17 skin without causing any cutaneous adverse effects (Gomes et al., 2015).

18

## 19 **4. Conclusions**

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

27

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31

## 32 **Conflict of interest**

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

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1 **Tables**

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

4  
5 Table 2. Size (nm) and appearance of unloaded NPs prepared with different CH concentrations and  
6 CH/TPP weight ratios (mean  $\pm$  SD,  $n = 3$ ).

7  
8 Table 3. Size (nm) and appearance of loaded NPs prepared with different CH concentrations and  
9 CH/TPP weight ratios (mean  $\pm$  SD,  $n = 3$ ).

10  
11 Table 4. Yield, Encapsulation efficiency (EE) and Drug loading (DL) of loaded NPs obtained with 5  
12 mg/mL CH concentration (mean  $\pm$  SD,  $n = 3$ ).

13  
14 Table 5. Zones of inhibition (mm) of Spanish Broom fibers and vancomycin (VM) evaluated by agar  
15 plate diffusion method and overlay method. (mean  $\pm$  SD,  $n = 3$ )..

## Figure captions

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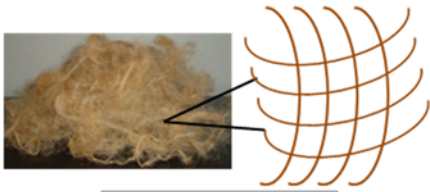
Figure 1. Mechanical characterization of single fibers, prepared with both A and C extraction methods: a) Young's modulus, b) elongation at break, c) tensile strength. The differences are not significant: for Young's modulus  $p=0.591$ , for elongation  $p = 0.294$ , for tensile strength  $p = 0.250$

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

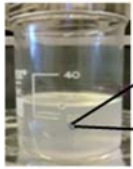
Figure 3. Stability of CH/TPP 4:0.5, CH/TPP 4:1 and CH/TPP 4:2 nanoparticles (5 mg/mL CH concentration) in water over the time (mean  $\pm$  SD,  $n = 3$ ).

Figure 4. *In vitro* VM release from NPs (CH/TPP 4:1, 5mg/mL CH concentration), cotton fibers impregnated 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).  $M_t$  = drug amount released over time,  $M_0$  = drug amount at time zero (mean  $\pm$  SD,  $n = 3$ ).

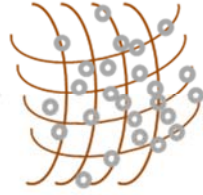
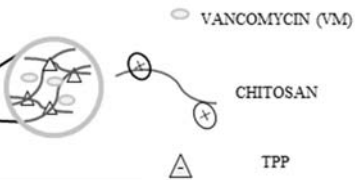
Figure 5. Viability of HaCaT human keratinocytes cells cultured with different extracts (mean  $\pm$  SD,  $n = 3$ ).



Spanish Broom fibers



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  $\pm$  SD,  $n = 3$ ).

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

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

CH concentration (mg/mL)	CH/TPP weight ratios				
	4/0.5	4/1	4/2	4/3	4/4
10	160 $\pm$ 5 <sup>a</sup>	180 $\pm$ 9 <sup>a</sup>	b	b	b
5	159 $\pm$ 6 <sup>a</sup>	162 $\pm$ 4 <sup>a</sup>	197 $\pm$ 5 <sup>a</sup>	b	b
2.5	c	c	c	285 $\pm$ 7 <sup>d</sup>	393 $\pm$ 10 <sup>a</sup>

<sup>a</sup>= opalescent

<sup>b</sup>= precipitation occurred

<sup>c</sup>= clear

<sup>d</sup>= weakly opalescent



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

CH concentration (mg/mL)	CH/TPP weight ratios				
	4/0.5	4/1	4/2	4/3	4/4
10	256 $\pm$ 3 <sup>a</sup>	313 $\pm$ 5 <sup>a</sup>	b	b	b
5	239 $\pm$ 6 <sup>a</sup>	247 $\pm$ 4 <sup>a</sup>	287 $\pm$ 5 <sup>a</sup>	b	b
2.5	c	c	c	335 $\pm$ 8 <sup>d</sup>	453 $\pm$ 12 <sup>a</sup>

<sup>a</sup>= opalescent

<sup>b</sup>= precipitation occurred

<sup>c</sup>= clear

<sup>d</sup>= weakly opalescent

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

Weight ratios CH/TPP	Yield %	EE%	DL %
4/0.5	41.45 $\pm$ 4.88	24.13 $\pm$ 5.90	52.32 $\pm$ 12.28
4/1	34.04 $\pm$ 5.71	22.86 $\pm$ 7.69	59.69 $\pm$ 5.48
4/2	35.35 $\pm$ 3.88	22.74 $\pm$ 7.61	55.92 $\pm$ 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  $\pm$  standard deviation (mean  $\pm$  SD,  $n = 3$ ).

<b>Treatments</b>	<b>Agar plate diffusion method</b>	<b>Overlay method</b>
Spanish broom fibers	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
VM	17.98 $\pm$ 0.21	14.35 $\pm$ 0.43
Spanish broom fibers + VM	17.96 $\pm$ 0.37	14.63 $\pm$ 0.62
Spanish broom fibers + unloaded chitosan NPs	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Spanish broom fibers + VM loaded chitosan NPs	22.57 $\pm$ 0.23	17.80 $\pm$ 0.48

Figure 1

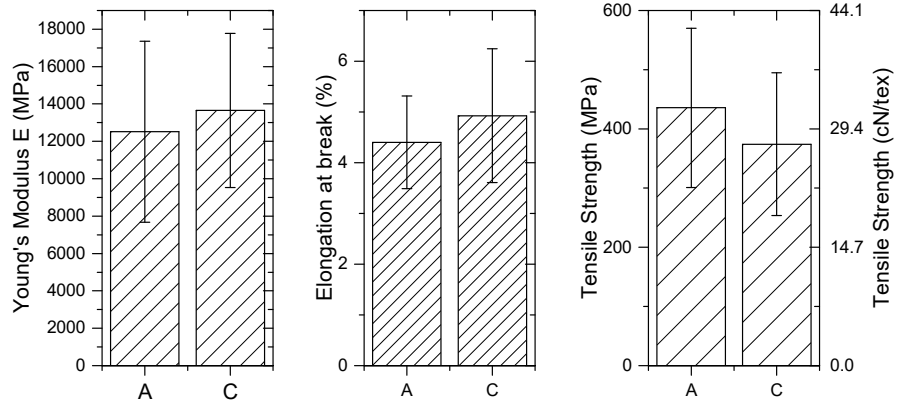


Figure 2

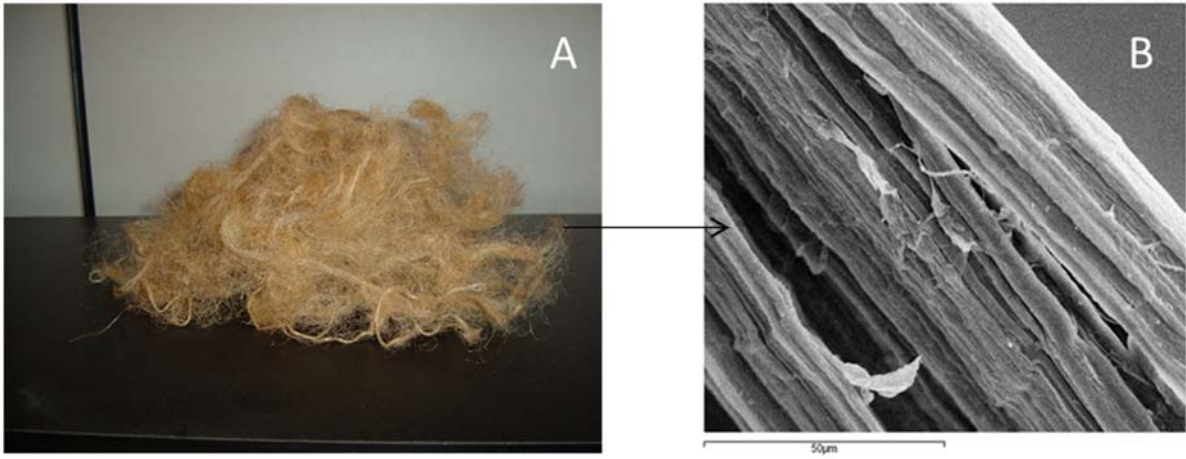


Figure 3.

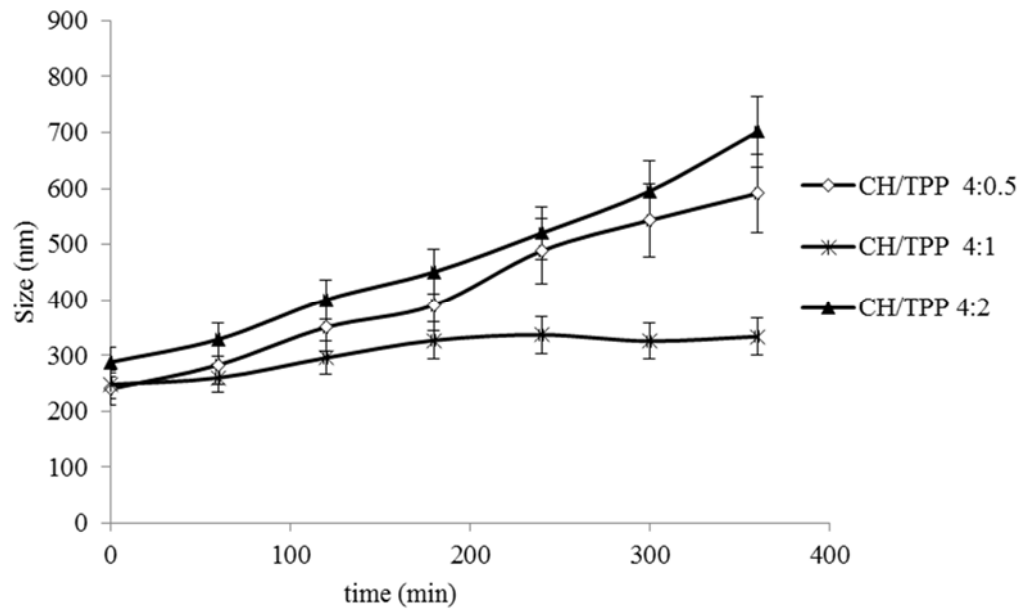


Figure 4.

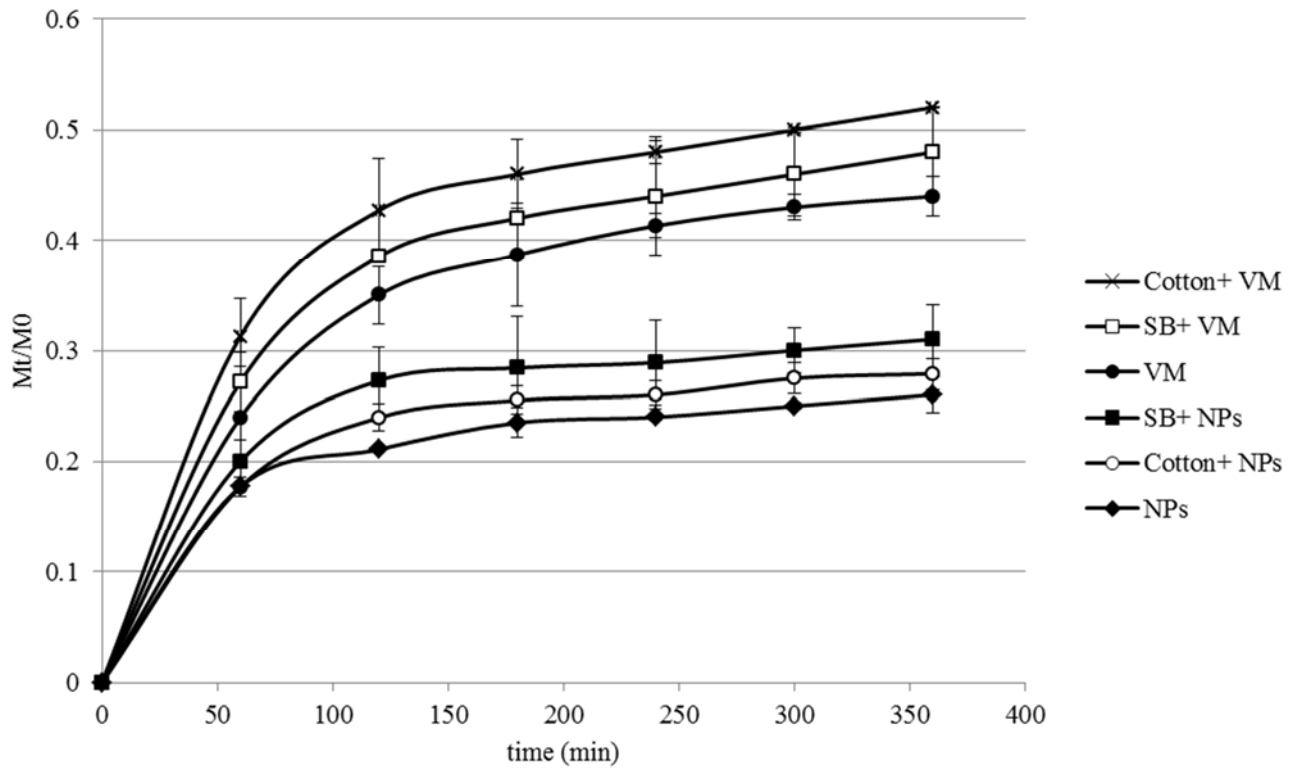


Figure 5.

