

Manuscript Details

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Title	Intermolecular interaction and solid state characterization of abietic acid/chitosan solid dispersions possessing antimicrobial and antioxidant properties.
Article type	Research Paper

Abstract

The aim of this work was to prepare and characterize solid dispersions of abietic acid (AB) and chitosan (CS) to investigate how formulation of the mixture may help in the battle against microbial colonization in different areas, such as the biomedical field or the food industry. Solid dispersions were characterized by differential scanning calorimetry, infrared spectroscopy, Raman spectroscopy, polarized optical microscopy, zeta potential and size analysis. The data showed that the dispersion/solvent evaporation method formed solid dispersions in which abietic acid was molecularly dispersed in the carrier. A synergistic effect between the two components in terms of antioxidant and antimicrobial properties was found, especially in the formulations obtained with 1/1 AB/CS molar ratio. Interestingly, the aggregation state (amorphous/crystalline) of AB seemed to affect the antimicrobial activity of the formulation, suggesting increased bioactivity when the drug was in the amorphous state. These findings, together with the demonstrated biocompatibility of the formulations, seem to open promising perspectives for a successful application of the developed AB/CS formulations in the biomedical field or in the food industry.

Keywords	Chitosan; abietic acid; solid dispersion; antimicrobial formulations; antioxidant formulations
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Suggested reviewers	Alexandra Muñoz-Bonilla, Birthe Venø Kjellerup, Fabiana Quaglia

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To the Editor

European Journal of Pharmaceutics and Biopharmaceutics

Dear Prof. Nicolas Bertrand,

Thank you for considering our work for publication in European Journal of Pharmaceutics and Biopharmaceutics.

We have addressed the reviewer's comments and revised the manuscript accordingly. All revisions are in red in the manuscript.

I hope that now the manuscript is suitable for publication.

Kind regards,

Iolanda Francolini

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Reviewer 1

This manuscript presents the comparison between two methods of mixing chitosan (CS) and abietic acid (AB). The first method is based on mechanical mixing, whereas the second method is based on dissolution. The two series of blending were characterized by DSC, IR, Raman and POM. Moreover, the antibacterial and antioxidant properties of those mixing were evaluated. The manuscript is well written and the language is good. However, several points are risen through this manuscript.

1) The major point is the characterization of the CS/abietic acid blends obtained by dissolution. Chitosan was first solubilized in acetic acid before to be mixed with abietic acid. Chitosan was protonated acetic acid and a solution of chitosan acetate was obtained. Chitosan acetate was further mixed with abietic acid and the resulting solution was freeze dried. No purification was realized. The content in acetate was not determined as well as the ratio of protonated and non-protonated abietic acid. The exact composition of these blends is not known and will depend of the experimental conditions. The lack of characterization affects the discussion through the manuscript. As example, what are the antibacterial properties of abietic acid, abietate and acetate ions?

We apologize to have left out the information about the purification of CS/AB blends during the description of their preparation. As correctly pointed out by the reviewer, the excess of acetic acid (used to solubilize CS) must be removed from the mixture because it may affect the properties of the resulting blends. We have, indeed, purified CS solution from the excess of acetic acid by dialysis before the abietic acid dispersion. We added these experimental details in the manuscript (Page 6 lines 119-127).

As for the antimicrobial properties of abietic acid and abietate, we have only determined the MIC of sodium abietate because abietic acid is not water soluble. This issue has been clarified in the manuscript (Page 8, line 171-172).

The antimicrobial activity of acetate ions was not tested. We believe that acetate content in the solution is very low since the excess was removed by dialysis. That is reflected in the high MIC value of the (CS/AB)_{SD} 0.5/1 solid dispersion, situation in which we expect the highest release of acetate ions from CS since AB is in molar excess (exchange with abietate).

2) Comparison with literature of the glass transition temperature of chitosan should be discussed.

The discussion of CS glass transition temperature in the framework of the literature was added (Page 10 lines 235-238, Page 11 lines 239-240). Four references were also added [31-34].

3) The IR bands of abietic acid between 3000 and 2800 cm^{-1} are related to C-H stretching and not to O-H. O-H stretching bands for carboxylic acid are very broad from 3600 to 2200 cm^{-1} .

We agree with the reviewer. We changed the text and added a more in depth explanation of the abietic acid IR spectrum bands (see Page 11, lines 261-264) also with the help of new reference "V. Beltran et al. Anal. Bioanal. Chem. 408 (2016) 4073-4082".

The band at 1690 cm^{-1} is related to C=O stretching of carboxylic group. Where the C=O stretching band of carboxylate is observed? Carboxylate band should be at much more lower value.

The carboxylated band of abietic acid is, indeed, at lower value compared to C=O stretching of carboxylic group, that is 1554 cm^{-1} (Page 12 line 278).

Contrary to line 617, the deacetylated repeating units of chitosan also possess aliphatic moieties that adsorb about 2800-3000 cm^{-1} .

The mistake was corrected. Both deacetylated and acetylated units of chitosan contribute to the adsorption at 2800-3000 cm^{-1} (Page 12, line 267)

What is the assignment of the band at 1554 cm^{-1} ? This band was used in Fig 8. Quantification by ATR on solid sample is difficult to achieve due to the variation in contact between the sample and the probe. The authors did not mention if a calibration curve was performed. What is the accuracy on the A1554/A1690 ratio?

The band at 1554 cm^{-1} is related to the carboxylated abietic acid. The shifting of the peak of AB carboxylic acid from 1690 to 1554 cm^{-1} was considered an indication of the formation of a salt between AB and CS. The (1690/1554) intensity ratio was used to highlight a trend of the AB-CS salt formation with the increase in CS/AB molar ratio. The ratio gives just a qualitative estimation of such phenomenon and is not an absolute value (Page 12, lines 285-286). The curves reported in Figure 8 can be considered calibration curves if used to estimate the composition of an unknown mixture.

4) The introduction section mentioned the importance of microbial biofilms, but all antibacterial experiments were realized in solution.

We revised the introduction to give less emphasis to the biofilm issue that was not faced in the experimental phase of this work. Future studies will be planned to test our blends towards *S. epidermidis* microbial biofilm in order to collect evidence on their activity vs sessile-growing bacteria.

5) Identification of curves in Fig. 4 and 6 is not clear.

We added markers on each curve to permit a clear identification of the samples.

-Reviewer 2

This manuscript details the development of antimicrobial compounds based on solid dispersions of abietic acid (AB) and chitosan (CS).

Comments:

1. The abstract states that these compounds also could be anti-biofilm compounds, however no evidence or results were shown throughout the manuscript and statements like this should be removed from the manuscript.

We removed in the abstract the concept of microbial biofilm and we focused on microbial colonization in general.

2. The text is well written, but there are MANY abbreviations and letter combinations that all together make it hard to follow and understand, since the reader must concentrate on this instead of understanding the science itself. Therefore I recommend that the authors revise the paper so the majority of these abbreviations/letter combinations are removed and the most important ones left behind.

We made an effort to reduce the number of abbreviations along the manuscript.

3. The figures are of poor quality and cannot be published as they appear. In particular Figures 4 and 6 are difficult to understand, since all the lines are black and the different styles do not differentiate. The rest of the figures appear pixelated.

We added markers on Figures 4 and 6 curve to permit a clear identification of the samples. We also improved the resolution of figures, where needed.

4. Figure 10 legend: "Comparison of the MIC values of (CS:AB)PM 1:1 and (CS/AB)SD 1:1.". The descriptions underneath the 2 bars in the graph do not reflect this.

For sake of clarity, Figure 10 and the description underneath the 2 bars have been changed.

5. Figure 9 - why are these figures in color?

Different colors were used only to permit a good identification of the samples.

6. Figure 2. The microscopic images should be explained in more detail in the legend as well. It is really hard to see anything in panel C. Should it be removed?

In the capture of Figure 2, explanation of the POM images concerning the crystalline or amorphous state of the drug have been included. We believe that Panel C is needed because is the only image showing the amorphous state of the drug (no birifrangence).

7. Table 4: The concentrations should be added as a column in this table. It is not enough to say "2 x MIC".

As suggested, the concentrations have been added in Table 4

8. References: Bacterial names must be italicized. Other spelling mistakes should also be corrected.

Done

9. Conclusions: This was stated: "It is difficult to be sure of the effects of the various parameters.". Please revise this statement and other in the conclusion, so you write what it IS that you can say and what you can conclude does not occur. The conclusion must be stronger and based on the results in the paper. Other wise it must be characterized as Future experiments.

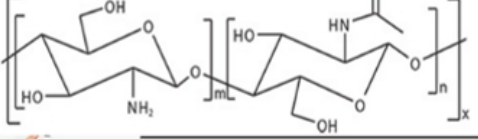
As suggested, Conclusions were rewritten to highlight the significant results obtained in the work.

10. The authors claim that the AB and CS compounds are antimicrobial. Limited experimental results have been shown to support this and none of these were referred to in the conclusion. I recommend that the authors revise the manuscript to reflect that they are interested in "antimicrobial properties" and not only the chemical characteristics and whether they dissolve or form solids. This should be discussed in the context of how and why this would make the compounds more/less antimicrobial.

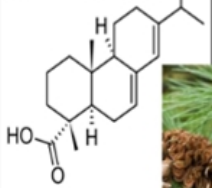
The introduction was revised to reflect the real goal of the work, that is the development of formulations with improved antimicrobial activity.



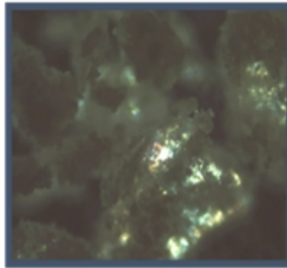
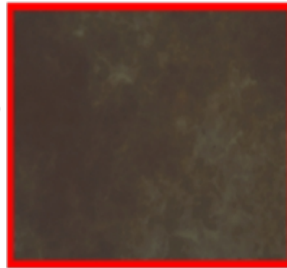
Chitosan



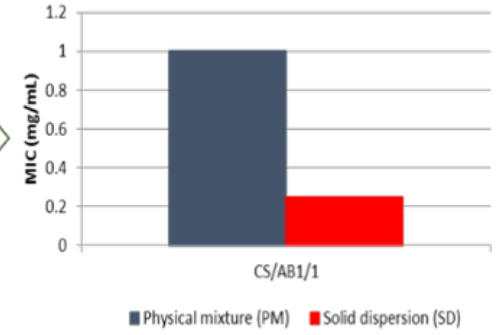
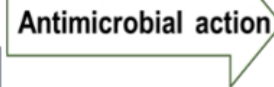
Abietic Acid



Amorphous State



Semi Crystalline State



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3 1 **Intermolecular interaction and solid state characterization of abietic acid/chitosan solid**
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5 2 **dispersions possessing antimicrobial and antioxidant properties.**
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10 4 Valentina Cuzzucoli Crucitti^{a,1}, Luisa Maria Migneco^a, Antonella Piozzi^a, Vincenzo Taresco^b,
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12 5 Martin Garnett^b, Richard H. Argent^b, Iolanda Francolini^{a*}
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62 **Abstract**
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64 24 The aim of this work was to prepare and characterize solid dispersions of abietic acid (AB) and
65 25 chitosan (CS) to investigate how formulation of the mixture may help in the battle against microbial
66 26 **colonization** in different areas, such as the biomedical field or the food industry. Solid dispersions
67 27 were characterized by differential scanning calorimetry, infrared spectroscopy, Raman
68 28 spectroscopy, polarized optical microscopy, zeta potential and size analysis. The data showed that
69 29 the dispersion/solvent evaporation method formed solid dispersions in which abietic acid was
70 30 molecularly dispersed in the carrier. A synergistic effect between the two components in terms of
71 31 antioxidant and antimicrobial properties was found, especially in the formulations obtained with 1/1
72 32 AB/CS molar ratio. Interestingly, the aggregation state (amorphous/crystalline) of AB seemed to
73 33 affect the antimicrobial activity of the formulation, suggesting increased bioactivity when the drug
74 34 was in the amorphous state. These findings, together with the demonstrated biocompatibility of the
75 35 formulations, seem to open promising perspectives for a successful application of the developed
76 36 AB/CS formulations in the biomedical field or in the food industry.
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94 **Keywords**
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96 39 Chitosan; abietic acid; solid dispersion; antimicrobial formulations; antioxidant formulations
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121 **42 Introduction**
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125 ~~44 Microbial biofilms are defined as microbial populations irreversibly attached to a surface,~~
126 ~~45 embedded in an extracellular matrix, mainly composed of polysaccharides, produced by the~~
127 ~~46 microorganisms themselves [1]. The advantages of this highly cooperative community are mostly to~~
128 ~~47 do with survival, since microorganisms in biofilms are up to 1000 times more resistant to~~
129 ~~48 antibiotics with respect to their planktonic counterparts [2]. The ability of many microbial species to~~
130 ~~49 form biofilms has important implications in various sectors, especially in the biomedical field [3,4]~~
131 ~~50 and in the food industry [5,6].~~

132 51 ~~Due to the emergence of antibiotic-resistant microorganisms,~~ The understanding of mechanisms
133 52 by which living organisms defend themselves from invasion by pathogens has become a major
134 53 source of inspiration for the development of new antimicrobial formulations particularly for finding
135 54 solutions to the emergence of antibiotic-resistant microorganisms [1,2]. Similarly there has been
136 55 increased interest in natural antimicrobial agents. In the last decade, chitosan (CS) has been
137 56 recognized as a versatile antimicrobial agent displaying excellent biocompatibility, physical
138 57 stability and processability [3]. In the food industry, chitosan is used as a preservative for
139 58 improvement of quality and shelf life of foods [4] and can be either added in the food or applied to
140 59 the surface to provide an edible protective coating [5]. In the biomedical field, chitosan is mainly
141 60 used for drug/gene delivery [6]. Recently, to improve its antimicrobial activity, chitosan has been
142 61 blended with different antimicrobial agents including antibiotics [7,8] and natural antimicrobial
143 62 extracts [9,10]. Plants are known to be able to produce a variety of small antimicrobial molecules
144 63 (MW <500 g/mol), generally classified as "phytoalexins", among which the most common belong
145 64 to the classes of glycosteroids, flavonoids, terpenes, di-terpenes, terpenoids and polyphenols
146 65 [11,12]. In this framework, the di-terpene abietic acid (AB) has been recently recognized as a
147 66 substance with important biological activities [13]. Abietic acid is the major component of rosin
148 67 that is the non-volatile portion of the resin produced mostly by conifers [14]. The production of

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180 68 such resin is associated with a defense mechanism against attack by insects or fungal infections in
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182 69 presence of a tissue injury and that in part explains why AB possesses antimicrobial activity against
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184 70 some Gram-positive bacteria, including *Staphylococcus aureus*, one of the most important
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186 71 pathogenic bacteria [15]. That has prompted research into potential applications as an antibacterial
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188 72 agent [16-18].
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191 73 A limiting factor in the application of AB is its poor solubility in an aqueous environment
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193 74 resulting from the strong hydrophobicity of the hydrophenanthrene skeleton (Fig. 1a). To increase
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195 75 AB water solubility, AB has been either functionalized with quaternary ammonium groups or linked
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197 76 to hydrophilic polymers [19]. Acrylic and methacrylic polymers based on AB have also been
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199 77 synthesized [19] from monomers obtained by reaction of AB with hydroxyl ethyl methacrylate,
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201 78 hydroxyl ethyl acrylate and hydroxyl ethyl butyl acrylate. Although these strategies have had some
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203 79 benefits, they can be time consuming and expensive.
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206 80 In this work, for the first time, ~~antimicrobial~~-solid mixtures based on abietic acid and chitosan
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208 81 were developed and characterized in order to improve AB water solubility and produce
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210 82 antimicrobial formulations with improved activity compared to pure components. The hypothesis is
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212 83 that the hydrophilic chitosan may interact with AB, reduce the size of drug particles, change the
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214 84 drug crystalline state and increase drug wettability. Indeed, CS possesses amine groups (Fig. 1b)
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216 85 potentially involved in acid/base interaction with the AB carboxylic group, thus favoring the
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218 86 intimate interaction between the drug and the carrier [20]. In the whole, the interaction between CS
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220 87 and AB could increase availability of the drug and its antimicrobial efficacy towards
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222 88 microorganisms. Additionally, being CS intrinsically antimicrobial, CS could have the dual function
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224 89 of allowing AB dissolution and to explicate a biocidal action as the same time as AB. That is of
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226 90 course beneficial for increasing the chances of success of the formulation and reducing the risk of
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228 91 selecting drug resistant microorganisms.
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231 92 In order to find out the best condition promoting CS/AB interaction, different CS/AB molar
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233 93 ratios and different preparation methods were investigated. The resulting systems were
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239 94 characterized by IR and Raman spectroscopy, differential scanning calorimetry, polarized optical
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241 95 microscopy, zeta potential and size analysis. The biological properties of the formulations were also
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243 96 evaluated. Particularly, the antimicrobial activity was determined versus a reference strain of
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246 97 *Staphylococcus epidermidis*, chosen because of its involvement in numerous nosocomial infections,
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248 98 such as wound infections and medical device-related infections. The antioxidant property of CS/AB
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250 99 formulations was also determined. This feature, in combination with the antimicrobial one, may be
251
252 100 relevant since free radicals, produced during the inflammatory response of the body to a pathogen,
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254 101 have been shown to favor diversity and adaptability in biofilm communities [21]. Finally, a
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256 102 hemolysis test was performed.
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260 104 **2. Experimental part**

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266 106 **2.1 Materials and Methods**

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269 107 Chitosan (CS, deacetylated 85%, low molecular weight) was obtained from Sigma-Aldrich. Abietic
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271 108 Acid (AB, 85%) was supplied by Acros Organics. 2,2 diphenyl-1-picrylhydrazyl radical (DPPH),
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273 109 sodium hydroxide, acetic acid, methanol were purchased from Sigma-Aldrich. The regenerated
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275 110 cellulose membrane (Spectrapor membrane BIOTECH) had a cut-off of 3500 Da. The Gram-
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277 111 positive *Staphylococcus epidermidis* ATCC 35984, grown in Muller Hinton (MH, Oxoid) medium,
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279 112 was employed for the microbiologic tests.
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285 114 **2.2 Preparation of drug-polymer solid mixtures**

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287 115 Drug-polymer solid mixtures were prepared by incorporating AB within CS in varying molar
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289 116 ratios(CS:AB 0.5:1, 1:1, 2:1, 4:1 and 6:1), corresponding to AB weight percentages equal to 80%,
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291 117 65%, 50%, 33% and 25%. Two methods were used for preparation of drug-polymer mixtures: (i)
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298 118 physical mixing of the powders by grinding them in a mortar for about 10 min and (ii) dispersion of
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300 the drug in a water solution of protonated CS. In this latter method, the following procedure was
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302 used. First, CS was solubilized in an aqueous solution of 1% acetic acid. Subsequently, the solution
303 120
304 was dialyzed against DI water, using a cellulose membrane with a 3500 Da cut-off, and the
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306 protonated CS was recovered by freeze-drying and re-dissolved in water. AB was dispersed to this
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308 latter solution using the desired amount according to the targeted molar ratio. ~~in 1% acetic acid~~
309 123
310 ~~followed by lyophilization.~~ The formulations obtained with the first method were named as
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312 (CS:AB)_{PM} while the second ones as (CS:AB)_{SD}, where the subscript PM stands for physical
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314 mixture and SD for solid dispersion. All the dispersions were left stirring overnight in order to get
315 126
316 an intimate drug:polymer interaction.
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321 322 129 **2.3 Characterization of drug-polymer solid mixtures**

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324 130 Infrared analysis in attenuated total reflection (IR-ATR) was accomplished by using a Thermo
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326 131 Nicolet 6700 instrument equipped with a Golden Gate diamond single reflection device (Specac).
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328 132 Spectra were acquired at a resolution of 2 cm⁻¹, in the range 4000–650 cm⁻¹. Differential Scanning
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330 133 Calorimetry (DSC) was performed using a METTLER TA-3000 calorimeter with 3-5 mg of sample,
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332 134 in the 25-250°C temperature interval, at a heating rate of 10K/min, under nitrogen.

334 135 The electrophoretic mobility was measured by the electrophoretic laser Doppler technique using
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336 a NanoZetaSizer (Malvern, UK) equipped with a 5 mW HeNe laser. The zeta potential of the
337 136
338 particles was obtained from the measurement of mobility v , by using the Smoluchowski equation:

$$341 \quad v = 4\pi \varepsilon_0 \varepsilon_r \frac{\xi}{6\pi\mu} (1 + kr)$$

342 138 where ε_0 and ε_r are the relative dielectric constant and the electrical permittivity of a vacuum,
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344 respectively, μ is the solution viscosity, r is the particle radius, and k is the Debye-Hückel
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346 parameter defined as:
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$$k = \frac{2n_0 z^2 e^2}{\sqrt{\epsilon_0 \epsilon_r k_B T}}$$

where n_0 is the bulk ionic concentration, z is the valence of the ion, e is the charge of an electron, k_B is the Boltzmann constant, and T is the absolute temperature.

An advanced Polarising Optical Microscope (POM, HS1 microscope), Prior Lux POLTM with 12V and 30W halogen lamp with variable brightness control, was employed to analyze the crystalline state of the drug in the different drug-polymer mixtures.

UV–Vis spectra were obtained by using a HP U2000 singular beam spectrophotometer working in the 190–1100 nm wavelength range and with a resolution of 0.004 nm.

A confocal spectroscopy system (Horiba-Jobin-Yvon Ltd, Middlesex, UK) was used to collect Raman spectra of raw materials and the drug-polymer formulations, in the wavelength range of 40–1800 cm^{-1} . The experiments were performed with a near-IR laser (785 nm) of 250 mW power. Spectra were acquired using a 50 \times objective and a 300 μm confocal hole. A 600 lines/mm rotatable diffraction grating was used to simultaneously scan a range of frequencies.

2.4 Evaluation of the antioxidant activity of the CS:AB solid mixtures

The antioxidant activity of the solid mixtures was determined by using DPPH as a free anionic radical [22]. For each sample, different concentrations were tested (expressed as the molar ratio between the antioxidant agent, in our case AB, and DPPH). Firstly, a 0.2 M MDPPH stock solution in methanol was prepared. Then, an aliquot of this solution (2 ml) was added to an acetic acid solution (1%, 2 ml) containing the different CS:AB solid mixtures at varying concentrations. CS and AB were also tested alone as control samples. The variation in absorbance was determined at room temperature at 520 nm after 30 min. The amount of residual DPPH was evaluated from a previously obtained calibration curve at the same wavelength. The antioxidant activity of each solid mixture was expressed in terms of Effective Concentration (EC_{50}), which is the amount of

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416 166 antioxidant agent necessary to decrease the initial DPPH concentration by 50%.EC₅₀ values were
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418 167 extrapolated from a graph obtained by plotting the residual DPPH as a function of antioxidant
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421 168 agent:DPPH molar ratio.

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424 425 170 **2.5 Evaluation of the antimicrobial activity of the CS:AB solid mixtures**

426
427 171 The antibacterial activity of the CS:AB solid mixtures was assessed against *S. epidermidis*. The
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429 172 minimum inhibitory concentration (MIC) of each sample was determined as previously described
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431 173 [23]. The activity of pure CS and AB was also evaluated, as controls. Specifically, due to AB
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433 174 insolubility in water, the MIC of sodium abietate was determined. Briefly, a bacterial inoculum at
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436 175 1×10^6 CFU/ml in tryptic soy broth (TSB) with an optical density of 0.05 at 550 nm was first
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438 176 prepared. Subsequently, sample (1 ml)at various concentrations was added to test tubes containing
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440 177 bacterial inoculum (1 ml). A control tube containing bacterial inoculum and TSB was also prepared.
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442 178 Control and test tubes were incubated at 37°C for 24 h. Following incubation, bacterial growth was
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444 179 determined by measuring the absorbance at 550 nm and the percentage of bacterial inhibition (I%)
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446 180 was calculated as follows:

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$$450
451 182 I\% = 1 - \frac{A_s - A_0}{A_{control} - A_0} * 100$$

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456 184 where A_0 is the absorbance of the inoculum before incubation, $A_{control}$ is the absorbance of the
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459 185 inoculum after incubation and A_s is the absorbance of the sample after incubation. All the
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461 186 experiments were performed in triplicate. Differences were considered significant for $P < 0.05$.

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464 465 188 **2.6 Evaluation of the haemolytic activity of the solid mixtures**

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189 For the hemolysis assay, blood was collected into heparinised tubes and erythrocytes harvested and
190 washed in Phosphate-Buffered Saline (PBS) as described [24]. The pure materials and CS:AB solid
191 mixtures were diluted in PBS (100 μ l) and added to 48-well plates followed by erythrocyte
192 suspension (150 μ l) and incubated for 1 h at 37°C, before centrifugation at 500 rpm for 5 min.
193 Supernatant (100 μ l) was carefully transferred to a clear 96-well plate and release of hemoglobin
194 determined using a TECAN Spark 10M plate reader at 450 nm. PBS was used as the negative (no
195 lysis) control and 0.2% Triton X-100 used as the positive (complete lysis) control, and percentage
196 hemolysis was calculated relative to these controls:

$$\%Hemolysis = \frac{(Abs_{test} - Abs_{PBS})}{(Abs_{TX} - Abs_{PBS})} * 100$$

199 3. Results and discussion

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201 Several methods have been employed in the literature for the preparation of drug-polymer solid
202 mixtures [25] in the formulation of water-insoluble drugs, among which the commonest are:(i) the
203 physical mixing of the drug and polymer powders, and (ii) the dispersion of the drug into a polymer
204 solution. These methods are simple and can be used for all kinds of drugs, even thermolabile ones,
205 since the drugs do not need special treatments.

206 When developing drug-polymer solid mixtures, it is interesting to understand if the drug is
207 molecularly dispersed (or not) in the polymer carrier since this condition is usually associated with
208 a better drug solubility. Due to the complexity of the drug-polymer intermolecular interactions, it is
209 not always trivial to delineate the differences between molecularly dispersed and not molecularly
210 dispersed solid mixtures. In the case of drugs which are capable of crystallization, a formulation
211 lacking ordered crystalline structures, is commonly considered as a molecularly dispersed mixture
212 [26,27].In the present work, to evaluate the level of drug-polymer interaction in the solid mixtures

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534 213 different analytical techniques were employed, namely POM, FT-IR, Raman spectroscopy and DSC
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536 214 analysis [28,29].
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538 539 215 *POM observation of the solid mixtures* 540 541 216 542 543 217 544

545 218 The observations of the samples by POM were conducted to evaluate the state (crystalline or
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547 219 amorphous) of the drug, qualitatively and rapidly in different solid mixtures. Indeed, the
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549 220 observation of birefringence indicates the presence of a crystalline phase. AB alone is a crystalline
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551 221 compound as shown in Figure 2A. When AB is physically mixed with CS in any of the employed
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553 222 molar ratios, it keeps crystallinity, at least in part, as shown in Figure 2B where the POM image
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555 223 obtained for (CS:AB)_{PM} 1:1 is reported. On the contrary, the (CS:AB)_{SD} mixtures, obtained by drug
556 224 dispersion in the polymer solution, did not show any birefringence for all CS:AB molar ratios equal
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558 225 to or greater than 1:1 (Fig. 2C), suggesting a good drug-polymer interaction for these samples.
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560 226
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564 227 *Differential scanning calorimetry* 565 566 228 567

568 229 In solid drug-polymer systems, either the decrease, shift or disappearance of the endothermic
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570 230 peak usually indicates that the drug is present in an amorphous state rather than its crystalline form,
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572 231 or in an amorphous-latex mixture [29]. In Figure 3, the thermograms of pure AB and CS are
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575 232 reported. In the AB thermogram (Fig. 3A), an exothermic band at about 160°C is followed by an
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577 233 endothermic peak at 168°C indicating an initial partial drug crystallization during the DSC
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579 234 experiment followed by the melting of the whole crystalline phase. The enthalpy of melting (ΔH_m)
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581 235 was found to be 36.8 J/g. The CS thermogram in the first scan (Fig. 3B) shows a wide endothermic
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583 236 band centered at about 100°C likely due to the presence of water. In the second cycle, however, a
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585 237 step at 116°C is observed relative to the glass transition of the amorphous portion of the
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587 238 polymer. The observation of this transition by DSC is not always easy to observe due to the rigidity
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593 239 of CS that involves a low free volume associated with the chains [30]. The CS T_g value can be
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595 240 influenced by different factors such as crystallinity, molar mass, and degree of de-acetylation, as
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598 241 well as by the source and method of extraction. Different T_g values are, therefore, reported in the
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600 242 literature. Dhawade et al. [31] and Rotta et al. [32] obtained T_g values around 115 °C by DSC
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602 243 measurements while higher values (150-160 °C) were obtained by the dynamic mechanical analysis
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604 244 (DMA) [33,34].

606 245 The DSC thermograms of the $(CS:AB)_{PM}$ 1:1, 2:1, 4:1 and 6:1 samples are reported in Figure 4 in
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608 246 comparison to AB, in the temperature range of interest. Each thermogram was normalized as a
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610 247 function of the AB content and by keeping constant the total weight of each sample to 5 mg.

612 248 A progressive decrease of the AB melting peak with increasing CS:AB molar ratio was
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615 249 observed. This trend is evident if the enthalpy of melting of each sample, normalized for the AB
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617 250 content in the sample, is reported as a function of the CS:AB molar ratio (Fig.5). This finding
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619 251 indicates the occurrence of drug-polymer interactions that hinder drug crystallization. The
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621 252 interactions are especially promoted for high CS contents ($(CS:AB)_{PM}$ 4:1 and 6:1). A decrease in
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623 253 the melting temperature (T_m) with the increase of the CS:AB molar ratio was also observed, further
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625 254 underlining the presence of drug crystals with reduced order as a result of the interaction with CS.

627 255 As expected, the CS:AB interactions were more pronounced in the solid dispersions. Indeed, in
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629 256 these samples the drug was present substantially in the amorphous state in all the $(CS:AB)_{SD}$ ratios,
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632 257 and in the DSC thermogram an endothermic band rather than a sharp drug melting peak was
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634 258 observed (Fig. 6). This finding was in accordance with POM observations that showed the absence
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636 259 of birefringence associated with the crystalline state of the drug in all of the $(CS:AB)_{SD}$ ratios.

640 261 *FT-IR Spectroscopy*

644 263 FT-IR measurements were used to estimate the type and extent of drug-polymer interactions. In
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646 264 Fig. 7, the FT-IR spectra of AB, CS, $(CS:AB)_{PM}$ and $(CS:AB)_{SD}$ are reported. The IR spectrum of

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AB (Fig. 7A) shows a large band at 3400 cm⁻¹ and the two bands at 2650 and 2534 cm⁻¹ correspond to the group –COOH [35]. Specifically, the first band to free OH and the last two to bonded OH related to the formation of dimers in the solid state. The C–H stretching absorption bands in the 3000-2800 cm⁻¹ spectrum range. The band at 1690 cm⁻¹ is related to the stretching of C = O. In the IR spectrum of CS (Fig 7B), the absorption related to the OH and NH stretching are present in the range between 3750 and 3000 cm⁻¹, while the absorption peak of the aliphatic moieties, ~~related to the fraction of acetylated CS,~~ is present at about 2800 cm⁻¹. The absorptions at 1645 and 1590 cm⁻¹ correspond to the C = O stretching of the secondary amide of acetylated repeating units (amide I) and the NH bending of the secondary amine of residues of chitin, respectively. The stretching C-O-H and C-O-C are in the range between 1150 and 1000 cm⁻¹.

In the IR spectra of the (CS:AB)_{PM} formulations (Fig. 7C), the presence of the two components in the formulations was confirmed by the absorption at 1690 cm⁻¹, related to the AB carbonyl group, and at ca. 1100 cm⁻¹, related to the chitosan C-O-H and C-O-C stretching. No significant shifting of the bands at all the CS:AB ratios was observed.

In contrast, important changes in specific absorption bands were observed in the IR spectra of the (CS:AB)_{SD} formulations (Fig. 7D). Specifically, a significant reduction in the absorbance of the peak at 1690 cm⁻¹, related to the AB carboxylic acid group, accompanied by a corresponding increase in the absorbance of the peak at 1554 cm⁻¹ (carboxylate C=O of AB) was observed as the CS content in the formulation increased. The shifting of the peak of AB carboxylic acid from 1690 to 1554 cm⁻¹ is likely to be attributed to the formation of a salt between AB and CS by electrostatic (acid/base) interactions.

To qualitatively estimate the magnitude of the electrostatic drug-polymer interactions as a function of CS:AB molar ratio, the ratio between the absorbance of the peak at 1554 cm⁻¹(A₁₅₅₄) and that of the peak at 1690 cm⁻¹(A₁₆₉₀) was plotted vs CS:AB molar ratio for both series of samples

(Fig. 8). The $\frac{A_{1554}}{A_{1690}}$ ratio is a relative value, not an absolute parameter, and was used to highlight the

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711 trend of the CS-AB salt formation with variation in CS:AB molar ratio. Only for the (CS:AB)_{SD}
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714 samples a significant increase of the $\frac{A_{1554}}{A_{1690}}$ ratio was observed, suggesting that CS:AB interactions
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717 were promoted by the increase of CS:AB ratio.
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721 *Raman spectroscopy*
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725 In order to have a better insight into the AB level of structural interaction in the formulations and
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727 to reinforce the POM, IR and DSC observations, Raman spectroscopy analysis was carried out on
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729 some selected samples. Particularly, this technique allowed evaluation of the presence of drug
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731 crystals or their different polymorphs (Raman phonon-region) [36]. Indeed, the phonon region
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733 pattern of crystalline forms, generally, presents defined peaks, while amorphous materials are
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735 characterized by broad features [37]. As shown in Figure 9A and 9B, AB Raman spectrum presents a
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737 precise pattern in the range between 40 and 400 cm⁻¹. On the contrary, CS does not show any peaks
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739 in this region. In Figure 9A, the Raman traces of (CS:AB)_{PM} samples are compared to AB and CS.
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741 The phonon regions show the same patterns which are weakened as the CS/AB ratio increased,
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743 confirming a crystalline AB order throughout the physical formulations. Moreover, observing the
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745 whole range of wave number (from 40 to 1800 cm⁻¹) all the formulations, apart from (CS:AB)_{PM} 6:1,
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747 show the same pattern as AB alone with sharp and well-defined peaks. A less defined Raman
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749 spectrum can explain the reduction in crystallinity of AB in the (CS:AB)_{PM} 6:1 mixture and it is in
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751 agreement with the DSC observations. As for the (CS:AB)_{SD} samples, only the phonon region of
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753 (CS:AB)_{SD} 1:1 (Fig. 9B) shows the same pattern as free AB, likely due to a partial re-crystallization
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755 of the drug in the blend. This possible AB order was not detected by DSC. Instead, (CS:AB)_{SD}
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757 2:1 and (CS:AB)_{SD} 6:1 samples do not show any peaks in the AB phonon region, suggesting the
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759 complete lack of order and thus drug amorphization in the formulations. This latter evidence
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770 314 supports the lack of birefringence of those samples and lack of thermodynamic activity in the DSC
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772 315 traces.

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777 317 *Bioactivity of the formulations*

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781 319 On the basis of the results of the formulation physical characterization, the biological tests were
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783 320 performed only on the solid dispersions because in these samples, unlike the physical mixtures, the
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785 321 abietic acid was molecularly dispersed in the polymer carrier. It is, therefore, reasonable to assume
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787 322 that the solid dispersions can show better performance than the physical mixtures. Amongst the
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789 323 investigated CS:AB ratios, the 0.5:1, 1:1 and 6:1 samples were chosen in order to investigate the
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791 324 effects on the biological properties of CS being equimolar, in deficit or in large excess with respect
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794 325 to AB.

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798 327 *Antioxidant activity of the formulations*

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802 329 An inflammation process is often concomitant with the infectious disease. This process causes an
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804 330 oxidative stress that seems to have some effects on the course of the infection. In general, the role of
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806 331 free radicals in infections is two-fold. On one hand, free radicals protect against invading
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808 332 microorganisms. On the other hand, they can accumulate during the infection disease, cause tissue
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811 333 damage and, sometimes, have fatal consequences. Though specific experiments on the effects of
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813 334 oxidative stress on the severity of infections have not been carried out yet, some authors claim that
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815 335 the mitigation of oxidative stress using exogenous compounds appears to be a suitable
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817 336 complementary approach to treat infections [38]. Free radicals have been also shown to promote
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819 337 antibiotic resistance in biofilm-growing bacteria, as recently demonstrated in different biofilm
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821 338 communities [21]. Specifically, in cystic fibrosis patients the oxidative stress was shown to be
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823 339 associated with the occurrence of antibiotic resistant bacteria in the lung [39]. In addition, in an

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829 340 animal model of wound infection, Dhall et al. reported on the role of high levels of reactive oxygen
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831 341 species (ROS) in establishment of chronic wounds [40,41]. Consequently a reduction in oxidative
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834 342 stress during antibacterial therapy would be an advantage.

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836 343 To evaluate a possible antioxidant activity of the formulations, DPPH was used as the model free
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838 344 anionic radical and the activity was expressed in terms of EC₅₀ and compared to the raw materials
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840 345 (Table 1). As expected, CS has a low antioxidant activity showing an EC₅₀ of about 11 mg/ml, in
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842 346 agreement with literature data [42]. In contrast, AB showed a higher antioxidant activity, with an
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844 347 EC₅₀ value of 1.65 mg/ml (5.4 x 10⁻³ M), even if less effective than common antioxidant
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846 348 polyphenols [43].

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849 349 Looking at Table 1, for the SD formulations we can see that there is an inconsistency in the
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851 350 apparent EC₅₀ for the formulations and their components in that two of the EC₅₀ occur at a lower
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853 351 concentration, than we may expect. The relative contribution of each of the components is also
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855 352 unclear. However, if we calculate the amount of each component (CS and AB) present in the assay
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857 353 at the amount of EC₅₀, a clearer picture emerges. We can see that the concentration of AB
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859 354 component at the EC₅₀ is similar (range 0.40-0.60 mg/mL) in all of the formulations and has a value
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861 355 that is about one quarter the EC₅₀ of the pure AB. Considering the CS component, for the 0.5:1 and
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863 356 1:1 CS/AB formulations the CS component is less than 2% of the EC₅₀ and so unlikely to
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865 357 contribute significantly to antioxidant activity by its usual mechanism, but in the 6:1 formulation
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867 358 where it is 20% of the EC₅₀ there may be some CS contribution. Overall therefore it seems that the
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870 359 presence of AB in the amorphous form is a more effective antioxidant.

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872 360 There are a number of aspects which may account for this formulation advantage some of which
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874 361 may depend on the mechanism of the AB antioxidant activity. The antioxidant activity seems to be
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876 362 related solely, to the double bonds [44], with a proposed mechanism providing two alternative
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878 363 oxidative pathways, which can occur individually or simultaneously [44]. One pathway sees the
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880 364 production of an epoxy structure, the other one, instead, involves the production of peroxides and
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888 365 free radicals [44]. Some authors have synthesized a catechol-derived AB to increase the antioxidant
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890 366 activity [45].
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893 367 For the formulations, firstly, the amorphous versus the crystalline nature of the drug may have
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895 368 some effect in terms of solubility and availability of drug. Secondly, it is possible that a stabilization
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897 369 of the radical form of AB occurred thanks to interaction with CS. In fact, it is known that the AB
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899 370 radical is unstable and reacts with oxygen to form peroxides. Such oxidation is the cause of the
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901 371 bitter taste that can result from chewing gum which has 90 % of ester compounds made of AB [43],
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903 372 for a prolonged time. Usually, to avoid this oxidation, a second antioxidant, α -tocopherol, is added
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905 373 in the chewing gum which decreases peroxide levels and thus the AB degradation leading to the
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907 374 sensory perception. Therefore, in the formulations, chitosan may act similarly to α -tocopherol,
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909 375 stabilizing the AB radical.
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911 912 376 913 914 377 *Antimicrobial activity* 915

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918 379 To evaluate the effect of the new drug-polymer formulations on antibacterial activity compared
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920 380 to the raw materials, a broth dilution assay was performed using *S. epidermidis* as the model
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922 381 microorganism. MICs of both AB and CS were determined to be 0.8 and 0.5 mg/ml, respectively.
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924 382 The activity of CS is related to the presence of partially protonated NH_2 that can interact with the
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926 383 anionic bacterial cell membrane [46]. The AB activity is mainly attributable to the carboxylic
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929 384 functionality, which interacts with the lipid component of the bacterial cellular membrane allowing
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931 385 this molecule to penetrate inside the membrane, altering the membrane functions [47].
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933 386 A preliminary screening of the antimicrobial activity of $(\text{CS:AB})_{\text{SD}}$ and $(\text{CS:AB})_{\text{PM}}$ 1/1 formulations
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935 387 was performed (Fig. 10) to assess whether the state of AB (amorphous or crystalline) might
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937 388 influence the antimicrobial activity. As determined from the physical-chemical characterization of
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939 389 the formulations, the state of AB in the two formulations tends to be partially crystalline in the
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941 390 $(\text{CS/AB})_{\text{PM}}1:1$ while is completely amorphous in the $(\text{CS/AB})_{\text{SD}} 1:1$, so this state may affect the
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947 391 solubility and thus the bioactivity. As can be observed in Figure 10, the physical mixture
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949 392 (CS:AB)_{PM} 1:1 showed a MIC of 1 mg/mL significantly higher than (CS:AB)_{SD} 1:1 (0.25 mg/ml),
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952 393 suggesting that the amorphicity or dispersion state of the drug may significantly affect the
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954 394 bioactivity of the drug itself.

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956 395 To shedlight on the low value of MIC of (CS:AB)_{SD} 1:1 in Figure 11, the percentage of bacterial
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958 396 inhibition (I%), defined as described in Materials and Methods, is reported as a function of
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960 397 component concentration for (CS:AB)_{SD} 0.5:1, (CS/AB)_{SD} 1:1 and (CS/AB)_{SD} 6:1. The biological
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962 398 tests showed that the MIC values (i.e. the first concentration for which there was complete
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964 399 inhibition of bacterial growth)varied with the CS:AB molar ratio. When CS was in a molar excess
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966 400 with respect to AB, the sample (CS:AB)_{SD} 6:1,the CS concentration in the formulation exceeded the
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968 401 CS MIC value (Table 2), so this result would be as predicted. Similarly, when CS was in a molar
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970 402 deficit with respect to AB, i.e. in the sample (CS:AB)_{SD} 0.5:1, the formulation showed antimicrobial
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972 403 activity at 1 mg/ml (Fig. 11), that is when the AB component is at its MIC (Table 2). The most
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974 404 interesting situation was found in the formulation(CS:AB)_{SD} 1:1. In this case, the MIC of the
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976 405 formulation was equal to 0.25 mg/ml, where both CS and AB were at concentrations below the
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978 406 MICs of either CS or AB alone, and below that which may be expected for an additive effect(Table
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980 407 2). This finding strongly suggests a synergistic effect between the two components.

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983 408 To understand reasons behind this synergy, size and zeta potential of the formulations were
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985 409 determined (Table 3), since these two features may influence the nanoparticle/bacteria interaction.
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988 410 In general, small nanoparticulate size and positive charge promote interaction with cells [48]. In our
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990 411 case, sizes and PDI values decreased as AB content increased. In particular, a reduction in average
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992 412 sizes and a narrower size distribution were observed with AB excess. This probably results from the
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994 413 polymer being involved in ionic interactions with AB as suggested from the change in zeta potential
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996 414 data. The decrease of repulsive interactions between the CS chains, and the presence of
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998 415 hydrophobic interactions probably most likely amongst AB rings is likely to lead to a compaction of
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1000 416 aggregates to a state more like a defined nanoparticle. The near neutrality of the 1:1 complex might

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417 be a further demonstration of the equimolar ratio of the two molecules. The complex 0.5:1, where
418 AB is in a molar excess, showed a negative Zeta Potential. In contrast, the 6:1 complex is positively
419 charged due to the large molar excess of CS.

420 It would be expected that the net negative charge may interfere with the binding of the
421 CS/AB0.5:1 formulation to the bacterial cells, and that this may reduce the effectiveness of this
422 formulation. However, the AB content is equivalent to the MIC of AB, so this component appears
423 to have retained its normal effectiveness.

424 More difficult is to find an explanation for why (CS/AB)_{SD} 1:1 had higher activity than
425 (CS/AB)_{SD}6:1. Indeed, from size and charge data, we would have expected a better activity for
426 (CS/AB)_{SD} 6:1 that has a size similar to (CS/AB)_{SD} 1:1 (370 vs 440 nm) but a positive charge, unlike
427 the (CS/AB)_{SD} 1:1 that is essentially neutral (zeta potential = -5). Indeed, the positive charge of
428 (CS/AB)_{SD} 6/1 should confer this formulation with a higher binding ability towards the bacterial
429 cell membrane and, therefore, with an antimicrobial activity higher than that of the (CS/AB)_{SD} 1/1
430 formulation. Additionally, with the charges balanced between CS and AB, we may expect that this
431 would affect the ability of each component to act by its normal mechanism through these charges
432 being less available. To explain this odd behavior, we have called into question the role of the
433 hydrophylic/hydrophobic balance on the antimicrobial activity of polymer formulations. It is known
434 that the activity of cationic polymers is also related to the balance between cationic and
435 hydrophobic moieties. Specifically, hydrophobic moieties can improve polymer activity since they
436 promote the insertion of the polymer chain in the lipid bilayer of the cell membrane [49]. Therefore,
437 it's reasonable to hypothesize that the neutral (CS:AB)_{SD} 1:1 formulation possesses a suitable
438 hydrophylic/hydrophobic balance that causes the disruption of the bacterial membrane not primarily
439 by establishing electrostatic interactions with the membrane but mainly by insertion of the AB
440 hydrophenanthrene ring (and maybe also of the polymer chain) into the lipid bilayer.

442 *Hemolysis assay*

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To determine the biocompatibility of the most promising formulation, (CS:AB)_{SD} 1:1, a hemolytic assay was carried out. The hemolysis activity of this sample was compared to that of CS, AB and (CS:AB)_{PM}1:1 formulation. For each sample, the test was performed at the MIC, below the MIC (0.25*MIC) and above the MIC (5*MIC). The two tested formulations as well as pure CS and AB showed negligible lytic activity (Table 4). This activity was lower than 1%, in the concentration range of 0.25*MIC up to 5*MIC for each formulation, indicating that the formulations presented have good biocompatibility.

Conclusions

Solid mixtures based on AB and CS were developed in order to produce antimicrobial formulations with improved activity. Results obtained by IR spectroscopy, thermal and size analysis as well as by zeta potential measurements showed the importance of acid/base interactions between AB and CS to achieve an homogeneous dispersion of AB in CS and promote the stabilization of the amorphous state of the drug. These two conditions positively affect drug antimicrobial activity. Indeed, the (CS:AB)_{SD} 1:1 sample, that possessed a good dispersion of AB in the amorphous state, was found to be the best in terms of MIC, also showing a synergy between the two components. Therefore, the (CS:AB)_{SD} 1:1 formulation is promising for a potential application against microbial colonization in different areas, including the food industry and the biomedical field.

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Conflicts of Interest

The authors declare no conflict of interest.

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590 **Caption to figures**
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592 **Figure 1.** Chemical structure of abietic acid (A) and chitosan (B). In chitosan, $m=0.8$ and $n=0.2$.

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593 **Figure 2.** POM images of AB (A) and solid mixtures $(CS:AB)_{PM}$ 1:1 (B) and $(CS:AB)_{SD}$ 1:1 (C).
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594 (A) and (B) images show the phenomenon of light birefringence due to the presence of drug
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595 crystalline domains in both the samples. Lack of light birefringence in (C) indicates the amorphous
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596 state of the drug in the solid dispersion.
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597 **Figure 3.** DSC thermogram of abietic acid (A) and chitosan (B), this latter in the first and second
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598 cycle of heating.

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599 **Figure 4.** DSC thermograms of $(CS:AB)_{PM}$ 1:1, 2:1, 4:1 and 6:1 compared to AB.

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600 **Figure 5.** Enthalpy of melting of $(CS:AB)_{PM}$ as a function of CS:AB molar ratio.

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601 **Figure 6.** DSC thermograms of $(CS:AB)_{SD}$ 1:1, 2:1, 4:1 and 6:1 compared to AB.

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602 **Figure 7.** IR spectra of abietic acid (A), chitosan (B), $(CS:AB)_{PM}$ formulations (C) and $(CS:AB)_{SD}$
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603 (D) formulations.

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604 **Figure 8.** Ratio between the absorbance of the peak at 1554 cm^{-1} (A_{1554} , CS) and that of the peak
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605 at 1690 cm^{-1} (A_{1690} , AB) vs CS:AB molar ratio for $(CS:AB)_{PM}$ and $(CS:AB)_{SD}$ formulations.
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606 **Figure 9.** Raman traces for $(CS:AB)_{PM}$ (A) and $(CS:AB)_{SD}$ (B) formulations in comparison with
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607 pure CS and AB.

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608 **Figure 10.** Comparison of the MIC values of $(CS:AB)_{PM}$ 1:1 and $(CS/AB)_{SD}$ 1:1.

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609 **Figure 11.** Inhibition of bacterial growth for $(CS:AB)_{SD}$ 0.5:1, $(CS:AB)_{SD}$ 1:1 and $(CS:AB)_{SD}$ 6:1 at
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Sample (molar ratio)	CS Weight (%)	AB Weight (%)	[CS] mg/ml	[AB] mg/ml	EC ₅₀ mg/ml
CS	100	0	11	-	11
AB	0	100	-	1.65	1.65
(CS:AB) _{SD} 0.5:1	20	80	0.104	0.416	0.52
(CS:AB) _{SD} 1:1	35	65	0.214	0.396	0.61
(CS:AB) _{SD} 6:1	75	25	1.95	0.60	2.40

Table 1. Analysis of formulation component antioxidant activity. Amounts of component present in each formulation at the EC₅₀ are calculated.

Sample (molar ratio)	CS Weight* (%)	AB Weight* (%)	[CS] [§] mg/ml	[AB] [§] mg/ml	MIC mg/ml
CS	100	0	-	-	0.5
AB	0	100	-	-	0.8
(CS/AB) _{SD} 0.5:1	20	80	0.20	0.80	1.0
(CS/AB) _{SD} 1:1	35	65	0.088	0.162	0.25
(CS/AB) _{SD} 6:1	75	25	0.562	0.188	0.75

* Weight percentage of each component in the formulation

§ Concentration of each component in correspondence of the MIC

Table 2: MIC values for pure AB, pure CS, and (CS/AB)_{SD} formulations. For each formulation, the weight percentage and the concentration of the two single components in relation to the MIC were calculated.

Sample	Size (nm)	PDI	Zeta Potential (mV)
Chitosan	1000	0.70	+22
(CS/AB) _{SD} 0.5:1	190	0.16	-35
(CS/AB) _{SD} 1:1	370	0.38	-5
(CS/AB) _{SD} 6:1	440	0.67	+11

Table 3. Size, polydispersity index (PDI) and zeta potential of selected (CS/AB)_{SD} samples

Sample	Concentration (expressed as multiplex MIC) [‡]	Real concentration (mg/mL)	Hemolysis (%)
CS	5*MIC	2.50	0.04
	1*MIC	0.50	0.01
	0.25*MIC	0.13	0.08
(CS/AB) _{SD} 1:1	5*MIC	1.25	0.15
	1*MIC	0.25	0.06
	0.25*MIC	0.06	0.17
(CS/AB) _{PM} 1:1	5*MIC	5.00	0.08
	1*MIC	1.00	0.18
	0.25*MIC	0.25	0.20
AB	5*MIC	4.00	5.83
	1*MIC	0.80	0.10
	0.25*MIC	0.20	0.10

[‡]MIC values: CS = 0.5 mg/ml; AB = 0.8 mg/ml; (CS/AB)_{SD} 1/1 = 0.25 mg/ml; (CS/AB)_{PM} 1/1 = 1 mg/ml

Table 4. Hemolysis values for CS, AB, (CS/AB)_{SD} 1:1 and (CS/AB)_{PM} 1:1.

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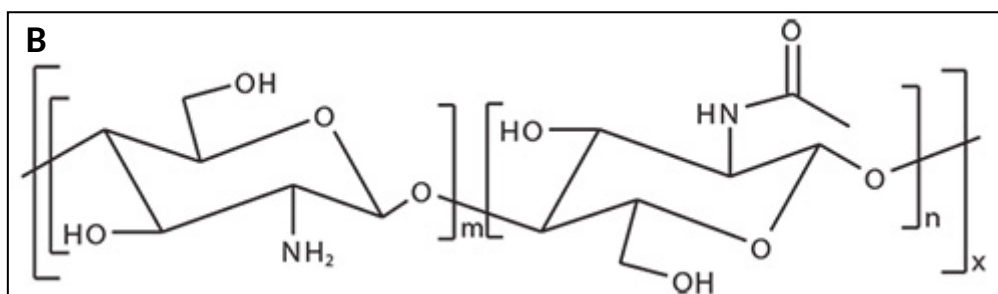
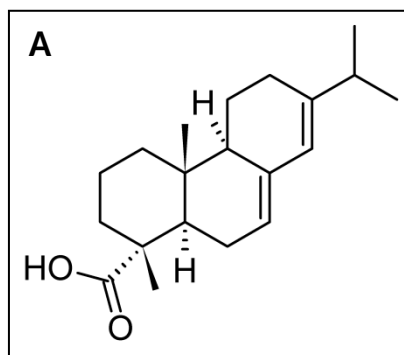


Figure 1. Chemical structure of abietic acid (A) and chitosan (B). In chitosan, $m=0.8$ and $n=0.2$.

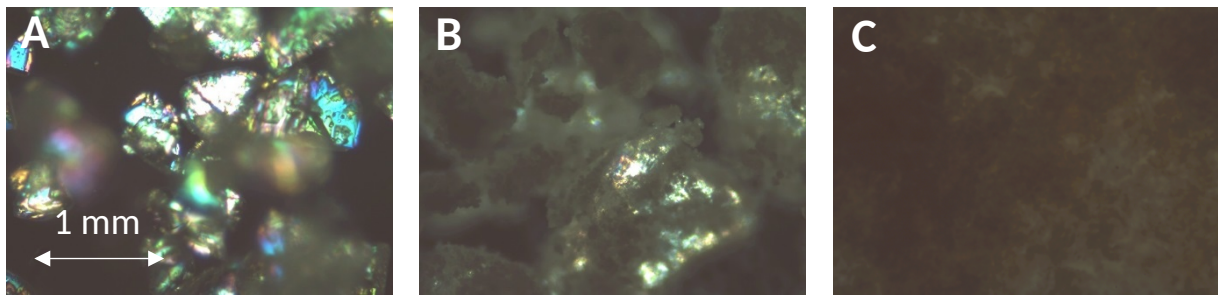


Figure 2. POM images of AB (A) and solid mixtures $(CS:AB)_{PM}$ 1:1 (B) and $(CS:AB)_{SD}$ 1:1 (C).

(A) and (B) images show the phenomenon of light birefringence due to the presence of drug crystalline domains in both the samples. Lack of light birefringence in (C) indicates the amorphous state of the drug in the solid dispersion.

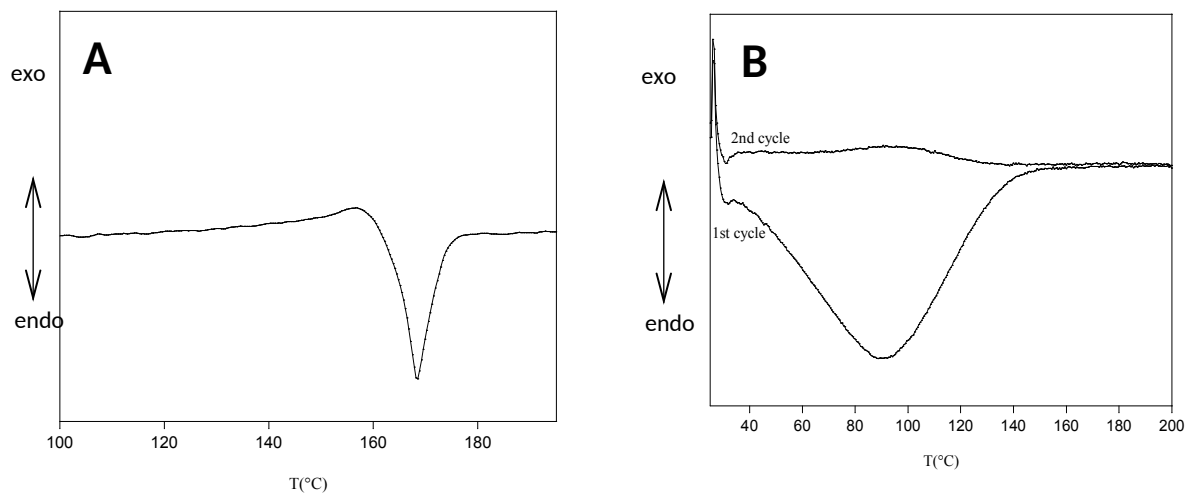


Figure 3. DSC thermogram of abietic acid (A) and chitosan (B), this latter in the first and second cycle of heating.

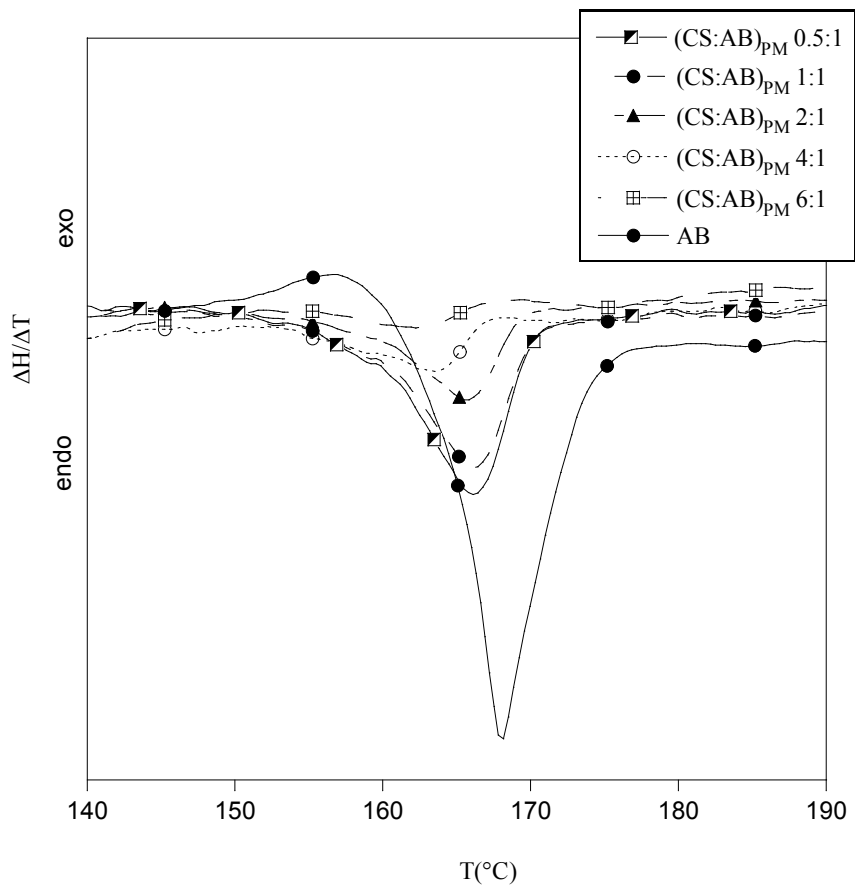


Figure 4. DSC thermograms of (CS:AB)_{PM} 0.5:1, 1:1, 2:1, 4:1 and 6:1 compared to AB.

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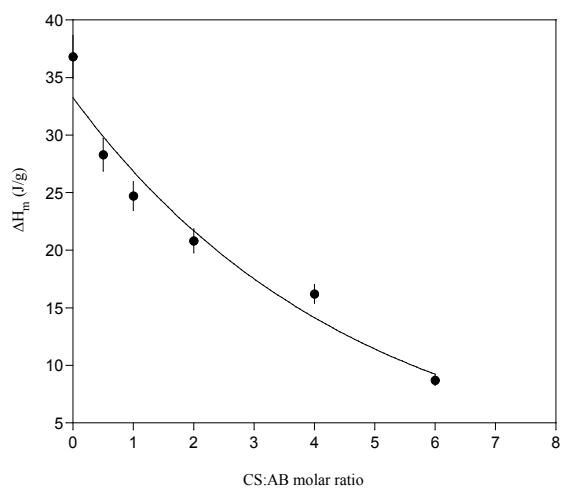


Figure 5. Enthalpy of melting of $(\text{CS:AB})_{\text{PM}}$ as a function of CS:AB molar ratio.

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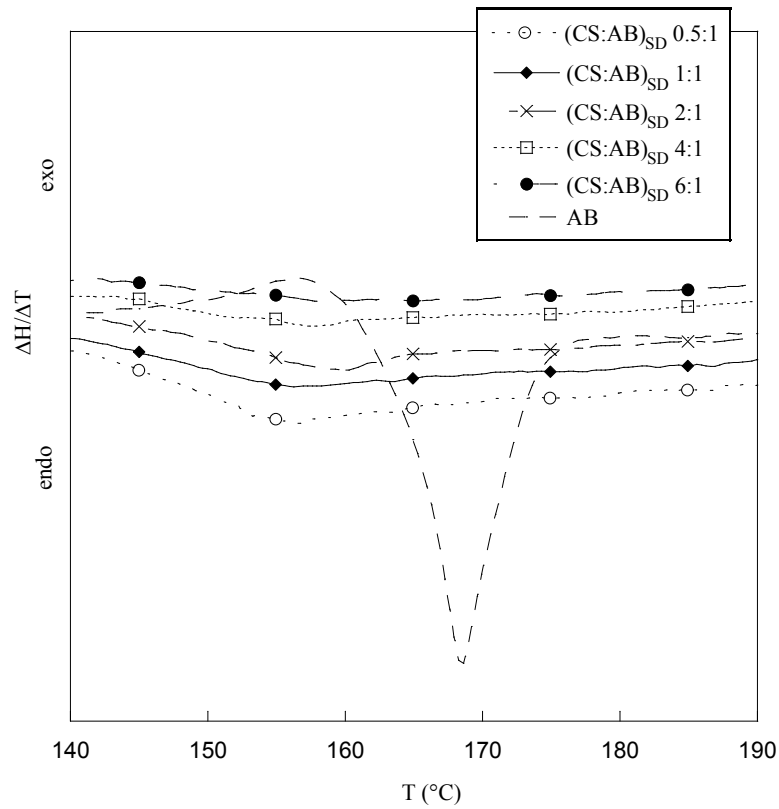


Figure 6. DSC thermograms of $(CS:AB)_{SD}$ 0.5:1, 1:1, 2:1, 4:1 and 6:1 compared to AB.

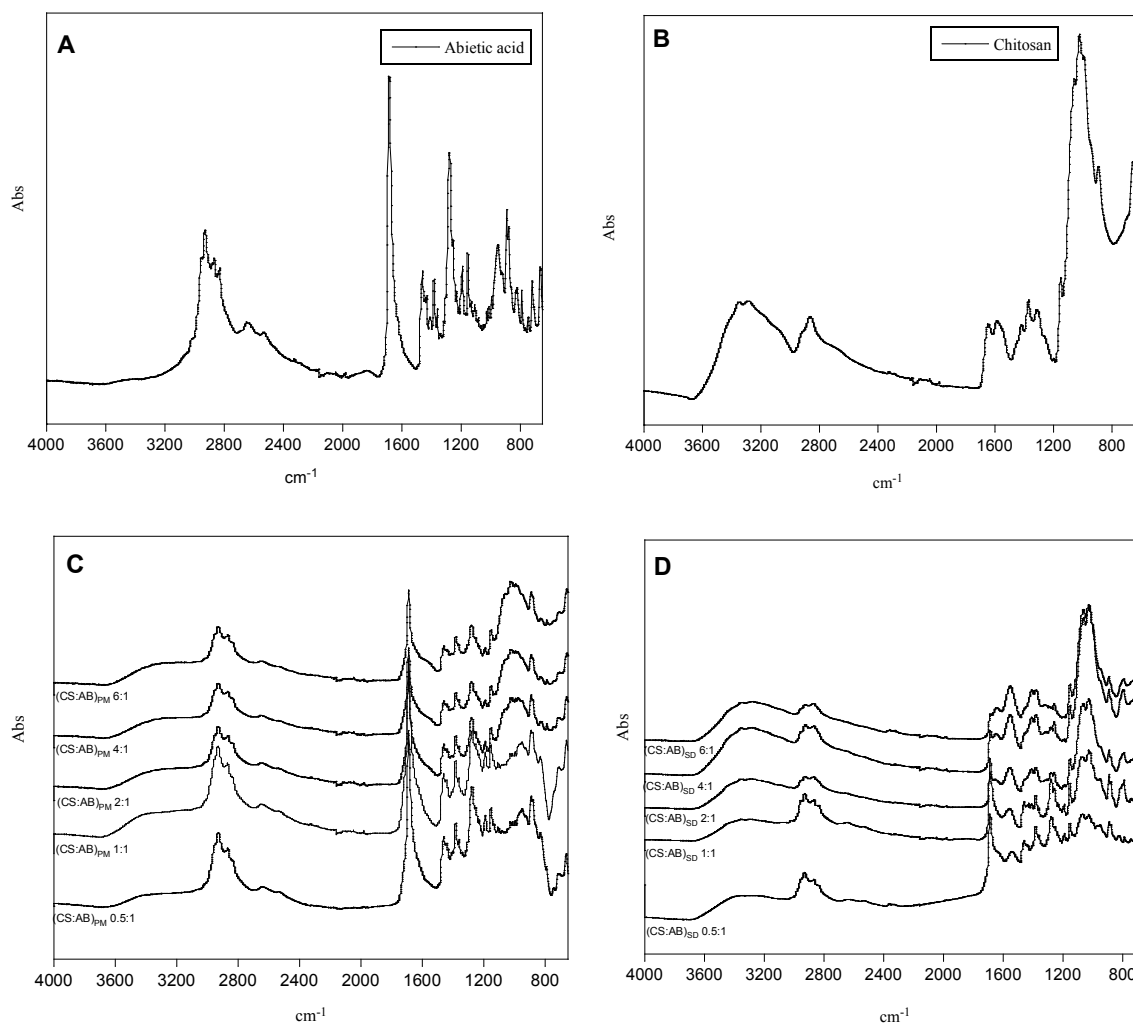


Figure 7. IR spectra of abietic acid (A), chitosan (B), (CS:AB)_{PM} formulations (C) and (CS:AB)_{SD} (D) formulations.

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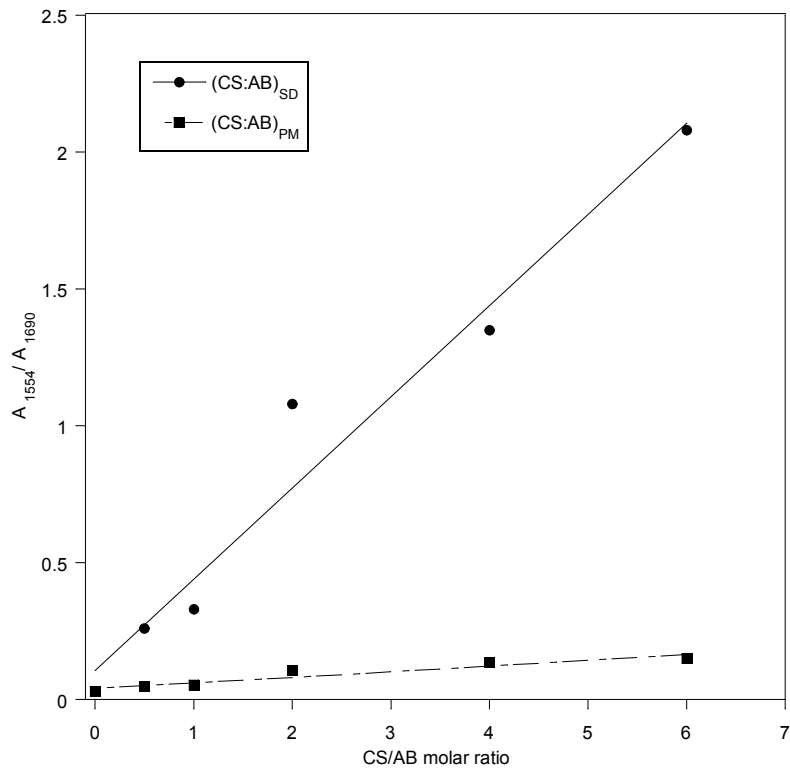


Figure 8. Ratio between the absorbance of the peak at 1554 cm^{-1} (A_{1554} , CS) and that of the peak at 1690 cm^{-1} (A_{1690} , AB) vs CS:AB molar ratio for $(CS:AB)_{PM}$ and $(CS:AB)_{SD}$ formulations.

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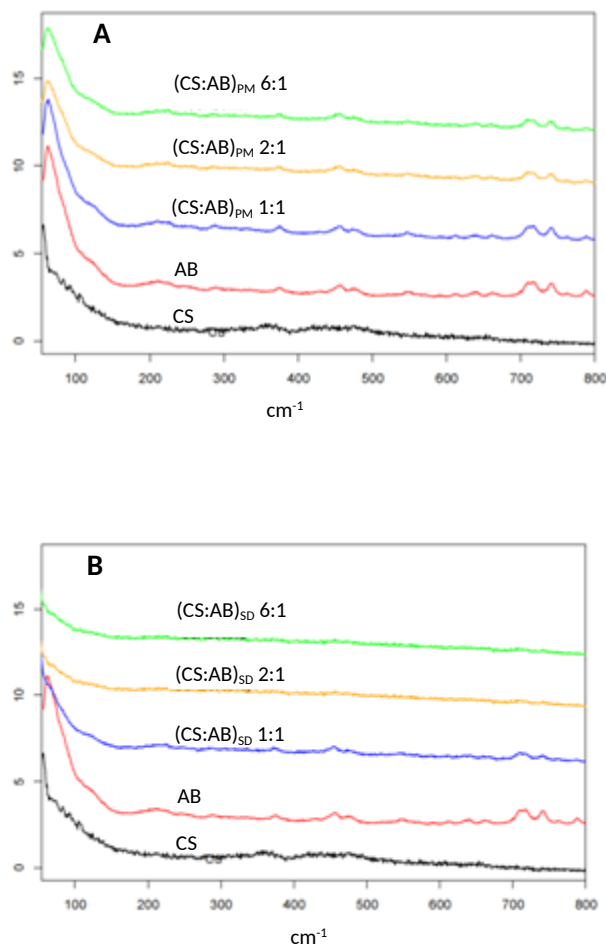


Figure 9. Raman traces for (CS:AB)_{PM} (A) and (CS:AB)_{SD} (B) formulations in comparison with pure CS and AB.

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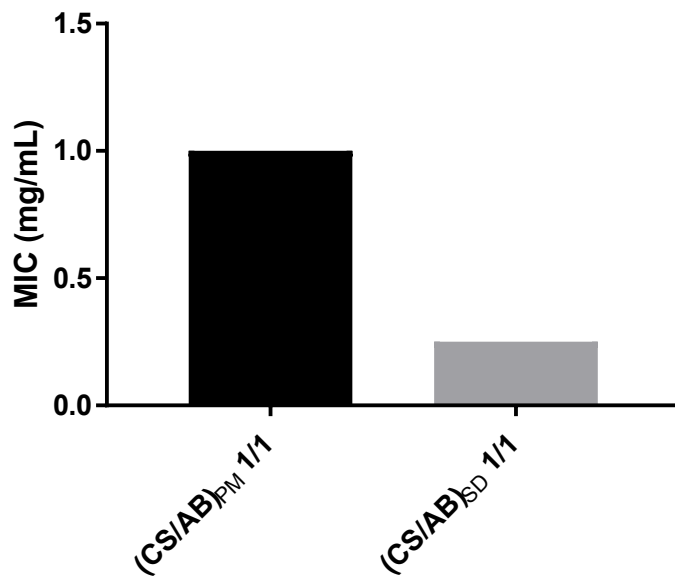


Figure 10. Comparison of the MIC values of (CS:AB)_{PM} 1:1 and (CS/AB)_{SD} 1:1.

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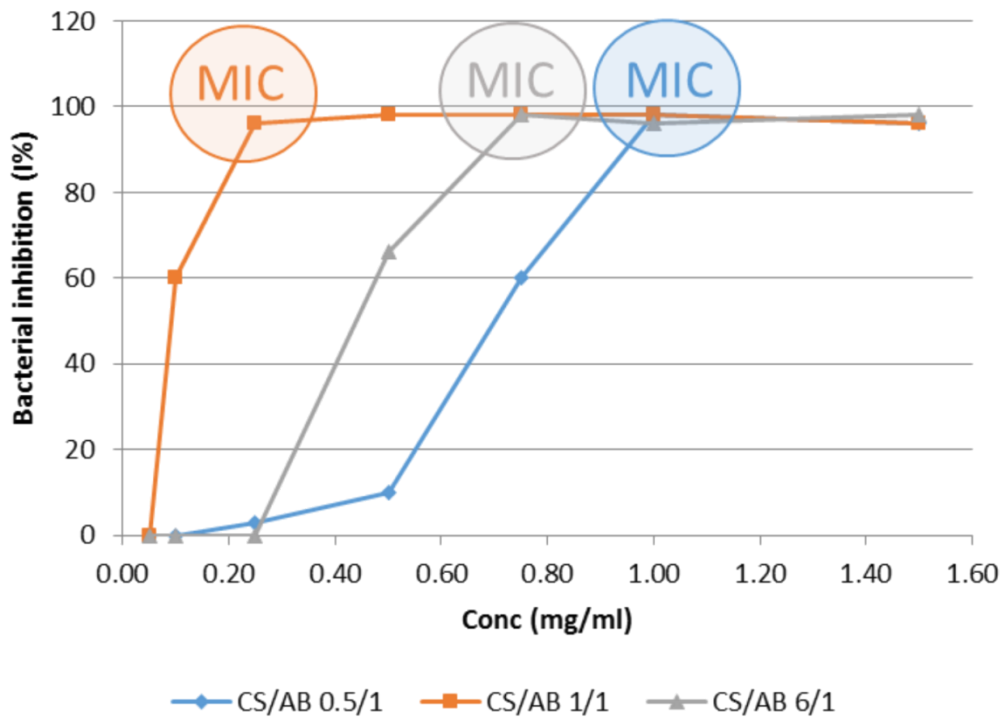


Figure 11. Inhibition of bacterial growth for (CS:AB)_{SD} 0.5:1, (CS:AB)_{SD} 1:1 and (CS:AB)_{SD} 6:1 at different concentrations.