ANTIMICROBIAL PROPERTIES OF THE BIOPRODUCTS FORMULATED WITH CHITOSAN AND COLLAGEN

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Three types of formulations (stable suspensions) based on collagen, chitosan, limonene and an imidazole derivative were studied through *in vitro* tests, from the point of view of antimicrobial activity. The results obtained on 4 standardized microorganisms, namely *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus aureus* MRSA, *Candida albicans*, demonstrated that most of the obtained bioproducts have a local effect (inhibition diameters below 15 mm are obtained) and moderate effect (inhibition diameters located under 20 mm), except bioproducts containing collagen, chitosan, limonene and an imidazole derivative (antibiotic reagent) in mass ratio Col:Chit:Lim:CT=1:1:1:0.1, Col:Chit:Lim:CT=1:1:0:0.1 which exhibit a significant antimicrobial effect on *Staphylococcus aureus* and *Staphylococcus aureus* MRSA. These two formulations also exhibit significant antimicrobial effects for *Candida albicans*, for which the average inhibition diameters obtained are greater than 34 mm.

Keywords: gels, chitosan, collagen, antimicrobials

INTRODUCTION

A variant of counteracting antibiotic resistance is the finding of pharmaceutical formulations, which contain biologically active molecules, for which pathogenic microorganisms do not have resistance genes. Among the molecules of interest are limonene and chitosan. Limonene is mainly found in essential oils obtained from citrus peels which result as a by-product from the food industry (Mohammed *et al*., 2022). As far as chitosan is concerned, it is currently obtained from chitin, which is currently extracted from microfungi and crustaceans (α-chitin), by deacetylation (Babeanu *et al*., 2022). In the world, research is currently being done to obtain pharmaceutical chitosan from natural sources where chitin is in the beta form. The best sources of β-chitin are some species of squid (cephalopods of the order *Teuthida*). These species contain an endoskeleton, called a gladius, which is made of β-chitin and has the shape of a pen or a sword (Cuong *et al*., 2016). Regarding the antimicrobial activity of chitosan obtained from squid endoskeletons, Moduto *et al*. (2019) demonstrated that the solutions obtained from deacetylated squid chitin have antimicrobial action on gram-negative microorganisms involved in stomatologic diseases. Such a study carried out on *P. gingivalis* demonstrated the effectiveness of some culture media in which the concentration of the chitosan solution was greater than 10.75%, in which case the development of the microorganism is totally inhibited. The antimicrobial activity of chitosan solutions was highlighted on pathogenic microorganisms (Ibanez-Peinado, 2017; Zaghoul and Ibrahim, 2019) and on phytopathogenic fungi too (Abirami *et al*., 2020).

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Chitosan membranes obtained from acetic acid solutions, by evaporation of the solvent and chitosan-based nanomaterials containing silver, plant extracts, clays or antibiotics such as tetracycline, gentamicin, ciprofloxacin have antimicrobial activity (Escárcega-Galaz, 2017; Al-Zahrani, 2021). Gels obtained from chitosan and sodium tripolyphosphate (TPP) containing 0.25% chitosan and 0.1% TPP show antimicrobial activity against *S. aureus, P. aeruginosa, E. coli* (Al-Zahrani, 2021). Using as raw material chitin extracted from squid, Yusharani *et al*. (2019) have obtained chitosan with a degree of deacetylation of 82-84%. With chitosan with a high degree of deacetylation, obtained from squid chitin, Cuong *et al*. (2022) have formulated chitosan-tripolyphosphate nanomaterials, using chitosan, TPP, and acetic acid as raw materials. These nanomaterials were included in the culture medium in different concentrations (50ppm; 100ppm; 150ppm; 200ppm; 250ppm) and were tested on fungal phytopathogens. The best results were obtained for concentrations higher than 200 ppm, for microorganisms of the type *A. alternata, P. digitatum* and *Lasiodiplodia sp*. The mechanism of action was highlighted by SEM studies and showed that under the action of nanomaterials with chitosan, the plasma membrane of mycelia and spores is destroyed (Xing *et al*., 2009; Cuong *et al*., 2022). Taking into account the need to create pharmaceutical formulations in which the phenomenon of antibiotic resistance is completely eliminated, in the conducted study we proposed to test the antimicrobial activity of some biopreparations, made with biomolecules obtained from natural resources such as chitosan, collagen and reagents with the potential for use in regenerative medicine, respectively limonene and clotrimazole (Mohammed *et al*., 2022). Reagents used: 2% collagen solution (from INCDPI Bucharest); glacial acetic acid, propylene glycol, pharmaceutical chitosan (DVR Pharm Brasov, Romania) limonene 97% (Merck, Bucharest, Romania), and clotrimazole (Biofarm Bucharest Romania) according to the methodology established by Babeanu *et al*. (2022). Clotrimazole dilutions were made with propylene glycol.

MATERIALS AND METHODS

The Kirby Bauer method was used to evaluate the antimicrobial activity of the produced bioproducts. The microorganisms used were: *Candida albicans* ATTC 10231, *E. coli* ATTC 11303, *P. aeruginosa* ATTC 1338, *S. aureus* ATTC 25923, *S. aureus* MRSA ATTC 33592. The microbial inoculum was made from 24-h cultures old, in physiological serum solution, according to the standard McFarland 0.5 ($1.5x10^8$UFC). The inoculum made in this way was sterilely distributed on the surface of Petri plates with a diameter of 90 mm, with a cotton swab, in the microbiological hood. After 20 minutes, between 3-5 discs of cellulose with a diameter of 6 mm, soaked in each studied bioproduct were deposited on the surface of each Petri plate. The bioproducts tested and their coding is presented in Table 1.

The evaluation of the antimicrobial activity was carried out by comparison with the antibiotic specific to each microorganism tested (Babeanu *et al*., 2022; Zaharie *et al*., 2022; Radu *et al*., 2010; Ioan *et al*., 2020). The evaluation of the antimicrobial activity was carried out on the basis of an empirical scale, established as follows: 6.1≤inhibition diameter≤15 mm=local antimicrobial activity; 15<inhibition diameter≤20 mm=moderate antimicrobial activity; inhibition diameter >20 mm = significant antimicrobial activity.

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RESULTS AND DISCUSSIONS

Studies performed *in vitro* showed that biopreparations synthesized with collagen, chitosan, limonene and or clotrimazole have no effect on *P. aeruginosa*, which is why the results for this microorganism are not presented. Regarding *E. coli*, the results obtained revealed that the bioproducts made have local activity, the inhibition diameters obtained being smaller than 9 mm (Fig. 1), results which are in agreement with those obtained by other researchers (Abirami *et al*., 2021; Zaghloul and Ibrahim, 2019). The studies carried out on *S. aureus* highlighted a local antimicrobial activity for most of the bioproducts, except the bioproduct obtained at the mass ratio Col:Chit:Lim:CT = 1:1:1:0.1 (Fig. 2), for which an activity is obtained significant antimicrobial (inhibition diameter=20.9 mm).

Table 1. Bioproducts used in antimicrobial tests (sources: own studies)

Bioproducts	Appearance	Concentration, % /mass ratio between the components	Code
Clotrimazole	Clear	1%	CT 1%
Clotrimazole	Clear	0.1%	CT 0.1%
Chitosan 1%	Viscous, homogenous	Chitosan 1%	Chit 1%
Solution colagen:chitosan = 1:1	Viscous, homogenous	1:1	Col:Chit =1:1
Solution collagen:chitosan= 3:1	Viscous, homogenous	1:3	Col:Chit =3:1
Solution collagen:chitosan: clotrimazole= 1:1:0.1	Viscous, homogenous	1:1:0.1	Col:Chit=3:1
Solution collagen:chitosan:limonene:clotrimazole= 1:1:1:0.1	Viscous, homogenous	1:1:1:0.1	Col:Chit:Lim: CT=1:1:1:0.1
Solution collagen:chitosan:limonene: clotrimazole=1:1:1:0.5	Viscous, with particle of solid chitosan	1:1:1:0.5	Col:Chit:Lim: CT=1:1:1:0.5

Similar results are also obtained for *S. aureus* MRSA (Fig. 3), where one bioproduct has a moderate antimicrobial effect, respectively the bibioproduct named Col:Chit:Lim:CT=1:1:1:0.1, for which is obtained an average inhibition diameter of 16.18 mm; another one bioproduct exhibit a significant antimicrobial effect respectively the bioproduct named Col:Chit:Lim:CT=1:1:0:0.1, for which correspond an average inhibition diameter of 21 mm. In the case of the two bioproducts, it is interesting to note that the chitosan solution practically has no effect on *S. aureus* MRSA, and on *S. aureus* it only has a local antibacterial effect (inhibition diameter 8.8 mm).

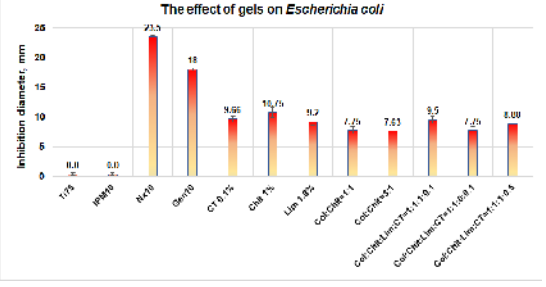

Figure 1. The effect of bioproducts made on *Escherichia coli* (sources: own studies)

Analyzing the experimental data, it can be stated that the bioproduct Col:Chit:Lim:CT = 1:1:1:0.1 has a synergistic effect on *S. aureus* MRSA, synergism due to the presence of chitosan and the antibiotic reagent derived from imidazole (clotrimazole).

Similar results were obtained by Zaghloul and Ibrahim (2019), on chitosan

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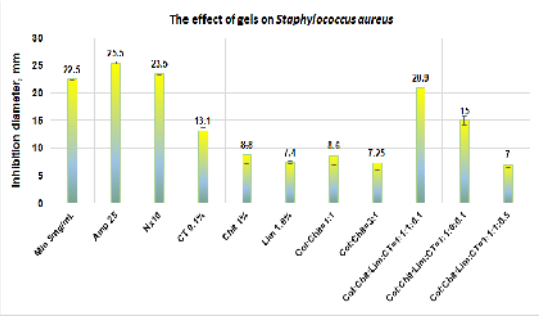

Figure 2. The effect of bioproducts made on *S. aureus* (sources: own studies)

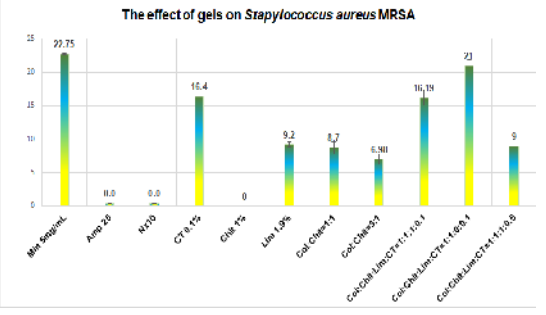

Figure 3. The effect of bioproducts made on *S. aureus* MRSA (sources: own studies)

solutions made in dilute acetic acid. Two types of chitosan were used in the experiments on *S. aureus*: chitosan obtained by the two authors from shrimp exoskeletons and respectively commercial chitosan.

In both cases, the results obtained were the same: a moderate antimicrobial effect corresponding to an inhibition diameter of 16 mm for *S. aureus* (Zaghloul and Ibrahim, 2019). Asli *et al.* (2017) have made the tests on *S. aureus* and *S. aureus* MRSA 1158c with a different type of chitosan, respectively chitosan with different average molecular mass, (1.3 kDa and 4KDa), different degrees of depolymerization less than 3, and degrees of depolymerization situated between 3 and 6.

The results obtained by them for all tested microorganisms showed that at low degrees of depolymerization, a minimum inhibitory concentration (MIC) of 16 µg/mL is obtained, while for solutions made with chitosan with degrees of depolymerization >3, is obtained a value of MIC less than 1 (Asli *et al*., 2017). Asdapoor *et al*. (2021) showed that solutions containing chitosan and clindamycin (antibiotic reagent) act synergistically on *S. aureus*, inhibiting the formation of biofilms. The mechanism of action consists of the electrostatic interaction between chitosan and the charged components of the extracellular microbial matrix the result is the loss of cell wall integrity followed by cell death (Asdapor *et al*., 2021). The mechanism of action was previously confirmed in 2009 by Xing *et al*., through the studies carried out on *S. aureus* with mixed solutions of chitosan (with a degree of deacetylation = 82%) and oleic acid, in which the destruction of the cell wall was confirmed by TEM analysis (Xing *et al*., 2009). The results obtained from the tests carried out on *C. albicans* (Fig. 4), showed that significant antimicrobial activities are obtained in the case of the bioproduct Col:Chit:Lim:CT =1:1:1:0.1, for which obtained an inhibition diameter of 34.7mm, and respectively for the codified bioproduct Col:Chit:Lim:CT =1:1:0:0.1, for which is obtained an inhibition diameter of 35.67 mm. These values are superior to the standard antibiotic used for *Candida sp.*, respectively the clotrimazole 1% solution (inhibition diameter corresponding = 31 mm). For the rest of the bioproducts, local antimicrobial activities are obtained, because the inhibition diameters obtained are below 15mm).

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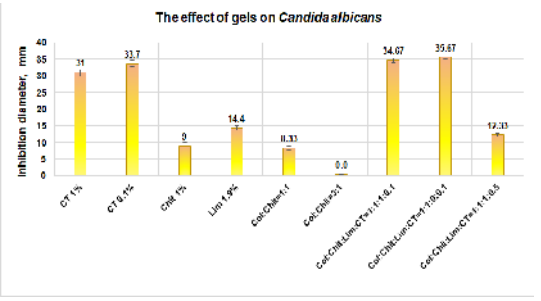

Figure 4. The effect of bioproducts made on *C. albicans* (sources: own studies)

The obtained effect does not seem to be a synergistic one, because in the presence of the 1% chitosan solution, an average inhibition diameter of 9 mm is obtained, and in the presence of the 0.1% clotrimazole solution, is obtained an average inhibition diameter of 33.7%.

In the case of the 0.1% CT solution, the large inhibition diameter obtained is probably due to the solvent in which the antibiotic was diluted, namely propylene glycol, which has the effect of reducing the degree of colonization of *C. albicans* (Henry-Stanley, 2009), and inhibiting the adhesion of pathogenic cells to the substrate (Klotz *et al*., 2004). Similar results were obtained by Zaghloul and Ibrahim (2019), in studies carried out with 1% chitosan gels, for which they obtained inhibition diameters of 15 mm for *C. albicans.* Regarding the mechanism of action, the studies carried out by Pu *et al*. by fluorescence microscopy and respectively by scanning electron microscopy (SEM) demonstrated that the exposure of *C. albicans* to chitosan solutions has the effect of permeabilizing the fungal cell membrane. This action is the basis of the anti-biofilm activity of chitosan solutions (Pu *et al*., 2014). Alburquenque *et al*. in the studies carried out with chitosan solutions on some species involved in candidiasis (microorganisms isolated from patients) such as *C. albicans, C. galabrata, C. krusei, C. tropicalis, C. lusitaniae, C. parpsilopsis*, they obtained minimal inhibitory concentrations (MIC) located between 2-128 mg chitosan /mL, at a pH = 4-4.5 (Alburquenque *et al*., 2010).

CONCLUSIONS

The bioproducts obtained with collagen and chitosan, in the presence of limonene and or an antibiotic derived from imidazole (i.e. clotrimazole) have no effect on bacteria of the *Pseudomonas aeruginosa* type but have a local antimicrobial effect on *E. coli*. In the case of *S. aureus*, only the bioproduct obtained at the mass ratio Col:Chit:Lim:CT=1:1:1:0.1 show a significant antimicrobial effect. The rest of the obtained biopreparations have a local effect. In the case of *S. aureus* MRSA, the bioproduct obtained at the mass ratio Col:Chit:Lim:CT=1:1:0:0.1 has a significant antimicrobial effect. The rest of the bioproducts act similarly to *S. aureus*, with the exception of the 1% chitosan solution, to which *S. aureus* MRSA shows resistance. In the case of *C. albicans*, significant antimicrobial activities are obtained in the case of two bioproducts: Col:Chit:Lim:CT=1:1:0:0.1 and respectively Col:Chit:Lim:CT=1:1:1:0.1, for which the inhibition diameters obtained are greater than 34%.

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