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Synthesis and Characterization of Modified Chitosan with Aminophosphonic Groups and Zn(II) Ions and Assessment as Potential Antibacterial Adsorbent

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Abstract: *Chitosan is a natural biopolymer, being a cationic polysaccharide, which is generally obtained by deacetylation of chitin. Aminophosphorylated chitosan is of interest due to the presence of its multiple functional groups of aminophosphonate type that can serve as chelating sites and their interesting biological and chemical properties. This paper presents the achievement of antibacterial adsorbent based on modified chitosan with aminphosphonic groups and Zn(II) ions. The new aminophosphonic adsorbent supported on chitosan was modified by impregnation with Zn(II) ions using the hydrothermal reaction. It was prepared from the natural biopolymer of chitosan type. The obtained product was characterized by different techniques: FTIR, SEM / EDX, XPS and thermogravimetric analysis. This research aimed to test modified chitosan against the strains (Staphylococcus aureus and Pseudomonas aeruginosa). In order to highlight the effect of the presence of Zn(II) ions, both the chitosan functionalized with aminophosphonic groups and impregnated with Zn(II) ions (code: ChitPZn) and the chitosan functionalized with aminophosphonic groups (code: ChitP) were tested. It was found that Zn(II) ions impregnation on chitosan functionalized with aminophosphonic groups increases the antibacterial effect in both St. aureus as well as at Ps. aeruginosa.*

Keywords: *chitosan, aminophosphonic groups, modified chitosan-Zn(II)ions, antibacterial agent*

1. Introduction

Chitin is one of the biomaterials that have attracted the attention of many researchers because it is one of the most abundant polysaccharides in natural macromolecules [1]. Chitosan is a natural biopolymer, being a cationic polysaccharide, which is generally obtained by deacetylation of chitin [1, 2]. Chitin and chitosan are of making importance due to the high percentage of nitrogen (6.89%) [3, 4]. Chitosan and chitosan derivatives are recommended as functional materials because these biopolymers have excellent properties such as hydrophilicity, biocompatibility, biodegradability, antibacterial action, non-toxicity, adsorption properties and remarkable affinity for many biomacromolecules [1-5].

The presence of amino and hydroxyl groups on chitosan are considered interesting functional pendant groups to modify with desired properties. There are two methods in modification process of -NH_2 group of chitosan, i.e. physical and chemical method [6].

Chemical modifications of chitosan to the -NH_2 or -OH groups are studied, as the functionalization with amidoxime [7], thiosemicarbazide [8], thiourea [9], ethylenediaminetetraacetic acid [10], thymine [11], and aminophosphonate [12-14], and after these modifications, it can be applied in the diverse field.

Phosphorylated chitosan [12-15] gains importance because of its interesting biological and chemical properties. In our previous papers we have presented the obtaining of functionalized chitosan with aminophosphonic groups by the "one-pot" Kabachnik-Fields chemical modification of chitosan.

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It is widely involved in wastewater treatment due to the presence of its multiple functional groups of aminophosphonate type that can serve as chelating sites [12-15].

Nowadays, infections caused by bacteria and viruses are a major problem because they are one of the leading causes of death worldwide [16, 17]. Biopolymer-based materials by type chitosan-metal biopolymer have attracted great interest for its potential use in biomedical domain [18-20]. Zinc (Zn) has a disinfecting and bactericidal effect and is the easiest to bind to chitosan among metal ions. Thus Zn ions bind to chitosan through nitrogen, oxygen or a combination of these atoms [21]. Following the binding of Zn ions, it is possible that some atoms remain free as potential donors, and these free donor atoms increase biological activity [21].

This work was aimed at preparing and characterizing functionalized chitosan with aminophosphonic groups and Zn(II) ions (code: ChitPZn) and testing its antimicrobial effects against pathogens.

The antibacterial effect was confirmed, both for the chitosan functionalized with aminophosphonic groups and impregnated with Zn(II) ions, and for the chitosan functionalized with aminophosphonic groups (raw material, with code: ChitP). Our study brings as a novelty the presence of phosphorus atoms due to the existence of aminophosphonic groups grafted on chitosan and the impregnation of Zn(II) ions. It is observed that the impregnation of Zn(II) ions on chitosan functionalized with aminophosphonic groups increases the antibacterial effect both in *St. aureus* as well as at *Ps. aeruginosa*.

2. Materials and methods

2.1. Materials and apparatus

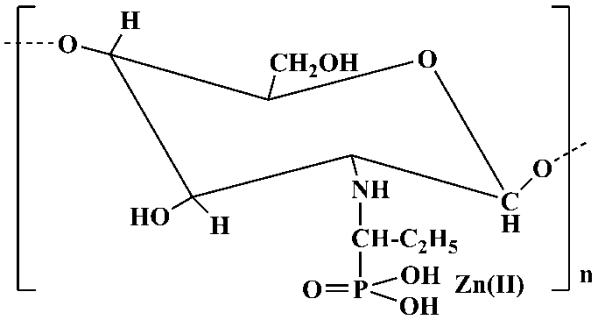
Chitosan (the degree of *N*-deacetylation (%) of chitosan is 75-85% DD, the degree of *N*-acetylation (%) of chitosan is 15-25% DA, molecular weight is 190,000-310,000 DA, Sigma-Aldrich), chitosan functionalized with the aminophosphonic group (was previously obtained by "one-pot" Kabachnik - Fields, glacial acetic acid, $Zn(CH_3COO)_2x2H_2O$ (Zn(II)acetate dihydrate), were used in our work without further purification.

The FTIR spectrum (performed on KBr tables) of the chitosan functionalized with aminophosphonic groups with Zn(II) ions was recorded using a JASCO-FT/IR-4200 spectrophotometer within the wave number range of 4000-600 cm^{-1}. The surface morphology of the sample was studied by scanning electron microscopy (SEM) using a Quanta FEG Microscope equipped with EDAX ZAF quantifier-FEI Company (Nederland). The thermal stability of the sample was investigated by differential thermography (TG-DTA) by using a Mettler-Toledo device, in the temperature range 25-600^{o}C, at a heating rate of 10^{o}C/min, under a nitrogen atmosphere. X-ray photoelectron spectra (XPS) were collected on a Kratos Analytical Axis NOVA instrument. Optical images of the final product were recorded on a ZEISS STEMI 508 microscope.

2.2. The experimental procedure for obtaining modified chitosan-Zn(II)ions

In this paper, a procedure previously described in our works was used [13, 14] for obtaining aminophosphonic acid functionalized onto chitosan and impregnated with metal ions.

In an Erlenmeyer beaker 1.6 g of ChitP was dissolved in 50 mL of 1% glacial acetic acid solution under magnetic stirring for 24 h. In a Berzelius beaker, $Zn(CH_3COO)_2 \times 2H_2O$ was dissolved in 100 mL of distilled water and 0.5 g of urea was added with stirring until a clear solution formed with *p*H~4 is obtained. We worked with a 1:1 molar ratio of Zn(II)ions: phosphorus contained in the aminophosphonic group (phosphorus content 14.40% by mass). The final solution in the Erlenmeyer beaker was adjusted to *p*H=5 (by the addition of 0.1 N NaOH) and subsequently placed on the water bath at 80^{o}C for 60 h. The precipitate formed was filtered and washed with distilled water. The final product (Scheme 1; ChitPZn) was dried in an oven at 50^{o}C for 48 h.



Scheme 1. Functionalized chitosan with aminophosphonic group and impregnated with Zn(II)ions

2.3. Preparation of samples for antibacterial testing

The solutions were prepared in a volume of 30 mL [22], following the indications depicted for each sample in Table 1. The samples of chitosan functionalized with aminophosphonic groups and impregnated with Zn(II) ions requires a 1% acetic acid solution for complete dissolution.

Table 1. Composition of samples for the antibacterial assays

Sample code	Tested materials	Dissolving medium
P1	ChitPZn sol 1%(w/v)	0.3%HCl (+ 5 ml sol 1% acetic acid)
P1bis	ChitPZn sol 2%(w/v)	0.3%HCl (+ 5 ml sol 1% acetic acid)
P3	ChitP sol 1%(w/v)	0.3%HCl
P3bis	ChitP sol 2%(w/v)	0.3%HCl
P4	Chitosan sol 1%(w/v)	0.3%HCl
P5	$Zn(CH_3COO)_2x2H_2O$ sol 1%(w/v)	0.3%HCl
P6	Control for P3, P3bis, P4 and P5	0.3%HCl
P7	Control for P1 and P1bis	0.3%HCl (+ 5 ml sol 1% acetic acid)

2.4. Antimicrobial tests

The antibacterial activity of the samples was tested against *Staphylococcus aureus* (ATCC 25923) as a Gram positive bacteria and *Pseudomonas aeruginosa* (ATCC 27853), as a Gram negative bacteria.

The bacterial strains were obtained from the culture collection of the Laboratory of Microbiology in the Faculty of Veterinary Medicine within Banat's "King Michael I of Romania" University of Agricultural Science and Veterinary Medicine Timisoara. The ATCC strains are maintained in this laboratory at -50ºC.

For the tests, the selected ATCC strains were revived by inoculation in Brain Heart Infusion (BHI) broth (Oxoid, CM1135), and overnight growth at 37°C.

To perform the assay was prepared a 10^2 dilution of each fresh culture using a BHI broth. The optical density was established using McFarland standard at the value of 1.5×10^8 UFC/mL

The disc diffusion method was used to determine the antibacterial activity. For this purpose, 6 mm diameter filter paper discs were used. The discs were sterilized by autoclaving at 1 atmosphere, 120°C for 30 min. Both P1, P1bis samples and P3, P3bis samples were tested for concentrations 1% and 2%. The solutions used for chemical conditioning the samples included in the study were also tested, as control samples (P4, P5, P6, P7). In addition, gentamicin (0.1% gentamicin sulfate test discs – company Oxoid), was chosen and used as a positive control sample.

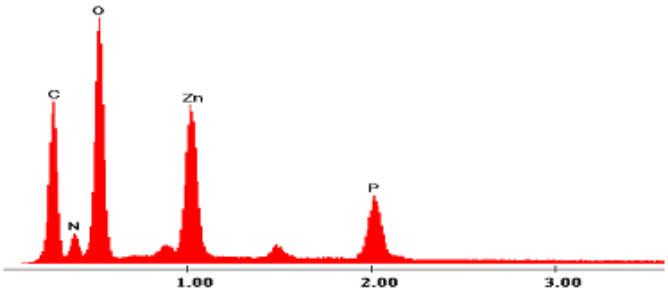
The sterile filter paper discs were soaked with the solutions and placed on the surface of the culture medium (nutrient agar media) poured into Petri dishes, which was previously inoculated with the bacterial suspensions from the selected strains. When placing the discs on the surface of the inoculated culture medium, a distance of 2.5 cm from the edges of the plate and between the discs was kept. The Gentamicin disc was placed in the middle of each Petri plate. The plates were incubated at 37°C for 24 h. Experiments were performed in several variants: A (P1 + P1 bis + P3 + P3 bis + P6), B (P1 bis + P3 bis + P6) and C (P1 bis + P3 bis + P4 + P5 + P6 + P7). In order to assess the antimicrobial activity, the

inhibition zones developed around each disc were measured using a micrometer (including the disc diameter - 6 mm).

3. Results and discussions

3.1. The characterization of modified chitosan with aminophosphonic groups and impregnated with Zn(II) ions

The values of weight (Wt%) for elements of the modified chitosan with aminophosphonic groups and impregnated with Zn(II) ions can be seen in the Figure 1. The EDX analysis confirmed the presence of P (1.69%) and Zn (0.12%) in sample, indicating that the chitosan modification took place.



Elem	Wt%	At%	K-Ratio	Z	A	F
C K	32.67	38.90	0.1401	1.0116	0.4238	1.0005
N K	13.43	13.71	0.0219	1.0027	0.1626	1.0009
O K	52.09	46.58	0.0906	0.9947	0.1748	1.0000
P K	1.69	0.78	0.0091	0.9154	0.5859	1.0000
ZnK	0.12	0.03	0.0011	0.8068	1.1459	1.0000
Total		100.00	100.00			

Element	Net Inte.	Backgrd Inte.	Error	P/B
C K	366.57	6.64	0.76	55.17
N K	69.05	11.05	1.99	6.25
O K	643.67	14.30	0.58	45.01
P K	244.15	21.75	1.00	11.22
ZnK	10.12	1.53	5.16	6.61

Figure 1. EDX of ChitPZn sample

The surface morphology of ChitP, raw material , has been presented in a previous paper [13]. The newly obtained product reaction ChitPZn was studied by scanning electron microscopy (SEM) at different magnifications (100x, 2500x, and 5000x) and it is shown in Figure 2. The SEM images indicate the impregnation of zinc ions onto chitosan surface under formation of zinc oxid nanflowers nuclei with a lot of numbers of branches [23].

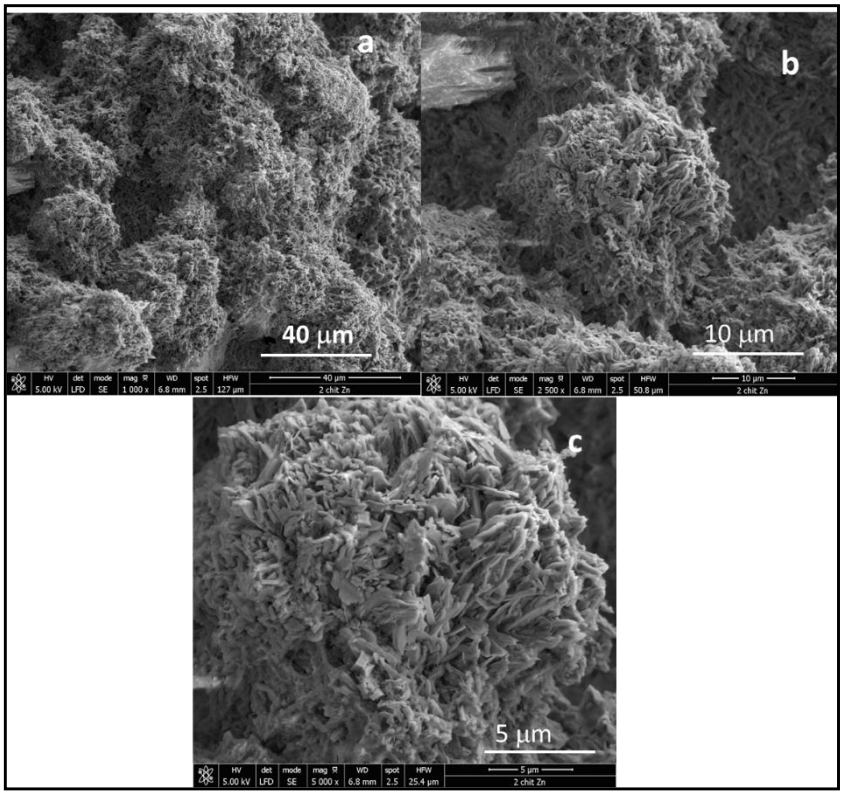


Figure 2. SEM images of ChitPZn sample

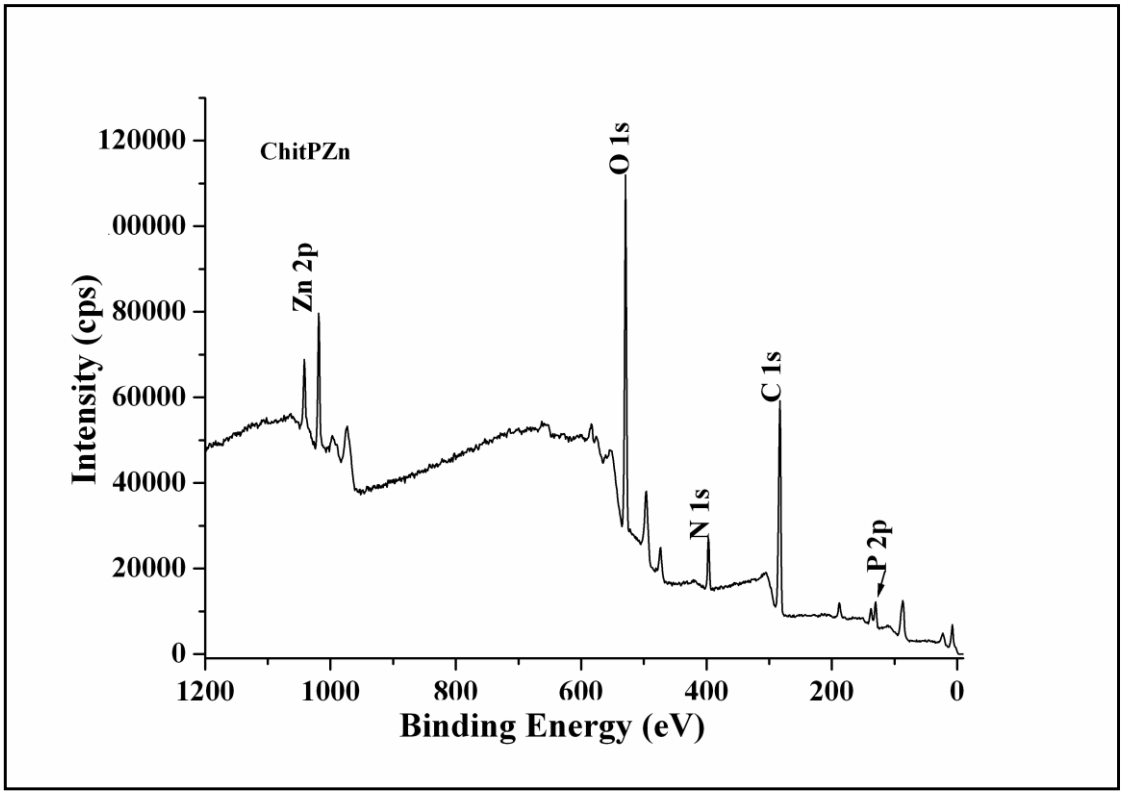


Figure 3. X-ray photoelectron spectroscopy (XPS) spectrum of ChitPZn sample

The XPS spectrum of ChitPZn (Figure 3) revealed the occurrence of characteristic peaks of C, N, O, P and Zn. From X-ray photoelectron spectroscopy (XPS) spectrum it was observed that ChitPZn exhibit for phosphorus and Zn ions comparable with the one mentioned in the literature [13, 24]. The XPS

analysis confirm the loading of chitosan surface with zinc ions as ZnO, in accordance with the SEM images, the peaks at binding energies of 1044.4 and 1021.3 eV are assigned to the Zn 2p peaks of Zn^{2+} [25].

The FTIR spectrum of functionalized chitosan with the aminophosphonic group and Zn(II) is presented in Figure 4. The presence of a band at 1103 cm^{-1} is attributed to the –P-O-H group. The stretching and banding bands of N-H appear at 1635 and 1562 cm^{-1}. A band 590-560 cm^{-1} is presented in the FTIR spectrum, which mainly arises from the stretching vibration due to the presence of N-Zn and Zn-O bonds [24, 26]. The formation ChitPZn sample is confirmed by absence of bands in the range 2700-2560 cm^{-1}, corresponding to the P-O vibration of the P-OH acid groups [27, 28].

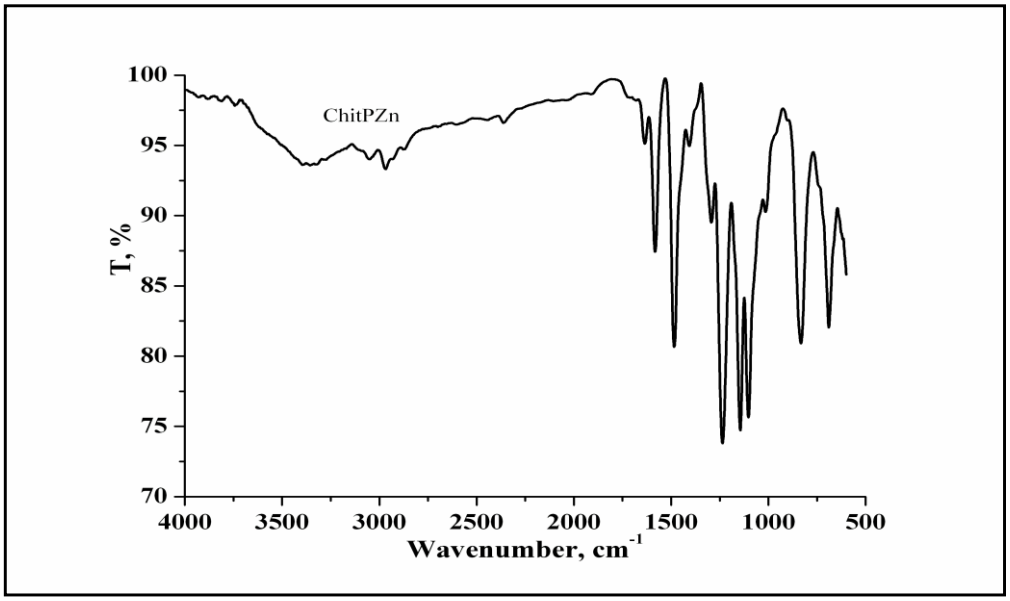


Figure 4. FTIR spectrum of ChitPZn sample

In Figure 5 TG-DTG curves are presented for ChitPZn. They were carried out from 25 to 600°C under a nitrogen flow, in 10°C/min heating step.

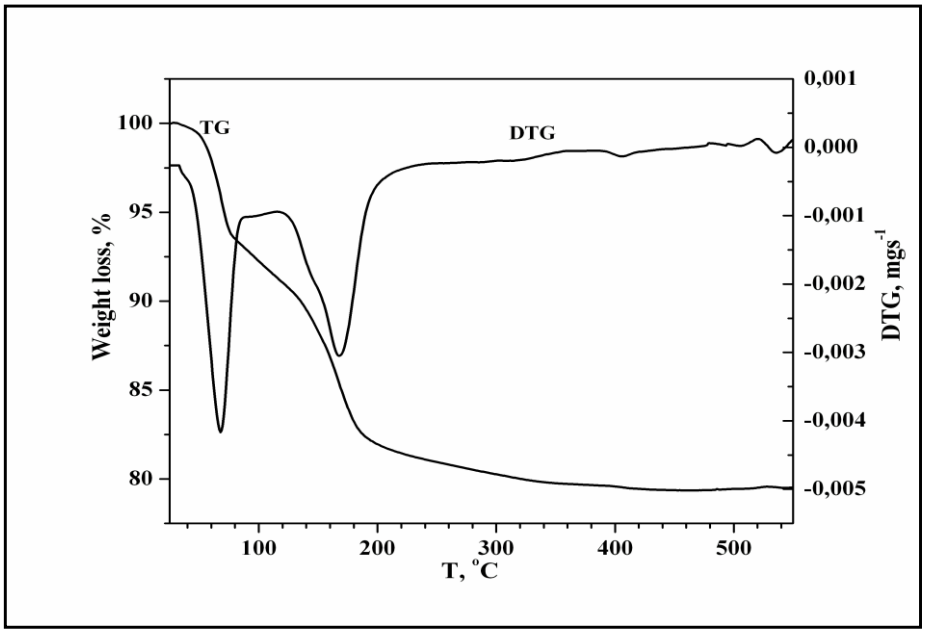


Figure 5. TG /DTG in nitrogen of ChitPZn sample

The TG/DTG curves of ChitPZn sample (Figure 5), recorded under nitrogen atmosphere, show three distinct mass losses: (i) at 40-120^{o}C with a weight loss of ~8.7% due to the loss of residual water from the material, (ii) at 120-250^{o}C, with a weight loss of ~12.3% that corresponding the decomposition of organic compounds, the degradation of the N-C-P bond between chitosan and the aminophosphonic group and (iii) in the temperature range 250-600^{o}C with a mass loss of ~2.2%, which is attributed to the degradation of the P-O structure and the degradation of the carbohydrate nucleus [28].

3.2. Evaluation of antibacterial activity

Table 2. Antibacterial studies on Gram positive and Gram negative bacteria

Staphylococcus aureus									
	Diameter of the inhibition zone (cm)								
Experimental variant of tested samples[a]	Control antibiotic	P1	P1bis	P3	P3bis	P4	P5	P6	P7
A (P1+P1bis+P3+P3bis)	2	1.7	2	0.8	1.3	-	-	-	-
B (P1bis+P3bis+P6)	2	-	1.9	-	1.2	-	-	0.8	-
C (P1bis+P3bis +P4+P5+P6+P7)	2	-	1.9	-	1.2	0.9	0.9	0.8	0.6
Pseudomonas aeruginosa									
	Diameter of the inhibition zone (cm)								
Experimental variant of tested samples[a]	Control antibiotic	P1	P1bis	P3	P3bis	P4	P5	P6	P7
A (P1+P1bis+P3+P3bis)	2	0.9	1.3	0.7	1.1	-	-	-	-
B (P1bis+P3bis+P6)	2	-	1.2	-	1.2	-	-	0.8	-
C (P1bis+P3bis +P4+P5+P6+P7)	2	-	1.3	-	1.2	0.8	1	0.8	0.6

[a.] The tested samples were described in Table 1.

The results obtained from the antimicrobial effect of the tested samples (P1-P7) are presented in Table 2. These results (Table 2) showed that both samples P1, P1bis and P3, P3bis have an antibacterial effect against Gram-positive bacteria (*St. aureus*) and Gram-negative bacteria (*Ps. aeruginosa*). Sample P1, provides zones of inhibition of 1.7 cm against *St. aureus* and 0.9 cm from *Ps. aeruginosa*. Sample P1bis, provides areas of inhibition larger than P1, it has values of 1.9 - 2 cm against *St. aureus* and 1.2 - 1.3 cm at *Ps. aeruginosa*. Sample P3, provides inhibition zones of 0.8 cm from *St. aureus* and 0.7 cm from *Ps. aeruginosa*. The P3bis sample, offers areas of inhibition larger than the P3 sample, it having values of 1.2 - 1.3 cm compared to *St. aureus* and 1.1 - 1.2 cm for *Ps. aeruginosa*. The samples, P1, P1bis show antibacterial effect against the tested strains. The antibacterial effect increases as the concentration increases, both in *St. aureus*, as well as *Ps. aeruginosa*. The samples P1, P1bis is much more effective against *St. aureus*, compared to *Ps. aeruginosa*. The antibacterial effect expressed against *St. aureus* is equal to that indicated by the antibiotic used in the positive control (Gentamicin). it is still effective against tested bacteria. The antibacterial effect against the tested strains (*St. aureus* and *Ps. aeruginosa*) in samples P3, P3bis is relatively similar, the areas of inhibition being relatively equal.

Knowing that the solutions used for the preparation - dilution of the tested samples may have antibacterial effects, it was considered necessary to compare the results obtained.

The control samples, represented by the solutions used to condition the samples studied in this experiment, showed a weak antimicrobial effect, the ranges of inhibition being dependent on their composition, from 0.6 cm (for P7) to 0.8 (for P6) and 0.9. cm (for P4 and P5) against *St. aureus* and from 0.6 cm (for P7) to 0.8 cm (for P6 and P4) and 1 cm (for P5) against *Ps. aeruginosa*.

Acetic acid has been suggested in the literature [29, 30] as a decontamination agent. Decontamination is a bactericidal treatment applied to reduce pathogens and spoilage [31]. The antimicrobial activity of chitosan-based materials has been observed under acidic *p*H with hydrochloric acid and acetic acid [31].

Fraise and co-workers have demonstrated that acetic acid (solution 5% acetic acid) has good activity against both the strains of *P. aeruginosa* (the minimum inhibitory concentration (MIC) of 0.166%) and *S. aureus* (the minimum inhibitory concentration (MIC) of 0.312%) [32].

Tian Ding and co-workers [33] observed using flame atomic absorption spectrophotometry at 766.5 nm that the 0.1% HCl solution induced the percent intracellular potassium (K^+) leakage of 0.84% on *S.*

aureus. After treatment with 0.1% HCl of *S.aureus* culture, it is observed that the cell wall has shrunk, the nucleus has partially cracked and is no longer intact and the vacuoles are larger with more agglutinated particles [33]. The use of hydrochloric acid solution with its low pH value may be responsible for most cytoplasmic disorders [33]. The intracellular potassium (K^+) is known to be essential for the permeability of the cell membrane of microorganisms [33].

The best results were obtained for samples P1, P1bis prepared by dissolving in hydrochloric acid and acetic acid, which have antimicrobial properties. The antimicrobial activity of the tested materials was compared to the controls which were the hydrochloric acid and acetic acid solutions (P7).

The control P7 has no antibacterial effect, the value 0.6 cm obtained in the antibacterial test for P7 is corresponds to the disc diameter (0.6 cm). P7 was obtained by mixing hydrochloric acid with acetic acid. In several articles it has been shown that no synergistic activity was obtained on *S. aureus* when acetic acid was combined with hydrochloric acid [34, 35]. These studies [34, 35] could explain the lack of antibacterial effect of P7 control. Our study showed a good incorporation of the ChitPZn sample in acetic acid and hydrochloric acid and obtaining the best effect in antimicrobial actions.

4. Conclusions

A new chitosan-type adsorbent modified with aminophosphonic groups and impregnated with Zn(II) ions was developed by hydrothermal reaction. The obtained product has characterized by different techniques: FTIR, SEM, EDX, XPS and thermogravimetric analysis.

The antimicrobial effect was tested on the solutions prepared from the final product of the hydrothermal reaction (ChitPZn) and the raw material (ChitP), respectively. The chitosan functionalized with the aminophosphonic groups and impregnated with Zn(II) (P1 and P1 bis) is better than chitosan functionalized with the aminophosphonic groups (P3 and P3bis). It can be concluded that the zinc impregnation of chitosan functionalized with aminophosphonic groups increases the antibacterial effect both in *St. aureus* and *Ps. aeruginosa*.

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