Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 27 issue 2, 2017, pp 111-114

ANTIMICROBIAL STUDIES OF CHITOSAN EXTRACTED FROM PACHYGRAPSUS MORMORATUS

Alef Mustafa[1] *, Rodica Sirbu[2] , Corina Dalia Toderescu[1]
[1]UMF Carol Davila Bucharest, Faculty of Pharmacy, Traian Vuia Str.Nr.6, Sec.2, Bucharest, Romania
[2]Ovidius University of Constanta, Faculty of Pharmacy, 900527, Constanta, Romania.

ABSTRACT: The study and design of a new formulation based on marine resources can provide a useful alternative for the synthetic drugs with antifungal and antibiotic properties that have various side effects. The aim of this study was to analyze the physicochemical parameters of the chemicaly extracted chitosan and to test the antimicrobial activity against pathogens. The antimicrobial activity was tested against Escherichia coli, Staphylococcus aureus and Candida albicans. The results have shown that chitosan has an inhibiting property against the proliferation and development of pathogens.

Keywords: natural marine resources, chitosan, antimicrobial activity

INTRODUCTION:

Due to the increasing incidence, in the last years of the bacterial rezistance and the appearance of new infectious diseases lead to the necesity of researching new active substances that have antimicrobial activity, mainly from natural products and sources like, biopolimers, marine algae and plants. These natural raw materials serve as a source for new bioactive molecules and biopharmaceutical products, with non-toxicity, no side effects and no risk of accumulation in the body. Also, the rezistance of the pathogen is considerably reduced against the antimicrobial effect of these substances. It is a known fact that wounds and burns have a high risc of infection, if they are not treated correctly and also hard to heal. Biopharmaceutical formulations used in preventing bacterial infections must present some important properties, such as, biodegrdability and biocompatibility with the human body tissues (A. Francesko *et al*.,2011). Chitosan is a natural polysaccharide that presents antimicrobial and antifungal properties depending on the values of the main parameters: the degree of deacetylation and molecular weight. This natural product, of marine source, has some vital properties that prevent the wound infections, that include: permeability to oxygen, antifungal and bactericidal properties and tissue regeneration (Ijaz Bano *et al.*,2017). Chitosan and its derivatives have been transformed in various forms and showed very effective antimicrobial activity against different types of microorganisms, such as, viruses, bacteria and fungi (Krishna Rao KSV *et al.,* 2012), (Ma GP *et al.,* 2008). The main chracateristics that influence the antimicrobial activity of chitosan are the molecular weight (Mw) and the polimer concentration. Chitosans with low and medium molecular weight have a stronger antifungal effect than chitosans with high Mw and are harmless to the human body. The efficacy rate of the chitosan formulations may depend on many factors, like the microorganism species from which the chitosan was extracted, concentration of chitosan and of the alkaline solution used for deaceylation, chelating capacity, hydrophilic and hydrophobic characteristic and solubility (Onishi H *et al.*, 1999)(Ijaz Bano *et al*., 2017)(Xu X *et al.*, 2006). The external stimuli, such as, temperature, pH and electric fields, can have an influence on the polimer, due to its cationic nature (V. Patrulea *et al*., 2015). Recent studies have shown that chitosan presents two antimicrobial mechanisms. The first mechanisms suggests that there may be a bond between the interaction of the anionic groups on the cell surface and the cationic groups from the polimeric chain of chitosan (M.B. Dreifke *et al.,* 2015). A great influente upon the bonding capacity of the chitosan molecules to the cell walls of bacteria is the increase in the number of positive charge in the form of $-NH_3^+$ on chitosan, and hence the high antibacterial activity. This interaction leads to the formation of a impermeable layer around the bacterial cells and inhibits the transport of vital solutes to the bacterial cell (J.S. Boateng *et al.*, 2008). Another mechanism involves the invasion of low molecular weight chitosan and chitosan oligomers into the bacteria cell nucleus and inhibit the RNA and synthesis of protein (R.A. Muzzarelli *et al.*, 1999).

Chitosan has demonstrated a high antifungal activity, even at low concentrations against various bacteria types, such as: *Escherichia coli and Staphylococcus aureus* (O. Felt *at al.*, 2000).

Chitosan is a biomaterial, a biodegradable polysaccharide and a coplymer of N-acetyl-D-glucosamine and D-glucosamine (Onishi H *et al.*, 1999). It is obtained by the alkaline deacetylation of chitin, extracted from the exosckeleton of invertebrates (Ocloo *et al.*, 2011). Chitosan is an abundant natural biopolimer, the second most plentiful polimer in nature with many remarkable biological properties like: nontoxicity, biodegradability and biocompatibility. It's an environmental-friendly and a cheap raw material with many medical and industrial applications (Jigar MJ *et al.*, 2007).

MATERIAL AND METHOD:

Chitosan isolation from raw material

Chitosan was extracted from the shells of the stone crab, *Pachygrapsus mormoratus*, collected from the shores of the Black Sea. The chemical extraction followed 4 reaction steps: demineralization, using HCl 1N solution (1:15 w/v), at a temperature of 30^0C, for

Correspondence*: *Alef Mustafa, UMF Carol Davila Bucharest, Faculty of Pharmacy, Traian Vuia Str.Nr.6, Sec.2, Bucharest, Romania e-mail mustafa_alef@yahoo.com*

6h; deproteinization, using NaOH 3,5% (1:10 w/v), at 65^0C , for 2h; decolouration with NaOCl 0,315%, and deacetylation, using concentrated solution of NaOH 50% (1:10 w/v) for 5h, at 100^0C. The obtained chitosan was washed with distilled water until a pH of 6,5 and dried to constant mass.

The chitosan solution was obtained by disolving the sample in acetic acid 0.2M/ sodium acetate 0.1M solution, using a magnetic stirrer, at 50^0C.

Sample characterization

Chemical analysis was carried out for the determination of the degree of deacetylation, molecular weight, ash content and pH.

Determination of degree of deacetylation (DD)

The degree of deacetylation of the sample of chitosan was determined by pH-metric titration of a acid solution of chitosan with an aqueous solution of sodium hydroxide (NaOH). The pH measurements were made using a Consort C861 Multi-parameter analyzer with a SP10B type pH electrod.

Determination of the molecular weight (Mw)

The molecular weight of the chitosan samples was determined by measuring the intrinsic viscosity of the solutions, in 0,2M acetic acid/0,1M sodium acetate buffer using a Ubbelohde (SCHOTT, Mainz) capillary viscometer, with the capillry constant of 0,009056 mm^2/s^2.

Determination of the ash content

The ash content was determined by thermogravimetric analysis (TGA) using a Simultaneous Thermal Analyzer STA 6000, in air atmosphere (20mL/min), in a range of temperature of 25^oC-950 ^{o}C, at 10^oC/min.

Determination of pH

The pH measurement of the chitosan solutions were carried out using a microprocessor pH meter.

FTIR studies of chitosan

The FTIR spectra was recorded using a TENSOR 37 model FTIR spectometer over the frequency range 400-4000 cm^{-1}.

Antimicrobial studies of chitosan

The agar medium was prepared in Petri dishes, by adding 1mL of inoculated culture yeast extract and 80mL sterilized unspecific nutritiv agar. The concentration of the yeast extracts was 3 x 10^{-8}. After solidification of the agar media, sterile disks were made on the inoculated plated by using sterile molds for each volume of solution. The chitosan solutions were prepared in 3 different concentrations: 0.025%, 0.05% and 0.1% and these solutions were added to the disks in 3 different volumes (0,25mL, 0,5mL, 1mL). The plates were incubated at 37^0C for 24 in a bacterial incubator. The inhibition halos that appeared around the discs were measured and recorded as the inhibition zones of the bacterial proliferation under the influence of the chitosan solutions.

RESULTS AND DISCUSSION:

The extraction of chitosan involves a series of chemical steps. First of all, the chitin is isolated from the raw shells of the crabs, than the proteins are removed – in the deproteinization step. The next step is the demineralization for the removal of carbon and other salts present in the raw shells. The final step is the deacetylation, when chitin transform into chitosan. The recorded results of the chitosan extraction are shown in Table 1. Each step is described by method and materials.

Table 1.
The extraction process of chitosan

Step	Method
Demineralization	HCl 1N (1:15 w/v), t=30^0C, 6h
Deproteinization	NaOH 3,5% (1:10 w/v), t=65^0C , 2h
Decolouration	NaOCl 0,315%
Deacetylation	NaOH 50% (1:10 w/v), t=100^0C, 5h

The physicochemical results of the studies made for the determination of the extracted chitosans characteristics are shown in Table 2.

Table 2.
Characteristics of the Chitosan extracted from *Pachygrapsus mormoratus*

Characteristic	**Result**
Degree of deactylation (DD)	60,1%
Ash	2,23%
pH	8,2
Molecular weight	9,56 x 10^5 g/mol

Fourier transform infrared spectroscopy analysis

Figure 1. shows the FTIR spectrum of chitosan with Mw=9,56 x 10^5 g/mol and DD=60,1% over the frequency range 400-4000 cm^{-1}. The absorbtion band observed at the 1027 cm^{-1} peack represents the free amino group (-NH_2) at C_2 position of glucosamine. The 1422 cm^{-1} peack represents the –C-O stretching of primary alcoholic group (-CH_2-OH). The absorbance bands at 2865, 1653, 1442 cm^{-1} indicated the N-H stretching, symetric CH_3 stretching and asymetric CH_2 stretching.

In this study, the absorbance bands that were observed are the same as the absorbance bands of the standard chitosan, at 2865, 1653, 1442, 1027, 899 și 775 cm^{-1} which confirms the structure of chitosan (Puvvada YS *et al.*, 2012).

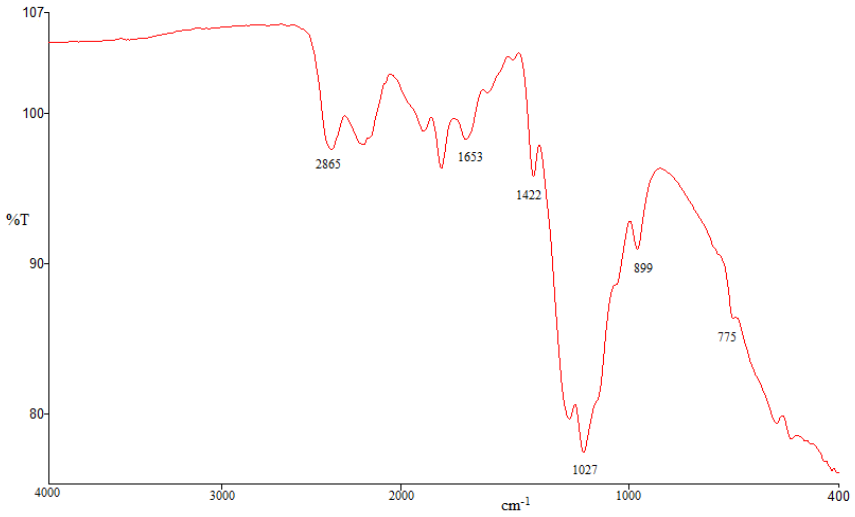

Fig.1.The FTIR report of the chitosan extracted from *Pachygrapsus mormoratus*

Antibacterial activity studies

The study was developed upon the antimicrobial activity of chitosan extracted from *Pachygrapsus mormoratus* against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*.

The start mechanism was the inhibition property of chitosan against microbial cells; due to its polycationic character, chitosan interacts with the anionic groups found on the surface of the cells and forms an waterproof layer, that stops the flow of essential fluids. The zone of action is the external membrane of the gram-negative bacteria (Helander I *et al.*, 2001).

The inhibition effect was more pronounced against gram-negative bacteria (*E.coli*) than gram-positive (*S.aureus*), because gram-pozitive bacteria present a thick cell wall formed by high amounts of peptidoglycan. (Hu B *et al.*, 2008). Figure 2 presents the inhibition zones formed by the chitosan solutions in different concentrations and volumes on the contaminated plates.

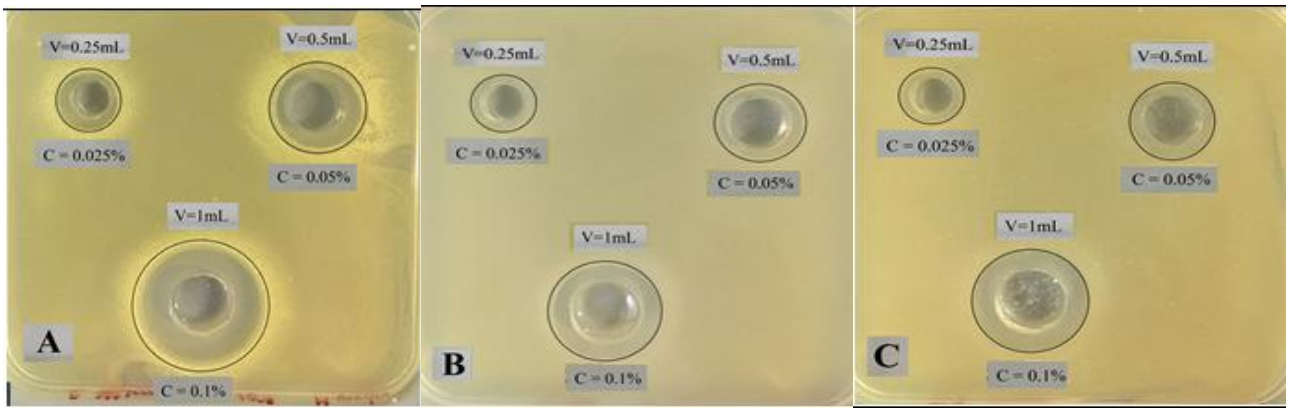

Fig. 2. The inhibition zones of the antibacterial tests. A. *Escherichia coli*; B. *Candida albicans*; C. *Staphylococcus aureus*

The inhibitions of the development of bacteria is observed at a minimal concentration of 0.025% chitosan solution, especially on the *Escherichia coli* germ, but also at the rest of the studied bacteria. We can conclude that the cationic amino gropus of chitosan bond with the anionic groups of the micriorganisms and present a growth inhibition of the pathogen (Laka M *et al.*, 2006).

CONCLUSIONS:

Natural polymers and marine bioresources have demonstrated a high efficiency in the antimicrobial activity and the prevention of wound and burn infections, due to their similar characteristics and properties to the sintetic products. Additionaly, these substances are free of toxicity and side effects, present major biodisponibility and biodegradability, some of the most important aspect to consider when chooseing the right material for the formulation of a safe pharmaceutical product for human consumption. The study showed the similarity of the chemicaly extracted chitosan from the shells of *Pachygrapsus mormoratus* to the commercial chitosan and the antimicrobial activity was as effiecient as the sintetic drugs against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. It was observed that the inhibition effect was more pronounced against gram-negative bacteria (*E.coli*) than gram-positive (*S.aureus*).

REFERENCES:

A.Francesko, T. Tzanov, 2011. Chitin, chitosan and derivatives for wound healing and tissue engineering, Adv. Biochem. Eng. Biotechnol. 125, 1–27.

Helander I, Nurmiaho-Lassila E, Ahvenainen R, Rhoades J, Roller S, 2001. Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. Int J Food Microbiol; 71: 235-44.

Hu B, Wang SB, Wang K, Zhang M, Yu SH, 2008. Microwaveassisted rapid facile ''green'' synthesis of uniform silver nanoparticles: self-assembly into multilayered films and their optical properties. J Phys Chem C 112:11169–11174.

Ijaz Bano, Muhammad Arshad, Tariq Yasin, Muhammad Afzal Ghauri, Muhammad Younus, 2017. Chitosan: A potential biopolymer for wound management. International Journal of Biological Macromolecules. 380–383

J.S. Boateng, K.H. Matthews, H.N.E. Stevens, G.M. Eccleston, 2008. Wound healing dressings and drug delivery systems: a review, J. Pharm. Sci. 97, 2892–2923.

Jigar MJ, Sinha VK, 2007. Ceric ammonium nitrate induced grafting of polyacrylamide onto carboxymethyl chitosan. Carbohydr Polym 67:427–435

Krishna Rao KSV, Ramasubba Reddy P, Lee YI, Kim C, 2012. Synthesis and characterization of chitosan–PEG–Ag nano composites for antimicrobial application. Carbohydr Polym 87: 920–925

Laka M, Chernyavskaya S, 2006. Preparation of chitosan powder and investigation of its properties. Proc Estonian Acad Sci Chem; 55(2):78-84.

M.B. Dreifke, A.A. Jayasuriya, A.C. Jayasuriya, 2015. Current wound healing procedures and potential care, Mater. Sci. Eng. C 48; 651–662.

Ma GP, Yang DZ, Zhou YS, Xiao M, Kennedy JF, Nie J, 2008. Preparation and characterization of water-soluble N-alkylated chitosan. Carbohydr Polym 74:121–126

O. Felt, A. Carrel, P. Baehni, P. Buri, R. Gurny, 2000. Chitosan as tear substitute: a wetting agent endowed with antimicrobial efficacy, J. Ocul. Pharmacol. Ther.16, 261–270.

Ocloo, F.C.K., Quayson, E.T., Adu-Gyamfi, A., Quarcoo, E.A., Asare, D., Serfor-Armah, Y., Woode, B.K., 2011. Physicochemical and functional characteristics of radiation-processed shrimp chitosan. Radiat. Phys. Chem. 80, 837–841.

Onishi H, Machida Y, 1999. Biodegradation and distribution of water-soluble chitosan in mice. Biomaterials 20:175–182

Puvvada YS, Vankayalapati S, Sukhavasi S, 2012. Extraction of chitin from chitosan from exoskeleton of shrimp for application in the pharmaceutical industry. Int Curr Pharm J;1(9):258-63.

R.A. Muzzarelli, M. Mattioli-Belmonte, A. Pugnaloni, G. Biagini, 1999. Biochemistry, histology and clinical uses of chitins and chitosans in wound healing, EXS 87, 251–264.

V. Patrulea, V. Ostafe, G. Borchard, O. Jordan, 2015. Chitosan as a starting material for wound healing applications. European Journal of Pharmaceutics and Biopharmaceutics 97,417–426

Xu X, Yang Q, Wang Y, Yu H, Chen X, Jing X, 2006. Biodegradable electrospun poly(L-lactide) fibers containing antibacterial silver nanoparticles. Eur Polymer J 42:2081–2087