Antibacterial Activity of Chitosan Based Nanoparticle Films Derived from *Aspergillus niger*

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ABSTRACT

Chitosan a deacetylated derivative of chitin has been reported for its various food and pharmacology industries. Chitosan was extracted from the fungal mycelia, *Aspergillus niger*. The physical-chemical nature of fungal chitosan was characterized by FTIR, MALDI-TOF and solid-state NMR with a 70.56% Degree of Deacetylation. Chitosan-mediated inhibition is affected by several factors included such as intrinsic, environmental, microorganism and physical state. Numerous studies presented chitosan with some biological properties such as antibacterial activity, antitumor activity, and antifungal activity paving the way to solid state forms such as beads, films, fibers, and hydrogels. Hence the present study was focused on preparation of chitosan based nanoparticle film were prepared using ionic gelation method followed by casting plate technique. SEM micrograph image showed the size of nanoparticles varied approximately from 200 nm to 400 nm. The films were further subjected for antibacterial action against Gram positive and Gram negative bacteria.

KEYWORDS

Antibacterial activity, Degree of Deacetylation, FTIR, Fungal chitosan, Nanoparticle film.

INTRODUCTION

Chitin is a naturally occurring mucopolysaccharide present in the exoskeleton of crustaceans, insects and fungal cell walls. It is one of the most abundant polysaccharides on earth and ranks second to cellulose in terms of abundance. Chitin consists of a sugar backbone with β-1, 4-linked glucosamine units and a high degree of acetylation. It is a derivative of cellulose with the hydroxyl groups replaced by amine groups thereby making it polycationic. Chitosan obtained typically by the slightly alkaline deacetylation of polysaccharide. Owing to its negligible toxicity and biodegradability, chitosan has found considerable application in the pharmaceutical industry. Moreover, chitosan itself has been rumored to possess numerous useful medical specialty properties viz. wound-healing properties, antiulcer properties, anti-acid properties, hypocholesterolemic action, spermicidal activity, anti-tumor, and hemostatic properties.

The role of chitosan in the pharmaceutical industry has been extensively explored. It is mainly utilized as an excipient for tablets, as a controlled release dosage form, gel, absorption enhance, for drug dissolution, in wound healing products and developing micro/nanoparticles.

Today, wound healing is found in a wide range of applications in the pharmaceutical industry. Due to new advances in nanotechnology, it is now possible to produce drug nanoparticles that can be utilized in a variety of innovative ways (Mohammad pour Dounighi *et al*., 2012). However, among the range of polymers that were used for drug-loaded nanoparticles, chitosan has received nice attention in each of the medical and pharmaceutical fields (Shahbazi *et al*., 2008). Chitosan has been widely applied as a useful biopolymer in food and medical specialty. Chitosan is understood to own varied biological activities together with immune enhancing effects, antitumoral, antifungal, and antimicrobial activities (Li-Feng ki *et al*., 2005).

The effectualness of the many medications is commonly restricted by their potential to succeed in the location of therapeutic action. In most cases solely a tiny low quantity of administered dose reaches the target web site, while the majority of the drug distributes throughout the rest of the body following its physicochemical and biological properties. The developing of drug system that optimizes the pharmaceutical action of drug while reducing its toxic side effects. One of the approaches is the use of mixture drug carriers that may offer website specific or targeted drug delivery combined with optimum drug unharnesses profiles. Among these carriers, liposomes and nanoparticles have been the most extensively investigated. Liposomes gift some technological limitations together with poor reliableness and stability, low drug defense potency. Polymeric nanoparticles that possess a higher reliableness and stability profiles than liposomes are projected as various drug carriers that overcome several of those issues. Nanoparticles are solid colloidal particles with diameters ranging from 1- 100nm. They accommodate drug carriers within which the active ingredient is dissolved, dispersed, entrapped, encapsulated, adsorbate or with chemicals connected. Polymers accustomed

type nanoparticles are often each artificial and natural polymers. Most of the polymers ready from water-insoluble polymers area unit concerned heat, organic solvent or high shear force that can be harmful to the drug stability. Moreover, some preparation ways such an emulsion chemical process and solvent evaporation area unit complicated and need a variety of preparation steps that area unit longer and energy-intense. In contrast, water-soluble polymers offer mild and simple preparation methods without the use of organic solvent and high shear force. Among water-soluble polymers available chitosan is one of the most extensively studied.

This is a result of chitosan possess some ideal properties of compound carriers for nanoparticles like biocompatible, biodegradable, non-toxic and inexpensive. Furthermore, it possesses positively charge and exhibits absorption enhancing effect. These properties render chitosan a engaging material as a drug delivery carrier. In the last 10 years, Chitosan wound healing nanoparticles have been extensively developed and explored for pharmaceutical application.

Chitosan nanoparticles can easily be prepared by the ionic gelation method using Tripolyphosphate as a crosslinking agent. The advantage of this technique was attributed to its gentle conditions achieved while not applying harmful organic solvent, heat or vigorous agitation that are damaging to sensitive proteins. Moreover, it may with efficiency retain the bioactivity of macromolecules (such as deoxyribonucleic acid, proteins, etc.) throughout preparation. It has been reported that chitosan nanoparticles have an excellent capacity for associating protein (Pan *et al*., 2002 & Gan *et al*., 2007).

MATERIALS AND METHODS

SOURCES

Chitosan was isolated from the fungus *Aspergillus niger* obtained from the Culture Collection Centre, Centre for Advanced Studies in Botany, University of Madras, Chennai Tamilnadu, India.

EXTRACTION OF CHITOSAN

Chitosan extraction was carried out by a modified method of Shajahan. A *et al*., (2017). The whole biomass were ground, suspended with 1M NaOH solution and autoclaved at 121°C for 30 min. Alkali-insoluble fractions (AIF) were collected after centrifugation at 10000 rpm for 15 min, washed with distilled water and recentrifuged to a neutral pH. The residues were further extracted using 1% acetic acid at 95°C for 8h. The extracted slurry was centrifuged at 10000 rpm for 15 min. The supernatant was separated from the precipitated chitin, adjusted to pH 8.5 - 9, and centrifuged. The obtained precipitate, chitosan air-dried at 60 °C to a constant weight.

PREPARATION OF CHITOSAN NANOPARTICLES

Chitosan nanoparticles are prepared by ionic gelation method. This method is based on the conjugation of oppositely charged macromolecules for preparing chitosan nanoparticles. Tripolyphosphate is nontoxic,

multivalent and able to form gelate through ionic interaction between positively charged amino groups of chitosan and negatively charged Tripolyphosphate used to prepare chitosan nanoparticles. Sodium tripolyphosphate solution is added dropwise with a syringe to chitosan solution while stirring. The resulting suspension is subsequently centrifuged at 10000 rpm for 15 min. The pellets obtained are re-suspended in deionized water by sonication, centrifuged and dried at room temperature. Drug-loaded chitosan nanoparticles are formed spontaneously upon dropwise addition of 12 ml of 0.4 % aqueous sodium tripolyphosphate solution to 20 ml of 0.35%w/v chitosan solution containing 3 – 8 mg/ml of the drug under magnetic stirring, followed by sonication. The resulting nanoparticle suspensions are centrifuged at 10000 rpm washed with distilled water and dried (Shajahan. A *et al*., 2017).

CHARACTERIZATION OF EXTRACTED CHITOSAN BY FOURIER TRANSFORMS INFRARED SPECTROMETRY

Extracted lyophilized chitosan was ground into powder and further subjected to Fourier Transform Infrared Spectrometer analysis. FTIR analysis was performed with Perkin Elmer through lambda software. FTIR analysis for chitosan was performed through KBR Disc method. Potassium Bromide (KBr) was preground and desiccated at 500°C for 12hrs. 2mg of chitosan sample was ground along with 100mg of Potassium Bromide (KBr) and Disc was prepared by Hydraulic pressure method.

NUCLEAR MAGNETIC RESONANCE (NMR)

15mg of chitosan were added to a 5 mm NMR tube containing 0.8 mL of 25% HCl solution in D2O for chitosan, and 0.5 mL of 2.5% HCl solution in D2O for chitosan. Samples are heated at 80°C for 30 minutes to speed up the dissolution. NMR spectra were recorded using a Bruker AVANCE 300 spectrometer at 80°C.

DETERMINATION OF WEIGHT AVERAGE MOLECULAR WEIGHT

The average molecular weight is calculated using MALDI-TOF spectrophotometer. So using the molecular weight of chitosan we can predict the length of the chitosan chain concerning some monomers in the chain.

DEGREE OF ACETYLATION (DAC)

^{13}C CP/MAS NMR spectra were recorded at 100.62 MHz Bruker MSL 300 Spectrometer. Degree of deacetylation (DA) was calculated using the following equation,

$$\boldsymbol{Degree\ of\ deacetylation\ (DA)} = \frac{I_{CH_3}}{\frac{(I_{C_1}+I_{C_2}+I_{C_3}+I_{C_4}+I_{C_5}+I_{C_6})}{6}}.$$

Where I_{C_1} to I_{C_6} be the intensities of C1, C2, C3, C4, C5 and C6 ring of carbons and I_{CH_3}, intensity of the methyl carbon.

MORPHOLOGICAL CHARACTERIZATION

The surface morphology of the chitosan micro-particle was observed with the scanning electron microscope (SEM). The micro-particle was vacuum dried, sputter-coated with gold-palladium for 5 minutes and observed microscopically.

PREPARATION OF CHITOSAN NANOFILM

The Chitosan Nanoparticle films were prepared and modified according to the Azeredo H M C *et al.*, (2009) method.

A quantified amount of chitosan was dissolved in water containing acetic acid. The solution was continuously kept under stirrer at 40^0C for one hour. The impurities were removes. To this solution quantified amount of glycerol was added under stirring. The solution was degassed with aspirators, to remove gas bubbles. Glass plates which are cleaned with ethyl alcohol are taken and covered with diaxial oriented polypropylene film (BICOR B-306) (Mobil chemical Co., Pittsford, New York) to facilitate the removal of film from plates. The solution was cast in the platter around the circular area and spread manually without any formation of air bubbles. The setup was dried for 24 hours at ambient conditions and in the environmental chamber at 60 ^{0}C and 15%relative humidity to complete drying. Films were peeled from oriented polypropylene film after drying. They are soaked in basic solution like sodium hydroxide solution to remove the acidic nature of the film. Further they were washed twice with glass distilled water for 2 times. They are stored in darkness at ambient condition (23^0C and 50% relative humidity) in an environmental chamber.

ANTIBACTERIAL ACTIVITY

Antibacterial activity for chitosan nanofilms: Well Diffusion Assay

The preliminary screening of crude extract of fungi for antibacterial activity against three bacterial species was done by agar well diffusion method (Patel *et al.*, 2007).

Bacterial Strains

The bacterial culture used was obtained from National Chemical Laboratory, Pune, India.

Gram-Positive Bacteria

- *Staphylococcus aureus* ATCC25923
- *Streptococcus mutans* ATCC700069

Gram-Negative Bacteria

- *Escherichia coli* ATCC25922
- *Klebsiella pneumonia* ATCC29665

RESULTS

FTIR SPECTRUM OF EXTRACTED CHITOSAN

The infrared spectra of isolated chitosan from *Aspergillus niger* (figure 1 and 2). Chitosan exhibits main characteristic bands of carbonyl (C=O-NHR) and amine group (-NH2) at 1642 cm-1. The characteristic absorption bands of chitosan are at 1056.09–1145.61 cm−1, confirms that the monomers in chitosan are linked by d-glycosidic linkages, the same holds for the bonds between the monomers in the glucan component. Absorption band at the region of 1368-1622 cm−1, this absorption band is due to associated with water or amide band. The shoulder amide band in the spectra of alkaline soluble and insoluble glucan might be contributed from the chitosan polymer.

The samples were analyzed by FTIR and the graph depicting wave number versus % transmittance is shown in figure 1 and figure 2.

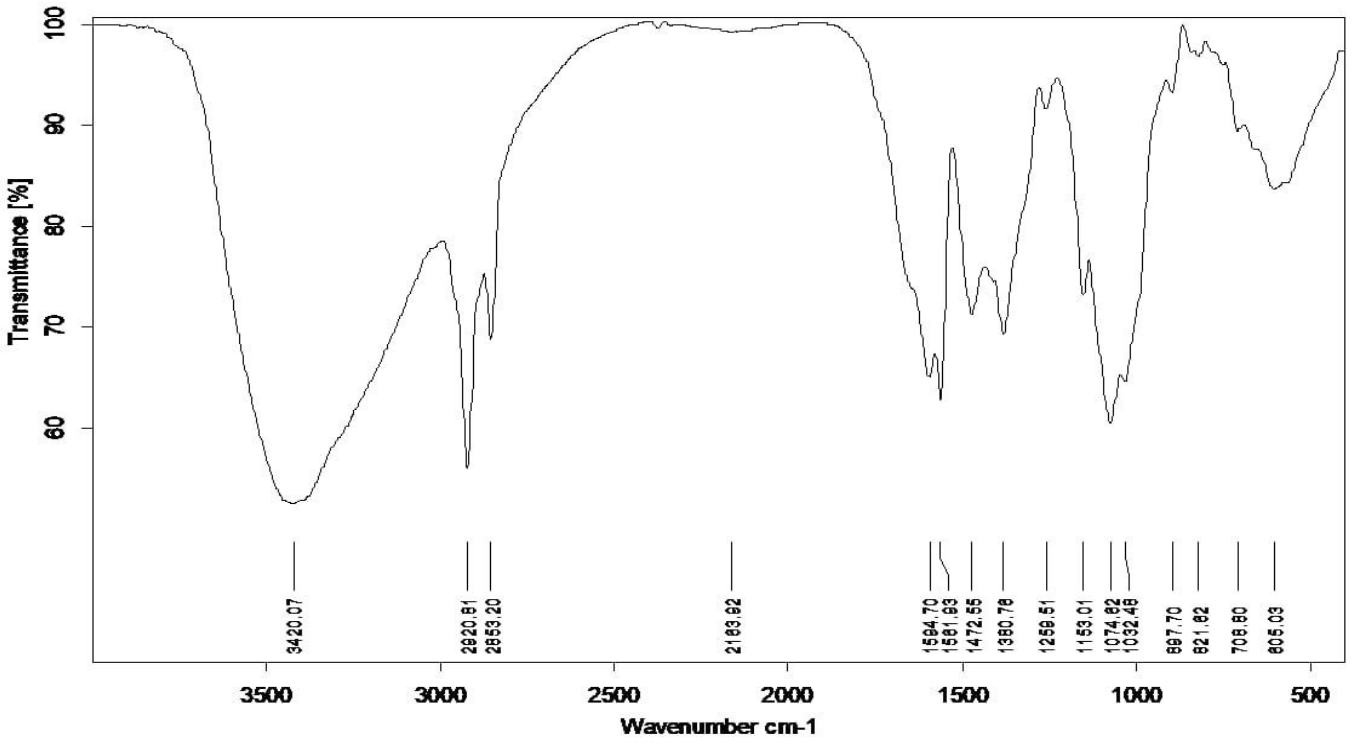


FIGURE 1: FTIR CHARACTERIZATION OF PRACTICAL GRADE CHITOSAN

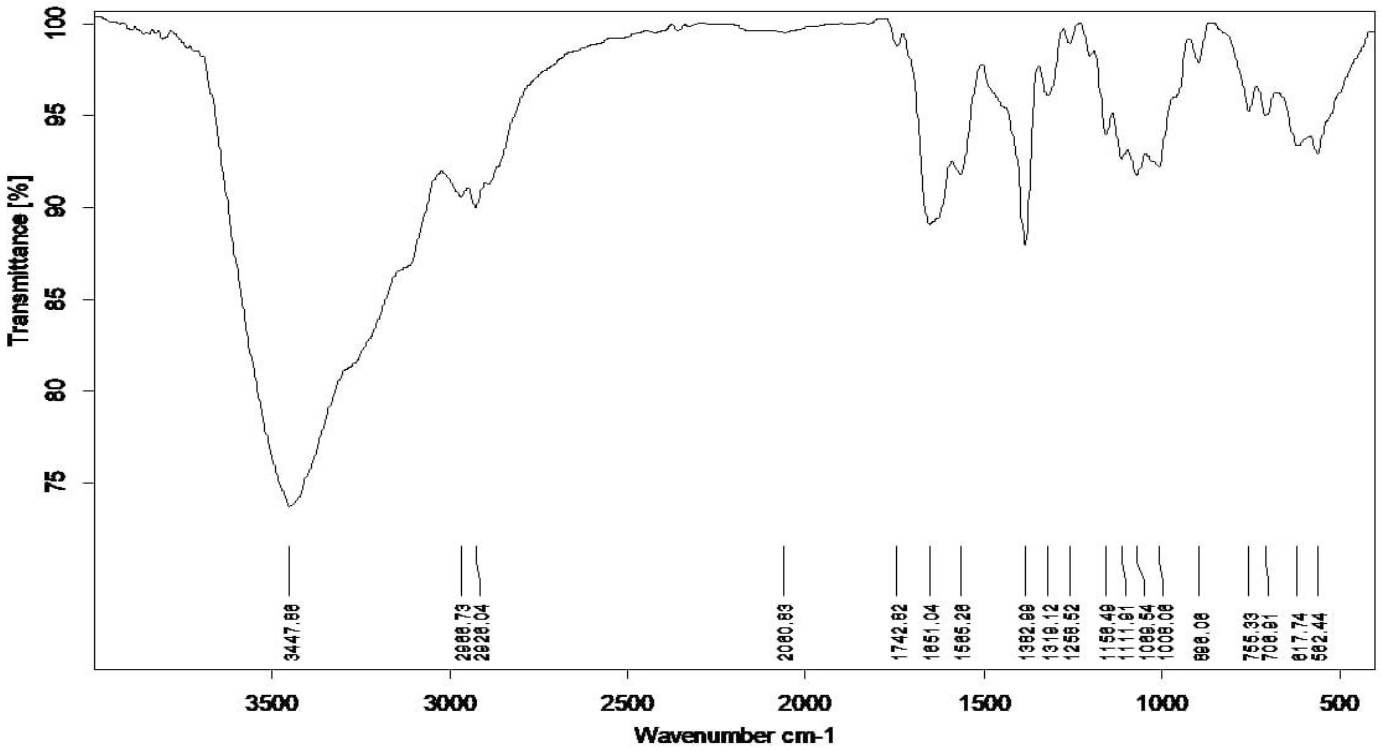


FIGURE 2: FTIR CHARACTERIZATION OF EXTRACTED FUNGAL CHITOSAN

NUCLEAR MAGNETIC RESONANCE (NMR)

NMR stands for the samples placed in a powerful magnetic field that empowers the molecular structure was analyzed by observing and measuring the interaction of nuclear spins. The analyzed samples were shown in figure 3. The degree of deacetylation was measures as 70.56 %.

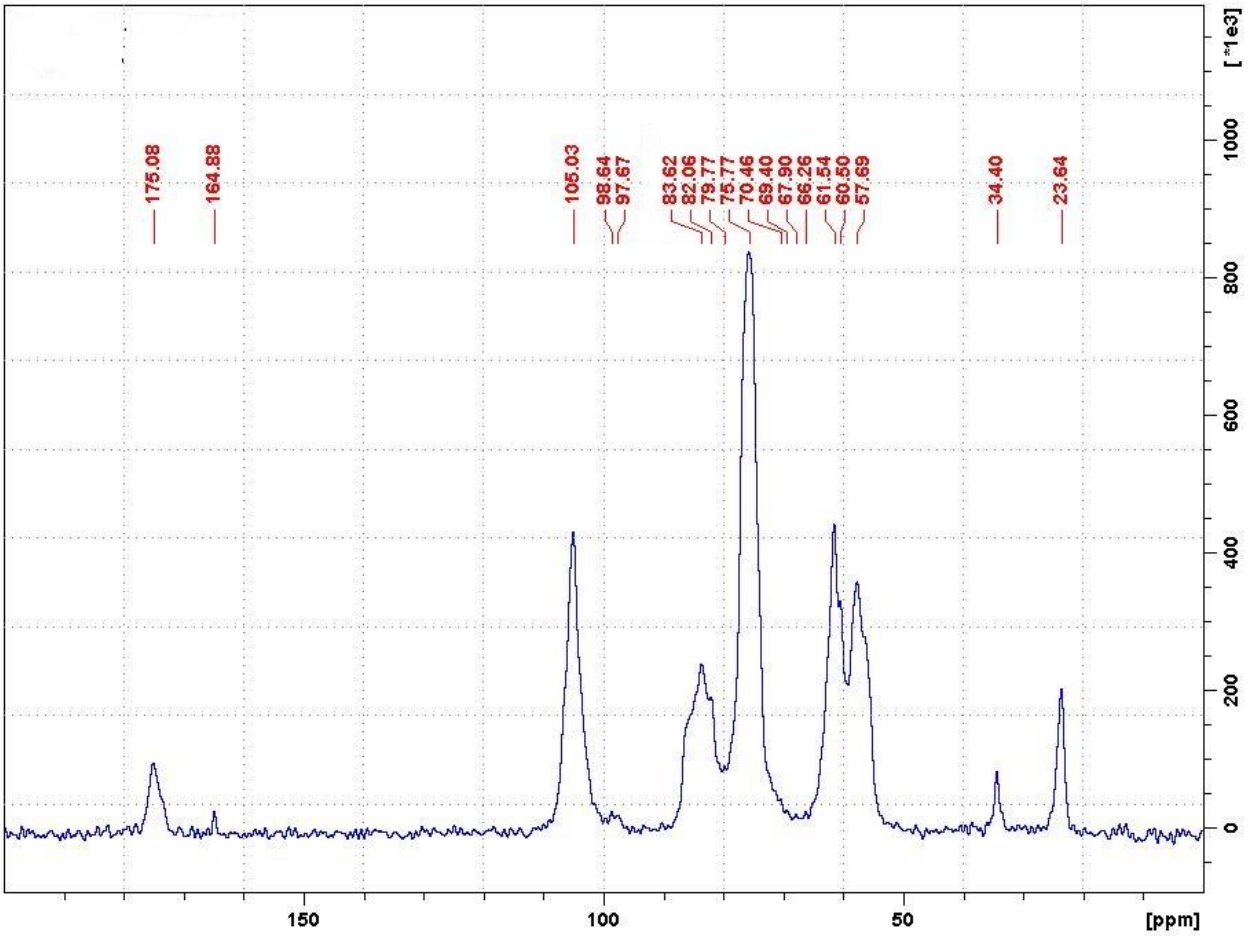


FIGURE 3 ^{13}C CP MAS SOLID STATE NMR SPECTROSCOPY OF EXTRACTED CHITOSAN

MALDI-TOF SPECTROPHOTOMETER

MALDI- TOF involves a laser strike of matrix for small molecules to make the analyzed molecules into the gas phase without fragmenting or decomposing them. The analyzed sample's results showed in figure 4 and the reading of samples shown in table 1.

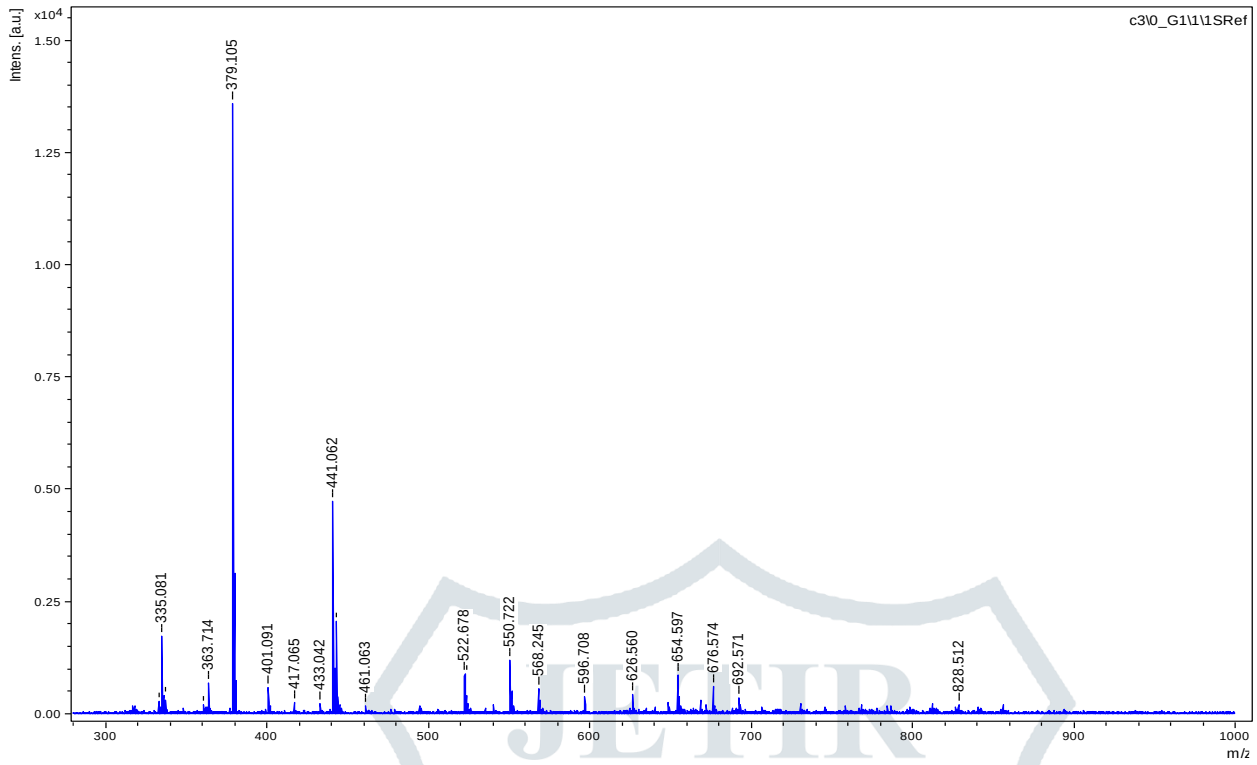


FIGURE 4: MALDI-TOF

TABLE 1: READING FOR MALDI-TOF SPECTROPHOTOMETER

m/z	SN	Quality Fac.	Res	Intens	Area
333.041	12.1	99	3354	243.71	31
337.082	85.3	7110	3259	1754.84	223
336.094	9.9	196	3607	207.41	22
351.083	8.7	40	4577	193.74	20
367.724	26.0	1209	3174	601.99	95
399.165	522.4	76674	3492	13676.93	1980
406.081	30.7	1168	3981	546.34	73
417.065	13.1	435	4458	226.78	26
433.042	9.8	325	5494	208.54	27
441.062	181.2	42109	4640	4547.96	609
443.066	70.0	1444	4890	1702.30	216
461.063	10.1	115	5463	161.99	21
535.679	35.3	494	5016	816.73	118
534.231	10.8	141	6559	250.99	28
560.732	47.7	1403	6228	1170.43	162
578.247	22.8	775	5054	593.40	93
569.652	17.7	473	6008	465.06	63
596.708	16.1	687	5140	355.66	63
626.560	16.2	931	8894	400.13	42
654.597	31.1	1647	6975	824.01	117
676.574	20.7	1303	7919	556.22	70
682.571	13.9	540	7959	350.60	45
828.512	7.6	204	8760	171.76	28

SCANNING ELECTRON MICROSCOPE

The observed chitosan nanoparticles in the film were showed in the figure 5. The sizes of nanoparticles were measured using image J software with a size ranging from 200 nm to 400 nm. The nanoparticles are spherical in shape.

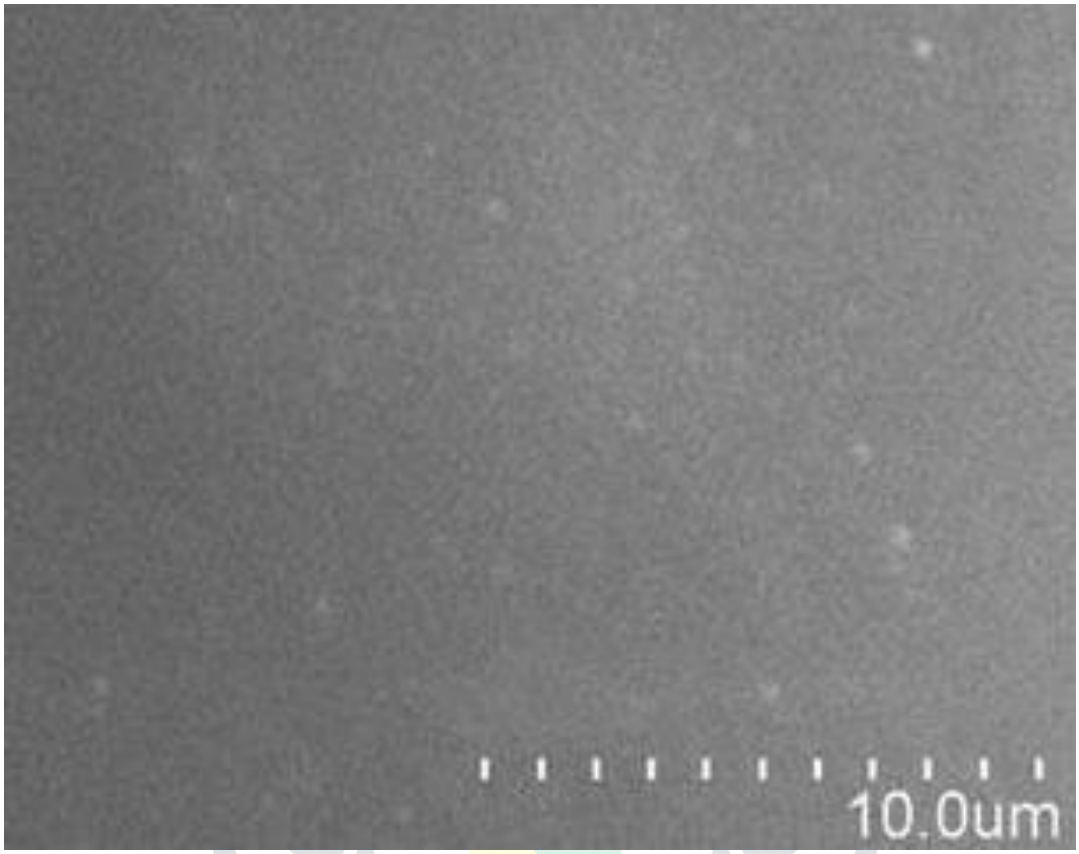
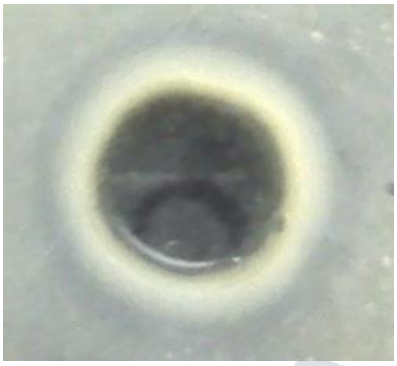


FIGURE 5: SEM MICROGRAPH OF CHITOSAN BASED FILM

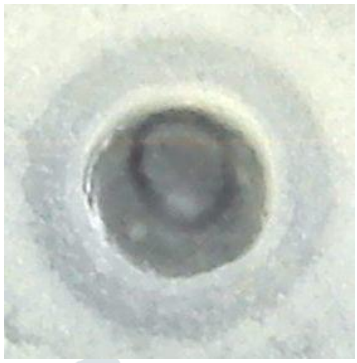
FIGURE 6: THE CHITOSAN BASED NANOFILM

ANTIBACTERIAL ACTIVITY

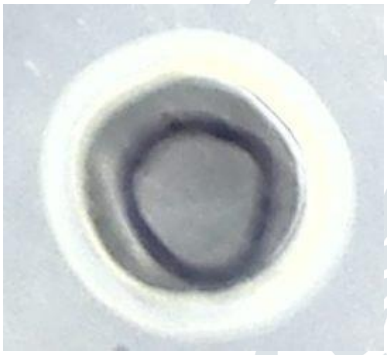
Inhibitory effect of chitosan based antibacterial activity against *S.Aureus*, *S.mutants* and *Klebsiella pneumonia* and *E.coli* in Well Diffusion Assay.



S.Aureus



S.mutans



Klebsiella pneumonia

E.coli

SUMMARY AND CONCLUSION

Over the last decades, considerable interest and attention have been focused on chitosan ascribing from its potential and advantages as antimicrobial agent. Investigations on its antimicrobial property originated from typical morphology observation to micrometer and submicron inner structural metabolism study. The methods and technologies used to evaluate the phenomena and results have gone beyond sole biological conception, but yet incorporate a combination of disciplines involving chemistry, physics, informatics, nanotechnology, and genetic engineering. The focus will persist and more thorough comprehension about the mode of action, which is also beneficial for the exploitation of new generation of antimicrobial agents and the development of new biomedicine. The present study showed chitosan based nanoparticle film were prepared using ionic gelation method followed by casting plate technique. The electron micrograph image indicated the size of nanoparticles varied approximately from 200 nm to 400 nm. The data elucidates the inhibition of nanoparticle films against Gram positive as well as Gram negative bacteria. However significant results were observed against Gram negative bacteria. In the case of the antimicrobial mode of action, future work should aim at clarifying the molecular details of the underlying mechanisms and their relevance to the antimicrobial activity

of chitosan. Moreover, further investigations in this area, in particular concerning bacterial resistance mechanisms against this compound, are warranted.

REFERENCES

1. Domard. A and Rinaudo. M. Preparation and characterization of fully deacetylated chitosan. International Journal of Biological Macromolecule, 1983; 5:49–52.
2. Gan Q, Wang T. Chitosan nanoparticles as protein delivery carrier-systematic examination of fabrication conditions for efficient loading and release. Colloids Surfaces B. 2007;59(1):24-34.
3. Hancock, S., In: Mozes, N., Handley, P.S., Busscher, H.J., Rouxhet, P.G. (Eds.), Cell surface analysis. VCH Publishers, Weinheim, 1991; p. 23.
4. Jeon, Y.J., Park, P.J., Kim, S.K., Antimicrobial effect of chitooligosaccharides produced by bioreactor. Carbohydrate Polymers 44, 2001;71–76.
5. Jucker, B.A., Zehnder, A.J.B., Harms, H., Quantification of polymer interactions in bacterial adhesion. Environmental Science & Technology 32, 1998; 2909–2915.
6. Kong, M., Chen, X.G., Liu, C.S., Yu, L.J., Ji, Q.X., Xue, Y.P., Cha, D.S., Park, H.J., Preparation and antibacterial activity of chitosan microspheres in a solid dispersing system. Frontiers of Materials Science in China 2, 2008; 214–220.
7. Li-Feng Qi, Zi-Rong Xu, Yan Li, Xia Jiang, Xin-Yan Han. In vitro effects of chitosan nanoparticles on proliferation of human gastric carcinoma cell line MGC803 cells. World J Gastroenterol 2005; 11(33):5136-5141.
8. Mohammadpour Dounighi N, Eskandari R, Avadi MR, Zolfagharian H, Mir Mohammad Sadeghi A, Rezayat M. Preparation and in vitro characterization of chitosan nanoparticles containing Mesobuthus eupeus scorpion venom as an antigen delivery system. The Journal of Venomous Animals and Toxins including Tropical Diseases ISSN 1678-9199, 2012, volume 18, issue 1, pages 44-52.
9. Moore.G.K and Roberts. A.F., Determination of the degree of N-acetylation of chitosan**.** International Journal of Biological Macromolecule, 1980; 2:115–116.
10. Ozbas Turan. S, Akbuga. J, Aral. C. Controlled release of interleukin -2 from chitosan microspheres, J.Pharm. Sci.91 2002; 1245-1251.
11. Pan Y, Li YJ, Zhao HY, Zheng JM, Xu H, and Wei G, et al. Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin in vivo. Int J Pharm. 2002; 249(12):139-47.
12. Patel, J.K., Jivani, N.P. Chitosan based nanoparticles in drug delivery. Int. J. Pharm. Sci. Nanotech. 2, 2007: 517-52.

13. Sathiyaseelan. A, Shajahan. A, Kalaichelvan. P.T., Kaviyarasan. V. Fungal chitosan based nanocomposites sponges—an alternative medicine for wound dressing. 2017: Centre for Advanced Studies in Botany, University of Madras, Guindy Campus, Chennai, 600025, India
14. Shahbazi MA, Hamidi M, Peymani P. Interaction of chitosan, a natural polymer used in Nanodrug/gene delivery, with non-steroidal anti-inflammatory drugs (NSAIDs). Internet J Nanotech. 2008;2(2).
15. Shajahan A, Shankar S, Sathiyaseelan A, Karthik S. Narayan, Narayanan V, Kaviyarasan V, Ignacimuthu S. Comparative studies of chitosan and its nanoparticles for the adsorption efficiency of various dyes. International Journal of Biological Macromolecules. 2017.
16. Shweta Kalyan, Pramod Kumar Sharma, Vipin Kumar Garg, Nitin Kumar, Jonish Varshney. Recent advancement in Chitosan based formulations and its pharmaceutical application. Der Pharmacia Sinica, 2010, 1 (3): 195-210.