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In Vitro Evaluation of Antimicrobial Activity of Chitosan Against *Mycobacterium smegmatis* MTCC 994

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ABSTRACT

Tuberculosis (TB) presently represent one of the biggest world health problems and hence it is urgent to find new drugs that allow better control of the outbreak and arrest the emergence of patients with multiple drug resistance tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB) cases which have arisen alarmingly. There is a general consensus that antimicrobials from natural products might prove very effective and must be validated so that the same can be exploited for human well-being. The present study provides a scientific validity to chitosan, a natural and readily available compound as chitin over the crustacean body. The chitosan is biodegradable and biocompatible in nature and hence causes minimum hazards to the body. Chitosan solution have shown very effective antimicrobial property against *M. smegmatis* MTCC 994 which was selected for this study as this bacterium is non-pathogenic in nature, has fast growing rate and have similarity in cell wall composition with that of *Mycobacterium tuberculosis.* The disc diffusion assay as well as resazurin microtiter assay (REMA) methods were used to evaluate the antimicrobial activity of chitosan. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the chitosan solution were found around 117.18 µg/ml. This finding suggests that even at lower concentrations, chitosan is very effective and hence can be potent antimicrobial agent in future for *Mycobacterium tuberculosis* as the MDR and XDR TB are evolving rapidly. The current study recommends similar research against *Mycobacterium tuberculosis* to evaluate the potential of chitosan to be used as anti-tuberculosis agent.

HIGHLIGHTS

- We studied antimicrobial activity of chitosan against *Mycobacterium smegmatis* MTCC 994.
- The results are encouraging and chitosan can prove an efficient tool to fight TB in future for which further investigation is required.

Keywords: *Mycobacterium tuberculosis, Mycobacterium smegmatis*, Chitosan, REMA, MBC, MIC.

Tuberculosis (TB) is a common and deadly infectious disease caused by *Mycobacterium tuberculosis* discovered in 1882 by Robert Koch. Tuberculosis most commonly attacks the lungs (as pulmonary TB) but can also affect the other body parts which is known as extra pulmonary TB (Smith, 2003). Tuberculosis is also referred as the "white plague" (Ananthanarayan and Paniker, 2007). It usually spread through air by expulsion of respiratory fluids or bacterial droplets through the air by coughing and sneezing of the patient having an active TB infection. TB has very ancient origins: it has survived over 70,000 years and it currently infects nearly 2 billion people worldwide (MacDonald, 2015); with around 10.4 million

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new cases of TB each year. According to World Health Organization (WHO) almost one third of the world's population are carriers of the TB bacillus **(**WHO, 2016) and are at risk for developing active disease and subsequent death during their lifetime (Caminero, 2005; Global tuberculosis report, 2018). Over 95% of cases and deaths are in developing countries. People who are infected with human immunodeficiency virus (HIV) are 20 to 30 times more likely to develop active TB (Global tuberculosis report, 2018**).** The situation is getting worse because of development of drug resistant by TB bacilli. Globally, 3.5% of new TB cases and 18% of previously treated cases had MDR/RR-TB. Three countries account for almost half of the world's cases of MDR/RR-TB: India (24%), China (13%) and the Russian Federation (10%).

Due to this increase in drug resistant strains of *M. tuberculosis,* there has been renewed interest in natural products as potential sources of novel antibiotics (Cragg and Newman, 2013). The use of natural products as medicines is well known in rural areas of many developing countries. The natural products are cheaper, more effective and impart least side effects as compared to synthetic medicines. The World Health Organization reported that 80% of world's population depends on traditional medicine, and a major part of the traditional therapies involve the use of plant extracts and other active constituents (Rates, 2001). In this regard, increasing investigation has been given to chitosan and its derivatives.

Chitosan is modified form of chitin which is the second most important natural polymer in the world. Chitin occurs in nature as ordered crystalline microfibrils forming structural components in the exoskeleton of arthropods or in the cell walls of fungi and yeast. In recent years, chitosan and its derivatives have attracted much attention as antimicrobial agents against fungi, bacteria, and viruses and as elicitors of plant defence mechanisms. The investigation of chitosan as suitable anti-tuberculosis agent needs a suitable system in which we can test the efficacy of this drug and the results can be directly applied for the tuberculosis. One of its kinds of bacterium in which we can do preliminary studies is *Mycobacterium smegmatis* MTCC 994 which is "fast growing" bacterium (having short doubling time) and is non-pathogenic to workers. This species shares more than 2000 homologous genes with *M. tuberculosis* and hence these properties make this bacterium a suitable agent to study active compound having antimicrobial property against tuberculosis.

MATERIALS AND METHODS

Chemicals and reagents

The chemicals and reagents were purchased from different National and International firms i.e. Sigma Chemicals (USA), Himedia (India) and BD (USA). Middlebrook 7H9 medium and OADC were purchased from BD (USA) while glycerol and Tween 80 were purchased from Himedia (India). The sterile polystyrene 96 well plates were purchased from Nunc. Resazurin salt and Rifampicin were purchased from Sigma (Germany). The chitosan prepared from shrimp shell containing 75% degree of deacetylation (DD) was purchased from Himedia. Acetic acid was of HPLC grade procured from Merck. All other chemicals used in this study were of the molecular grade. We have use bench top centrifuge– REMI (India), Microcentrifuge, Magnetic stirrer– REMI (India) and Micropipettes– Eppendorf, (Germany). Glassware used was obtained from Borosil (India) and Schott Duran (Germany). Glassware were thoroughly washed and sterilized wherever necessary following the recommended procedures.

Maintenance of *Mycobacterium smegmatis* MTCC 994 culture

Fresh inoculums were prepared and used for this study. Older cultures may result in unreliable susceptibility test results. The strain of *M. smegmatis* MTCC 994 was maintained by sub culturing on Mueller-Hinton agar plates incubated at 37°C for 2-3 days. The bacterial strain was sub-cultured in liquid media by transferring a loop full of bacterial growth using sterile loop in sterile screw cap tube with glass beads containing 2-3 ml 7H9 broth. The tubes were incubated at 37ºC for 2-3 days. In liquid culture, Tween 80 is added in the initial cultures to minimize clumps. For REMA, *M. smegmatis* MTCC 994 strains were grown in bulk in 7H9 medium containing 0.05% Tween 80 supplemented with 10% oleic acid albumin-dextrose complex (OADC).

Preparation of chitosan solution

The main difference between chitin and chitosan lies in their solubility; chitosan is therefore said to be chitin that has been *N*-deacetylated to such an extent that it becomes soluble in dilute aqueous acids. Pure, native chitosan (pKa ~ 6.3) is insoluble in water, in alkaline medium and even in organic solvents. However, water soluble salts of chitosan may be formed by neutralization with organic acids (e.g. 1–10% aqueous acetic acid, formic acid, succinic acid, lactic acid, glutamic acid and malic acids) or inorganic acids such as hydrochloric acid (Henriksen *et al*., 1996)**.** The pH-dependent solubility of chitosan is attributed to its amino groups (—NH_2), which become protonated upon dissolution at pH 6 or below to form cationic amine groups (—NH^{3+}), increasing intermolecular electric repulsion and resulting in a polycationic soluble polysaccharide, with a large number of charged groups on a weight basis. Here we standardised the solubility of chitosan by trial and error method by dissolving a constant amount of chitosan at different pH. Finally, we standardised it at a concentration of 7500 µg/ml for this study. Thus 15 mg of chitosan was dissolved in 2 ml of 2% aqueous acetic acid solution under magnetic stirring at 600 rpm using an octagonal stirring bar for maximum solubility of chitosan at pH 4.

Disc diffusion assay

The required quantity of chitosan solution was prepared as mentioned above. Freshly grown *M. smegmatis* MTCC 994 culture was plated over the Mueller Hinton Agar (MHA) plate. Two discs dipped in the chitosan solution containing approx. 10 µl chitosan solution were placed over the MHA plates. Standard disc of Rifampicin was also placed over it to compare the zone of inhibition. A disc dipped in of 2% acetic acid was also placed over it as acetic acid control. The plate was then incubated at 37°C for 2 days. The protocol was repeated thrice.

Minimum inhibitory concentration (MIC)

REMA was performed to determine the MIC of chitosan solution against *Mycobacterium smegmatis* MTCC 994. The test is based on colorimetric method using REMA performed in a 96-well microtiter plates (Martin *et al*., 2003). Briefly, 100 µl of 7H9 broth was dispensed in each well of a sterile flat-bottom 96-well plate and serial two fold dilutions of chitosan solution were performed directly in the plate. The standard *M. smegmatis* MTCC 994 bacterial suspension of No. 1 McFarland standard was prepared and diluted 1:100 in 7H9 broth and 100 µl inoculums was used to inoculate into each well of the plate. Different controls including a growth control, Rifampicin standard drug control (positive control) and a sterile control without inoculums were also included. Plates were sealed and incubated at 37ºC for 2 days. For MIC, after the incubation for 2 days, 30 µl of resazurin 0.02% w/v was added to each wells, sealed and re-incubated at 37°C for 12 h for colour development. A change in color from blue to pink indicated the growth of bacteria. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the drug that prevented this change in color. A change in color of growth control well to pink indicated the proper growth of the isolate and no change in color of media control indicated absence of contaminants.

Minimum bactericidal concentration (MBC)

The primary steps for calculation of minimum bactericidal concentration (MBC) are similar to that of REMA for MIC. Here, before addition of resazurin, 10 µl of solution from each well were placed over MHA plates and incubated for 2 days. If the compound has antimicrobial property, there will be no growth in the MHA plates at a particular lowest concentration and above it. This lowest concentration is the minimum bactericidal concentration (MBC).

RESULTS AND DISCUSSION

At present, tuberculosis is a major health hazard due to the rise of multi drug resistant (MDR) and extensively drug resistant (XDR) strains. Even the presently used drugs are having lot of side effects. Hence it is very important to find out new drugs from natural sources to fight effectively against this dreaded disease tuberculosis. A number of studies have explored a wide range of natural products with strong activity against tubercular bacilli (Cantrell *et al*., 2001; Newton *et al*., 2000). Various plant extracts have also reported for anti-TB activity (Erdemoglu *et al*., 2003; Jimenez-Arellanes *et al*., 2003; Patil and Kumbhar, 2018). There are various group of secondary metabolites reported in plant extracts namely glycosides, saponin, alkaloid etc. having antimicrobial activity (Newton *et al*., 2000). The researchers are now searching for the biocompatible and

biodegradable compounds having potential antimicrobial properties. Chitosan is an undisputed biomolecules of great potential due to the polyelectrolite properties, including the presence of reactive functional groups, gel-forming ability, high adsorption capacity, complete biodegradability, antimicrobial, antifungal, and even having anti-tumor influence. The activity of chitosan depend on the various factor that help to increase its antimicrobial activity (Fouad, 2008). Though comparison of chitosan and chitosan nanoparticles showed that inhibition zone for nano-chitosan was higher than that of chitosan (Qi *et al*., 2004) in our study we have not prepared nanoparticles as the preparation of nanoparticles is a cumbersome event and hence we have just tried with the crude form of chitosan by simply dissolving it in acidified aqueous solution. The same will prove very beneficial in terms of outcome in future as no sophisticated instruments and skills needed for this formulation. The present work evaluated the antimicrobial potential of chitosan against *M. smegmatis* MTCC 994, a close relative of *M. tuberculosis*. The *M. smegmatis* MTCC 994 is a rapidly multiplying non-pathogenic organism hence easier to handle and gives quick results which is not possible with that of *M. tuberculosis* which is slow grower and highly pathogenic and require Biosafety Level-3 (BSL-3) facility to perform the experiments. The chitosan in this study was in the form of white flakes and are 75% deacetylated. The degree of deacetylation has great influence in the activity of chitosan **(**Singla and Chawla, 2001). In this study we purchased, pre-formed chitosan, prepared from chitin that has been extracted from shrimp shell by the manufacturer (Himedia). The required quantity of chitosan was dissolved in 2 ml of 2% aqueous acetic acid (7500 µg/ml final concentration) and disc diffusion was performed first. There were clear zone of inhibition in MHA plate around the chitosan as well as around positive control discs (Rifampicin). Whereas no zone of inhibition were there around the disc dipped with 2% aqueous acetic acid solution. The zone of inhibition by chitosan discs were approx. 11 mm (Fig. 1).

Though the zones of inhibition were smaller than that of standard (Rifampicin), the chitosan posses strong antimycobacterial activity. It has been previously demonstrated that chitosan nanoparticles were effective in inhibiting *M. tuberculosis* growth (Wardani and Sudjarwo, 2018). Even though the present study does not involve the preparation of nanoparticles, the inhibition by chitson solution suggest that chitosan solution itself can be utilized for anti-mycobacterial treatment in Countries with poor economic resources.

Fig. 1: Disc diffusion assay showing antimicrobial activity of chitosan against *M. smegmatis* MTCC 994 **(1)** Disc 1 and 2: Chitosan solution, **(2)** Central disc: RIF (Positive control) **(3)** 2% AA Disc (Acetic acid control)

The result of disc diffusion encouraged us to further investigate the antimicrobial property of chitosan solution and hence we calculated MIC and MBC by using microtiter assay. MIC of chitosan was noted to 117.18 µg/ml (Fig. 2) proving its significant antimicrobial property against *M. smegmatis* MTCC 994.

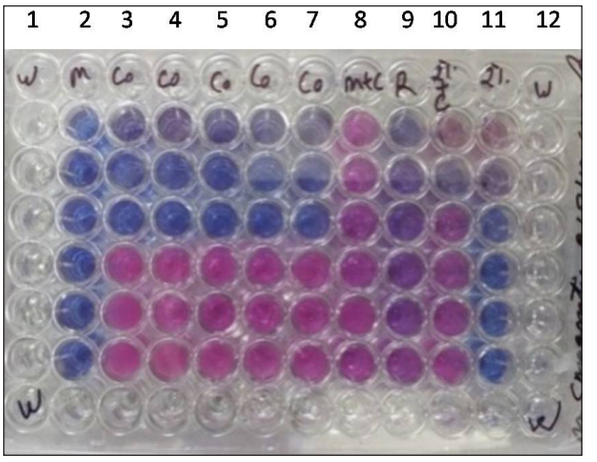

Fig. 2: Minimum inhibitory concentration (MIC) by chitosan solution against *M. smegmatis*. **(1)** Column 1, 12 and Row 1, 8: Water **(2)** Column 2: Media control **(3)** Column 3,4,5,6 and 7: Media + Chitosan + culture **(4)** Column 8: Media + culture **(5)** Column 9: Rifampicin (Positive control) **(6)** Column 10: 2% Acetic acid +culture **(7)** Column 11: 2% Acetic acid

Further decrease in concentrations of chitosan resulted in increase in bacterial growth as a result colour changed from blue to pink after addition of resazurin. Similar results were obtained for MBC (Fig. 3). When 10 µl of culture from each well of microplate were kept over (MHA) and incubated for 2 days, there were no growth in the plate from concentration 117.18 µg/ml and above in the chitosan treated groups. The controls showed the expected results (Fig. 3).

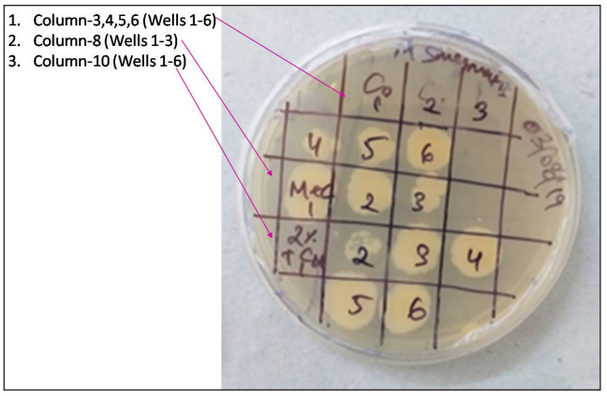

Fig. 3: Minimum bactericidal concentration (MBC) by Chitosan solution against *M. smegmatis*.

We achieved better results in our study and even at lower concentrations of chitosan solution than observed by other researchers (Aliasghari *et al*., 2016). Though the study by Aliasghari *et al*. (2016) utilized Streptococci therefore, the results observed can not be directly interpolated for mycobacteria. However, the finding suggests that chitsoan possess anti-bacterial and anti-mycobacterial activity and further experiments will be required to confirm its activity against drug susceptible and drug resistant tuberculosis.

CONCLUSION

In conclusion, we can say that chitosan which is one of the most abundant polymer in nature and is biodegradable and biocompatible in nature possessing anti-mycobacterial activities. The observation that chitosan inhibit the growth of mycobacteria even at lower concentrations indicate its potential for use in future as anti-mycobacterial agent and may serve as an arsenal in our fight against tuberculosis.

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