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Chitosan and antibiotic coated 40S cotton with synergistic effect for enhanced bioefficacy against nosocomial pathogens

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Abstract: The current study focuses on the bioefficacy potency of chitosan against the nosocomial pathogens. For the effective application in hospital environment, chitosan was coated on the surface of 40s cotton to exhibit antibacterial activity against the bacterial pathogens *Escherichia coli* and *Staphylococcus aureus* prevailing in hospital zones. Antibiotics with similar mode of bacterial killing action like chitosan were analysed for the synergistic effect using checker board assay. Using pad-dry-cure method the 40s cotton fabric was coated with chitosan along with an anchoring agent cyanuric chloride. Similarly the reactive dye binding method was used to coat the synergistic drug Cefixime on the surface of chitosan coated cotton fabric. Scanning Electron Microscopy (SEM) analysis was done for the normal and coated cotton fabrics. Both qualitative and quantitative bioefficacy analysis of the coated cotton fabric were done by using antibacterial activity assay-AATCC 100 test methods and wash fastness test-AATCC 124 test methods respectively. The results showed that the antimicrobial finish of the 40s cotton fabric was effective against *E. coli* and *S. aureus* and will be active for up to 50 home launderings. Even though the growth and colonizing ability of both the bacterial pathogens used in this study were effective, a better killing effect was observed against *S. aureus* than *E. coli.*

***Key words*:** Chitosan, Cefixime, Synergistic drugs, Pad-dry-cure, Reactive dye binding, Wash fastness test

I. Introduction

Hospital environment plays a vital role in determining the comfort and health of patients. The suspended particles in the hospital environment harbouring infectious microbes can be a potent infection spreading agent which gets deposit on hospital fabric surface. Thus a fabric can act as an effective substrate for microorganisms to get colonise on its surface. Nearly about 45% of the nosocomial infection outbreak is due to the dissemination of pathogenic microorganism from hospital used fabrics. The most prevalent nosocomial bacterial pathogenic species in hospital environment are *Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli* (Bharadwaj, *et al.*, 2003).

Fabric surface coated with antimicrobial substances can retard microbial growth and act as protective clothing. An essential need of such protective fabric will be a good support for the hospitalized immuno-suppressive in-patients along with their medications (Kurita, K. 1998). Many biopolymers are available from the natural resources and one such biopolymeric polysaccharide is Chitin, present in the shells of marine crustaceans (Bodmeier, *et al.*, 1989). Chitin is one of the most abundant biopolymers in nature and the main composition of shrimp and crab shells which is found usually as food wastes (Somashekar and Joseph, 1996). Basically these biopolymers are not easily biodegradable and be as remains in the soil sub-layers (Shahidi, *et al.*, 1998). It is considered as an important landfill disturbing the agricultural practice (Schulz, *et al.*, 1998). The farmers are experiencing great difficulty in farming because of the presence of marine crustacean shells as landfills (Nam, K.S., 2001).

Chitin and its derivative chitosan are of commercial interest due to their excellent antibacterial, biocompatibility, biodegradability, non-toxicity, chelating and adsorption power (Monllor, *et al.*, 2010). Chitosan is reported to be having high antibacterial activity than chitin, which is formed by deacetylation of chitin polymer (Mohanraj and Chen, 2006). In the present study, the chitosan was used to prepare bio-medical cotton fabrics for hospital in-patient's use. Using reliable pad-dry-cure method, chitosan was coated on the cotton surface. Its antibacterial effect after coating and its persistence with the coated cotton fabric surface was analyzed for its multiple usages (Boeckh, *et al.*, 1990) and (Mir, *et al.*, 2008).

II. Materials and Methods

The experiments were carried out in the Microbiology Laboratory, Department of Microbiology, CMS College of Science and Commerce, Coimbatore. The FTIR analysis was carried out in PSG College of Arts and Science, Coimbatore and SEM analysis was done at Karunya University, Coimbatore.

Chitosan was commercially procured from Sigma- Aldrich. All the other chemicals used were analytical grade purchased from Merck and Hi Media.

The bacterial cultures of clinical isolate used in this study were purchased from Microbiology Lab, R.S. Puram, Coimbatore.

2.1 Sample Collection

40S Cotton material was purchased from Lakshmi Mills, Coimbatore with the specifications like; Count: Warp - 40^s, Weft – 40^s, Ends per inch (EPI) – 60, Picks per inch (PPI) – 56 and plain weaving.

2.2 Bacterial Strain and Inoculum Preparation

The overnight culture of *E. coli* and *Staphylococcus aureus* strain were diluted with autoclaved ISO Sensitest broth to get the final bacterial inoculums of approx. 7 x 10^5 CFU/ml in each tube. The tubes were incubated at 37˚C for 20 to 24 hours in ambient air before interpretation as described by CLSI (Clinical and Laboratory Standards Institute) guidelines.

2.3 Chitosan

Medium molecular weight chitosan (75 – 85 % deacetylated) was purchased from Sigma- Aldrich, and the degree of deacetylation was measured by FT-IR spectroscopy. Acetate buffer of pH 4.65 was used as solvent for the preparation of stock solution of chitosan.

2.4 Antibiotics

Antimicrobial powder of Clarithromycin (assay value >95% HPLC), Chloramphenicol, Ofloxacin (assay value 99%), Polymycin B (assay value 60-70%), Sulfamethoxazole (assay value 99.9% HPLC), Streptomycin (assay value 98%), Tobramycin (assay value 98% TLC), Tetracycline (assay value 99%), Cefixime (assay value 99% HPLC) and Trimethoprim (assay value 99% HPLC) were purchased from Sigma – Aldrich.

2.5 Antimicrobial Assay

Following the method of San Tin, *et al.* (2010), minimal inhibitory concentration (MIC) for the antibiotics and chitosan were determined in duplicate by the spot inoculation method in nutrient agar plates. Antimicrobial activity of chitosan and antibiotics were tested against the pathogens, using inoculum concentration of 10^6 CFU/ml. The effect of acetate ion concentration in acetate buffer against the pathogens was also determined using the spot inoculation plating method.

2.6 Population Analysis

From the Checkerboard method (San Tin, *et al.*, 2010), the concentrations for the antibiotics and chitosan that show synergism were identified. The response of the bacterial pathogens to these concentrations was investigated by subjecting them to incubation for 48 hrs at 37˚C in the concentrations. The colonies in the plates were then counted and quantified to find the log concentration of bacterial colony forming units per ml.

The checkerboard method was performed in a series of sterile test tubes. Ten twofold dilutions of antibiotics were made with ISO Sensitest broth. Every antibiotic was placed in sterile tubes in descending concentrations starting at two times the MIC and ending at zero MIC. The other antibacterial compound (Chitosan) was similarly distributed among the tubes with antibiotics. Thus, each of the 100 tubes (10 different antibiotics combination with chitosan at 10 different concentrations) held a unique combination of concentrations of the two antibacterial compounds. An inoculum of 50 µl of every pathogen per plate was used at a concentration of about 5 x 10^5 CFU/ml. The plates were incubated overnight, and the MIC was recorded according to the first dilution plate without any bacterial colonies. ΣFIC was calculated and interpreted as below:

Synergism = ΣFIC ≤ 0.5
Antagonist = ΣFIC 2 - 4
Additive = ΣFIC 0.5 - 1
Indifference = same concentration for all the two drugs

2.7 Preparation and finishing of cellulosic with chitosan

For permanent finishing of 40^s cotton, 1% solution of chitosan was prepared using acetic acid, followed by pH-adjustment with Na-carbonate to the final desired value (pH 5 to 6.5) and anchoring chemical was added. Cyanuric chloride (with addition of co solvent dioxane) was used as the anchoring chemical of chitosan with cellulosic cotton. The amount of anchoring chemical used was 5 to 8 X 10^{-4} mol g^{-1} of cotton fabric used (Ashenafi, *et al.*, 2020).

The textile was impregnated for 5 to 10 min and squeezed between rollers to a liquor uptake of ~100 %. Fixation was done at 170°C for 3 min after pre-drying at 80°C (5 min). Washing was done using phosphate buffer solution of pH 4.66 at 40°C (Desislava Staneva, *et al.*, 2023).

2.8 Reactive dye method

This approach in the study was to modify two well described antibacterial drugs (fluoroquinolone and nitroimidazole compounds) for direct attachment to textile fabrics following the method suggested by Chun and Gamble, 2007.

2.8.1 Synthesis of reactive synergistic drugs

To convert the drug reactive and imparting to cotton fabrics, the reactive dye exhaust method was performed. Synthesis of reactive Cefixime drug was accomplished by suspending 0.02 mole each of Cefixime in 20 ml deionized water and maintained in an ice bath at 5°C for 15 min. To this suspension, 0.04 mole cyanuric chloride (2,4,6-trichloro-1,3,5-triazine was added during the drop-wise addition of 0.04 mole (40 ml,1.0 N) sodium hydroxide solution by maintaining at 5°C. Synthesis of reactive nitroimidazole drug was similarly prepared suspending 0.03 mole each of the drug in 20 ml deionized water in an ice bath at 5 °C. To this suspension, 0.03 mole cyanuric chloride was added. The suspension was maintained at 5 °C during the drop-wise addition of 0.03 mole (30 ml 1 N) sodium hydroxide.

2.8.2 Exhaust dyeing method to bind reactive antibacterial agents to textile materials

An exhaust dyeing method was used to bind the synthesized reactive antibiotic to the 40^s cotton fabric. The dye bath was prepared by adding 0.5 ml of Triton-X 100, 75 g of sodium sulfate, and 6.5 g of the reactive antibacterial drug to 1.2 L of deionized water. To the suspension the carrier cyanuric chloride was added at a concentration of 2% as a cross-linking agent. Three, 20 g squares of the test fabric (40^s cotton) were submerged in the dye bath heated to 60 °C. After 30 min of incubation, 12 g Na OH that had been dissolved in 100 ml of deionized water was added. The temperature was then raised to 80 °C, and the fabrics heated for another 30 min. The fabric was then rinsed in deionized water and heated for 10 min at 80 °C in deionized water, then rinsed and kept in a convection oven at 105 °C until dried.

2.9 SEM Analysis for morphology

The morphology of 40^s cotton fibres coated with chitosan and cefixime was investigated by scanning electron microscopy (SEM), H-6009 IV, Hitachi, Japan (Goldstein, *et al.*, 1992). Specimens were dissected and coated with gold and the microstructures of the fabric sponges were analysed by geometrical measurement on the scanning electron micrographs (Zhang, *et al.*, 2010).

2.10 Antibacterial persistance test of drug loaded 40^s cotton fabric

The chitosan-cefixime coated cotton was cut into discs and was placed in pathogen (3hrs grown culture of *E. coli*, and *S. aureus*, lawn cultured Muller Hinton (MH) agar plates and incubated at 37°C for 24 hrs. An uncoated cotton disc was used as control to differentiate the efficacy efficiency of the coated cotton material.

After zone formation, both coated and control cotton discs were transferred aseptically to fresh pathogen lawn cultured MH agar plates and incubated as the previous day. This procedure was followed till there was no visual zone formation of all the two disc samples against the used pathogens (Bayston *et al.*, 2009).

2.11 Bioefficacy of biomedical cotton fabrics against selected bacterial pathogens

2.11.1 *Assay for antibacterial properties (AATCC 100 Method; Version-1999)*

The antimicrobial activity was quantitatively evaluated against the standard strains of *Staphylococcus aureus* and *Escherichia coli* according to AATCC 100 test method. The fabric samples both treated and untreated with 4.25 ± 0.1 cm in diameter were placed in a 250 ml glass jar with screw cap and absorbed 1.0 ± 0.1 ml of bacterial inoculum. After incubation over a contact period of 24 hrs, 100 ml of sterilized distilled water was added into the jar and vortex vigorously for 1 min. The solution was then serial diluted from 10^{-1} to 10^{-8}. The diluted solution was spread plated on nutrient agar plates and incubated for 24 hrs at 37 ± 2°C (Walker, 1996).

2.11.2 *Wash fastness test (AATCC Test Method 124; Version-1996)*

AATCC Test Method 124-1996, proposed in the reference quoted by Chun & Gamble (2007) was used for performing the wash fastness test. The test result ensures the bioefficacy ability of the bound chitosan to cotton fabrics and the number of washes it can withstand in the textile.

AATCC Test Method 124; Version-1996

Wash condition Version 1996	
Cycle	Normal/Cotton Sturdy
Wash water temperature	60 ± 3ºC
Rinse water temperature	Less than 29ºC
Water level	18 ± 1gal
Agitation speed	179 ± 2 spm (speed per minute)
Wash Time	12 minutes
Spin Speed	630-660 rpm
Final spin cycle	6 minute

To evaluate the durability of antibacterial effect after washing, the treated fabrics were washed according to AATCC 124-1996 test method with AATCC Standard Reference Detergent WOB (without bleaching agent). One cycle of laundering by this method is equal to five typical careful hand launderings at temperature of 40 ± 3ºC.All the treated samples were subject to 3 cycles consecutive laundering. At the end of the 3rd cycle, the samples were rinsed with warm water & air dried and tested for antibacterial activity based on AATCC 100 method (mentioned above).

2.12 Bacterial Reduction Percentage in Cotton Blended with Chitosan

Colonies of bacteria recovered on the agar plate for both untreated and treated cotton fabrics before and after washing were counted and the per cent reduction of bacteria (R) was calculated by the following equation:

$$R (\%) = (B – A) \times 100/ B \quad \ldots\ldots(1)$$

Where, A is the number of bacterial colonies from treated specimen after inoculation over 18 hrs contact period and B is the number of bacterial colonies from untreated control specimen after inoculation at 0 contact time.

III. Results & Discussion

3.1 Specification of 40S cotton

40S cotton material purchased from Lakshmi Mills, Coimbatore analysed for physical parameters like, warp and weft. Their count shows 40 numbers of threads and each which is a major specification of 40S cotton. Similarly, the EPI and PPI were 60 and 56 respectively. The method of cotton weaved was found as plain.

40S cotton material because of its good air permeability and wet pick up it is a preferred textile material in many working environment and especially for the patients in hospital and for other medical applications. A similar piece of sample with similar physical parameter was used in this research work.

3.2 Bacterial strain and inoculum preparation

According to the work of San Tin, *et al*., (2010), their investigation reports that the microbial strains inoculums prepared for the analysis of MIC must be used in a proper concentration that can be indicated in CFU/ml.

Based on the above reference, the bacterial inoculums were prepared with a final concentration of approximately 7 X 10^5 CFU/ml. The reason for maintaining the inoculums concentration was to ensure proper exposure of bacterial cells to the antimicrobial substances (chitosan and cefixime) used in this research work.

3.3 Chitosan

Chitosan the polymeric substance was available in 3 different molecular weight namely, high, medium, and low. The investigations done by San Tin, *et al.*, (2010) reveal the low molecular weight chitosan showed much antimicrobial activity than the remaining. But in this study, the medium molecular weight chitosan was used because it got coated on the surface of 40S cotton which will undergo repeated laundering for reuse.

The low molecular weight chitosan even though has high antimicrobial activity when coated on the surface of cotton fabric can get degrade faster because of laundering which will minimize its persisting ability. In order to prepare a permanent antimicrobial cotton fabric, the medium molecular weight chitosan was used to get increased persistence and re usage.

3.4 Antibiotics

Different types of antibiotics with different mode of action were used along with chitosan for increased antimicrobial activity. The purity of the used antibiotic was given importance as it plays a major role in its mode of action against bacteria (Chen, *et al.*, 2020).

The antibiotics Clarithromycin, Streptomycin and Tobramycin used has an irreversible binding effect over the 50S and 30S bacterial ribosome respectively and there by inhibiting the translocation of peptidyl tRNA and inhibit protein biosynthesis. Polymycin B, Trimethoprim, Sulfamethoxazole and cefixime interact with the Gram negative bacterial outer membrane and cytoplasmic membrane. Tetracycline inhibits the binding of aminoacyl t-RNA to m-RNA-ribosome complex.

From the above bacterial growth inhibiting action of different antibiotics, those which act on the bacterial membrane where chosen for analysing the synergistic effect with chitosan as it has similar mode of action (Chung, *et al.*, 2004).

3.5 Antimicrobial Assay

The lowest concentration of the antimicrobial agent that will inhibit the bacterial growth was analysed using 10 different concentrations of antibiotics in chitosan (0.015 µg to 10 µg). The spot inoculation assay was done for the bacterial species *(E. coli* and *Staphylococcus aureus*) to identify the MIC of the drug and polymer and the result was tabulated in table-1 and fig-1.

Table 1 Antimicrobial Assay

S. No.	Name of antimicrobial agent	MIC Concentration(µg/ml)	
		Staphylococcus aureus	*Escherichia coli*
1	Clarithromycin	64	56
2	Streptomycin	4	6
3	Tobromycin	32	28
4	Polymycin B	8	5
5	Sulfamethoxazole	8	6
6	Trimethropim	1	3
7	Cefixime	4	4
8	Tetracycline	6	2
9	Chitosan	10	10

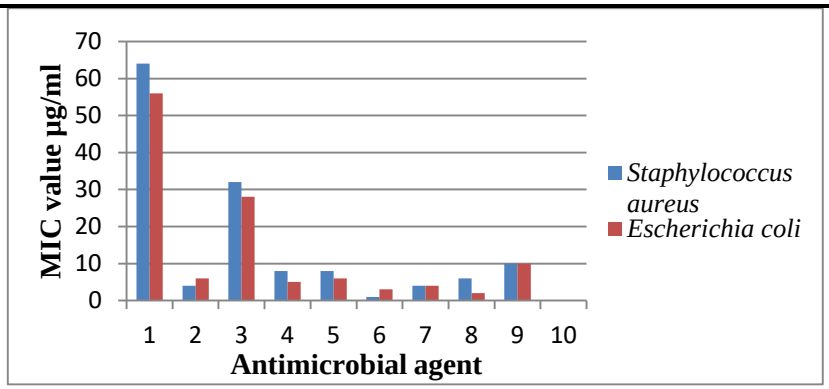


Fig:1 Antimicrobial assay against *S. aureus* and *E. coli*

The MIC of the different antimicrobial agents were recorded and analysed to choose the drug which will have effective synergistic action along with chitosan. Medium molecular weight chitosan is not a water soluble polymer, hence to make dissolution of the chitosan, acetate buffer was used. To ensure the action of acetate buffer on bacterial cells, different dilutions of the buffer was prepared and bacterial cells were exposed to it. The OD values mentioned in table 2 and fig. 2 shows the dilution of acetate buffer not lethal to bacterial cells.

Table 2 Analysis of non-lethal acetate buffer concentration

S. No.	Amount of acetate buffer(ml)	Amount of nutrient broth(ml)	OD value (600 nm)	
			S. aureus	*E. coli*
1	1	2	0.056	0.061
2	1	4	0.061	0.065
3	1	8	0.18	0.2
4	1	16	0.65	0.72
5	1	32	0.81	0.86
6	1	64	0.96	0.98
7	0 (Control)	10	0.96	0.98

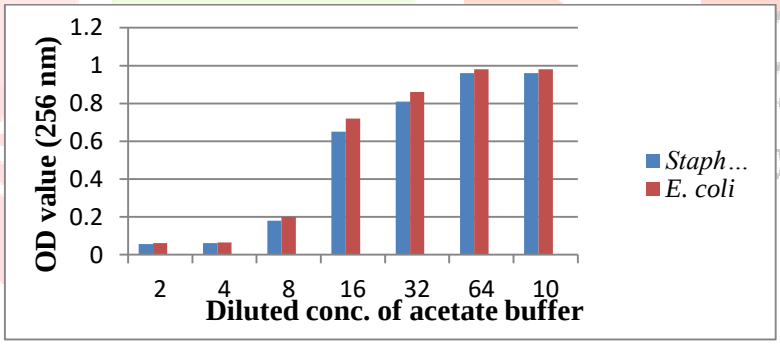


Fig.2 **Analysis of non-lethal acetate buffer concentration**

3.6 Checker board Assay

The checker board method was performed to analyse the MIC of polymer-antibiotic combination. Based on the mode of action and MIC of the drugs, drug with low MIC was chosen as a combination drug with chitosan for checking the synergistic activity. Sulfamethoxazole, the antibiotic which is not in use nowadays showed enhanced antibacterial effect when used in combination with chitosan. This result co-inside with the research work results of San Tin *et al.*, (2010) against *Staphylococcus aureus*.

Even though Sulfamethoxazole showed antimicrobial activity against *Staphylococcus aureus* with chitosan, the synergistic effect of cefixime with chitosan seems to be even more effective. Similarly, when compared with Streptomycin, Trimethoprim and Tetracycline, Cefixime was found to be a good synergistic drug with chitosan due to the similar mode of action on bacterial cell membrane. The plates with these drug combinations were spot inoculated, the growth was not observed in the plates with chitosan and cefixime drug combination. Remaining plate show mild bacterial growth and the FIC value of this drug combination was calculated and interpreted as synergism. The remaining drugs with chitosan showed the antagonist and additive effects rather than synergism. Table 3 and fig. 3 shows the FIC values of the drugs used in combination with chitosan.

Table 3 Checker board Assay

S. No.	Name of the synergistic drug		FIC value	Checker board result
	Polymer	Antibiotic		
1	Chitosan	Sulfamethoxazole	4.05	Antagonist
2	Chitosan	Streptomycin	4.9	Antagonist
3	Chitosan	Trimethoprim	12.1	Antagonist
4	Chitosan	Tetracycline	3.57	Synergism
5	Chitosan	Cefixime	3.53	Synergism

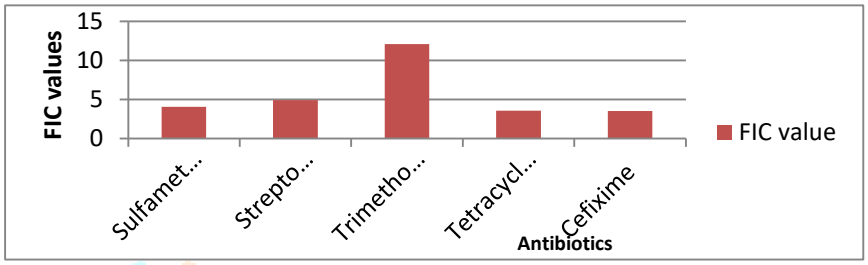


Fig.3 Checker board Assay

3.7 Preparation and finishing of cellulosic with chitosan

For the crosslinking of chitosan to cotton fabrics different anchors were used, among them cyanuric chloride was found to be one of the best cross-linker of chitosan and cellulose polymers. The cotton fabric padded with the mixture of synergistic drug and cross-linker by pad-dry-cure method influence the ionic bonding between the polymers and cyanuric chloride will act as a bridge between the drugs and cellulose fibres for firm attachment. The firmness of the attachment was checked using the laundering technique, AATCC 124 standard method.

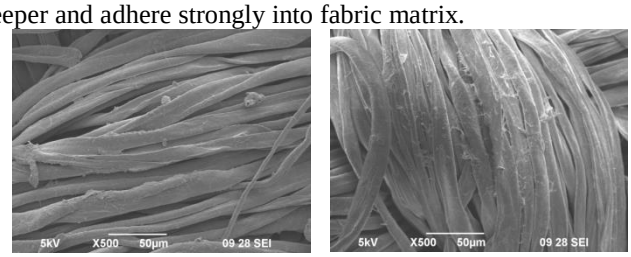
3.8 Reactive dye exhausts method

The approach in this study was to modify antibacterial drug, Cefixime previously or currently in use for treating diseases, for direct attachment to chitosan padded cotton fabric where treated fabric could act as a barrier against specific diseases or wound infections. Since these agents are antibacterial and by covalently attaching those to fabric the chance of imparting desired antibacterial properties to the fabric is expected to be high, this would provide specific medical, as well as general usage (Chun & Gamble, 2007).

3.9 Scanning electron microscopic analysis

The antibacterial drug coated implantable (silicone) and non-implantable (cotton) materials were observed visually and the topography of these samples was analysed using high resolution SEM with suitable accelerating voltage (10 KV), vacuum (below 5 Pa) and magnification (X 3500). One material from each category was analysed to meet the objective; so that silicone and cotton was selected as a representative implantable and non-implantable material.

The presence, binding and availability of chitosan and Cefixime on the fabric surface were analysed using SEM. The surface morphological features of cotton fabrics were depicted. Figure-4 showed the SEM photographs of cotton fabric treated with synergistic drugs using reactive dye method. The reactive bounded particles were well dispersed on the fibre surface and showed homogeneous distribution in the coating layer, thus making the coated fabrics to have uniform antimicrobial property [Fig. 4 (a) (b)]. It was clear from the photograph that reactive bounded drugs were of small spherical shape with a fairly uniform size distribution. The particle size plays a major role in determining their adhesion to the fibre molecules. It was clear that the micro-particles were present in intersections of the fibre assembly of fabric. It was also clear that the micro-structured drugs were firmly fixed on the fibre assembly of the cotton fabric which may be the reason for the durability of the antimicrobial effect. The sizes of the drugs were less than 10 µm. Generally agglomeration of large particles will get easily removed from the fibre surface, while the small particles will penetrate deeper and adhere strongly into fabric matrix.

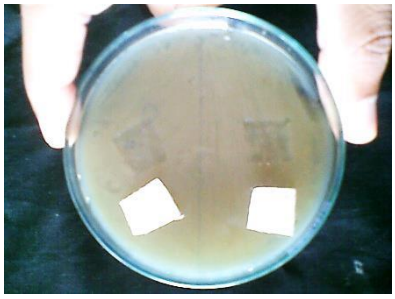


(a) Untreated cotton (b) Treated cotton with drugs

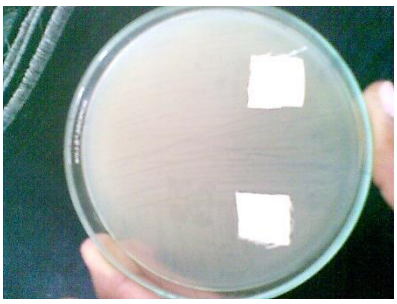
Fig.4 SEM picture of treated and untreated cotton

3.11 Persistence test of drug loaded cotton fabric

Initial testing determined whether the microcapsules would bond to the cotton fabric and impart antibacterial properties to the fabric. A pilot test was done with Cefixime and chitosan using both *Staphylococcus aureus* and *Escherichia coli* as challenge bacteria. The zone formed after 18hrs of incubation showed the antimicrobial effect of the treated cotton fabric (Fig.5).



No growth of *E. coli* under the cotton swatches

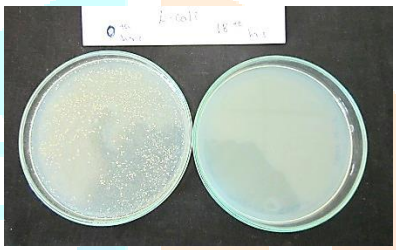


No growth of *S. aureus* under the cotton swatches

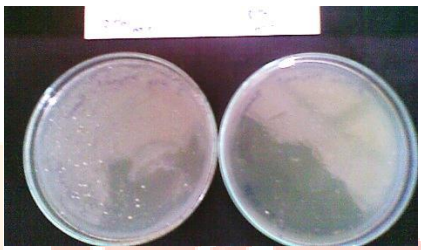
Fig-5 Persistence test of drug loaded cotton fabric

3.12 Bioefficacy of biomedical cotton fabrics (AATCC-100)

The reduction percentage of *S. aureus* and *E. coli* in untreated fabric was found to be 0, whereas with fabric treated with synergistic drugs showed 70% and 79% bacterial reduction against *S. aureus* and *E. coli* (Table- 4 & 5). The difference between the reduction percentage of control and the biopolymer-treated swatches were highly significant (Fig. 6). This indicated that the chitosan and cefixime were bound to the cotton fabric, and the antibacterial activity of the compounds was not affected.

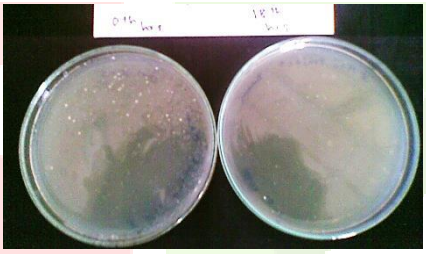


***E. coli* plates of AATCC 100 method**

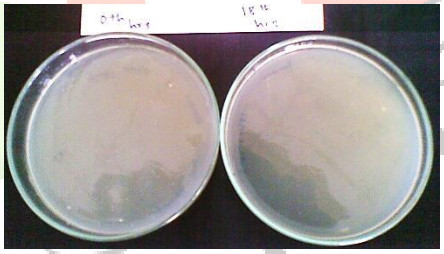


***S. aureus* plates of AATCC 100 method**

Fig-6 Bioefficacy of biomedical cotton fabrics



***E. coli* plates of AATCC 124 method**



***S. aureus* plates of AATCC 124 method**

Fig-7 Wash Fastness Test

Table.4 Reactive dye method against *S. aureus*

S. No	Sample	Number of colonies					
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1	Untreated (cotton)	TNTC	TNTC	TNTC	94	56	39
2	First wash	TNTC	TNTC	TNTC	95	58	42
3	Third wash	TNTC	TNTC	TNTC	95	58	42
4	Reactive dye exhaust cotton	126	97	83	67	38	23
5	First wash	99	78	67	59	26	19
6	Third wash	79	62	47	39	16	9

Table.5 Reactive dye exhaust method against *E. coli*

S. No	Sample	Number of colonies					
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1	Untreated (cotton)	TNTC	TNTC	TNTC	94	56	39
2	First wash	TNTC	TNTC	TNTC	95	58	42
3	Third wash	TNTC	TNTC	TNTC	95	58	42
4	Reactive dye exhaust cotton	112	87	71	64	31	18
5	First wash	89	68	57	36	19	11
6	Third wash	67	42	37	28	15	7

Table.6 Reduction percentage of Reactive dye exhaust method

S. No	Samples	Reduction of bacteria (%)					
		S. aureus			*E. coli*		
		Before wash	Single wash	Triple wash	Before wash	Single wash	Triple wash
1	Untreated sample	0	0	0	0	0	0
2	Treated sample	>70%	>75.56%	>82.2%	>79%	>74.14%	>79.31%

The large swatches of treated and untreated cotton fabric were washed 1 and 3 times to determine whether the biopolymer bound to the fabric would be durable through normal washing. After washing, these large swatches were cut into smaller swatches, sterilized, and then assayed for antibacterial properties. Using equation (1), the bacterial reduction percentage in the treated and untreated cotton fabric samples were calculated. Reduction percentage of *S. aureus* and *E. coli* after the third wash of fabric showed 82% and 79%, (Table-6) which provided the evidence for the antimicrobial activity persistence in the treated fabric after washes.

IV. Acknowledgement

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