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Smart functionalization cotton preparation using synergistic drug for enhanced bioefficacy against nosocomial pathogens

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ABSTRACT

Objective: This study focus on smart functionalization cotton fabric preparation by using the synergistic antimicrobial agents like, the antibiotic cefixime trihydrate and the biopolymer chitosan. The analysis provides a reliable method of coating the selected drugs permanently with the cotton fabric surface for reuse. **Methods:** The synergistic effect of the polymer chitosan and the antibiotic cefixime trihydrate were analysed using checker board method against nosocomial pathogens *Escherichia coli and Staphylococcus aureus*. The cotton fabrics were coated with the synergistic drug using the reactive dye exhaust method to prepare a permanent antimicrobial finished cotton fabric. The antibiotic cefixime was made reactive using cyanuric chloride which was an efficient linker of chitosan with cellulosic units in cotton fabrics. The antimicrobial efficiency of the finished cotton was analysed using the AATCC 124-1996 and AATCC 100 test methods. To ensure its biocompatibility, the coated cotton fabric was implanted in chorio-allantoic membrane of the embryonated eggs and analysed.**Results and Conclusion:** The qualitative and quantitative microbial analysis of the coated cotton fabric produced reliable results against the used pathogens. The bacterial reduction percentage after every wash of coated cotton was increasing and provides a proof for the permanent coating of synergistic drugs.

KEY WORDS: Synergistic drugs, chitosan, cefixime trihydrate, cyanuric chloride, chorio-allantoic membrane

1. INTRODUCTION

An exhaust dyeing method was used to bind the synthesized reactive antibacterial agents to the cotton fabric. The dye bath was prepared by adding 0.5 ml of Triton-X-100, 75 g of sodium sulphate, and 6.5 g of the reactive antibacterial drugs 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 (cotton) were submerged in the dye bath heated to 60°C. After 30 min of incubation, 12 g NaOH dissolved in 100 ml of deionized water was added. The temperature was then raised to 80°C, and the fabric swatches were heated for another 30 min. The fabrics were rinsed and heated for 10 min at 80°C in deionized water, then rinsed and kept in a convection oven at 105°C until dried.

The cotton fabrics with permanent antimicrobial finishes have been analysed many years by the researchers for retarding the microbial growth and colonization on the surface of fabrics. Exposing the pathogens to drugs at high concentration has the risk of forming antibiotic resistant species. Hence the usage of synergistic drugs were recommended, which are used in less concentration with high lethal effect against pathogens. Synergism was defined as the ability of a pair of drugs to produce a more rapid rate of bactericidal action within the first 24 hours of exposure than either member of the pair alone, and killing of greater numbers of bacteria than expected from simple summation of single drug effects. This definition is used because of its correlation with the therapeutic effects of combinations of antibiotics to treat infections in experimental animals and man.

Chitosan, ß-(1-4) linked 2-amino-2-deoxy-*D*glucose, is a natural biopolymer on earth after cellulose and deacetylated from chitin[1]. It is the main structural component of the cuticles of crustaceans, insects and molluscs and the cell walls of certain fungus and has been estimated that is produced in nature at a level of up to $1\times109^{-1}\times1010$ tonnes per year. Chitosan is a natural, positively charged polysaccharide with a p*K*a equal to 6.3-7.0[2], and it has a potential application in several areas, including food[3], pharmaceutical[4], biotechnology[5], and environment[6]. It exhibits various promising biological activities, including antimicrobial activity, antitumor activity, hemostatic activ-

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ity, and acceleration of wound healing[7]. Chitosan is biodegradable and biocompatible. Hence, extensive research has been conducted to explore its potential applications in various industries.

Cefixime trihydrate, is the third generation cephalosporin antibiotic. Cefixime is given orally in the treatment of susceptible infections including respiratory tract infections like acute exacerbations of chronic bronchitis, gonorrhoea, otitis media, pharyngitis, lower respiratory-tract infections such as bronchitis, and urinary-tract infections[8]. The permanent fixation of chitosan on a cotton surface has been investigated in many fields of application such as bioactive fibres for medicine, the creation of textiles with improved properties for wound dressing and textiles with ion binding capacities for many heavy metals. Therefore, an important task lies in the evaluation of the methods used to anchor chitosan permanently onto fibre surfaces in a way that the chitosan will retain their beneficial bulk properties.

The reactive dye method used to bind compounds (e.g., antibiotics) with natural or synthetic yarn or fabrics utilized two common antibacterial drugs (trimethoprim and sulfamethoxazole). The drugs were chemically converted to obtain a reactive dye type molecule, which could be applied to cotton fabric with the goal of imparting the antibacterial properties of the antibiotic compounds to the fabric. The drugs are often used together as a synergistic combination for the treatment and prevention of urinary tract infections, diarrhoea and respiratory infections. These two compounds were reacted with cyanuric chloride, and the resultant product was then cross-linked to cotton fabric using an exhaust methodology[9].

In the present study, the biopolymer chitosan and the antibiotic cefixime were used as synergistic drugs, considering their similar mode of action like altering the cell membrane permeability in nosocomial pathogens. The synergistic drugs were coated with cotton fabrics as a permanent finish and analysed for its antimicrobial property.

2. MATERIALS & METHODS

2.1 Sample Collection

Cotton fabric - mill scoured and bleached 100% cotton fabric was purchased from Lakshmi Mills, Coimbatore, Tamil Nadu, India.

2.2 Bacterial Strain and Inoculum Preparation

The overnight culture of *E. coli* (ATCC 43894) and *Staphylococcus aureus* (ATCC 29213) strain was diluted with autoclaved Luria Bertani broth to get the final bacterial inoculums of ~7 x 10^5 CFU/ml in each tube. The tubes were incubated at 37°C for 20 to 24 hrs in ambient air before interpretation as described by CLSI (Clinical and Laboratory Standards Institute) guidelines.

2.3 Chitosan and Cefixime Trihydrate

Chitosan (75 – 85 % deacetylated) was purchased from Sigma-Aldrich. Acetate buffer of pH 4.65 was used as solvent for the preparation of stock solution of chitosan. Antimicrobial powder of Cefixime (assay value 99% HPLC) was purchased from Ranbaxy, India.

2.4 Checkerboard Assay

Checkerboard assay was done by observing the minimal inhibitory concentration (MIC) of chitosan and cefixime. The fractional inhibitory concentration (FIC) of the synergistic drugs were also analysed and SFIC was calculated and interpreted as below.

Synergism = SFIC ? 0.5
Antagonist = SFIC 2 - 4
Additive = SFIC 0.5 – 1

2.5 Reactive Dye Method

This method suggests the modification of antibacterial drugs, cefixime and chitosan for direct attachment to textile fabrics[9].

2.5.1 Synthesis of Reactive Synergistic Drugs

To convert the drugs reactive and imparting to cotton fabrics, the reactive dye exhaust method was performed. Synthesis of reactive cefixime was accomplished by suspending 0.02 mole 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 NaOH by maintaining at 5°C. Synthesis of reactive chitosan was similarly prepared suspending 0.03 moles 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 NaOH.

2.5.2 Exhaust Dyeing Method to Bind Reactive Antibacterial Agents to Textile Materials

An exhaust dyeing method was used to bind the synthesized reactive antibacterial agents to the cotton fabric. The dye bath was prepared by adding 0.5 ml of Triton-X-100, 75 g of sodium sulphate, and 6.5 g of the reactive antibacterial drugs 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 (cotton) were submerged in the dye bath heated to 60°C. After 30 min of incubation, 12 g NaOH dissolved in 100 ml of deionized water was added. The temperature was then raised to 80°C, and the fabric swatches were heated for another 30 min. The fabrics were

rinsed and heated for 10 min at 80°C in deionized water, then rinsed and kept in a convection oven at 105°C until dried.

2.6 Quantitative Microbial Analysis of Synergistic Drug Loaded Cotton Fabric

The antimicrobial activity was quantitatively evaluated against the standard strains of *E. coli* (ATCC 43894) and *S. aureus* (ATCC 29213) according to AATCC 100 test method[9]. The C-C coated and uncoated cotton fabric samples with 4.25 ± 0.1 cm in diameter were inoculated with 1.0 ± 0.1 ml of bacterial inoculum. After 24 hrs of incubating contact period, the samples were added with sterile distilled water and serially diluted for plating in nutrient agar plates.

Bacterial reduction percentage was calculated using the following formula:

$$R = (A-B) / A \times 100 \ldots\ldots\ldots (1)$$

Where R is bacterial percentage reduction, A is the number of bacteria recovered from C-C coated cotton at zero time of bacterial contact and B is the number of bacteria recovered from the C-C coated cotton after 18 hours of bacterial contact.

2.7 Wash Fastness Test

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 subjected to 3 cycles of 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.

2.8 Biocompatability Analysis of Synergistic Drug Coated Cotton

The response of tissues against C-C coated cotton samples was analysed by standard HET-CAM test. To the surface of chorio-allantoic membrane (CAM) in 9days old embryonated chick eggs C-C coated cotton was implanted. The results were assessed by the tissue allergic reactions like pluritis, erythema, edema or necrosis caused by the coated chemicals on the membrane. After staining, inflammations could be indicated by the appearance of hue colours on the CAM. 0.1 N NaOH was used as positive control (produces inflammation) and 0.9 % NaCl as negative control.

3. RESULTS AND DISCUSSION

The MIC analysis of chitosan, cefixime and chitosan-cefixime (in combination) showed growth inhibition results at the concentration of 0.10µg/ml and 0.030µg/ml and 0.015µg/ml respectively. The FIC value of the drugs used was 0.2 and as indicated in the synergistic index the value obtained for FIC was > 0.5, the drug combination selected for the analysis showed synergistic action against *E. coli* (ATCC 43894) and *S. aureus* (ATCC 29213)[10].

Due to lack of solubility in water, chitosan was dissolved in acetate buffer. To avoid the interference of acetate ions in antimicrobial susceptibility testing, the buffer was serially diluted with broth media and the bacterial growth in the diluted buffer was measured at 600nm[10]. At a dilution of 1:16, (Table-1) little or no inhibition of bacteria growth was observed and was confirmed with the optical density value equivalent to the sterile control broth.

Table.I. Analysis of Non-Lethal Acetate Buffer Concentration

S. No.	Amount of acetate buffer (ml)	Amount of nutrient broth (ml)	OD (600 nm)
1.	1	2	0.056
2.	1	4	0.061
3.	1	8	0.18
4.	1	16	0.65
5.	1	32	0.81
6.	0 (Control)	10	0.66

The selected antimicrobial agents chitosan and cefixime show different minimal inhibitory concentration by micro-dilution method depending on their mechanism of action on *S. aureus.* Checkerboard micro-dilution method was applied to calculate the sum of FIC for the drug combination. In the drug combination used, chitosan with high molecular weight was preferred to get the maximum synergistic and antimicrobial effect.

The results obtained concur with earlier reports which state that high molecular weight chitosan can bind effectively to the Gram-positive cell membrane compared to the lower molecular weight chitosan. Thus, different molecular weights and degree of acetylation of chitosan can be synergistically effective[7].

The high molecular weight chitosan showed increased effect than low molecular weight, medium molecular weight or oligosaccharide chitosan molecules with most of the antibiotics. High molecular weight 90% deacetylated chitosan has the ability to block the entry of nutrients into the cell longer and thus may make the antibiotic effective[8]. The exhaust dye reactive method used for the fixation of cefixime and chitosan to cotton fabrics was done at the hot condition in the presence of a surfactant. The surfactant, Triton-X 100 showed much efficiency over chitosan for its mild washing and firm binding and also in the stability of cefixime trihydrate[6]. According to the previous researches, dye reactive method of coating synergistic drugs to cotton provides a constant and permanent coating on the surface. This

method has been used for years to dye the cotton with dyeing agents for permanent fixation without getting diffused during laundering[9]. 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[11].

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 microcapsules using both *E. coli* (ATCC 43894) and *S. aureus* (ATCC 29213) as challenge bacteria. The zone formed, 18mm in diameter for *E. coli* (ATCC 43894) and 12mm in diameter for *S. aureus* (ATCC 29213) after 18hrs of incubation showed the antimicrobial effect of the treated cotton fabric (Fig-1).

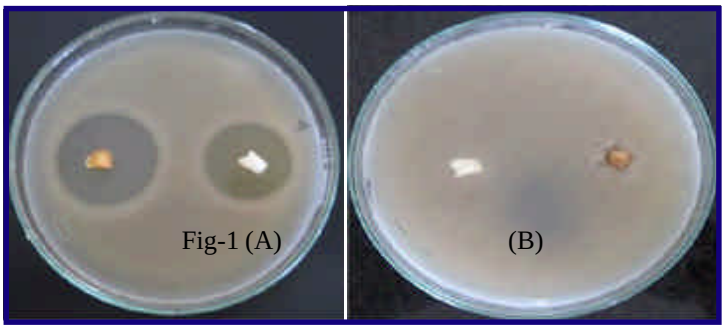

Fig-1(A) Zone of inhibition against *E. coli* (ATCC 43894) and *S. aureus* (ATCC 29213) exhibited by synergistic drug treated cotton. (B) Zone of inhibition against *E. coli* (ATCC 43894) and *S. aureus* (ATCC 29213) exhibited by untreated cotton

Fig-1 Qualitative Test of Treated and Untreated Cotton Fabric

The quantitative analysis of antimicrobial activity by treated cotton sample was analysed (using formula-1) in reduction percentage of *E. coli* (ATCC 43894) and *S. aureus* (ATCC 29213) using treated and untreated cotton fabrics. The reduction percentage of the bacterial pathogens in untreated fabric was found to be 0, whereas the synergistic drug treated fabric showed good reduction percentage (Table-2 & Fig-2). The difference between the reduction percentage of control and the synergistic drug treated swatches were highly significant. This indicated that the chitosan and cefixime were bound to the cotton fabric, and the antibacterial activity of the compounds was not affected. AATCC-124, the wash-fastness tests showed the bacterial reduction percentage after 2nd, 5th, 10th and 15th washes. The results tabulated in table-2 showed significant reduction percentage of bacterial pathogens even after many launderings.

Table.2. Reduction Percentage Test of Microbial Pathogens in Treated Cotton Fabric

Cotton fabric treated with drugs	Reduction of bacteria (%) *E. coli* (ATCC 43894)	*S. aureus* (ATCC 29213)
Before wash	100	100
After 2nd wash	81.9	80.2
After 5th wash	72.1	67
After 10th wash	50.3	45.1
After 15th wash	23.3	23.4

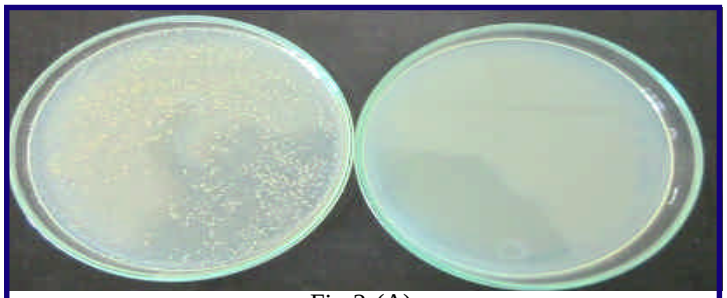

Fig-2 (A)

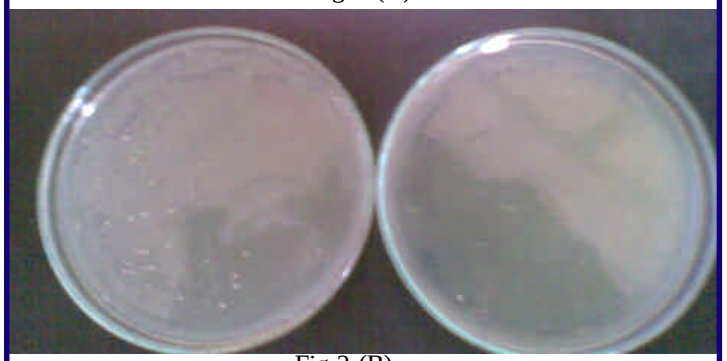

Fig-2 (B)

(A) *E. coli* plates of AATCC 100 method, (B) S. *aureus* plates of AATCC 100 method

Fig-2 Bioefficacy of Biomedical Cotton Fabrics

Determining the antimicrobial finish effect against *E. coli* (ATCC 43894), and *Staphylococcus aureus* (ATCC 29213) revealed that the synergistic drug coated cotton was bioactive for up to 50 home launderings as one laundering by AATCC-124 method is equal to 5 careful home laundering (Fig-3).

Fig-3 (A)

Fig-3 (B)

(A) *E. coli* plates of AATCC 124method, (B) *S. aureus* plates of AATCC 124 method

Figure-3 Wash Fastness Test

The chitosan and cefixime loaded cotton fabric was implanted in the nine-day old embryonated chick eggs by placing it on the chorio-allantoic membrane (CAM) portion and incubated for 7days (Fig-4). During and after the exposure period (9, 12 and 17 days), the eggs were observed for hypersensitive reactions-edema and erythema.

No such reactions on the CAM surface of the chicks were observed after the specified incubation period (Fig-4). The Hematoxylin and Eosin stained membrane showed the normal blood vessel formation without any pluritis and angiogenesis symptoms (Fig-4), hence the treated cotton fabric samples prepared in this experimentation were considered biocompatible.

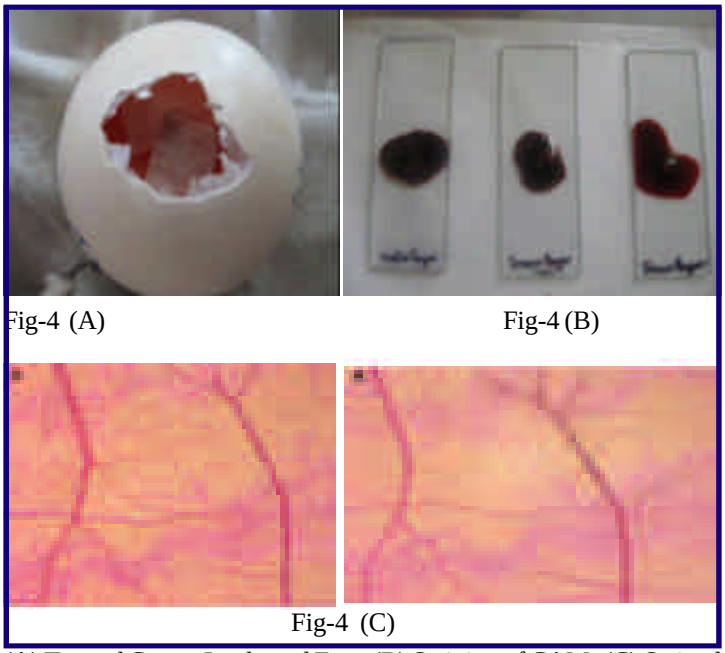

Fig-4 (A) Fig-4 (B)

Fig-4 (C)

(A) Treated Cotton Implanted Egg , (B) Staining of CAM (C) Stained CAM showing normal blood vessels formed in both Control (Top) and Cotton sample implanted (Bottom)

Fig-4 Tissue Responses in Chick Chorio-Allantoic Membrane

4. CONCLUSION

The cotton fabrics selected were coated with the synergistic drug using the reactive dye exhaust method to prepare a permanent antimicrobial finish. The antibiotic cefixime was made reactive using cyanuric chloride which was an efficient linker of chitosan with cellulosic units in cotton fabrics. The antimicrobial efficiency of the finished cotton was analysed using the AATCC 124-1996 and AATCC 100 test methods. To ensure its biocompatibility, the coated cotton fabric was implanted in chorio-allantoic membrane of the embryonated eggs and analysed. The qualitative and quantitative microbial analysis of the coated cotton fabric produced reliable results against the used pathogens. The bacterial reduction percentage after every wash of coated cotton was increasing and shows the slow and sustainable release of the coated drugs.

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