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In vitro activity of chitosans in combination with antibiotics against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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Abstract

Chitosan and its derivative water soluble chitosan oligosaccharide are used in a variety of applications in pharmaceutical preparations. In this study, 2 wild (ATCC 15729, PAO1) and 2 mutant strains (PT121, PT149) of *Pseudomonas aeruginosa* and one strain of MRSA *Staphylococcus aureus* (ATCC43300) are investigated for drug-drug interactions *in vitro*. Ten antimicrobial agents (antibiotics) are combined with different degree of deacetylated chitosans and chitosan oligosaccharide. All the chitosans exhibit synergistic activity with sulfamethoxazole against all *P. aeruginosa* strains. Chitosan oligosaccharide-sulfamethoxazole and high molecular weight chitosan-sulfamethoxazole combination are more effective than other chitosan preparations against *S. aureus*.

Microscopic studies of bacteria cell membrane show evidence of the collapse of membrane integrity after treating with the combined drugs. This suggests the membrane disruption function of the chitosan and disruption of cell membrane synthesis function of sulfamethoxazole act synergistically.

In conclusion, chitosan and chitosan oligosaccharide are effective against both gram-negative and gram-positive bacteria in combination with sulfamethoxazole.

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List of figures

List of tables

Contents

Chapter One

Introduction

The use of antimicrobial agents is critical to successful treatment of infectious diseases. Although there are numerous classes of drugs that are routinely used to treat infections in humans, there are several reasons why the discovery and development of new antimicrobial agents are important. Over the past decade there has been an increased development of resistance in organisms that are typical pathogens in humans. Among them *Pseudomonas species* and *Staphylococcus species* are common.

1.1 Antimicrobial Resistance

Since the discovery of the cure of infectious diseases by drugs which are toxic to the microorganism, selective antimicrobial chemotherapy has played an important role in battle against infection. Starting with azodye Nagana red to Penicillin, the effectiveness of antimicrobial agents is remarkable in treatment of infectious diseases. Once Penicillin was started to use widely, there were a number of set-backs. Some microorganisms became resistant to it. The number of resistant bacteria increased from a few to nearly 100%. Resistant strains are developing over the time.(1) To combat this problem, a number of antimicrobial chemotherapeutic compounds are developed in. Drug resistance was first reported in the hospitals where critically ill patients were treated with latest and sophisticated antibiotics. In day care centers and homes for elderly, the antibiotics were prescribed as

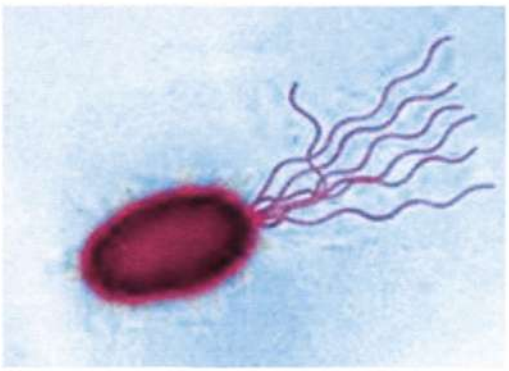
maintenance dose regularly. From there the resistant strains spread insidiously. The biochemical aspects of drugs resistance include:

1. Modification of the target site or enzyme where the drug binds
2. Prevention of the access of antibiotics
3. Production of the enzymes that destroy or inhibit the antibiotics.

The genetic basic of antibiotic resistance in bacteria is due to production of new gene from genetic mutation or acquiring a new gene from the resistant strain.(1)

1.2 Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative bacillus, motile by means of a single polar flagellum. *Pseudomonas* bacterium can be seen in nature as a biofilm, attached to some surface or substrate or in a planktonic form.(2)

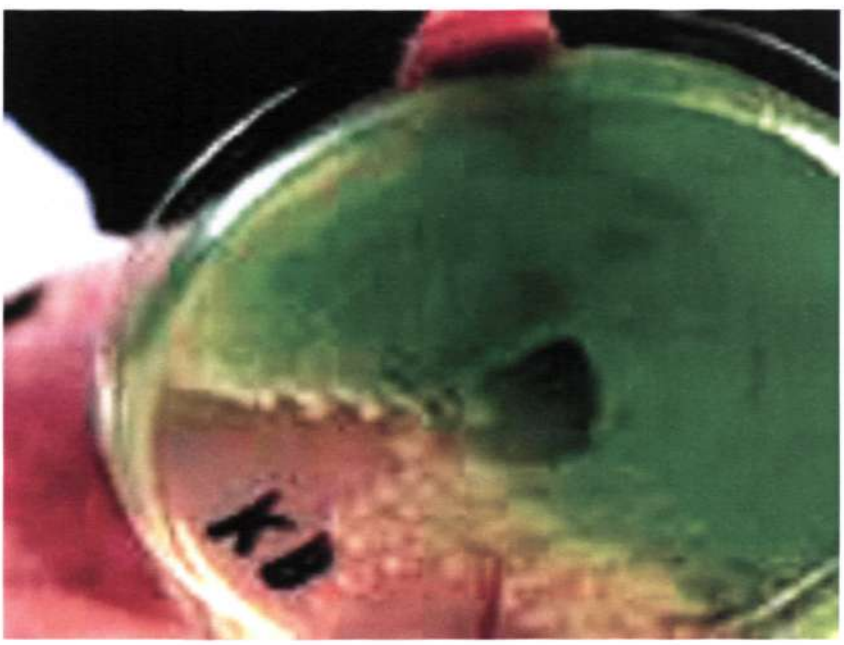


www.ehagroup.com/resources/patho...uginosa/

Fig 1. Structure of *Pseudomonas aeruginosa*

P. aeruginosa is an obligatory aerobe and can grow in ordinary culture media at an optimum temperature of 37°C, and it is able to grow at temperatures as high as 42°C.

P. aeruginosa characterizes as large flat colonies with distinctive smell due to aminoacetophenone production. Due to the secretion of a variety of pigments – pyocyanin, fluorescein and pyorubin, *P. aeruginosa* colonies are pigmented with bluish green to red brown. It can also be grown in MacConkey agar and other bile salt containing media and also on Cetrimide agar. When in Kigler iron agar, it has a characteristic pink-red slope and butt.(2)

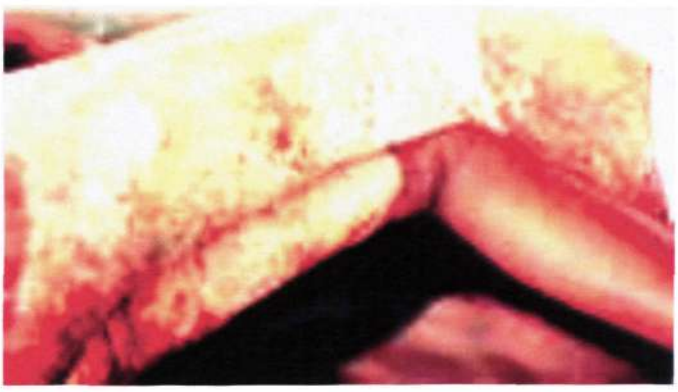


www.textbookofbacteriology.net/t...ora.html

Fig 2. Pigmented colonies of *Pseudomonas aeruginosa*

P. aeruginosa is an opportunistic pathogen for immunocompromised individuals. It typically causes the infection of respiratory tract, urinary tract, burns, wounds and also septcaemic infections. The most common infection of the bacteria is in burns

and external ear infections. It is the most frequent colonizer of medical devices such as catheters. In rare circumstances, it can cause community acquired pneumonia and ventilator associated pneumonia. Among Hospital acquired infections, 10% of the infections are due to *P.aeruginosa*.(2) Alginate slime, the product of the mucoid exopolysaccharide of *P. aeruginosa*, forms the matrix for the *Pseudomonas* biofilm**.** Biofilm protects the bacteria from the host defenses such as lymphocytes, phagocytes, the ciliary action of the respiratory tract, antibodies and complement. Thus, biofilm mucoid strains of *P. aeruginosa* are also less susceptible to antibiotics than their planktonic counterparts.(2) Mucoid strains of *P. aeruginosa* are most often isolated from patients with chronic infections like lung tissue of cystic fibrosis and other immunocompromised individuals.(2)



www.podiatrytoday.com/article

Fig3. *Pseudomonas aeruginosa* infection in burn

P. aeruginosa is naturally resistant to a large range of antibiotics and demonstrates additional resistance after unsuccessful treatment, particularly through modification of a porin. It is usually possible to guide treatment according to laboratory sensitivities, rather than choosing an antibiotic empirically. If antibiotics are started empirically, then every effort is made to obtain cultures and the choice of antibiotic used should be reviewed when the culture results are available.(2)

The extensive use of antimicrobial agents and the evolutionary antimicrobial resistance strategies of bacteria have resulted in the emergence of pan-drug resistant bacteria.(3) The efficacy of many antibiotics for treatment of infections has become quite limited due to the development of resistance. These antimicrobial-resistant organisms are accumulating and accelerating resulting in treatment failure.(3) The development of resistance to monotherapy is a common problem and dual antimicrobial coverage is often a necessity in *Pseudomonas* infections.(4) Combination therapy has been attempted to cope with this problem.(5) Several investigators studied the interaction of several antimicrobial combinations with multiresistant planktonic strains of *P. aeruginosa*.(6,7) Recently, Černohorska demonstrated the *in vitro* effect of 8 antibiotic combinations in *P. aeruginosa* biofilms using biofilm susceptibility testing.(8) Earlier, Neu reviewed the data available on the combinations of fluoroquinolones with other antimicrobial agents against several bacteria including *P. aeruginosa*.(9) The synergistic pair of vancomycin and cephalosporin or penicillin reported to be effective against a number of gram-negative bacteria.(10) The discovery of new antibiotics makes a major advance in the treatment of Multi Drug Resistance bacteria infections. On

the other hand, the increasing in number of drug resistannt bacteria and incident of drug resistant bacteria infections are still common.(11, 12)

Aminoglycosides, quinolones, cephalosporins, ureidopenicillins, carbapenems, polymyxins, monobactams are the most common antibiotics used in treatment of pseudomonal infections either as monotherapy or combinetherapy.(13) The mechanism of action of these groups of common antibiotics for *P. aeruginosa* are grouped as follow.(13,14)

Table 1. Mechanism of action of common antibiotics against *P. aeruginosa* infections

ANTIBIOTIC CLASS	**ANTIBIOTIC(S)**	**MECHANISM OF ACTION**
Aminoglycosides	Streptomycin Gentamycin	Inhibits protein synthesis
Quinolones	Niladic acid	Inhibits Nucleic acid synthesis
Cephalosporins	Ceftazidime Cefepime	Inhibits bacteria cell wall synthesis
Penicillins	Penicillin Piperacillin	Inhibits bacteria cell wall synthesis
Cabapenems	Meropenem	Inhibits bacteria cell wall synthesis
Polymycins	Polymycin B	Alters cytoplasmic membrane permeability
Monobactams	Aztreonam	Inhibits bacteria cell wall synthesis

1.3 Objective

The development of resistance to monotherapy (single antibiotic) is a common problem and dual antimicrobial coverage (two antibiotics) is often a necessity in treatment of bacteria infections.

The extensive use of antimicrobial agents and the evolutionary antimicrobial resistance strategies of bacteria have resulted in the emergence of pan-drug resistant bacteria. Due to the development of resistance and the threat from accumulating and accelerating of antimicrobial–resistant organisms, the efficacy of many antibiotics for treatment of infections has become limited

Moreover, the difficulties in strategizing to prevent the emergence of drug resistance has been discussed by Amyes *et al.*, 2007 in the issue of a good principle for antibiotic usage to limit resistance development.(15) Thus, there is the need to find new ways to control multi drug resistance bacteria and embark on the need for continuing search for new antimicrobial compounds.

The objective of this study is to determine;

1. The antimicrobial activity of different chitosan preparations against different *P. aeruginosa* species and one strain was taken as model organism for its diverse drug resistance and common cause of various infections.
2. The possible synergistic effect of chitosan preparations with selected antibiotics (tetracycline, sulfamethoxazole, trimethoprim, clarithromycin, polymycinB, ceftriazone, chloramphenicol, tobramycin, ofloxacin, and streptomycin) against *P. aeruginosa* species is to be determined.

3. Attempts are made to suggest on the possible mechanism of action or effect of combination on the bacteria.
4. Effect of drugs combination against a gram positive bacterium, *Staphylococcus aureus*, was also tested to get a better understanding of antimicrobial activity of chitosan preparations either alone or in combination with antibiotics.

Chapter Two

Literature Review

There are several classification schemes for antibiotics, based on bacterial spectrum (broad, narrow) or route of administration (injectable, oral, topical), or type of activity (bactericidal, bacteriostatic). The most useful is based on chemical structure and their mechanism of action.

2.1 Antibiotics

2.1.1 Tetracycline

Tetracycline is a broad-spectrum polyketide antibiotic produced by the Streptomyces bacterium. It displays good activity against most Gram-positive and Gram-negative bacteria.(16)

Mechanism of action

It works by inhibiting action of the prokaryotic 30S ribosome, by binding the 16S rRNA thereby blocking the aminoacyl-tRNA to the A site on the ribosome. However, the mechanism of most common form of resistance against tetracycline and its derivates is by encoding a resistance operon. In eukaryotic cells, toxicity may be result of inactivation of mitochondrial 30S ribosomes.(16)

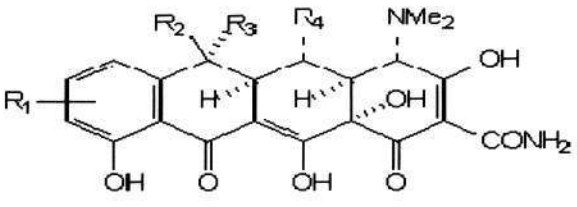


Fig 4. Structure of tetracycline

2.1.2 Sulfonamides

Sulfonamide drugs are the first antimicrobial drugs, and a major breakthrough in the chemotherapy of bacterial infections. It is a dye based drug and the first medicine ever discovered that could effectively treat a spectrum of bacterial infections inside the body. It has a strong protective action against infections caused by streptococci and a lesser effect on infections cause by other anaerobes. Sulfamethoxazole is a sulfonamide bacteriostatic antibiotic. It is most often used as part of a synergistic combination with trimethoprim in a 5:1 ratio.(16)

Mechanism of action

Sulfonamides are structural analogs and competitive antagonists of para–aminobenzoic acid (PABA)which is essential for bacterial folic acid synthesis. They inhibit the early stage of synthesis of folic acid, leading to failure in DNA synthesis. The effects seen are usually bacteriostatic in nature.(16)

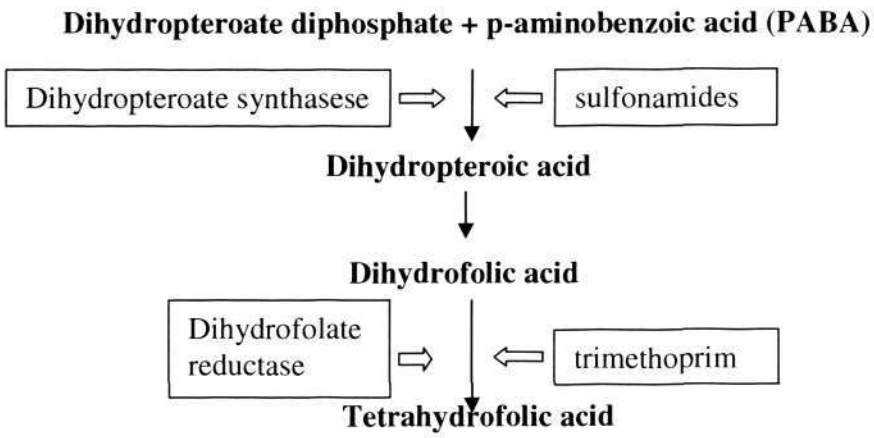


Fig 5. Mechanism of action of sulfamethoxazole and trimethoprim, the two sulfonamide antibiotics.

2.1.3 Macrolides

The macrolides are a group of antibiotics whose activity results from the presence of a *macrolide ring*, a large macrocyclic lactone ring. The antibiotics in this group share the similar molecular structure of usually 14, 15 or 16-membered macrocyclic lactone ring substituted with some unusual sugars. Macrolides are polyketide class of natural products. Some of the macrolides used as antibiotics are erythromycin, clarithromycin and roxithromycin. Microlides can be used as substitute for patients with penicillin allergy as its antimicrobial spectrum is wider than that of penicillin. Macrolides show effectiveness against not only Beta-hemolytic Streptococci, Pneumococci, Staphylococci and Enterococci, but also against Mycoplasma, Mycobacteria, some Rickettsia, and Chlamydia.(16)

Mechanism of action

The mechanism of action of the macrolides involves the inhibition of bacterial protein biosynthesis by binding reversibly to the subunit 50S of the bacterial ribosome. Subsequently, it inhibits translocation of peptidyl tRNA. Microlides are bacteriostatic agents, but it can also be bactericidal in high concentrations. The attractive feature of macrolides is well tolerated and better tissue penetration.(16)

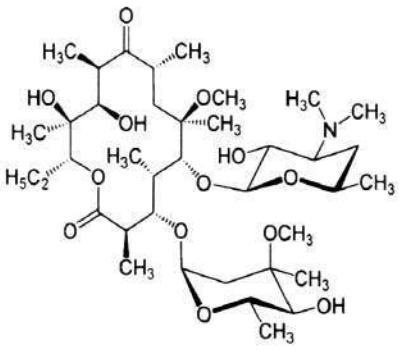


Fig 6. Structure of clarithromycin

2.1.4 Polymycin B

Polymyxin B is an antibiotic produced by *Bcaillus polymyxa* which is primarily used for resistant Gram-negative infections. Polymycins bind to the cell membrane and alters its structure making it more permeable resulting water uptake leads to cell death. They are cationic, basic proteins that act like detergents.(16)

Mechanism of action

Polymyxin alters cytoplasmic membrane permeability by binding to a negatively charged site in the lipopolysaccharide layer which has an electrostatic attraction for the positively charged amino groups in the cyclic peptide portion causing the leakage of essential cytoplasmic contents. (16)

2.1.5 Cephalosporins

Cephalosporin compounds were first isolated from cultures of *Cephalosporium acremonium* from a sewer in Sardinia in 1948. It has a wider spectrum than penicillin. The first cephalosporin, cephalosporin C is analogous to the penicillin

nucleus 6-aminopenicillanic acid. Unfortunately, it was not potent enough for clinical use. Modification resulted in the development of useful antibiotic. Cephalosporins are commonly described as "generations" by their antimicrobial properties. With the development of newer generation of cephalosporins, the antimicrobial activity for gram-negative bacteria is becoming more and more efficient. However, the effectiveness against gram-positive bacteria decreases in most cases. Fourth-generation cephalosporins, however, have true broad spectrum activity.(16)

Mechanism of action

Cephalosporins disrupt the peptidoglycan synthesis of the bacteria cell wall and lead to disintegration of the cell wall structure. They are beta-lactam antibiotics which interfere with the final transpeptidation step in the synthesis of the peptidoglycan. It is facilitated by transpeptidases known as penicillin binding proteins (PBPs).(16)

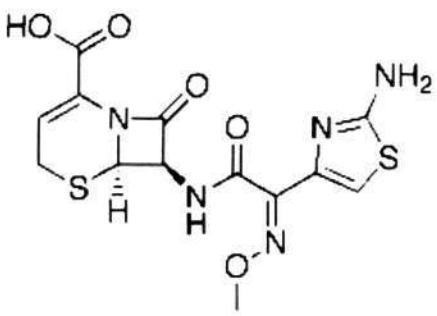


Fig 7. Structure of ceftriazone, a third generation cephalosporins

2.1.6 Chloramphenicol

Chloramphenicol is one of the first therappeutically bacteriostatic antimicrobial agent originally derived from the bacterium *Streptomyces venezuelae*. Chloramphenicol is effective against a wide variety of microorganisms. It is a simple, naturally occurring compound with relatively simple molecule. Chloramphenicol has a very broad spectrum of activity. It is active against Gram-positive bacteria (including most strains of MRSA), Gram-negative bacteria and anaerobes. One draw back for chloramphenicol is that it is a potential cause of fatal aplasticanaemia.(16)

Mechanism of action

Chloramphenicol inhibits the peptidyl transferase activity – the step at which the peptide bond is formed - of the bacterial ribosome.(16)

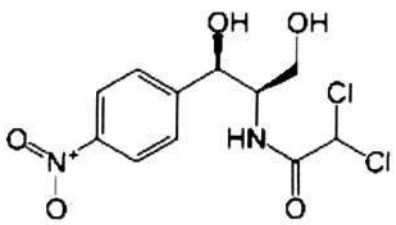


Fig 8. Structure of chloramphenicol

2.1.7 Aminoglycosides

Aminoglycosides are derived from bacteria of the *Streptomyces* genus and *Micromonospora*. Those derive from *Streptomyces* are named with the suffix *-mycin*, while those from *Micromonospora* are named with the suffix *-micin*. An aminoglycoside molecule contains a sugar group and an amino group. Different aminoglycosides have different effectiveness against various bacteria. Common aminoglycosides are amikacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, rhodostreptomycin, streptomycin, tobramycin, and apramycin.(16)

Aminoglycosides are broad spectrum bactericidal agents. They are effective against aerobic Gram-negative bacteria like *Pseudomonas*, as well as bacteria like *Acinetobacter* and *Enterobacter*. In addition, some Mycobacteria, that cause tuberculosis, are susceptible to aminoglycosides. Aminoglycosides are frequently used as empiric therapy for serious infections such as septicemia, complicated intra-abdominal infections, urinary tract infections, and nosocomial respiratory tract infections.(16)

Mechanism of action

Aminoglycosides work by binding to the bacterial 30S ribosomal subunit or binding to the 50S subunit, inhibiting the translocation of the peptidyl-tRNA from the A-site to the P-site. This causes misreading of mRNA, resulting in failure to synthesize proteins. Therefore, aminoglycosides are believed to kill bacteria by inhibiting protein synthesis and disrupt the integrity of bacterial cell membrane.

However, definitive mechanism and other paradoxical aspects of aminoglycoside action are still in dispute.(16)

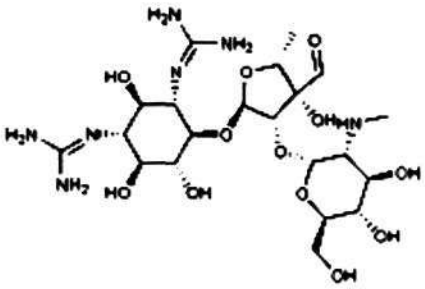


Fig 9. Structure of streptomycin

2.1.8 Quinolones

The quinolones are nalidixic acid family of broad-spectrum antibiotics. Fluoroquinolones are new groups of quinolones which possess significant pharmacological and antimicrobial properties. Fluoroquinolones have a fluoro group attached the central ring system, typically at the 6-position. Clinically used quinolones antibiotics are essentially fluroquinolones.(16)

Mechanism of action

Quinolones and fluoroquinolones are bactericidal drugs, actively killing bacteria. Quinolones inhibit DNA replication and transcription by inhibiting bacterial DNA gyrase or the topoisomerase IV enzyme.(16)

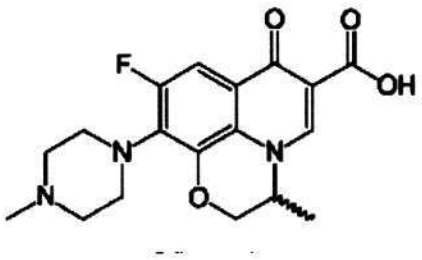


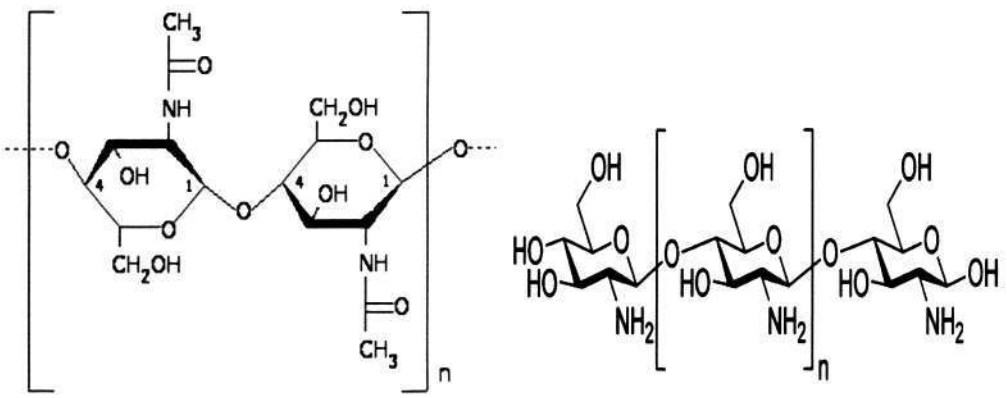
Fig 10. Structure of ofloxacin, a second generation fluroquinolones antibiotic

Table 2. Mechanism of action of selected antibiotics

ANTIBIOTICS	MECHANISM OF ACTION
Tetracycline	Inhibits prokaryotic 30S ribosome by binding aminoacyl-tRNA.
Sulfamethoxazole	Structural analogs and competitive antagonists of Para-aminobenzoic acid (PABA)
Trimethoprin	Interferes the action of bacterial dihydrofolate reductase, and inhibits synthesis of tetrahydrofolic acid
Clarithomycin	Binds to 50S bacterial ribosome and inhibits the translation of peptides.
Polymycin B	Alters cytoplasmic membrane permeability
Ceftriazone	Inhibits bacterial cell wall synthesis by binding to ribosome and thus inhibits the translation of peptides.
Chloramphenicol	Inhibits peptidyl transferase activity of the bacterial ribosome
Tobramycin	Binds to 30S and 50S bacterial ribosome and prevents the formation of the 70S complex
Ofloxacin	Inhibits DNA gyrase.
Streptomycin	Binds to bacterial 30S ribosomal subunit, and inhibits protein synthesis

2.2 Chitin

Chitin is a natural organic material which is the second most abundant to cellulose. It can be obtained from exoskeleton of animal sources particularly in crustacean, mollusks, insects and certain fungus. The chemical structure of chitin is poly [β (1-4) -2 acetamido 2- deoxy- D- glucopyranose. It is structurally similar to cellulose except that C (2)- hydroxyl group of cellulose is replaced by an acetamido group. This reflects the similarity in function as both materials are acting as structural and defensive for the organisms. Chitin is also structurally related to murein which is the main structural polymer of the cell wall of bacteria.(17)



(a) (b)

Fig 11. Structure of chitin (a), chitosan (b)

The main sources for laboratory preparation of chitin are usually from exoskletons of various crustacean, principally crab and shrimp. In these, chitin is closely associated with proteins, inorganic material, pigments and lipids. Demineralization by hydrochloric acid followed by treatment with sodium hydroxide to remove protein is the common practice for preparation of chitin.(17)

2.3 Chitosan

One of the main reactions carried out on chitin is deacetylation. The most common method for the deacetylation of chitin is the use of aqueous alkali. Sodium hydroxide is frequently used but potassium hydroxide and some other alkalis are also attempted and claimed to be suitable. When chitin is deacetylated, a group of polymers collective known as chitosan is obtained. The degree of deacetylation of chitosan range from 70% to 99%.The structure of chitosan is similar to that of cellulose. The chemical structure of chitosan is β- (1-4) -2 amino-2-deoxy-D-glucopyranose. Fig 11(b).(17) Both chitin and chitosan are insoluble in water, alkali and organic solvents but soluble in organic acids like acetic acid, especially, when the pH is less than 6.

A special attention to chemical modification of chitin has been paid to enable exploration of high potentials. Modification reaction of chitin is generally difficult due to the lack of solubility. Various attempts have been made to develop this rigid polysaccharide to increase the solubility in water. It is found that the water solubility occurs at an N-acetyl-D glucosamine: D- glucosamine ratio of approximately 1:1 when prepared under homogenous conditions. One of the methods of preparation of water soluble chitosan is by partial n-acetylation of chitosan in aqueous acetic acid methanol solution using acetic anhydride. Products having N- acetyl contents of approximately 46-55% are water soluble.(17)

2.4 Chitosan oligosaccharide

Chitosan oligosaccharide is a water soluble chitosan. Its chemical name is 2-mino-β-1, 4- glucose polymer and molecular formula is $(C_6\ H_{11}\ O_4N)_n$. Chitosan oligosaccharide is made from chitin and chitosan by chemical or enzymatic decomposition method. With its excellent physiological activity and function, it has wide usage.(17)

2.5 Antimicrobial activity of chitin, chitosan and chitosan oligosaccharide

Chitin, chitosan and their derivative compounds are biocompatible, biodegradable, and naturally regenerated from the shells of crab and shrimp. They posses diverse physiochemical properties – degree of deacetylation, molecular weight, viscosity, solubility, coagulation ability – depend on methods of preparation. Various applications of chitosan polymers are much related to these properties. The most common area of applications is water treatment, pulp and paper, pharmaceutical, cosmetics, agriculture, food, membrane and so on.(17)

Antimicrobial activity is also one of the attractive features of chitosan. Its antimicrobial activity is very wide spread including bacteria, fungus and virus in both animals and plants. The antimicrobial activity of chitosan varies depending on their physical properties (degree of deacetylation, DD, and molecular weight), solvent, microorganism species and source. The antimicrobial activity of chitosan varies with methods of preparation of DD and molecular weight.(18) The antimicrobial activity of chitosan decreases with decrease in molecular weight

when chitosan is prepared by oxidative-reductive degradation.(19) The result shows in reverse way when chitosan is prepared by ultrasonic degradation.(20) Molecular weight plays an important role in antimicrobial action. So the derivatives of chitosan like water soluble chitosan and chitosan oligomers have no significant antimicrobial activity unless their molecular weight reaches to a certain level. For water soluble chitosan, the molecular weight must be around 50KDa and more than 10KDa for chitosan oligomers.(21, 22) In terms of DD, the higher the % DD of chitosan, the better the antimicrobial activity will be.(23)

The mechanism of antimicrobial activity of chitosan is still not very clear. There are some proposals for its mechanism. In electron microscopic study, chitosan binds to the outer cell membrane of the microorganism resulting in the loss of barrier function.(24) In terms of molecular weight, chitosan prepared by enzyme degradation, high molecular weight chitosans are more affective against Gram-positive bacteria like *Staphylococcus aureus* as the chitosan forms a film which inhibit the organism from absorption of nutrients. Whereas, Gram-negative bacteria like *E. coli* behaves differently. The low molecular weight chitosan has the ability to enters the microbial cell wall easily and disturb the metabolism of the cell.(25) Chitosan oligosacchride is reported to have the ability to rupture the cell membrane of the *Actinobacillus actinomycetemcomitans* in EM study.(26) It is also postulated that binding of chitosan to teichoic acids, coupled with a potential extraction of membrane lipids (predominantly lipoteichoic acid) resulting in a sequence of events, ultimately leading to bacterial death.(27) Moreover, chitosan which shows diverse physical properties in combination with antimicrobial

activities make it and its derivatives applicable in different function in pharmaceutical preparations. The mucoadhesive properties make chitosan and its derivatives useful in control release formulation. The polycationic character, biodegradable, biocompatible and non-tocicity are useful for drug delivery, and synergistic with some antimicrobial agents. Tobramycin is one of the antibiotics which shows synergistic action with chitosan in planktonic culture of *P. aeruginosa*.(28) In the treatment of periodental diseases, the mucoadhesive, viscosity, bioadhesive and antimicrobial properties make chitosan affective against *Pseudomonas gingivalis* and *Streptococcus sanguinis* either alone or synergistically in combination with chlorhexadine.(29)

Chapter Three

Material and methods

In this study, chitosans/chitosan oligosaccharide – antibiotic combined drug activity was tested against both Gram-positive bacteria and Gram-negative bacteria.

3.1 Organism

Bacterial strain and inoculum preparation

ATCC 15729, PAO1 wild type clinical strain, PT121 (PAO mexE::ΩHg i.e. a PAO1 derivative inactivated by the insertion of a Ω Hg cassette in the coding region of MexE gene) and PT149 (MexEF-OprN overexpressor), strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus* ATCC43300 strain were used in this study.

3.2 Different degree of deacetylated chitosan and chitosan oligosaccharide

High molecular weight crab chitin was purchased from Bioline, Thailand. The crab chitin was deacetylated (75% - 85%deacetlated) by Technique of Horowitz(16) and the degree of deacetylation was measured by FT-IR spectroscopy.(Appendix II)

Medium molecular weight chitosan (75 – 85 % deacetylated) was purchased from Sigma- Aldrich.(Appendix II)

Medium molecular weight chitosan (>90 % deacetylated) was obtained from further deacetylation of medium molecular weight (75 – 85 % deacetylated) by

technique of Horowitz and the degree of deacetylation was measured by FT-IR spectrophotometry.(Appendix II)

Low molecular weight chitosan (75-85% deacetylated) was purchased from Sigma-Aldrich.(Appendix II)

Chitosan oligosaccharide lactate (>90% deacetylated) was purchased from Sigma-Aldrich. (Appendix II)

0.1%chitosan stock solutions were prepared by dissolving in acetate buffer pH4.6 (Sigma) filtered and keep in 2^{o} – 8^{o} C. Chitosan oligosaccharide stock solution of 5% w/v was prepared by dissolving in sterile deionized water, filtered and stored in 2^{o} – 8^{o} C.

3.3 Antibiotics

Antimicrobial powder of clarithromycin, chloramphenicol, ceftriazone, ofloxacin, polymycin B, sulfamethoxazole, streptomycin, tobramycin, tetracycline and trimethoprim were purchased from Sigma-Aldrich.

Stock antibiotic solutions of 10,000µg/ml were prepared, filtered, aloquted and kept in -20°C until the working solutions were prepared.

Manufacturer's guidelines were followed in preparing the stock solutions of antimicrobial agents. Sterile deionized water is generally used in the preparation of antibiotic solutions. Preparation of antibiotics require solvents other than water, a minimal amount of the recommended solvent was used and further diluted with water. The following formula was used to determine the amount of antimicrobial needed for the desired volume:

$$\text{Weight (mg)} = \frac{\text{Volume (ml) x desired concentration (µg/ml)}}{\text{Antibiotic potency (µg/mg)}}$$

The stock solutions were further diluted to the highest concentration needed to test the antibiotic by following the formula

$M_1V_1 = M_2V_2$.

Table 3. Preparation of chitosans and antibiotic solutions and their diluents used for working concentration

Antimicrobial agents	Solvent	Diluent
Chitosan	Acetic buffer (pH4.6)	Sterile deionized water
Chitosan oligosaccharide	Sterile deionized water	Sterile deionized water
Clarithromycin	Sterile deionized water	Sterile deionized water
Ceftriazone	Sterile deionized water	Sterile deionized water
Chloramphenicol	Sterile deionized water	Sterile deionized water
Ofloxacin	Minim volume of 0.1M NaOH and Sterile deionized water	Sterile deionized water
Polymycin B	Sterile deionized water	Sterile deionized water
Sulfamthoxazole	Minium volume of 2.5M NaOH and Sterile deionized water	Sterile deionized water
Streptomycin	Sterile deionized water	Sterile deionized water
Tetracycline	DMSO	Sterile deionized water
Tobramycin	Sterile deionized water	Sterile deionized water
Trimethoprim	Minium volume of glacial acetic acid and Sterile deionized water	Sterile deionized water

3.4 Microbiological Culture media

ISB-Sensitest broth and Agar were purchased from Oxoid. The culture media was prepared according to the manufacturer's instruction and autoclaved before the experiment.

3.5 Minimum Inhibitory Concentration (MIC) determination (Microdilution Method)

3.5.1 Preparation of Inoculum

4-5 colonies of overnight culture of *P.aeruginosa* were diluted into the ISB broth until the turbidity matched that of 0.5 McFarland turbidity standards (approximate turbidity 1.0x 10^8 CFU/ml). The suspension was further diluted 1: 100 by ISB broth to achieve 10^6 CFU/ml. The bacteria suspension was used within 15 minutes of preparation. (30)

3.5.2 Procedure

1. 50 µl of sterile ISB broth was dispensed into the well A2 to A12 of a microplate.
2. 50 µl of working diluted antibiotic solution or chitosan or chitosan oligosaccharide was dispensed to A1 and A2 well.
3. Serial dilution was made from well A2 to A10 and 50 µl was discarded from A10.
4. 50 µl of prepared bacteria suspension was added into well A1 to A11. (well A11 as growth control and A12 as negative control)

The microplate was incubated at 37°C for 20 to 24 hours in ambient air before interpretation. MIC was defined as the lowest concentration of the antimicrobial agent that completely inhibits growth of the organism in the wells as detected by the unaided eye. The growth of bacteria in the antimicrobial agents was compared with the growth control and negative control well.

3.6 Determination of drug interaction

3.6.1 Checkerboard Titration (Microdilution method)

Checker board titration is one of the most frequently used techniques to access drug interactions. Testing was performed utilizing 96 well microtiter plates. MICs were determined for each drug by broth microdilution according to standards of the CLSI (clinical laboratory standardization Institute).(30)

Preparation of Checkerboard Microdilution panels

1. MIC of individual antibiotics was determined for the test isolate.
2. A panel configuration was constructed by completing the worksheet in the following manner:
    a) MIC value of antibiotic was placed in cell A5 of the worksheet.
    b) MIC value of chitosan or chitosan oligosaccharide was placed in cell E l of the worksheet.

3. Two panels of sufficient quantities of four time's antimicrobial solutions were prepared. One was for combination of drugs panel (pnmel 1) and the other dilution of second compound (panel 2).

Panel 1

a) 50 µl of sterile ISB broth was dispensed to every well except those of column 1 and Column 8.

b) 50 µl of sterile ISB broth was added to well G12.

c) 50 µl of antibiotic solution was added to wells of column 7 and 8

d) Serial dilution was made from column 7 to 2. Discard 50 µl from column 2.

e) 50 µl from B1 to B 8 was taken from plate 2 and dispensed it in the corresponding row of the panel 1.

f) Step (e) was repeated for the next higher concentration.

Panel 2

a) 100µl of ISB broth was dispensed to all the wells of a microplate except Row A and Row H.

b) 100 µl of chitosan solutions or chitosan oligosaccharide was added to Row G and H.

c) Serial dilution was made from Row H to B. 100 µl was discarded from Row B.

Preparation of Inoculums

4-5 colonies of overnight culture of *P. areuginosa* were diluted into the ISB broth until the turbidity matched that of 0.5 McFarland turbidity standards (approximate turbidity 1.0x 10^8 CFU/ml). The suspension was further diluted 1: 100 by ISB broth to achieve 10^6 CFU/ml. The bacteria suspension was used within 15 minutes of preparation.

Inoculation and incubation of checkerboard panel

a) Using a multichannel pipette, 50 µl of diluted inoculum was added to every well.

b) 50µl of diluted inoculum was added to growth control well A1.

c) Purity plate and inoculum verification plate was prepared. The microplate, purity plate and inoculum verification were incubated plate at 37° C for 16- 20 hours.

Reading MIC Panels

a) The growth control well was examined for organism viability.

b) The purity plate and inoculum verification plate were examined for verification of pure culture and inoculum size.

c) Growth or no growth was recorded for all the wells on the worksheet.

d) The MIC results for antibiotics from well A2 to A8 were recorded.

e) The MIC results for chitosans and chitosan oligosaccharide from wells B to H in column 1 were recorded.

Calculations

1. The Fractional Inhibitory Concentration (FIC) for each antibiotic was calculated as follows:

$$\text{FIC for antibiotic} = \frac{\text{MIC of antibiotic in combination}}{\text{MIC of antibiotic alone}}$$

$$\text{FIC for chitosan/ chitosan oligosaccharide} = \frac{\text{MIC of chitosans in combination}}{\text{MIC of chitosans alone}}$$

2. Calculate the summation of FIC (Σ FIC) index for each combination as follow:

$$\Sigma\text{FIC} = \text{FIC for antibiotic} + \text{FIC for chitosans/ chitosan oligosaccharide}$$

Interpretation

1. If acceptable control parameters were obtained, the results were interpreted as follow: (Appendix II)

   Synergism = ΣFIC </ = 0.5

   Antagonist = ΣFIC >/= 4

   Additive = ΣFIC >0.5 and </=1

3.6.2 Population Analysis

Procedure

From the Checkerboard method, the end concentration of the antibiotics and chitosans which showed synergism was prepared and normal plate counting

method was performed to quantitate the log concentration of bacteria colony forming unit per militer.(30)

Preparation of inoculums

4-5 colonies of overnight culture of *P. areuginosa* were diluted into the ISB broth until the turbidity matched that of 0.5 McFarland turbidity standards (approximate turbidity 1.0x 10^8 CFU/ml). The suspension was further diluted 1: 100 by ISB broth to achieve 10^6 CFU/ml.

Preparation of Antimicrobial Dilutions in Melted Agar

1. Twelve 50ml polypropylene tubes were assembled for each culture dilution to be tested.
2. The tubes were labeled with the desired dilution of antibiotics alone or combination with chitosan preparations which showed synergistic action from checkerboard method.
3. The tubes were placed in a test tube rack.
4. The rack was placed in a waterbath at 50°C.
5. Using 25 ml sterile disposable pipette, 9.9 ml of melted ISB agar for single antibiotic and 9.8 ml for combination were dispensed.
6. 10 ml of melted ISB agar was dispensed to growth control tube and sterility control tube.
7. 0.1 ml of each prepared antimicrobial solution was added to the single antibiotic tubes.

   (e.g. 0.1 ml of 800µg/ml of sulfamethoxazole to the tube labeled as 8ug/ml)

8. 0.1 ml of antibiotics and 0.1 ml of chitosan preparations were added to the combination tubes. (e.g. 0.1ml of 800µg/ml of sulfamethoxazole and 0.1 ml of 800µg/ml of HMW chitosan solution to sulf+HMW tube.)
9. Voltex.

Inoculation and incubation

1. With the agar still melted, 1 ml of the culture from the prepared bacterial inculum was added to each tube.
2. The tubes were mixed by gentle inverting without causing airbubbles.
3. The mixture was poured into sterile petri dishes
4. Agar was allowed to solidify at room temperature.
5. Plates were incubated at 35°C for 48 hours.

Reading and recording of results

1. After incubation, the number of colonies was counted at each concentration.
2. The results were tabulated as CFU per millitier verses concentration of antimicrobial agents on semilog graph paper.
3. The antimicrobial agent concentration that shows a [$3\log_{10}$(CFU/ml]-fold decrease was determined when compared with the growth control.

Interpretation

1. Bactericidal activity at a given antimicrobial concentration relative to the control.

   Bactericidal activity = >/= 3 $\log_{10}$ CFU/ml decrease

   Bactericidal activity = </= 3 $\log_{10}$ CFU/ml decrease.

Effect of acetate ion concentration on *P. aeruginosa*

Two sets of test were performed in the determination of MICs for chitosans. One with chitosans dissolved in acetate buffer and second set (control) with same volume of acetate buffer without chitosan. *P. aeruginosa* strains suspension were incubated at 37°C for 20 to 24 hours at ambient air. The effect of acetate ion concentration in acetate buffer on *P. aeruginosa* was determined by analyzing bacterial growth by spectrophotometric analysis at 600 nm (checking the turbidity when compared with negative control)

Fluorescence microscopy

Bacteria suspension, serial dilution of chitosan and chitosan oligosaccharide up to their MIC and Propidium iodide were combined, and the resulting mixture was incubated for 10 h in a 37°C incubator. One tube of bacteria suspension without chitosan (control live cells) and a tube of heat killed bacteria suspension (control dead cells) were incorporated in the test.(31)

The tubes were centrifuged to get a pallet. The pallet was washed twice with PBS and wet mount preparation ware done on glass slides.

Bacteria stained with Propidium iodide were observed under Nikon Eclispe TE 2000-S inverted microscope equipped with UV excitation filter under 40Xplanfluor objective lens. Photographs were acquired with CCTV camera attached to the microscope.

Atomic Force Microscopy

The effect of the five different combinations of chitosans/ oligosaccharide-sulfamethoxazole on the bacterial cell surfaces was examined by AFM. Samples were prepared by applying 40 mL of bacterial suspension without treatment (control) or treated with drug combinations onto a clean glass surface, followed by airdrying. The samples were incubated in the presence of combine drugs for the same treatment times as for the antibacterial assays. The samples were then gently rinsed with deionized water to remove salt crystals, and air dried again before analysis. AFM was carried out with a Veeco Multimode IVa atomic force microscope (Veeco, Santa Barbara, CA), equipped with a j-type scanner (ca. 100_100_5 mm3 scan range). Bacteria morphology studies were carried out in the tapping mode in air, using silicon cantilevers with a resonant frequency of approximately 150 kHz (MikroMasch, Tallinn, Estonia). Two independently produced samples were analyzed, and several different areas were studied on each sample, but only characteristic images are shown here.(32)

Scanning Electron Microscopy

Bacteria suspension treated with drug combinations for the same treatment times as for the antibacterial assays were made smear on a clean glass coverslip (35mmx35mm). During the processing, great care was taken to avoid the development of artifacts or unrecognizable features. The samples were fixed in 5 % gluteraldehyde (5% v/v 0.1M phosphate buffer, pH7.4) for 3 hours, rinse twice in

0.1M phosphate buffer, pH7.4, post fixed in 2% gluteraldehyde -3% formaldehyde solution (v/v 0.1M phosphate buffer pH7.4) for 1 hour.

The samples were then rinsed twice with 0.1M phosphate buffer. Dehydration was achieved by sequentially for 20 minutes in 70%, 90% (both v/v 0.1M phosphate buffer, pH 7.4) and 100% acetone.

The air dried samples were mounted onto aluminum stubs using carbon tape and sputter coated with gold. The samples were view on the scanning electron microscope (JEOL JSM-5600LV). Two independently produced samples were analyzed, and several different areas were studied on each sample, but only characteristic images are shown here (33).

Chapter Four

Results and Discussion

P.aeruginosan and *S.aureus* showed similar reaction to the proposed drug combinations under standard controlled conditions and the complete analysis results are discussed in this chapter.

4.1 Result

Pseudomonas aeruginosa

Antimicrobial activity of acetate buffer against Pseudomonas aeruginosa

As chitosans solutions were prepared with acetate buffer as solvent, the antimicrobial activity of acetate buffer was investigated to exclude the antimicrobial effect of acetate ion interference. The antimicrobial activity of acetic buffer was observed at acetate ion concentration up to 0.25% (Fig 12). In preparation of chitosan working solutions that were used in this study, acetate ion concentration of less than 0.03% was used. This indicates that there was no interference of acetate ions. The antibacterial activity of the chitosan at culture media pH< 6 had earlier been studied and reported that there was no significant interference (34, 35).

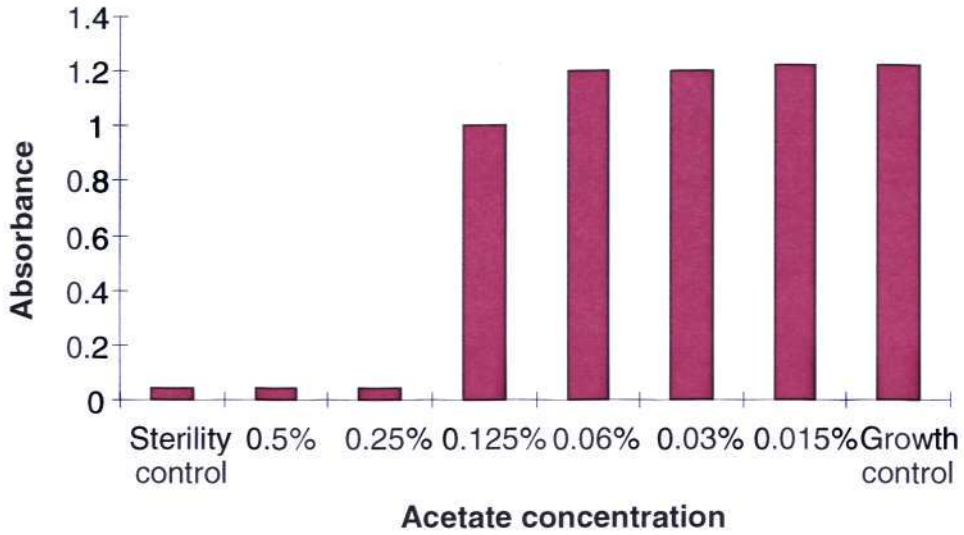


Fig 12. Antibacterial activity of acetate buffer showing different concentration of acetate ions versus OD at 600 nm.

Anti-pseudomonal activity of different antibiotics

The susceptibility range of the *P. aeruginosa* species were evaluated using 10 antibiotics (tetracycline, sulfamethoxazole, trimethoprim, clarithromycin, polymycinB, ceftriazone, chloramphenicol, tobramycin, ofloxacin and streptomycin. MIC values are shown in Figure 13. MIC value is found to be the lowest for tobramycin (0.25μg/ml) and the highest for chloramphenicol (128μg/ml).

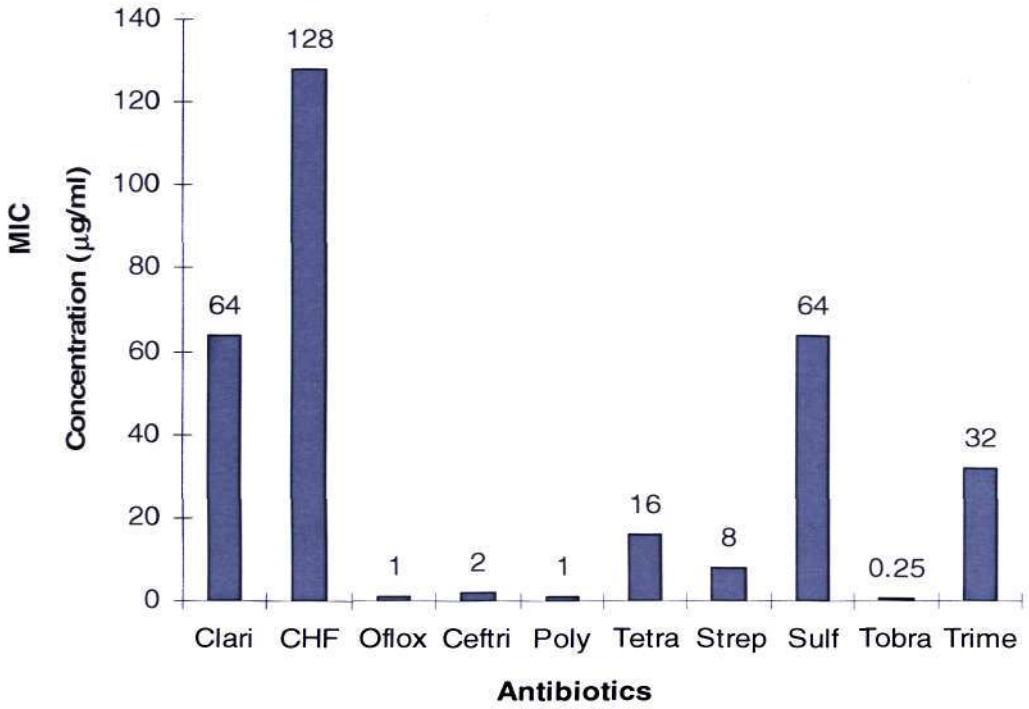


Fig 13 MICs(µg/ml) of different antibiotics for P.areuginosa ATCC15279

Anti-pseudomonal activity of different chitosans

Chitosans and chitosan oligosacchrides showed affective against *P. aeruginosa* (ATCC15729) planktonic culture in vitro and the MIC (minimum inhibitory concentration) of chitosan (32µg/ml) was much lower than chitosan oligosaccharide (4096µg/ml) (Fig 14).

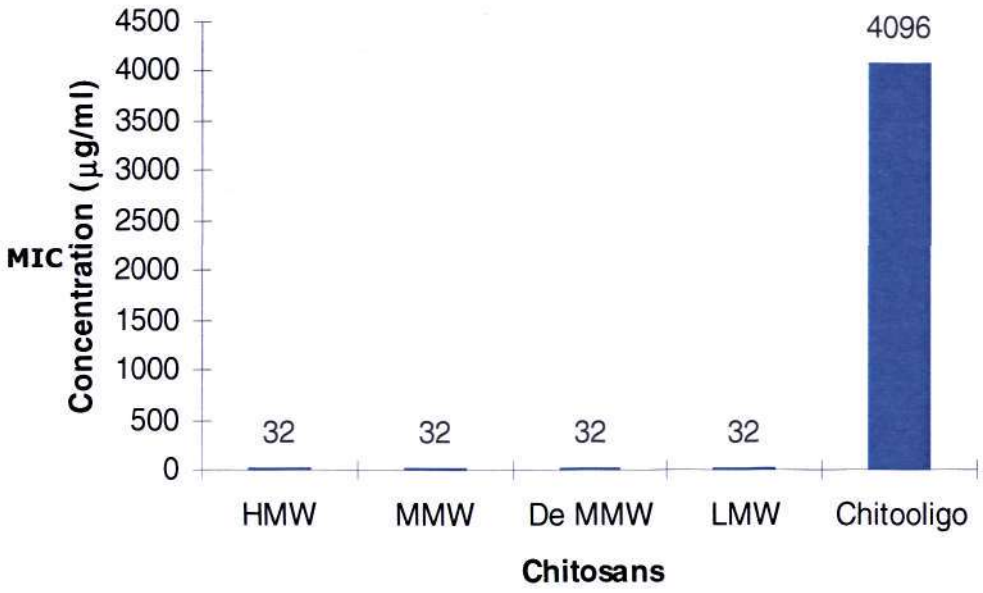


Fig. 14 MICs(µg/ml) of different chitosans for *P.aeruginosa* ATCC15279

Checkerboard assay results

In checkerboard assay, the combination with the above mentioned antibiotics and all the chitosan preparations and chitosan oligosaccharide showed synergistic action with sulfamethoxazole against *P.aeruginosa* ATCC15729. 8µg/ml of four chitosan preparations and 512 µg/ml of chitosan oligosaccharide effectively combined with 8 µg/ml of sulfamethoxazole (Fractional Inhibitory Concentration, FIC_{index} = 0.375). The remaining combinations showed additive effect in the checkerboard assay. (FIC_{index} 0.5 - 1.0).

In summary, the combinational affect of chitosan and chitosan oligosacharide can be summarized as follow:

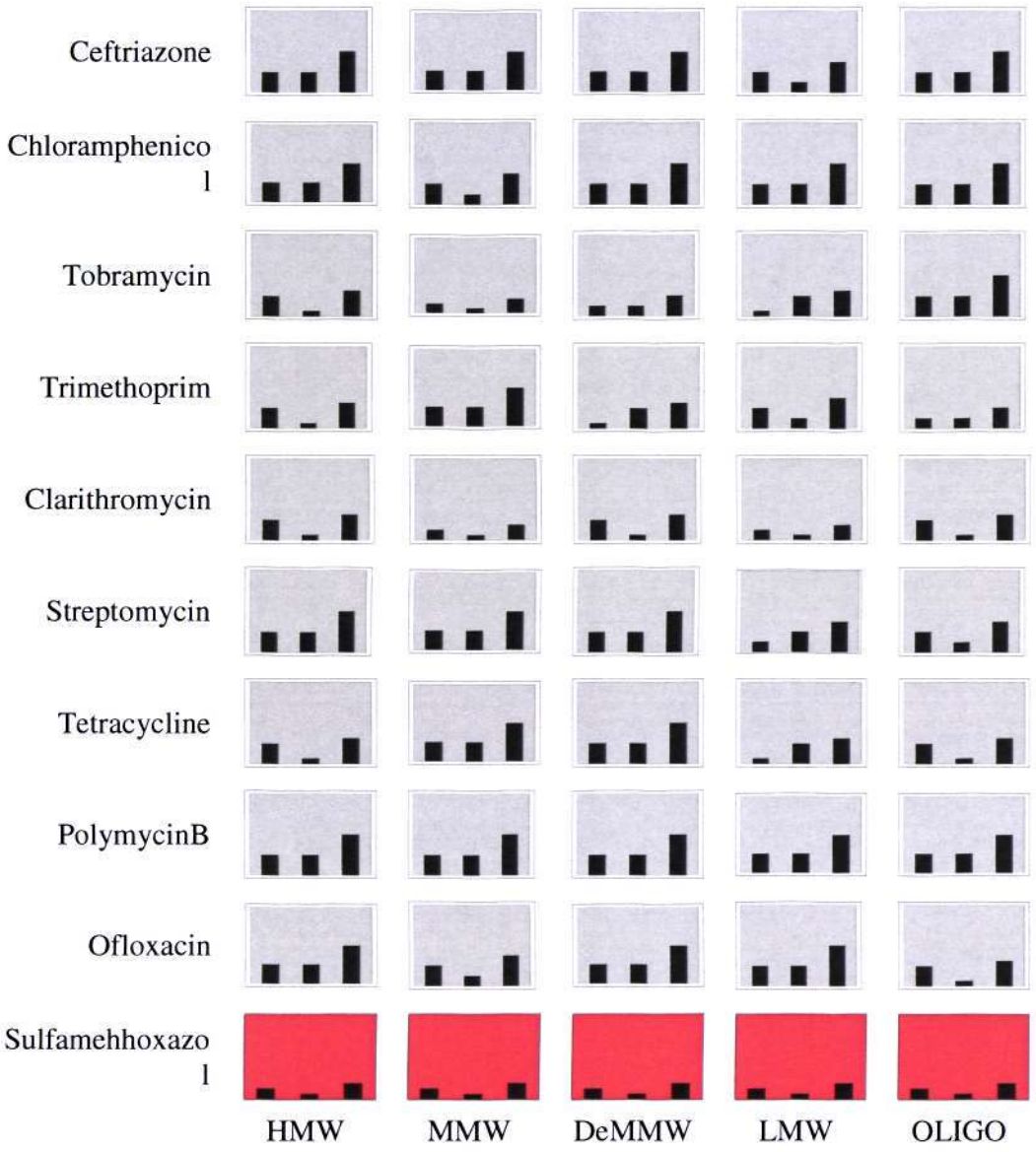


Fig. 15 Summary of combinational affect of chitosan, chitosan oligosaccharide and antibiotics Red = Synergism (FIC=0.375)
Grey= Additive (FIC </= 1)

Population analysis result

Population study of *P. aeruginosa* ATCC15729 was also performed. 1/4 MIC of chitosan/ chitosan oligosaccharide and 1/8 MIC of sulfamethoxazole combinations significantly reduced the log cfu/ml of the bacteria compared to that of chitosan preparations and sulfamethoxazole alone (Fig 16).

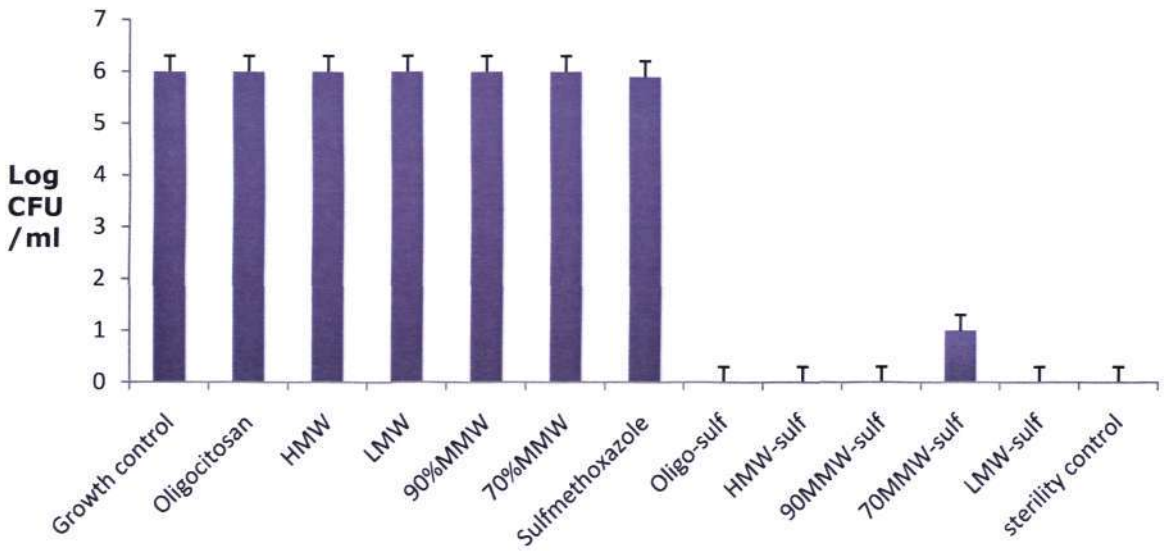


Fig 16. Population analysis of *P.aeruginosa* in combination of chitosans and sulfamethoxazole. (1/4 MIC of chitosan/ chitosan oligosaccharide and 1/8 MIC of sulfamethoxazole)

The identified synergistic combinations of sulfamethoxazole / chitosan and sulfamethoxazole / chitosan oligosaccharide were further investigated with three different clinical strains of *P. aeruginosa* PAO1 wild type, PT121 and PT149 strains (Please see materials and methods for details). Chitosan and chitosan oligosaccharide were found to have synergistic effect when combined with sulfamethoxazole against all the three *Pseudomonas* strains. 8 µg/ml of four chitosan preparations synergistically combined with 32 µg/ml of sulfamethoxazole (FIC_{index} = 0.25) and 512 µg/ml of chitosan oligosaccharide with 16 µg/ml of sulfamethoxazole against PAO1, PT121 and PT149 strain (FIC_{index} = 0.375). Fig 17, 18, 19, 20, 21 and 22 show the results of the single and combination effect.

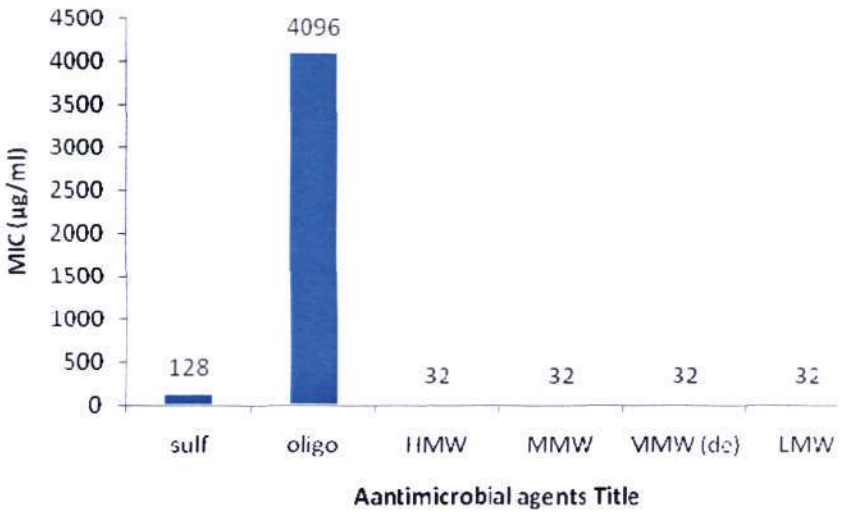


Fig 17 . MICs of *P. aeruginosa* mutant (PT121)

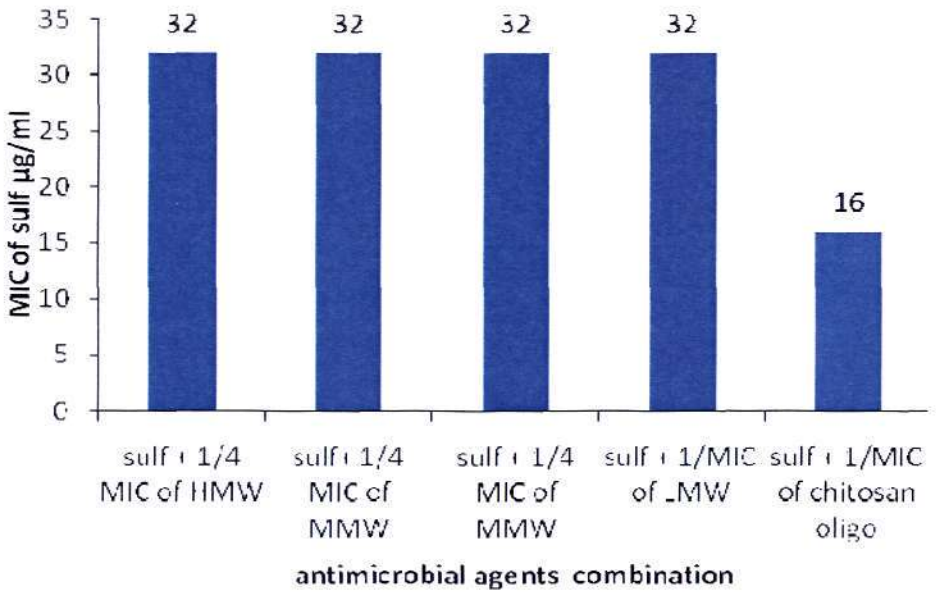


Fig 18. MIC of sulfamethoxazole after combination on *P.aeruginosa* mutant (PT121)

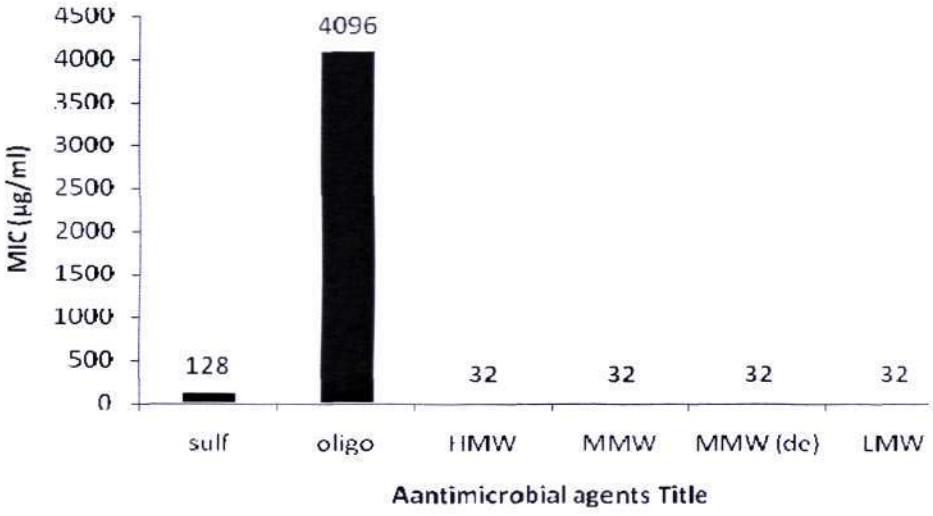


Fig 19. MICs of *P. aeruginosa* wild type (PA01)

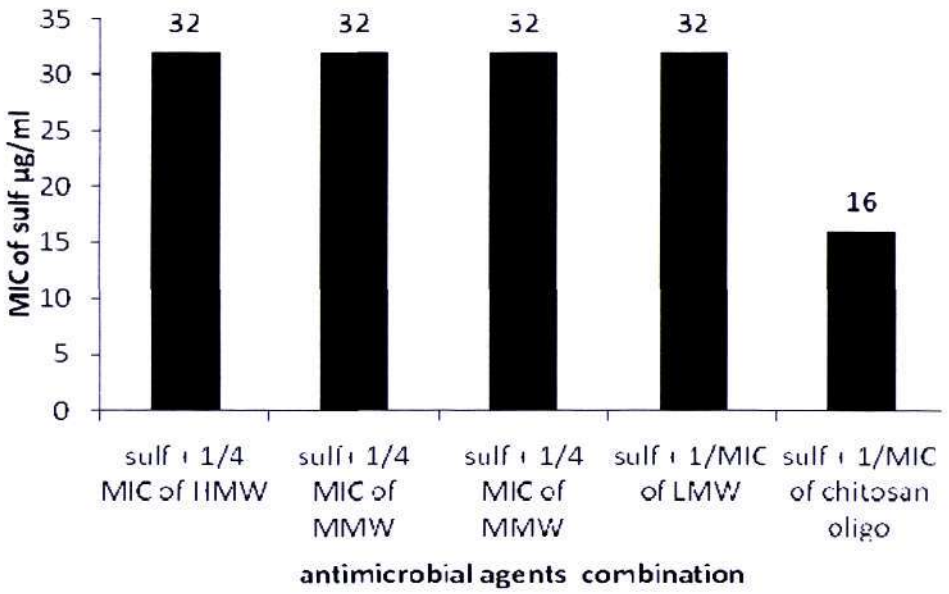


Fig 20. MIC of sulfamethoxazole after combination on *P.aeruginosa* mutant (PA01)

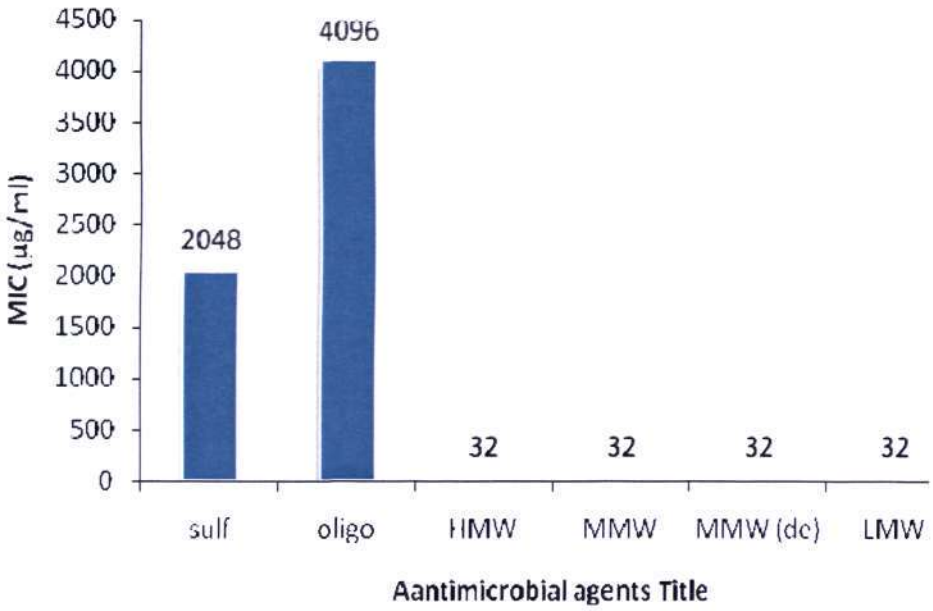


Fig 21. MICs of P.aeruginosa (PT149) chloramphenicol resistant strain

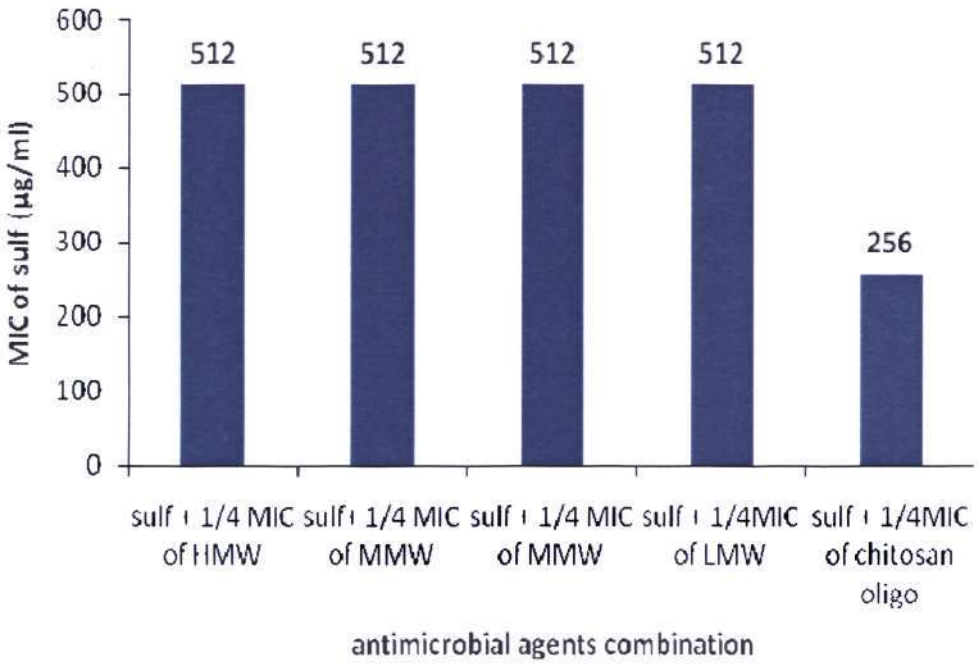


Fig 22. MICs of sulfamethoxazole after combination on*P.aeruginosa* PT149 chlroamphenicol resistant strain

Fluorescence microscopy results

The fluorescence staining of bacteria cells from chitosan and chitosan oligosaccharides starting from the dilution of ¼ MIC showing the same staining pattern as heat killed bacteria cells (Fig.23). Reference live cells are seen in Fig.24.The finding suggested that the antimicrobial activity of chitosan and chitosan oligosaccharides involve in disruption of the integrity of the cell membrane.

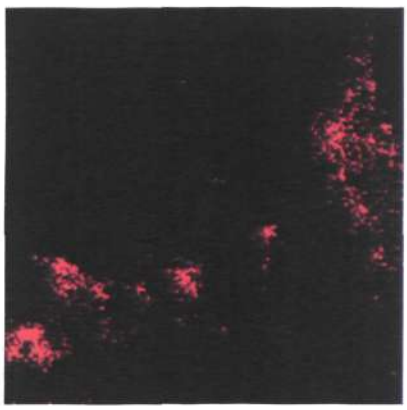


Fig. 23 Dead bacteria cell under 40X plan flour objective

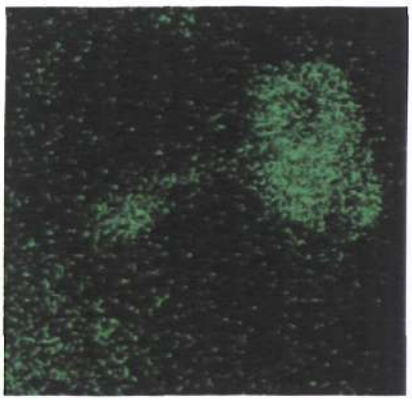
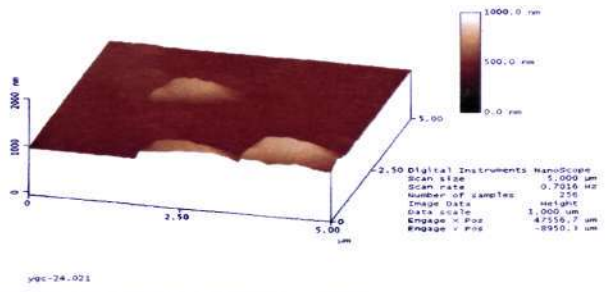
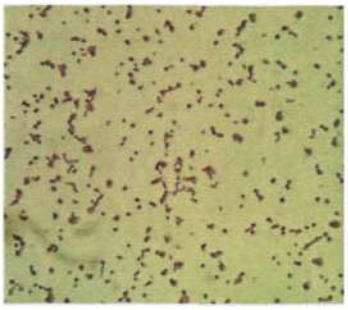


Fig. 24 Live bacteria cell under 40X plan flour objective.

Atomic Force Microscopy results

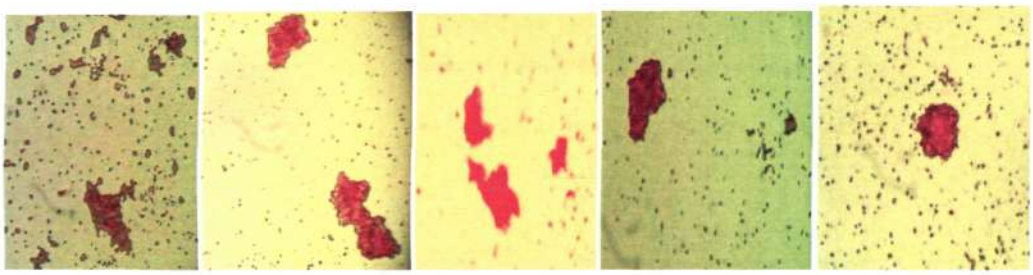




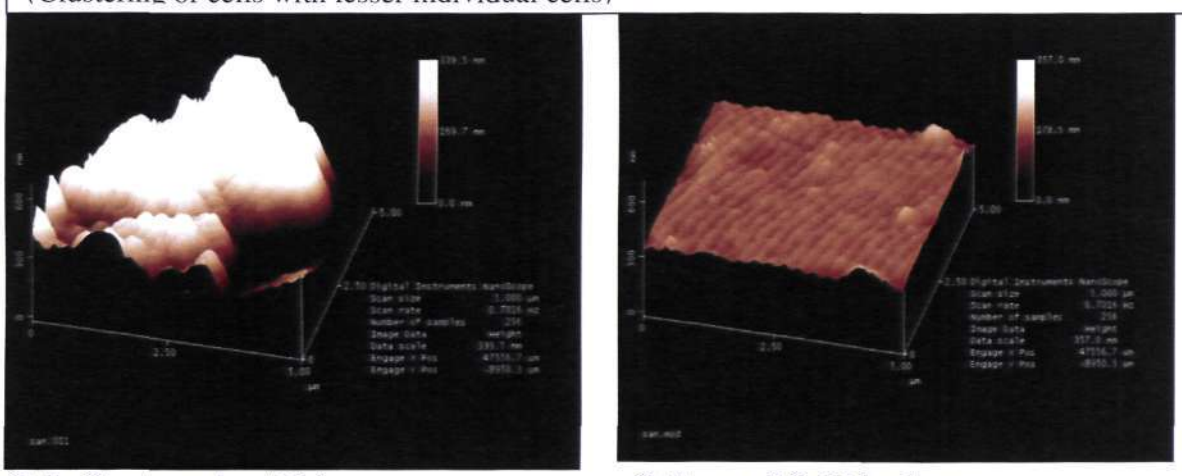
Untreated Bacteria (AFM)

Untreated Bacteria(Gram stain)

8 Hours incubation with drugs combination



1/4 MIC of sulfamethoxazole + 1/4 MIC of chitosan oligosaccharide, high molecular weight / medium molecular weight/ medium molecular weight deacetylated/ low molecular weight chitosan
(Clustering of cells with lesser individual cells)



Cells Cluster under AFM

Collapse of Cell Membrane under AFM

Fig 25. Effect of drug combinations on morphology of *P. aeuroginosa* under AFM

Scanning Electron Microscopy results

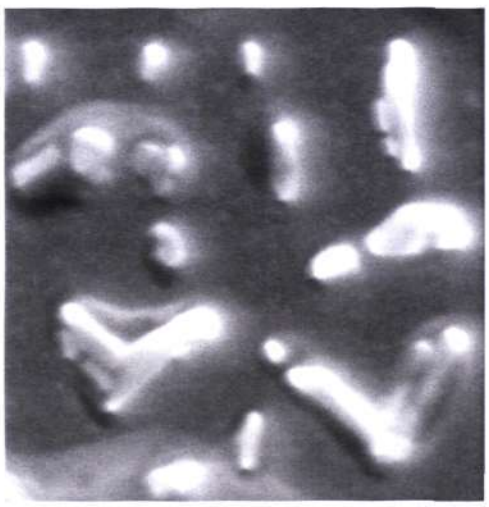


Fig. 26 Untreated *P. aeruginosa* under SEM

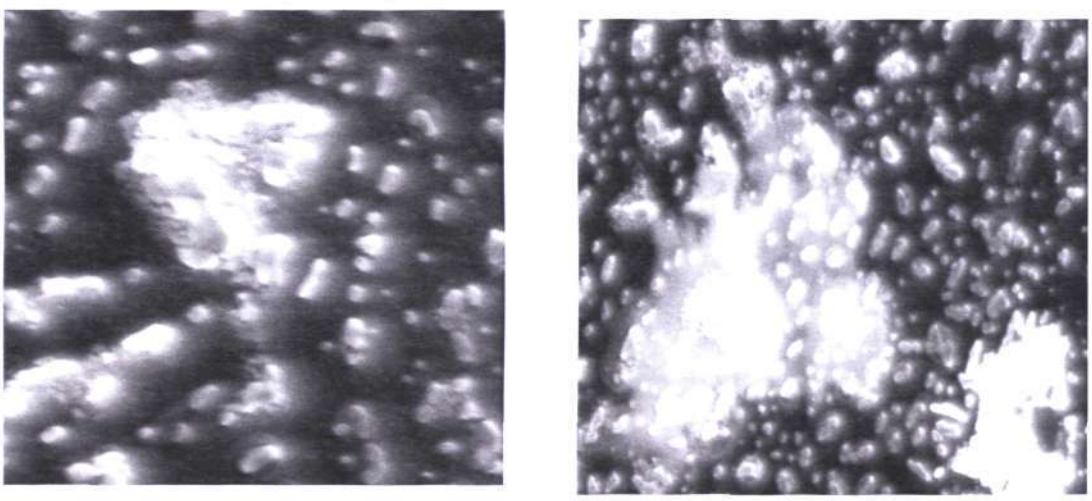


Fig. 27 Cell Clusters under SEM

Staphylococcus aureus

Anti-Staphylococcal activity of various antibiotics and chitosan preparations

Four chitosan preparations and water soluble chitosan oligosaccharide were also effective against S. aureus. chitosan was found to be more effective than chitosan oligosaccharide. The MIC values were shown in Fig. 28 and 29.

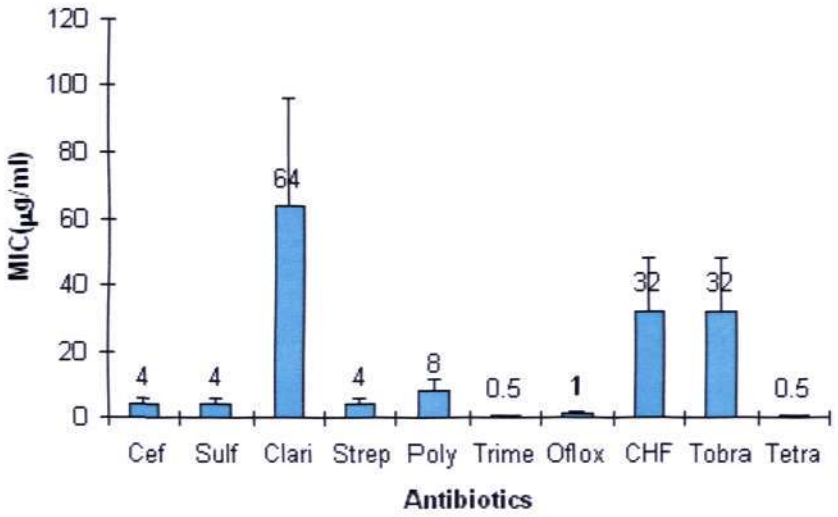


Fig. 28. MIC of ten different antibiotics

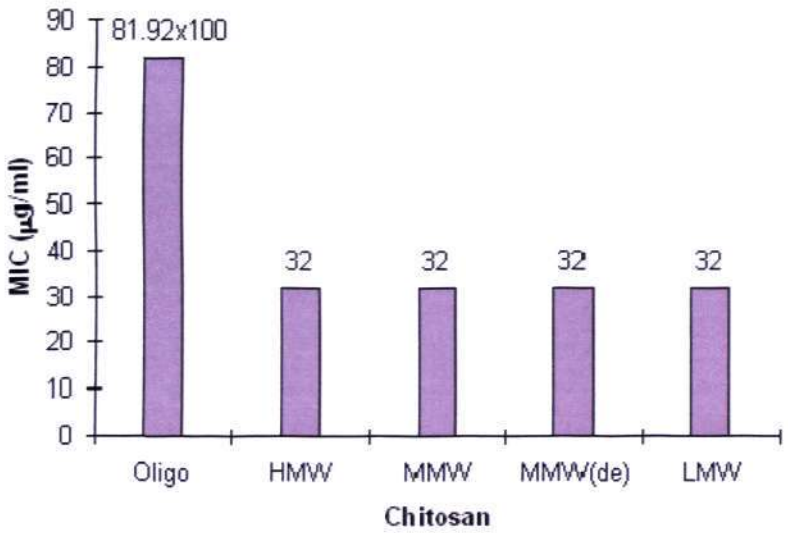


Fig. 29. MIC of chitosan and chitosan oligosaccharide

As in the case of *P. aeruginosa* the antibacterial effect of acetate ions is demonstrated in Fig 30.

At the dilution of 1 in 16, little or no inhibition was observed. Working chitosan preparations were prepared at 1 in 50 to 1 in 100 dilution of acetate buffer in this experiment. So the antimicrobial of chitosans had no interference of acetate ions concentration.

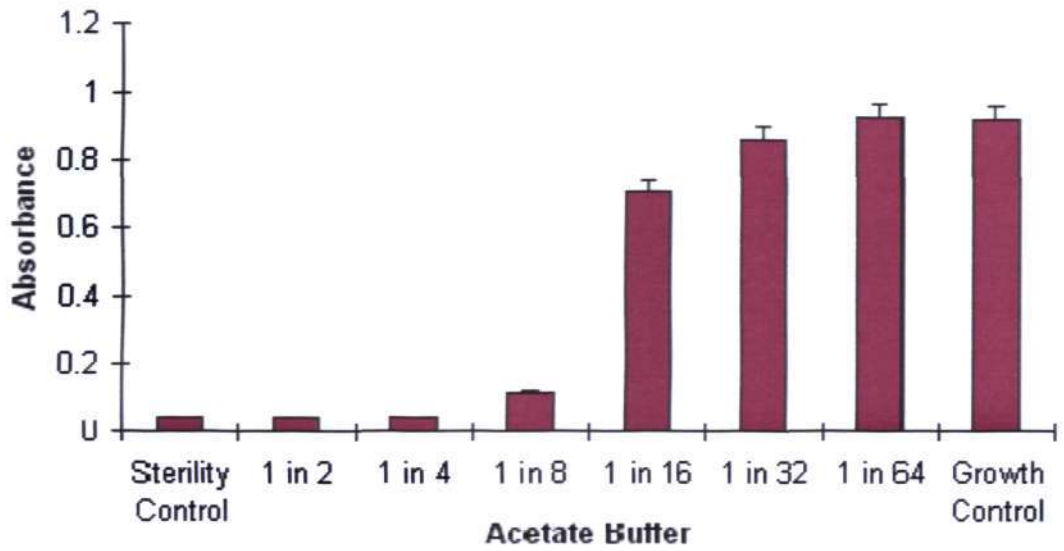


Fig.30. Inhibition of acetate buffer against the growth of Staphylococcus aureus

Drug interaction

The interaction of five different preparations of chitosan and ten antibiotics was tested against *S. aureus*. Checkerboard microdilution method was applied to calculate the sum of Fractional Inhibitory Concentration (ΣFIC) for each combination. The Checkerboard assay displayed ΣFIC = 0.375 when chitosan oligosaccharide combined with sulfamathoxazle, tetracycline and ceftriazone. In

other word, ¼ MIC of the above three antibiotics and 1/8 MIC of chitosan oligosaccharide combination acted synergistically against *S. aureus*.

However, chitosan preparations were not as effective as chitosan oligosaccharide. Only high molecular weight chitosan showed synergistic action against *S. aureus* ((ΣFIC = 0.5). The summary of (ΣFIC) was shown in table 4.

Table 4. Summary of (ΣFIC) of drug combinations

Antimicrobial Agents	Oligo ΣFIC	Remark	LMW ΣFIC	Remark	DeMMW ΣFIC	Remark	MMW ΣFIC	Remark	HMW ΣFIC	Remark
Clari	1	Additive	1	additive	1	additive	1	Additive	1	additive
Ceftri	0.375	Synergy	0.5	Additive	1	Additive	1	Additive	1	Additive
CHF	1	Additive	1	Additive	1	Additive	1	Additive	1.5	Additive
Oflox	1	Additive	1	Additive	1.5	Additive	1	Additive	1.5	Additive
Poly	1	Additive	1.5	Additive	1.5	Additive	1.5	Additive	2	Additive
Sulf	0.375	Synergy	1	Additive	1	Adittive	1	Adittive	0.5	Synergy
Strep	1	Additive	1	Additive	1	Additive	1	Additive	1	Additive
Tetra	0.375	Synergy	0.5	Additive	1	Additive	1	Additive	1	Additive
Tobra	1	Additive	1	Additive	1	Additive	1	Additive	1.5	Additive
Tri	1	Additive	1	Additive	1.5	Additive	1	Additive	1.5	Additive

Population analysis

Colony forming units were counted on the synergistic pair of the drug combination. A significant reduction in log colony forming units was observed when ¼ MIC of sulfamethoxazole or tetracycline or ceftriazone combined with MIC of chitosan oligosaccharide. Similar result was obtained in ¼ MIC of sulfamethoxazole and ¼ MIC of high molecular weight chitosan combination. The results are shown in Fig.31. This finding supported the finding of Checkerboard assay.

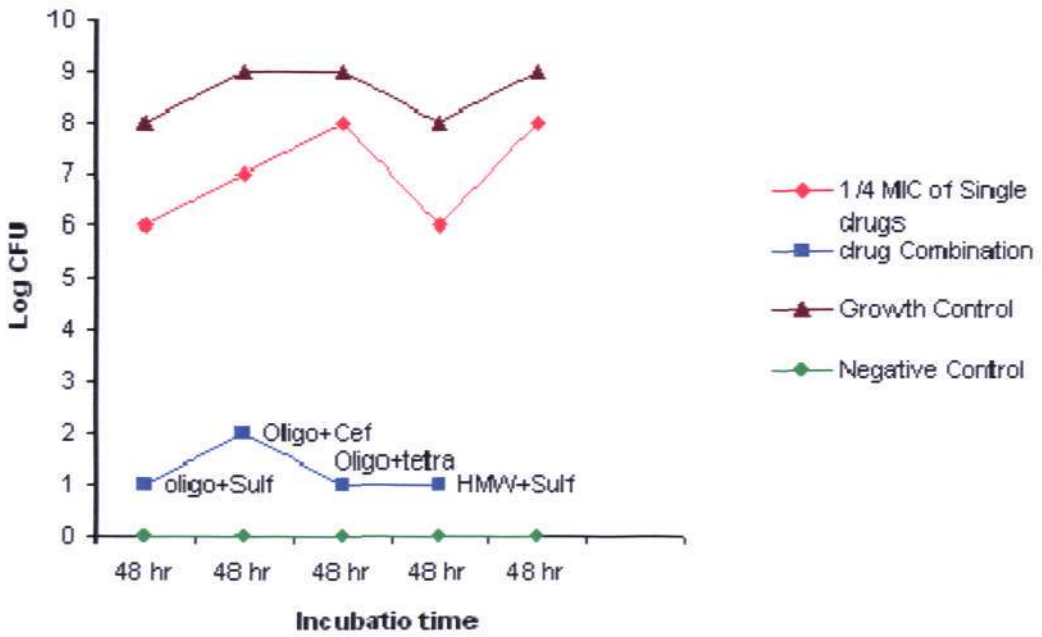


Fig 31. Synergistic Combination of Drugs in Population analysis

4.2 Discussion

P. aeruginosa is an important bacterial pathogen most frequently responsible for nosocomial infections. It is often resistant to many antibiotics used in causative therapy. The efficacy of many antibiotics for treatment of severe infections has become quite limited due to the development of resistance. Improving the effectiveness and decreasing the toxicity of antibiotics are the two basic objectives in the development of novel antimicrobial agents. Utilization of combination therapy is one of the contemporary approaches for successful modulation of existent antibiotics.(36)

Bioadhesive and antimicrobial properties make chitosan and their derivatives to become a novel material in antimicrobial drug delivery. Control release preparation of chlorhexidine and nystat in oral preparation (37, 38, 39) , control release preparation of ampicillin,(40) drug delivery system for ofloxacin(41) in ophthalmic preparation, and tobramycin sulfate gastrointestinal sustain release preparation(42) made chitosan become additional attractive features for combination with antimicrobial drugs.

Our results revealed the strong antibacterial activity of chitosan and its derivatives. Chitosan and its derivatives showed antibacterial activity against drug resistant strain of bacteria like *P. aeruginosa*. Furthermore, these chitosan derivatives are active against antibiotic resistant bacteria at very low concentration (32μg/ml). The MIC of chitosan oligosaccharide (2046μg/ml) was higher than that of other

chitosan preparations. It was noted that the MICs were much below the toxicity level. It had been reported earlier in an *in vivo* study, that chitosans have no evidence of toxicity (43) and the LD50 of chitosan in mice was 16g/kg of body weight (44).

When in combination with sulfamethoxazole, sub MIC concentrations of chitosan (8µg/ml) and chitosan oligosaccharide (512µg/ml) effectively inhibited the growth of various Pseudomonas strains. The presence of antibacterial activity against *P. aeruginosa* ATCC15279 was seen at sulfamethoxazole concentration as low as 8 fold (8 µg/ml) of its MIC. Antibacterial activity against three different clinical strains PAO1, PT121 and PT149, showed synergistic behavior suggesting that chitosan and chitosan oligosaccharide were promising compounds for combination antimicrobial therapy against drug resistant Pseudomonas infections.

In *P. aeruginosa* PT149, the MIC of sulfamethoxazole was found to be 5 fold higher than the remaining strains suggesting that sulfamethoxazole might be effluxed by the MexEF-OprN system, thereby reducing the effective concentration of the folic acid biosynthesis inhibitor. Thus, the reduction in drug accumulation was more apparent in the MexEF-OprN over-expresser. Although, sulfamethoxazole had high MIC, chitosan and chitosan oligosaccharide remained effective against this mutant strain either singly (MIC of 32 µg/ml and 4096 ug/ml respectively) or in combination (8 ug/ml of chitosans lower the MIC of sulfamethoxazole to 512µg/ml and 512µg/ml of chitosan oligosaccharide lower the MIC of sulfamethoxazole to 256µg/ml). These findings provided strong evidence

that chitosans and chitosan oligosaccharide are promising candidates for combination therapy against multi drug resistant *P. aeruginosa* infections.

From the results of FIC_{index}, chitosan oligosaccharide proved to be a better combination for sulfamethoxazole against *P. aeruginosa* , as the FIC_{index} were relatively lower than that of four different preparations of chitosan.

As to the mechanism of the synergistic effect of sulfamethoxazole-chitin and sulfamethoxazole-chitosan oligosaccharide combinations, the finding in fluorescence microscopy suggested that chitosan and chitosan oligosaccharide disrupted the integrity of cell membrane and enhanced the permeability of sulfamethoxazole into the cell. This finding supported the earlier study of *Helander* (24) who proposed that chitosan bound to the outer membrane of the organism and lead to the loss of barrier function.

For better understanding of the mechanism of action, Aatomic Force Microscopy study was proposed to explore the affect of chitosan-sulfamethoxazole combinations on *P.aeruginosa.* Under Atomic Force Microscope, untreated *P. aeruginosa* bacterium was seen as elongated rod shape structure. On the other hand, bacteria cells treated with drug combinations for 8 hours showed lesser discrete cells and tend to cluster under the light microscope. Morphologically under AFM, the bacteria cells were observed as spherical in shape rather elongated structure suggested that the mechanical barrier, cell wall, integrity was compromised. Many collapse rod shape cells were also seen under AFM in Fig. 27. The earlier report of

AFM study on the effect of chitosan and chitosan oligosaccharide against *E.Coli* showed similar morphology changes.(32)

Scanning Electron Microscopy was used to study on the cell clusters of the treated bacteria. The changes in morphology were observed. Most of the cells tend to be in spherical shape rather in their elongated structure as compared to the untreated cells.

These finding strengthened the fact that chitosan / chitosan oligosaccharide-sulfamethoxazole combination caused collapse and lyses of cell membrane which led to the cell death.

Chitosan and chitosan oligosaccharide were proved to be effective against Gram-positive bacteria, *S. aureus*. In *S. aureus* the MIC of chitosan oligosaccharide is much higher (8192 µg/ml).There was no significant in antimicrobial activity effect among different chitosan preparations against *S. aureus*.

The drug interaction study in *S. auerus* showed interesting behaviour. chitosan oligosaccharide effectively combined with ceftriazone and tetracycline in addition to sulfamethoxazole, whereas, other chitosan preparations did not show synergistic reaction with sulfamethoxazole. Only high molecular weight chitosan combined synergistically with sulfamethoxazole against *S. aureus*. This finding agreed with the earlier report which stated that high molecular weight chitosan can bind affectively to the Gram-positive cell membrane compared to the lower molecular weight chitosans.(32) No antagonist combination pair was observed in the selected pair of antibiotics and chitosan preparations.

It has been known that chitosan and chitosan oligosaccharide are effective antibacterial agents against both Gram-negative and Gram-positive bacteria by itself. In our combination study, chitosan oligosaccharide and high molecular weight chitosan are the promising compound for combined antimicrobial therapy against *S. aureus*. Although chitosan oligosaccharide has much higher MIC for *S. aureus*, it showed wider spectrum of effective combination with antibiotics. Chitosan oligosaccharide is believed to lyse the bacterial cell wall (45) and enhances the destructive activity of tetracycline, ceftriazone and sulfamethoxazole. In selection of chitosan for combined drugs preparation, high molecular weight chitosan (90% deacetylated) is the best candidate compared to the lower molecular weight preparations. It has the ability to block entering of nutrients into the cell longer (46) and make sulfamethoxazole easier to attack the cell. This finding encourages studying the detail mechanism of combined preparation against Gram-positive bacteria and animal study to evaluate the effectiveness in vivo.

Chapter Five

Conclusions

The results of the study help to understand the antimicrobial activity of chitosan and chitosan oligosaccharide interaction with antibiotics. Generally, Gram-negative bacterial are susceptible to various molecular weight preparations of chitosan in combination with sulfamethoxazole. High molecular weight chitosan chitosan preparation is more effective than its lower molecular weight ones when in combination with sulfamethoxazole, tetracycline and ceftriazone.

Atomic force microscopy and scanning microscopy study assisted in more in-depth understanding of the synergistic behaviour of chitosan and chitosan oligosaccharide-antibiotic combination.

FUTURE WORK

One of the causes of failure in antimicrobial therapy is the formation of biofilms. It is encouraged to explore the effect of the combination against biofilms in different biofilm forming bacteria.

Based on this finding, chitosan can be extensively applied to antimicrobial preparations against various microorganisms.

Potential for use of such combinations clinically is huge since it may be able to make some untreatable resistant infections treatable at currently recommended dosages that are often marginally effective against resistant strains when used alone.

These data encourages further studies with chitin derivatives plus other antimicrobial classes *in vivo* animal experiments to validate this interesting findings.

Publication

- Activity of chitosans in combination with antibiotics in *Pseudomonas aeruginosa*
  San Tin, Kishore R. Sakharkar, Chu Sing Lim, Meena K. Sakharkar
  Int. J. Biol. Sci. 2009, 5: 153-160

- Synergistic Combinations of Chitosans and Antibiotics in *Staphylococcus aureus*
  Tin, San; Lim, Chu S.; Sakharkar, Meena K.i.s.h.o.r.e.; Sakharkar, Kishore Ramaji *Letters in Drug Design & Discovery*, Volume 7, Number 1, January 2010 , pp. 31-35(5) Bentham Science Publishers

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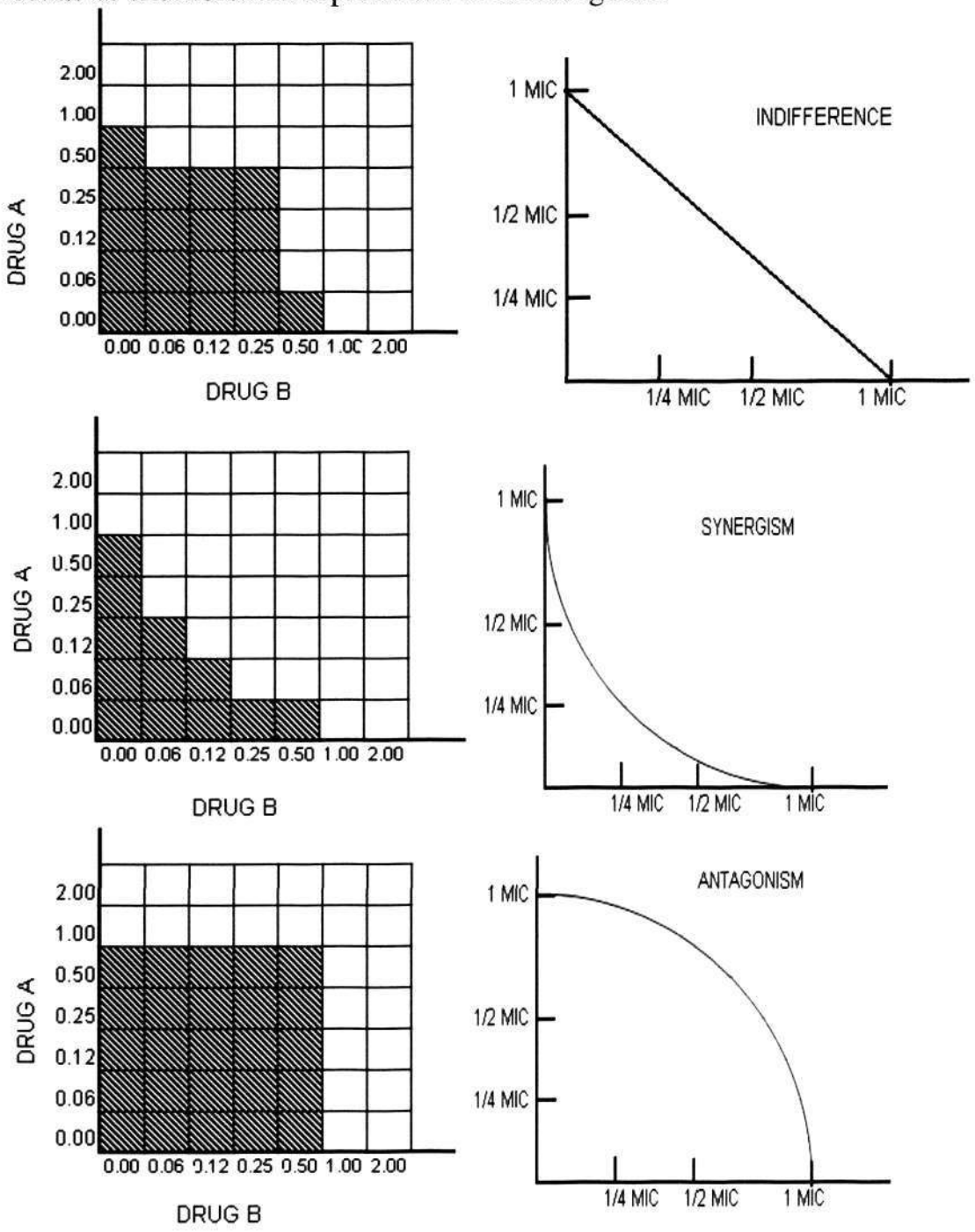
Appendix 1

Results of checkerboard represented as isobolograms



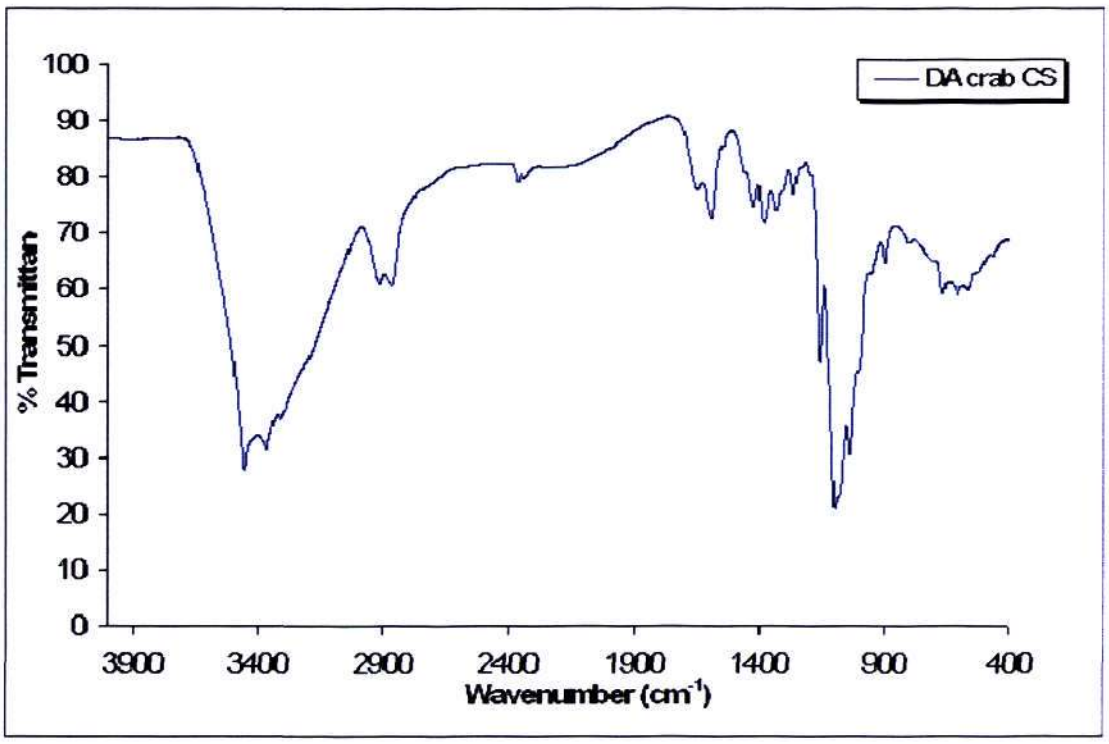
Appendix 2

Appendix 2 . FT-IR spectrum of different chitosans

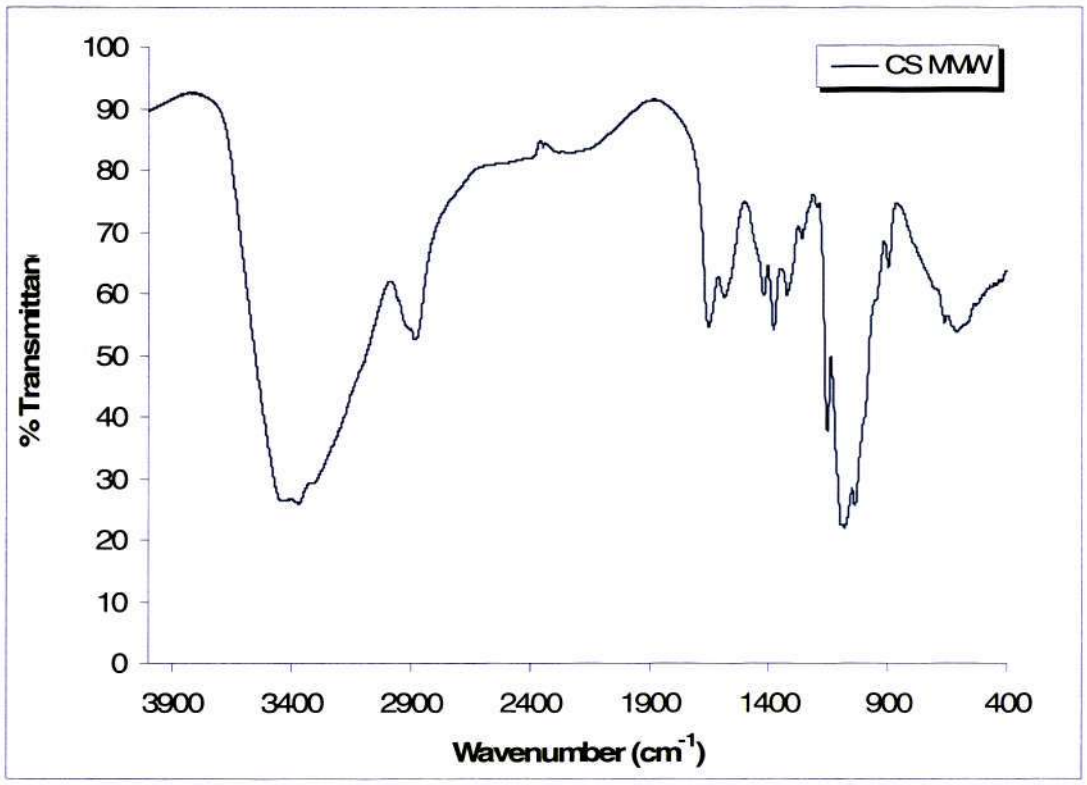
In IR spectrum, chitosan-*g*-PMA, has the characteristic peak at 3450 (3437) ($\nu_{N\text{-}H}$ and $\nu_{O\text{-}H}$) and 1655 (1638) cm^{-1} ($\nu_{\text{-CONH-}}$) confirmed the existence of chitosan moiety. The %DD is generally calculated by the following formula.

$DD = 100 \times (1 - (A_{1655}/A_{3450}) / 1.33$

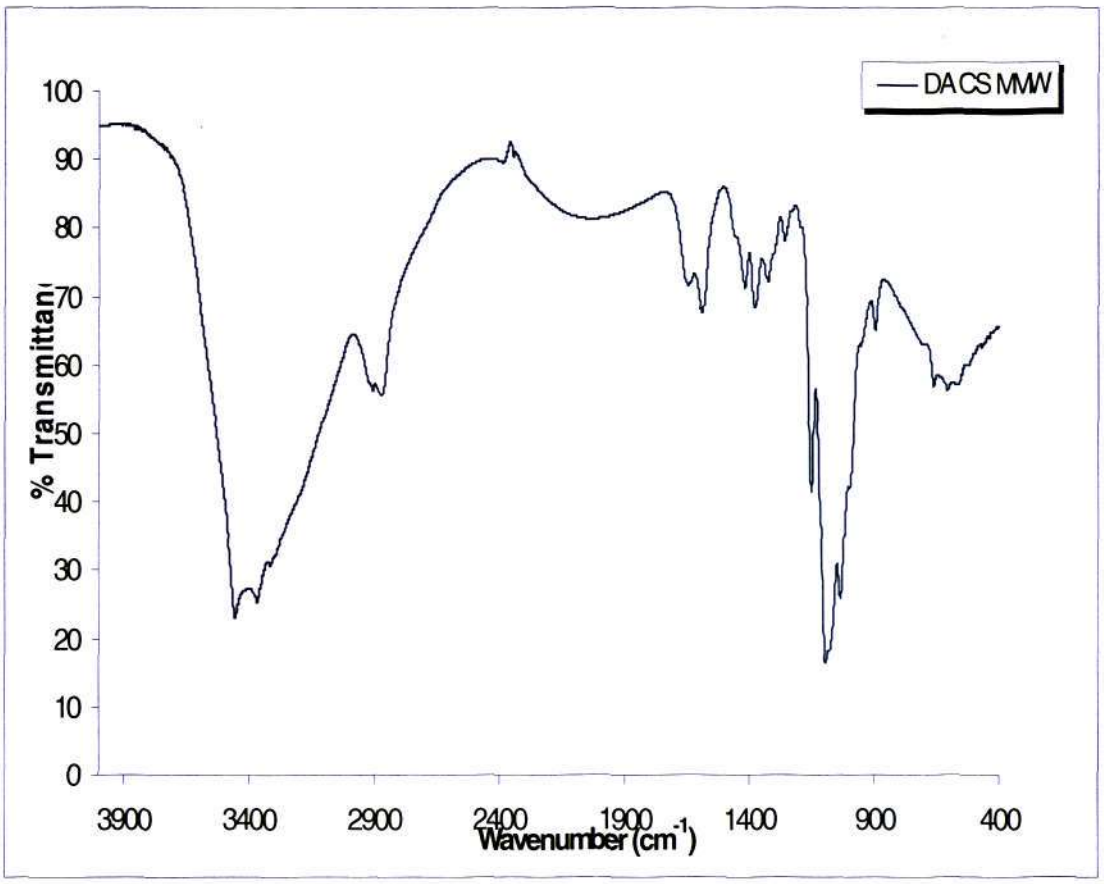
High molecular weight chitosan



Medium molecular weight chitosan



Medium molecular weight deacetylated chitosan



Low molecular weight chitosan