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Efficacy of Chitosan Against Selected Human Pathogens Under Different Environments

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The aim of the study is to evaluate the antimicrobial activity of chitosan in hydrochloric and lactic acids against nine different microorganisms: Methicillin Resistant *Staphylococcus aureus* (MRSA), Methicillin Sensitive *Staphylococcus aureus* (MSSA), *Salmonella typhi, S. paratyphi A, S. typhimurium, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Proteus vulgaris*. Using well diffusion method performed antimicrobial screening. Studies with chitosan- HCl, chitosan- lactic acid, chitosan-lemon and chitosan-garlic extracts indicated that the growth of tested organisms was markedly inhibited. Chitosan lactic acid enhanced the antimicrobial activity than chitosan-HCl, similarly chitosan-lemon and chitosan- garlic extracts exhibited higher inhibitory effect than the chitosan –HCl and lactic acid. Efficacy of chitosan under different environment conditions shows variable inhibitory activity against each bacterium tested. The present study finding confirms the antimicrobial action of chitosan against the drug resistant bacterial strains and typhoid bacteria.

Key words: Chitosan- HCl, Chitosan- lactic acid, synergistic effect- lemon and garlic extracts, antimicrobial activity.

Infectious diseases are the leading cause of death worldwide. The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant pathogens (Bandow *et al.,* 2003). Bacterial and fungal pathogens have evolved numerous defense mechanisms against antimicrobial agents, and resistance to old and newly produced drugs is on the rise. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of organic compounds like chitosan for their potential antimicrobial activity. Chitosan is a fiber like substance derived from chitin, a homopolymer of β- (1-4) linked N-acetyl- D-glucosamine. Chitin is the second most abundant organic compound in nature after cellulose (Tulin Oktem, 2003). Chitosan is currently obtained by the deacetylation of chitin that has been extracted from an abundant source of shrimp or crab shells (Choi *et al.,* 2004). Chitosan is a biopolymer of high molecular weight. The molecular weight and composition of chitosan varies with the raw material sources and the method of preparation (No *et al.,* 2002).

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Chitosan has advantage over chitin because of its high solubility in acidic solutions and its polycationic nature. In the last two decades application of chitosan have been developed in many industries; chitosan has emerged as a new biomaterial for food, pharmaceutical, textile and other industries as well as for waste water treatment. Chitosan oligomers can inhibit the growth of bacteria and fungi and exert antitumour activity (Choi *et al.,* 2004). Chitosan has been shown to have antibacterial activities on the growth of a wide variety of bacteria and their solution has been sold commercially for use as an antibacterial agent (Fujimoto *et al.,* 2006). Chitosan has several drawbacks to be utilized in biological applications, including poor solubility under physiological conditions (Kim *et al.,* 2000).

It is widely known that the antimicrobial property of cotton treated with chitosan is attributed to chitosan's amino group, which converts to ammonium salt in dilute solution; this salt then contacts with the negatively charged protoplasm of the microorganism and destroys the cell wall (Chung *et al.,* 1998). Chitosan is active against the wide range of target organisms. Its activity varies considerably with the type of chitosan, the target organism and the environment in which it is applied. Yeast and molds are the most sensitive group, followed by gram-positive bacteria and finally gram-negative bacteria (Rhoades and Rastall, 2003). Chitosan is expected to be one of the safest and most effective antimicrobial agents for hospital applications where many antibiotics are used (Tulin Oktem, 2003).

The emergence of methicillin resistant *Staphylococcus aureus* (MRSA) was reported just after the launch of methicillin (Quershi *et al.,* 2004). Many of these isolates are becoming multidrug resistant and are susceptible only to glycopeptide antibiotics such as vancomycin (Mehta *et al.,* 1998). Risk factors for nosocomial infections include the prior use of antimicrobial agents, a prolonged hospital stay, a serious underlying illness and immunosuppression. From a clinical point of view, *Klebsiella pneumoniae* is emerging as an important cause of neonatal nosocomial infection (Gupta *et al.,* 1993). *Escherichia coli* causes septicemias and can infect the gall bladder, meninges, surgical wounds, skin lesions and the lungs especially in debilitate and immunodeficient patients (Black, 1996). Infection caused by *Salmonella typhimurium* is a serious public health problem in developing countries and represents a constant concern for the food industry (Mastroeni, 2002). *Pseudomonas aeruginosa* is noted for its metabolic versatility and its exceptional ability to adapt to and colonize wide variety of antimicrobial agents. The bacillus almost never causes infection in healthy individuals and often infects the immunocompromised. Infections by *P.aeruginosa* are often difficult to treat because of its virulence and relatively limited choice for effective antimicrobial agents (Lidia *et al.,* 2004). *Proteus vulgaris* causes wound infections and urinary tract infections in the elderly and young males often following catheterization or cystoscopy, and it is a secondary invader of ulcers, pressure sores and so on (Cheesbrough, 2000).

The survey of literature reveals that chitosan has antimicrobial activity and to study the antimicrobial efficacy of chitosan in 2 % HCl and 1% lactic acid and also the synergistic effect of chitosan-lemon and garlic extracts on the test microorganisms, the present study was undertaken in the Post Graduate and Research Department of Microbiology, Dr.N.G.P Arts and Science College, Coimbatore, Tamil Nadu, India during the period from December 2005 to April 2006. The above experiments were performed in duplicates.

MATERIAL AND METHODS

Chitosan

Chitosan for this study was obtained from India Sea Foods, Cochin as a gift sample. It has a moisture content of 7.48 %, viscosity 54s and degree of deacetylation 84.93 % (According to the manufacturers).

Microorganisms

The microorganisms used in the study were obtained from Microbiology divisions of Kovai Medical Centre and Hospital, a 500-bed mutispeciality hospital in Coimbatore, India. Methicillin Resistant *Staphylococcus aureus* (MRSA), Methicillin Sensitive *Staphylococcus aureus* (MSSA), *Salmonella typhi, Salmonella*

paratyphi A, Salmonella typhimurium, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and *Proteus vulgaris*. Microorganisms were maintained at 4°C on nutrient agar slants.

Antibacterial activity testing using agar well method (cup plate method)

Efficacy of chitosan in 2% HCl

Chitosan oligomer can be prepared by digesting chitosan with HCl (Rhoades and Roller, 2000). 0.1 g of chitosan was dissolved in 100 ml of 2 % HCl. 10 ml from this stock solution yielded a concentration of 100 mg chitosan. 10-100 ml from this stock solution (100-1000 mg of chitosan) was used for this experiment. The selected strains of bacteria were inoculated in to 10 ml of sterile nutrient broth and incubated at 37^0C for 16-24 hrs. Using a sterile cotton swab, the nutrient broth cultures (equivalent to 0.5 McFarland's standard) were swabbed on the surface of sterile Mueller Hinton Agar (MHA) plates**.** Agar wells were prepared with the help of sterilized cork borer with 10mm diameter (Srinivasan, 2001). Using a micropipette, 10µl to 100µl were added into respective wells in the plate. The plates were then incubated in an upright position at 37°C for 24 hrs. The diameter of inhibition zones was measured in mm and the results were recorded. The inhibition zones with diameter less than 12 mm were considered as having no antibacterial activity.

Efficacy of Chitosan in 1% lactic acid against MRSA, MSSA, *S. typhi and S. typhimurium*

MRSA, MSSA, *S. typhi* and *S. typhimurium* were used to study the efficacy of chitosan in 1% lactic acid. Chitosan can be dissolved in lactic acid or sodium lactate solution (Devlieghere *et al.,* 2004). In this experiment, 1% lactic acid was used to dissolve the chitosan. 0.1g of chitosan in 10ml of 1% lactic acid solution was prepared and kept as stock solution. 50 – 100 ml from this stock solution yielded 500-1000mg of chitosan. All the four bacterial cultures (equivalent to 0.5 McFarland's turbidity) were swabbed onto sterile MHA plates. Agar wells were prepared with the help of sterilized cork borer with 10mm diameter (Srinivasan, 2001). Using a micropipette, 50µl to 100µl were added into respective wells in the plate. The plates were then incubated in an upright position at 37^0C for 24 hrs. The diameter of inhibition zones was measured in mm and the results were recorded. The inhibition zones with diameter less than 12 mm were considered as having no antibacterial activity.

Synergistic effect of Chitosan-garlic and Chitosan – lemon extracts

Garlic and lemon possessed antibacterial activity (Jiben Roy *et al.,* 2006; Seenivasan *et al.,* 2006). This work was specifically done for MRSA, MSSA, *S. typhi,* and *S. typhimurium.* Garlic extracts was made in a different way due to the difficulty to filter the crushed material. One hundred grams of the desiccated and cleaned garlic were taken and surface sterilized using ethanol. The ethanol was allowed to evaporate in a sterile laminar flow chamber and the garlic was homogenized aseptically using a sterile mortor and pestle. The extract was aseptically squeezed out using sterile cheesecloth and collected in a sterile beaker. Similarly, lemon extract was also collected in a sterile beaker. All these procedures were carried out under aseptic conditions. 10ml from each of these extract was added to 10ml of chitosan in 2 % HCl preparation and 10-100ml from each of this stock solution yielded 100-1000mg of chitosan-garlic and chitosan – lemon extracts respectively. All the four bacterial cultures equivalent to 0.5 McFarland's standard was swabbed onto sterile MHA plates. Agar wells were prepared with the help of sterilized cork borer with 10mm diameter (Srinivasan, 2001). Accurately measured volumes (10-100ml) of chitosan-garlic and chitosan-lemon extracts were added to the respective wells. The plates were then incubated in an upright position at 37°C for 24 hrs. The diameter of inhibition zones was measured in mm and the results were recorded. The inhibition zones with diameter less than 12 mm were considered as having no antibacterial activity.

RESULTS AND DISCUSSION

Efficacy of chitosan in 2% HCl

The efficacy of chitosan- HCl on the test bacteria is shown in Table 1. Chitosan in 2% HCl showed excellent antibacterial activity to both MRSA and MSSA. Gram-positive bacteria were found to be more sensitive. This shows that the concentration did not vary in inhibiting the growth

Table 1. Efficacy of Chitosan - HCl. (Zone diameter-mm)

Organism	Chitosan concentration (µg)									
	100	200	300	400	500	600	700	800	900	1000
MRSA	13	14	15	18	19	22	24	25	26	27
MSSA	14	15	18	20	21	24	25	27	28	30
S. typhi	-	-	-	I	11	12	13	14	15	17
S. typhimurium	-	-	-	-	-	-	12	15	16	17
S. paratyphi A	-	-	-	13	14	15	16	17	20	21
E. coli	-	-	-	-	-	-	-	I	13	14
P. aeruginossa	-	-	-	-	-	-	15	16	17	18
P. vulgaris	-	-	-	-	-	-	-	-	I	I
K. pneumoniae	-	-	-	-	-	-	-	-	-	-

I: No detectable zone of inhibition '-' : No zone of inhibition

Table 2. Efficacy of chitosan - lactic acid on MRSA, MSSA, *S. typ*hi and *S. typhimurium* (Zone diameter-mm)

Organism	Chitosan concentration (µg)					
	500	600	700	800	900	1000
MRSA	22	25	25	27	28	29
MSSA	20	22	22	25	27	27
S. typhi	24	24	26	26	27	28
S. typhimurium	20	21	23	23	26	29

Table 3. Synergetic effect of chitosan -garlic extracts specifically on MRSA, MSSA, *S. typhi* and *S. typhimurium.* (Zone diameter-mm)

Organism	Chitosan concentration (µg)									
	100	200	300	400	500	600	700	800	900	1000
MRSA	13	15	16	24	24	26	26	28	28	30
MSSA	14	15	15	25	25	28	28	31	31	32
S. typhi	12	13	13	19	21	22	22	24	26	29
S. typhimurium	0	13	15	18	18	19	19	21	21	22

Table 4. Synergetic effect of chitosan - lemon extracts specifically on MRSA, MSSA, *S. typhi* and *S. typhimurium.* (Zone diameter-mm)

Organism	Chitosan concentration (µg)									
	100	200	300	400	500	600	700	800	900	1000
MRSA	15	17	19	20	23	23	25	27	27	27
MSSA	17	21	23	24	26	26	27	28	29	30
S. typhi	13	15	16	17	18	20	23	24	26	26
S. typhimurium	15	15	16	18	19	20	22	22	24	24

of methicillin resistant and sensitive strains of *S. aureus*. Among the *Salmonella species* tested, *S. paratyphi A* was inhibited at lower concentration than *S. typhi* and *S. typhimurium*. When tested against the *Enterobacteriaceae* members included in the study, chitosan-HCl did not show any antibacterial activity against *P. vulgaris* and *K.pneumoniae,* but exhibited inhibitory action against *P. aeruginosa* and *E. coli*. Chitosan is found to disrupt the barrier properties of the outer membrane of the gram-negative bacteria and thus make them sensitive to chitosan. The presence of capsule in *Klebsiella* species can be one of the reasons for its resistance towards chitosan. Also there may be any plasmid responsible for the resistance nature.

Zhang *et al.* (2003) reported that in acidic solvents, the NH_2 group in chitosan becomes a quaternary amino group and allows the chitosan to inhibit the growth of many bacteria, including gram negative and gram positive ones. There are two proposed mechanisms of antibacterial activity by chitosan. In one mechanism, the polycationic nature of chitosan interferes with the bacterial metabolism by stacking at the cell surface. The other mechanism is the binding of chitosan with DNA to inhibit mRNA synthesis. In later mechanism chitosan must be hydrolyzed to a molecular weight less than 5000, which can easily penetrate the cells. It has also been shown that the antibacterial activity of chitosan solution increased with decreasing pH value (Fujimoto *et al.,* 2006). According to Tulin Oktem, (2003) the antimicrobial activity of chitosan is due to the antimicrobial action of the amino group at the C2 position of the glucosamine residue. The ability of chitosan to immobilize microorganisms derives from its polycationic character. Its protonised amino group blocks the protein sequences of microorganisms and thus inhibiting further proliferation. Chitosan binds to the negatively charged bacterial surface disrupting the cell membrane and altering its permeability. This allows materials to leak out of the bacterial cells resulting in cell death. Yadav and Bhise, (2004) in their study found that glucosamine does not show antimicrobial activity. The polycationic nature of chitosan might be responsible for interaction with the electronegative bacterial cell surface.

Efficacy of chitosan in 1% lactic acid against MRSA, MSSA, *S. typhi* and *S. typhimurium*

When the antimicrobial activity of chitosan in 1% lactic acid was studied against MRSA, MSSA, *S. typhi* and *S. typhimirium*, it was found that all the test organisms were inhibited at low concentration (500µg) showing inhibition zones of 22, 20, 24 and 20 mm respectively (Table 2). Comparing chitosan in 2 % HCl, lactic acid was found to enhance the antibacterial activity against MRSA, MSSA, *S. typhi and S. typhimurium*. Similarly, when comparing the efficacy of chitosan-HCl and chitosan-lactic acid, it was observed that at both conditions MRSA and MSSA were inhibited at 500µg, but against *S. typhi* and *S. typhimurium*, chitosan –HCl required higher concentration (600µg and 700µg) for inhibition than chitosan-lactic acid. Antibacterial activity was not observed at 500µg with the chitosan-HCl treated against *S. typhi* and *S. typhimurium*, but at the same concentration, chitosan lactic acid exhibited stronger activity. Devlieghere et al. (2004) reported that food items coated with chitosan by dipping the products in chitosan-lactic acid/ sodium lactate solution exhibited antimicrobial activity.

Synergistic effect of chitosan- garlic and chitosan- lemon extracts against MRSA, MSSA, *S. typhi* and *S. typhimurium*

From the results depicted in Table 3, it has become clear that the use of either garlic or lemon along with chitosan greatly increased its antimicrobial activity. The growth of MRSA and MSSA were inhibited at 200mg. But whereas the growth of *S. typhimurium* and *S. typhi* were inhibited at 300 mg and 400 mg respectively in chitosan-garlic combination, the requirement is low when compared to chitosan - HCl and lactic acid. Garlic oil components did not affect the physical and mechanical properties of chitosan films, as it does not have any interaction with the functional group of chitosan (Pranoto *et al.,* 2005). In garlic, allicin (diallyl thiosulfinate) is a major compound responsible for its antimicrobial activity. Concentrated water extract of garlic showed antimicrobial response to *S.aureus* and *E. coli*

(Jiben Roy *et al.,* 2006). So this can be further investigated that whether chitosan has been enhanced by this alison or due to any other characters of garlic. The present study also clearly suggested that lemon can also enhance the efficacy of chitosan against bacteria (Table 4). The growths of MRSA, MSSA and *S. typhimurium* were inhibited at 100mg but whereas for *S. typhi* inhibition occurred at 200mg. This may be because the citric acid in lemon may have some effect on the increasing the antibacterial activity of chitosan. This can be further studied using purified citric acid along with chitosan. Oil of lemon and lime exhibited significant inhibitory effects against bacteria. An important character of these essential oils and their components is their hydrophobidity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disrupting the cell structure and rendering them more permeable. Excessive leakage from bacterial cells or the exit of critical molecules and ions will lead to death. Lime was equally effective against gram positive and gram-negative bacteria (Seenivasan *et al.,* 2006). Thus it can be suggested that chitosan can be used for treatment against specific pathogens by either chitosan-HCL or lactic acid alone or better in combination with garlic or lemon.

Recently, multiple drug resistance has developed due to indiscriminate use of commercial antimicrobial drugs that are commonly used in the treatment of infectious diseases, making it a global growing problem. There is an urgent need to develop new antimicrobial drugs for the treatment of infectious diseases from chitosan, which may be less toxic to humans and possibly with a novel mechanism of action.

Because chitosan was highly soluble in acidic solutions, hydrochloric and lactic acids were used to study the antimicrobial efficacy of chitosan among the resistant microorganisms. They showed varying degrees of inhibitory effect but when compared to chiotsan –garlic and chitosan-lemon extracts, the latter showed an enhanced antimicrobial activity against the antibiotic resistant bacteria and typhoid bacteria. This study suggests that chitosan can be dissolved in natural extracts having acidic property and also exhibited excellent inhibitory action against the test microorganisms. This would help in the elimination and control of resistant bacteria and typhoid bacteria not only in the hospitals but also in the community settings.

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