*In vitro*Anti-microbial effect of Chitin MonomerN-Acetyl -D-glucosamine-A promising monomer for future anti-microbial therapy

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ABSTRACT:

As the incidence of microbial infections and antimicrobial resistance increases day by day there is a need for new anti-microbial agents, which has become global concern now a days. Polysaccharides have much importance because of their therapeutical potential against wide range of infectious diseases. The aim of present study is to determine the antimicrobial potential of N-Acetyl -D-Glucosamine by using *in vitro*methods.N-Acetyl D-glucosamine was subjected for screening of antimicrobial activity against various strains of bacterial species, Methicillin Resistant Staphylococcus aureus (MRSA), Bacillus subtilis, Salmonella typhimurium and Escherichia coli, using standard protocol of disc diffusion method (DDM) and well diffusion method. The antibacterial activities were determined by the zone of inhibition and MIC (Minimum inhibitory concentrations) values. The significant antibacterial activity of N-Acetyl-D-glucosamine was determined and compared with the standard antimicrobial agent Gentamicin (0.01mg/ml). The results obtained in the present study suggest that NAG can be used for treatment of various diseases caused by the test organisms used in this study.

KEY WORDS: NAG (N-Acetyl-D-Glucosamine), anti-microbial resistance, polysaccharides, MIC, Zone of inhibition.

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INTRODUCTION:

Pathogenic microorganisms are responsible for various infectious diseases and are the most common cause of deaths occur world-wide[1]. In order to treat these infectious diseases and to overcome the anti-microbial resistance of presently available antibiotics, discovery of newer antimicrobials with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases has become a global concern[2]. The clinical efficacy of many existing antibiotics becomes question mark because of the emergence of multidrug-resistant pathogens[3]. Therefore, researchers focusing more on natural polysaccharides for the development of new leads as better drugs against various microbial diseases[4]. Chitin is one of the most widely available polysaccharides on earth, arenewable biopolymer[5]. N-acetyl-D-glucosamine (NAG) is a monomer of chitin allied by beta-glyosidic bonding[6,7]and also a constituent of heterogenous oligosaccharides like murein[8]and hyaluronic acid (HA) [9,10].NAG is derived as an end product from the hydrolysis

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of chitin, which has now become an attractive alternate as food supplements and cosmetics[11–13].As it has sweet taste can also be used as substitute for similar applications[14].The aim of the present study is to determine the anti-microbial effect of N-Acetyl D-glucosamine,which is a monomer of chitin against various gram positive and gram negative microbial strains using *in vitro* methods.

MATERIALS AND METHODS:

Materials

Test drugs and chemicals

N-Acetyl-D-Glucosamine(≥99%), Gentamicin, werepurchased from Sigma Aldrich, Bangalore. Acetic acid(1%w/v),nutrient broth, casein hydrolysate, starch, agar was purchased from Supreme chemicals, Hyderabad.All the chemicals used in the present study were of analytical grade.

Test strains

The test microbial strains were obtained as gift samples from the Indian Institute of Chemical Technology (IICT), Tarnaka, Hyderabad.They were gram negative and gram positive strains of E. coli (MTCC452), MRSA (MTCC3103), P. aeruginosa(ATCC 27853)and Salmonella typhimurium (MTCC 1251). The test microbial strains were maintained at a temperature 4 ^{0}C in Brain Heart Infusion broth and purity, morphology and biochemical characters of test cultures were tested for every 15 days.

Preparation of bacterial culture

All the test bacterialcultures viz.,Escherichia coli, Methicillin resistant Staphylococcusaureus, Pseudomonas aeruginosa and Salmonella typhimurium were inoculatedinto Brain Heart Infusion broth and incubated at a temperature of 37^0 Covernight. Then the inoculum was centrifuged for 10 minutes at 8000 rpm. The supernatant was discarded, pellets were collected and mixed in sterile normal saline and centrifuged at 8000 rpm for 10 minutes. Then the cells were washed with normal saline twice and the concentration of the cells was adjusted10^9 cells /ml, which was used as inoculum to determine the antimicrobial effect of N-Acetyl -D-glucosamine (NAG).

Methods:

Mueller-Hinton

Nutrient broth: 300 ml, casein hydrolysate: 17.5 ml, starch: 1.5g, agar: 10 g and distilled water:1L were taken, the media was prepared according to the manufacturer instructions and the pH was adjusted to 7.6. Then the medium sterilizedat 121°C,15lbs for 15 minutes by using autoclave. Now the medium is used for antibiotic sensitivity tests[15].

a)Disk diffusion assay

Anti-microbial sensitivity of NAG was determined by using disc diffusion assay. In order to perform the test 0.1ml (approximately 10^{9}cells / ml) of test microbial cultures were taken and inoculated on Muller-Hinton growth media and spread over the whole surface of medium taken in Petri plates by using sterile swab.N-Acetyl D-glucosamine solution 1%w/v was prepared by using 1%w/v acetic acid. Then sterile Whatman filter paper discs of 6mm thicknesswere impregnated in various concentrations0.125mg/ml, 1.125mg/ml, 2.125mg/mlof test and 0.01mg/ml of standard solutions were placed on Muller-Hinton growth mediaand pressed gently. The plates were incubated at 35 $\pm 1^0$ C for 48 hours.After the incubation period the plates were removed from the incubator and zone of inhibition around each filter discs were measured in centimeters and noted. The experiment was triplicated[16, 17]

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b) Well diffusion method

Muller Hinton Agar (MHA) was taken and poured in Petri plates at a depth of 3-4 mm. The test culture was inoculated on the surface of Petri plates by using sterile cotton swab until to obtain an even inoculum. The plates were allowed for 3-5 min to get dry. After drying wells were made on the surface of Petri plate by cutting 5mm diameter with the help of borers.30 micro liter of different concentrations (0.125mg/ml, 1.125mg/ml, 2.125mg/ml) of test and standard (0.01mg/ml) were added in each well. Then the plates were allowed for incubation at 37^0c for 24hrs and zone of inhibitions were measured using antibiotic zone reader[18].

c) Minimum inhibitory concentration (MIC)

MIC is defined as the lowest concentration of the drug that inhibits the growth of bacteria. MIC was determined by using broth micro dilution method. Serial dilutions of NAG were made in Mueller-Hinton broth and One millilitre of standard inoculum of the test microorganism was added to both test tubes containing standard NAG and test tube containing only growth medium (without antimicrobial agent), which serves as a growth control. An uninoculated medium was taken in another test tube, which serve as a negative growth control. Then the plates were incubated at 37^0c for 24hrs. After incubation, all the tubes were observed for presence of turbidity or pellet formation indicating growth of the microorganisms. The lowest concentration of the NAG that inhibits growth of the test microorganism was designated the minimum inhibitory concentration[19, 20]. A loopful of inoculum was taken from each of the tubes of broth and was sub cultured on solid agar plates, which were divided into six sections. The lowest concentration of NAG that shows<0.1% of survival from original inoculum was considered as minimum bactericidal concentration (MBC).

RESULTS:

The antimicrobial potential of NAG was determined against various gram positive and gram-negative bacterial strains by using the parameters Zone of inhibition, minimum inhibitory concentration (MIC)and minimumbactericidal concentration (MBC).Zone of inhibitionof different concentrations of NAG was assessed by disc diffusion and well diffusionmethods and compared with the standard Gentamicin**.**The zone of inhibition, MIC and MBC are given in following tables (Table 1,2,3,4). All the values were expressed as mean±SD. Statistical analysis was done by one-way ANOVA using graph pod prism6 and $p<0.05$ is considered as statistically significant.

Table1: Anti-bacterial activity of NAG by Disc diffusion assay

Bacterial pathogens	Zone of inhibition(Disc diffusion assay)			
	Standard (Gentamicin 0.01mg/ml)	TEST1 (0.125mg/ml NAG)	TEST 2 (1.125mg/ml NAG)	TEST 3 (2.125mg/ml NAG)
Escherichia coli	13.66±0.495	7±0.5	9.83±0.763	12.00±0.45
Salmonella typhimurium	17.5±0.500	7.16±0.351	9.93±0.513	15±0.500
Methicillin resistant Staphylococcusaureus	22.66±0.288	13.66±0.288	18.5±0.5	20.83±0

Bacillus subtilis	25.33±0.577	11.1±0.360	19.5±0.5	23.83±0.288

Values are expressed asMean±SD; Statistical analysis was done by one-way ANOVA,

p <0.001is considered as significant.

Table2: Anti-bacterial activity of NAG by Well diffusion method

Bacterial pathogens	Zone of inhibition (Well diffusion method)			
	Standard (Gentamicin 0.01mg/ml)	TEST 1 (0.125mg/ml NAG)	TEST 2 (1.125mg/ml NAG)	TEST 3 (2.125mg//ml NAG)
Escherichia coli	16.86±0.404	9.06±0.513	13.6±0.529	14.33±0.288
Salmonella typhimurium	18.66±0.577	8.5±0.5	11.66±0.288	16.26±0.461
Methicillin resistant Staphylococcusaureus	24.66±0.288	9.66±0.577	17.5±0.5	20.26±0.404
Bacillus subtilis	26.66±0.288	9±0.5	18.43±0.513	24.33±0.577

Values are expressed asMean±SD; Statistical analysis was performed by one- way ANOVA,

p <0.001is considered to show significant difference.

Minimum inhibitory concentration and Minimum bactericidal concentration of NAG against various pathogens are as follows:

Table3: Minimum inhibitory concentration of NAG against gram positive and gram negative bacteria

Drugs	Gram positive		Gram negative	
	Methicillin resistant staphylococcus aureus	Bacillus subtilis	Escherichia coli	Salmonella typhimurium
NAG(µg/ml)	27.53±0.503	21±0.5	58.33±0.577	60.66±0.577
Gentamicin (µg/ml)	6.76±0.251	5.66±0.577	9.16±0.288	12±0.5

Values are indicated asMean±SD; Statistical analysis was done using one- way ANOVA,

p <0.001is considered significant.

Figure1:Minimum inhibitory concentration of NAG against various microorganisms

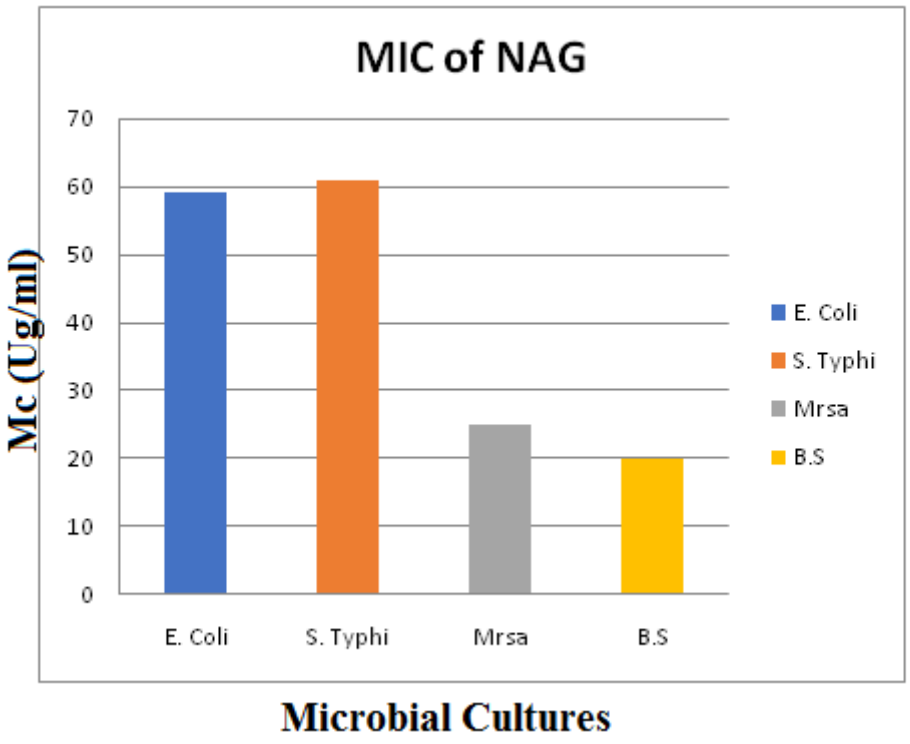

Table4: Minimum bactericidal concentration of NAG against gram positive and gram negative bacteria

Drugs	Gram positive		Gram negative	
	Methicillin resistant Staphylococcus	Bacillus subtilis	Escherichia coli	Salmonella typhimurium
NAG(µg/ml)	27.66±0.577	22±0.5	56.33±0.288	60±0.5
Gentamicin(µg/ml)	8.33±0.577	11.1±0.360	7.5±0.5	6.16±0.288

Values are expressed asMean±SD, Statistical analysis was performed by one- way ANOVA,

p <0.001is considered significant.

Figure2: Minimum bactericidal concentration of NAG against gram positive and gram negative bacteria

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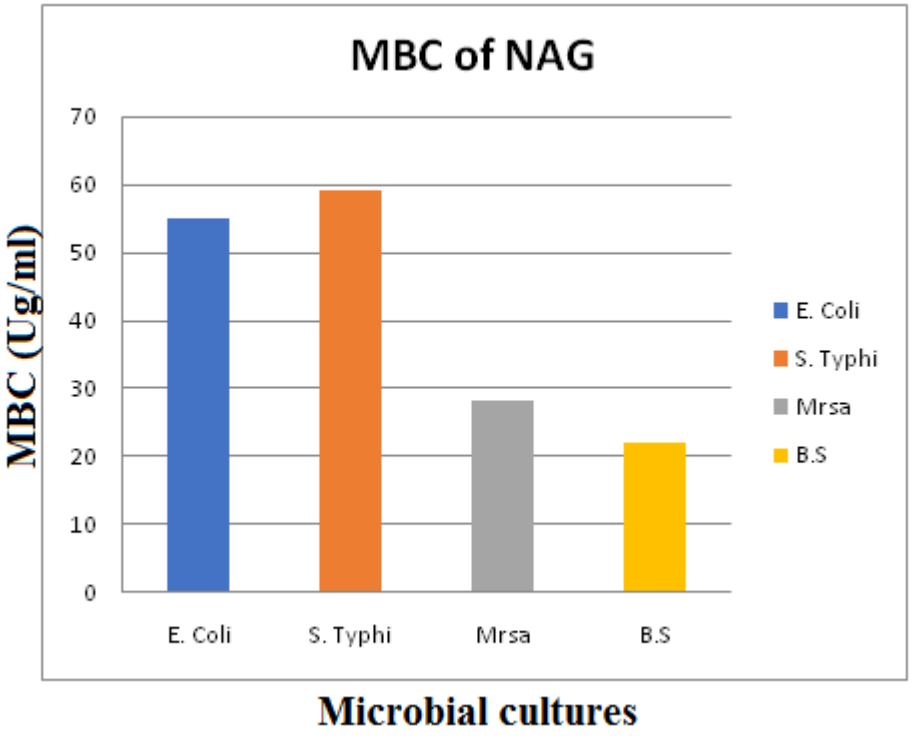

DISCUSSION:

N-Acetyl D-glucosamine is a monomer of chitin, which is a second most abundantly available polysaccharide. NAG is widely used for various diseases treatment. As the emergence of anti-microbial resistance enhances day to day there is a need for new antimicrobial agent. So in the present study N-Acetyl D-glucosamine was tested for anti-microbial potential. Based on the results obtained by the above methods it was proved that N. Acetyl D-Glucosamine has antimicrobial potential against various gram positive and gram-negative bacterial strains. It was also found that NAG has shown dose dependent rise in antibacterial potential.

CONCLUSION:

The present suggested that N-Acetyl D-Glucosamine, a nutraceutical polysaccharide has antibacterial activity against variety of gram positive and gram-negative bacteria. Further it requires more investigation to prove its activity by conducting *in vivo* studies to develop NAG as a potent anti-microbial agent alone or in combination with others.

CONFLICT OF INTEREST:

There is no conflict of interest between the authors.

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