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RESEARCH ARTICLE

CHITOSAN- CIPROFLOXACIN SCHIFF BASES: SYNTHESIS, CHARACTERIZATION AND *IN VITRO*ANTIMICROBIAL ACTIVITY EVALUATION.

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Abstract

In the present work, chitosan-ciprofloxacin Schiff bases (CHS-CFX-SB) containing azomethine group (–CH=N–) have been synthesized via condensing amino groups (NH_2) of chitosan and carbonyl group (CO) of ciprofloxacin by using different molar ratios (0.05, 0.1, 0.25, 0.5 and 1) to enhance the antimicrobial activity of ciprofloxacin to offer a great potential for medical applications. The obtained CHS-CFX-SBs were structurally characterized using FT-IR and ^{1}H NMR spectral analysis. Thus, the antimicrobial activities of these Schiff bases were assessed against six bacterial species, three gram-positive species (Methicillin resistant *Staphylococcus aureus* (MRSA), *Streptococcus pyogenus* and *Streptococcus pneumoniae*) and three gram-negative species (*Escherichia coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*) and one of fungus species; *Candida albican* by determining the microbial growth by measuring the inhibition zones in mm. The findings of this assessment showed that CHS-CFX-SB prepared by using equal molar ratio is having higher antibacterial activity against gram-positive bacteria than gram-negative ones. And thus, these chitosan derivatives have strong antibacterial activity against *Staphylococcus aureus*. Such Schiff bases had higher inhibitory efficiency against gram-positive bacteria than that of ciprofloxacin itself.

Introduction:-

Microbial infection the main cause of death of human worldwide and thus serious limitations and disadvantages of low molecular weight organic antimicrobial agents including antibiotics, such as cytotoxicity and short-term acting, in addition to emergence of the antibiotic resistant strains make development of new desirable antimicrobial agents is a vital issue in the present time, thence theantimicrobial polymer materials can be considered one of the most promising way for minimizing the environmental problems by reducing the residual toxicity of the conventional antimicrobial agents, increasing their efficiency and selectivity, and prolonging their lifetime[1].

Moreover, the use of such materials offers promise for rendering biomaterials resistance towards microorganisms that is a definite need in several areas, particularly in medical devices, drugs, health care and hygienic applications, water purification systems, hospital and dental surgery equipment, textiles, food preservation and packaging.

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Ciprofloxacin hydrochloride belongs to the second generation broad-spectrum fluoroquinolone antibiotic used to treat various bacterial infections. Fluoroquinolones target bacterial DNA gyrase, a type II topoisomerase in gram negative bacteria and topoisomerase IV in gram positive bacteria, an enzyme active in the process of DNA supercoiling, which allows the DNA strands within the bacterial cell to be compacted in an orderly fashion [2].

Inhibition of DNA gyrase permits the DNA strands to become entangled, thus preventing DNA replication, transcription, recombination, and repair, and resulting in bacterial death. It is highly active against various gram-positive and gram-negative bacteria [3].

Ciprofloxacin is used for the treatment of urinary tract infections, prostatitis, continuous ambulatory peritoneal dialysis infections, skin and skin infections, bone and joint infections, some diabetic foot infection, typhoid fever, etc. It shows anti-tumor activity against P388 leukemia [4]. However, relatively short serum half-life and certain adverse effects of ciprofloxacin, such as CNS effects, phototoxicity, tendonitis, hypoglycemia, and serious cardiac dysrhythmias are considered limiting factors for therapeutic potential of CFX 3-4 [5-8].

Recently, due to the increased resistance of some infections to ciprofloxacin, several trials were performed to modify the functional groups in this antibiotic to enhance its antimicrobial activity [9-18].

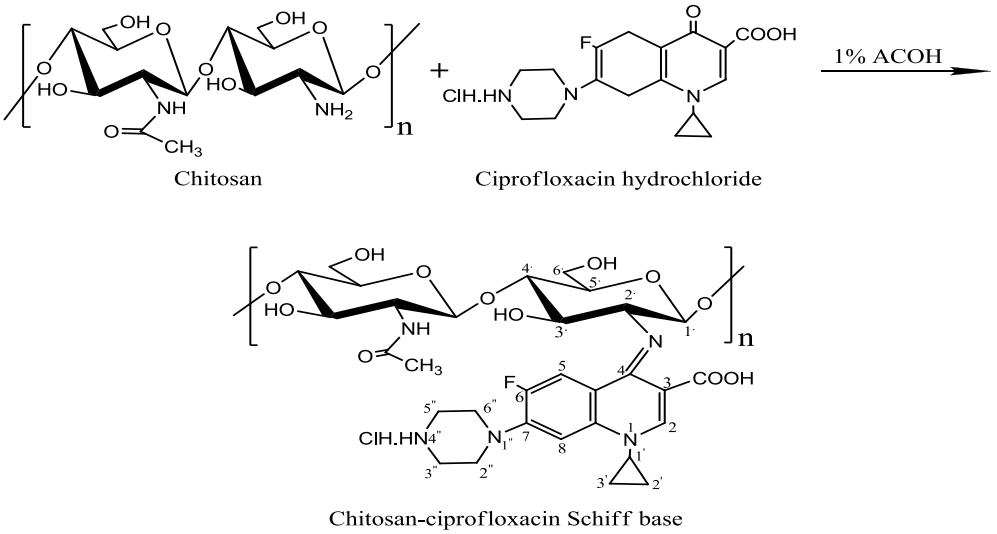
On the other hand, extremely potent activity and rapid bactericidal effects open the door for chitosan to be employed as contact disinfectants and sterile materials in many biomedical applications. Chitosan molecule bears free amino groups at C-2 position which can allow chemical substitution reactions to get various derivatives with a large spectrum of applications. Among these derivatives, chitosan Schiff-base which can be obtained by the reaction of these free amino groups of chitosan with active carbonyl compounds such as aldehyde or ketone with the created imine group (–RC=N–) on the Schiff-base product, these Schiff bases offer several biomedical applications [18-26] . In the light of excellent antimicrobial activities of ciprofloxacin and chitosan, the present study enlightens the development of polymeric materials with biocide activity via synthesis of chitosan-ciprofloxacin Schiff bases with their structural characterization by using spectroscopic analyses including IR and ^{1}HNMR and evaluation of their invitro antimicrobial activities.

Materials and Methods:-

Chitosan (CHS, Mη=620 kDa, degree of deacetylation = 85%, viscosity=115 cps) is a commercial product of from Acros Organics, New Jersey, USA. Ciprofloxacin hydrochloride (CFX, C17H18FN3O3.HCl.H2O) was purchased from Pharco company for pharmaceutical industry (Alexandria, Egypt). Glacial acetic acid was purchased from Alpha Chemika, Mumbai, India. All other chemicals were of analytical grade and used as received. The water used in all experiments was triple distilled.

Preparation of ciprofloxacin-chitosan Schiff bases:-

One gram of chitosan (5.6 mmole of glucosamine unit) was dissolved in 200 ml of 1% AcOH with stirring until forming a clear solution. Ciprofloxacin hydrochloride was added to chitosan at different molar ratios (0.05, 0.1, 0.25, 0.5 and 1) where, certain weight of CFX was dissolved in distilled water and then,aliquotsfrom this solution were added dropwise to the previously prepared chitosan solution with heatingat 55°C for 24 hours. The resultant precipitate was filtered off and dried at 50 °C (scheme 1).



Scheme 1:- Chitosan-ciprofloxacin Schiff bases preparation.

Spectral analysis:-

FT-IR:-

FT-IR spectroscopic analysis were performed for ciprofloxacin, chitosan and chitosan-derived Schiff bases (Shimadzu, Japan) after their preparation in form of KBr pellets usingShimadzu 8400S FTIR spectrometer. The crushed samples (~ 4 mg) were mixed with 200 mg of potassium bromide and was further ground in an agate mortar with pestle. The mixture was then transferred to a die and pressed into a disc in a vacuum press at 80 KN. The FTIR spectra were collected over the frequency range of 400-4000 cm-1. These spectra were Fourier-deconvoluted for 128interferograms with a resolution enhancement factor of 2 and a bandwidth of 15 cm-1 at room temperature and presented in absorption mode after the baseline correction [27].

1H NMR

^{1}H NMR spectroscopic analysis were carried outat 500.2 MHz using JEOL 500 MH NMR spectrometer (JEOL, Japan). Samples dissolved in DMSO/TFA were analyzed by a dual probe head at 25°C. The spectra were accumulated into 32K data points and processed using exponential multiplication with 2 Hz line broadening into 128K spectra. For the resulting spectra 25000–35000 scans were accumulated. All spectra were accumulated under identical conditions using power gated Waltz decoupling with 25-degree measurement pulse and 1s prepulse delay [28].

In Vitro antimicrobial assays:-

Antimicrobial activity of chitosan, ciprofloxacin and their derived Schiff bases were assessed against six bacterial strains, three gram-positive species (Staphylococcus aureus, Streptococcus pyogenus and Streptococcuspneumonia), three gram-negative bacteria (Escherichia coli, Klebsiella pneumonia and Pseudomonas aeruginosa) and one fungi species Candida albican. These strains are common contaminates of the environment in Egypt and some of which are involved in human and animal diseases. All microbial strains were kindly provided by Ain Shams University Microbiology Center (ASMC).

Bacterial strains were individually cultured for 48h in 100 ml conical flasks containing 30 ml nutrient broth medium. Fungi were grown for 7 days in 100 ml conical containing 30 ml Sabouraud's dextrose broth. One milliliter of cultured microbial suspension was added into tube containing mixture of10 ml of broth culture medium pre-sterilized by autoclaving at 121°C for 15 min and 2 ml of chitosan, ciprofloxacin or Schiff bases solution (0.5 % in an aqueous acetic solution (0.5%). These tubes containing bacterial or fungal strains were incubated in shaking incubator at 37 °C for 24 or 48 h, respectively. The growth of these organism strains in the presence of ciprofloxacin and its Schiff bases was detected by disc diffusion method [29].

Preparation of Agar Plates:-

For antibacterial test:-

10 g peptone, 10 g sodium chloride, 5 g yeast extract and 15 g agar were dissolved properly in 1000 ml distilled water in 1000 ml conical flask with stirring. Then mouth of conical flask was plugged with cotton. Conical flask was put in autoclave for sterilization for 15 lbs for 20 min. After autoclaving, warm 20-25 ml media was poured on Petri dish per plate in front of laminar flow. Leave it until media were solidified in Petri dish. Now agar plate was ready for use.

For antifungal test:-

5 g glucose, 5 g peptone, 2.5 g yeast extract and 4 g agar were dissolved properly in 250 ml distilled water in 250 ml conical flask with stirring. Then mouth of conical flask was plugged with cotton. Conical flask was put in autoclave for sterilization for 15 lbs for 20 min. After autoclaving, warm 20-25 ml media was poured on Petri dish per plate in front of laminar flow. Leave it until media were solidified in Petri dish. Now agar plates were ready for use.

Preparation of Compounds Solution:-

Ciprofloxacin and the five synthesized Schiff bases were dissolved in aqueous solution of 0.5 % aceticacid in test tube with vertex stirring, heated if required (each material was prepared at these concentrations 10, 15, 20 and 25 µg/ml) given the code of each tube. Divided the bottom of prepared plate into four quadrants with the help of marker. Given the same code to each quadrant as given to code to test tube containing solution of compound. Swab the one plate from one bacterial suspension with the help of cotton swab, coded the name of bacteria on each plate. Same procedure was adopted for all plates. With the help of micro pipette ,10, 15, 20 and 25 µg/ml solution of compound was dropped on same code of quadrant as given on the test tube containing solution of compound. Same procedure was adopted for all compounds in laminar flow. All plates were put in incubator for incubation for 24 hrs. After 24 hrs, view the plate. If the specific compound was sensitive for specific bacteria. Then growth was found in whole plate except where solution of compound was dropped. If the specific compound was not sensitive for specific bacteria. Then growth was found in whole plate including where solution of compound was dropped.

Results and Discussions:-

FT-IR spectral analysis:-

FTIR spectra of ciprofloxacin, chitosan and ciprofloxacin-chitosan based Schiff bases are presented in (figure 1). Ciprofloxacin spectrum exhibited two prominent super imposed characteristic peaks at 3514 and 3400 cm^{-1} which were assigned to the stretching vibration of O-H (of the carboxylic group at C_3 position) and N-H groups respectively, another band at 3064 cm^{-1} represented the alkene and aromatic C-H stretching vibration. The band at 1676 cm^{-1} represented the carbonyl C=O stretching vibration, the band at 1471 cm^{-1} represented C-O stretching vibration and at 1303 cm^{-1} suggested bending vibration of O-H group. A strong absorption peak at 985 cm^{-1} was assigned to C-F group. The IR spectrum of chitosan : 3427 cm^{-1} (O-H stretching overlapping the N-H stretching), 2902 cm^{-1} (C-H aliphatic stretching vibration), 1627 cm^{-1} (amide C=O , C-O stretching of the acetyl group), 1402 cm^{-1} (asymmetrical C-H bending of the CH_2 group). The peak appeared at 1060 cm^{-1} is the characteristic peak due to stretching of C–O pairing in β (1→4) glycosidic bonds of polysaccharide. However, the IR spectra of ciprofloxacin-chitosan Schiff bases (Figure 1) showed strong characteristic absorption peaks at 1618, 1600, 1598, 1608 and 1577 cm^{-1} are attributed to the vibration of C= N of imines formed in the condensation reaction of ciprofloxacin and with the molar ratios (0.05, 0.1, 0.25, 0.5 and 1) respectively. Further peaks appeared at the range from 1000 to 1100 cm^{-1} are attributed to the characteristic stretching of C–O pairing in β (1→4) glycosidic bonds of polysaccharides.

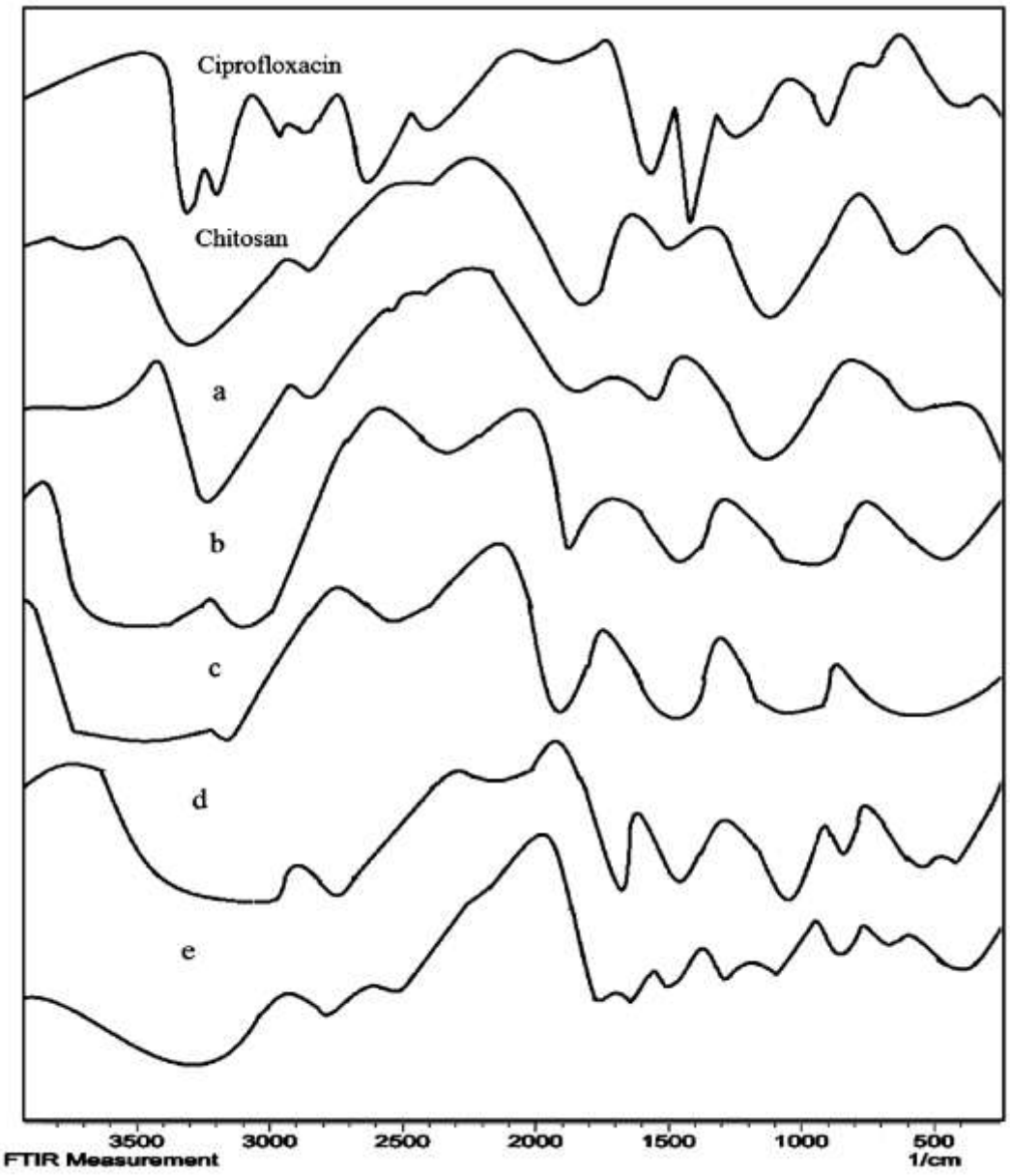


Figure 1:- FTIR spectra of ciprofloxacin, chitosan, derived Schiff bases; a,b,c,d and e synthesized with ciprofloxacin/chitosan at molar ratios of (0.05:1), (0.1:1), (0.25:1), (0.5:1) and (1:1) respectively.

^{1}H NMR spectral analysis:-

The chemical modification of ciprofloxacin can be obviously confirmed by ^{1}H NMR spectroscopy. The ^{1}H NMR signals of the synthesized ciprofloxacin-chitosan Schiff bases compared with ciprofloxacin and chitosan are shown in (figure 2).

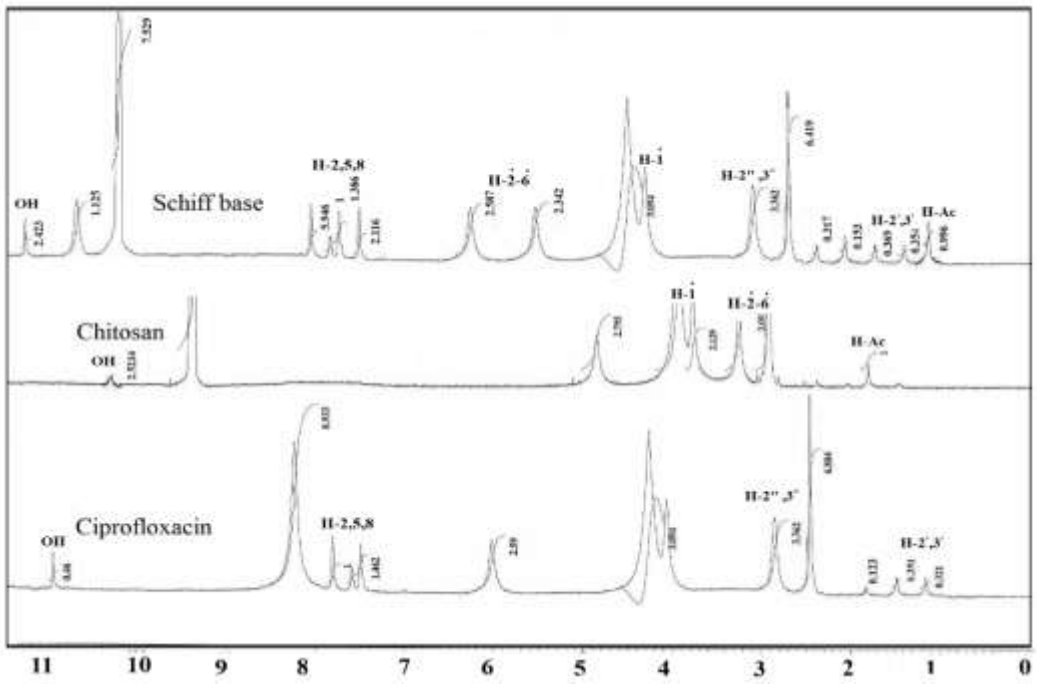


Figure 2:- ^{1}H NMR spectra of ciprofloxacin, chitosan and the corresponding Schiff base.

^{1}H NMR spectra of ciprofloxacin, chitosan and the corresponding Schiff bases are shown in (figure 2). In all cases, the peaks attributed to the protons along the backbones of ciprofloxacin and chitosan are numbered in (figure 2). The 3 proton assignments of ciprofloxacin were: 1.1-1.5 ppm (H-2',3'), 2.5-2.9 ppm (H-2'',3'') and 7-8 ppm (H-2,5,8). The proton assignments of chitosan were: 1.2 ppm (CH_3 of the acetyl group), 2.5-3.6 ppm (H-2'-6' of glucosamine ring) and 4.7 ppm (H-1'). The proton assignments of the synthesized Schiff base were: 0.9 ppm (CH_3 of the acetyl group), 1.2-1.5 ppm (H-2',3'), 2.4-2.9 ppm (H-2'',3''), 5.3 ppm (H-1'), 5.4-6.3 ppm (H-2'-6' of glucosamine ring) and 7-8 ppm (H-2,5,8).

The degree of substitution determination was based on the ratio of the areas of the aromatic protons (H-2,5,8) to the proton of the pyranose ring (H-2), as presented in the following equation:

DS (%) = ($A_{aromatic}$ / $A_{H\text{-}2'}$) × 100

where ;

DS = Degree of substitution.

A_{imine} = Area of aromatic protons peak.

$A_{H\text{-}2}$ = Area of H-2' proton peak of the pyranose ring.

DS values were calculated using the aforementioned equation. These results that presented in table 2 indicated that DS values ranged from 15.6 to 42.7 %. Moreover, it was indicated that degrees of substitution for all types of chitosan Schiff bases were close to each other. However, the yield of this process was calculated using the following equation:

Process yield (%) = Product (Schiff base) weight / reactants (chitosan + ciprofloxacin) weight × 100

The calculated yield values were tabulated in (table 1.) These values ranged from 72.9 to 85.0% based on the ratio of ciprofloxacin to chitosan and the degree of substitution.

Table 1: Substitution degree and yield of chitosan-ciprofloxacin Schiff bases.

Chitosan-ciprofloxacin Schiff base	DS %	Yield %
SB.1	15.6	72.9
SB.2	18.5	74.1

SB.3	20.8	72.7
SB.4	23.3	83.0
SB.5	42.7	85.0

Antimicrobial activity:-
The antimicrobial activities of the five Schiff's bases obtained in this study were evaluated using six bacterial strains. (cf. the experimental part). The results of this evaluation are compared with the activities of ciprofloxacin and chitosan itself were recorded in table 2.

Table 2:- Zone of inhibition (mm) of ciprofloxacin and their derivatives against various microorganisms.

Organisms	**CFX.**				**CHS.**				**SB.1**				**SB.2**			
(µg/ml)	**10**	**15**	**20**	**25**	**10**	**15**	**20**	**25**	**10**	**15**	**20**	**25**	**10**	**15**	**20**	**25**
S. aureus	10	12	14	16	5	8	9	12	8	12	14	15	10	11	19	22
S.pyogenus	8	10	12	13	10	12	13	16	5	8	10	15	8	10	12	14
S. pneumoniae	10	12	13	15	6	9	12	14	8	10	13	15	9	12	15	18
E. coli	15	18	20	22	0	5	8	10	6	10	12	18	5	8	13	16
K. pneumoniae	12	14	15	18	0	4	8	10	8	12	15	18	6	10	12	16
P.aeruginosa	12	14	15	18	0	7	10	13	6	10	14	18	5	8	12	15

SB.3				**SB.4**				**SB.5**			
10	**15**	**20**	**25**	**15**	**10**	**20**	**25**	**10**	**15**	**20**	**25**
8	10	15	18	14	19	20	24	18	24	28	33
5	10	13	16	5	10	12	14	10	15	22	27
5	10	12	15	8	13	18	20	10	12	25	28
4	7	12	15	6	10	15	20	10	15	20	31
5	8	12	16	7	10	13	17	12	15	23	30
3	10	13	19	5	6	8	14	13	18	25	31

Organism	**CFX.**				**CHS.**				**SB.1**				**SB.2**			
(µg/ml)	**20**	**30**	**40**	**50**	**20**	**30**	**40**	**50**	**20**	**30**	**40**	**50**	**20**	**30**	**40**	**50**
Cndida albicans	12	15	18	22	0	4	9	12	0	5	8	10	0	4	7	12

SB.3				**SB.4**				**SB.5**			
20	**30**	**40**	**50**	**20**	**30**	**40**	**50**	**20**	**30**	**40**	**50**
0	8	10	13	0	10	14	17	10	16	20	24

Ciprofloxacin-chitosan Schiff bases with molar ratios (0.05:1) SB.1, (0.1:1)SB.2, (0.25:1) SB.3, (0.5:1) SB.4 and (1:1)SB.5.

From these results, it can be generally indicated that the antibacterial activity of ciprofloxacin-chitosan Schiff bases is higher on gram positive bacteria comparing with that of ciprofloxacin itself. Schiff base (SB5) which formed by the condensation reaction of ciprofloxacin and chitosan with (1:1) molar ratio among all synthesized Schiff bases exhibited highest antibacterial activity especially against Staphylococcus aureus species at concentration 25 µg/ml compared with ciprofloxacin itself and all other Schiff bases. As indicated from this study, Schiff bases of ciprofloxacin and chitosan were exhibited a synergetic inhibitory effect against many species of bacteria especially Gram-positive ones.

The high antibacterial activity of ciprofloxacin-chitosan Schiff bases especially with (1:1) molar ratio shown in this study may be due to the synergistic effect of the mechanism of action against microorganisms of ciprofloxacin, chitosan and Schiff base.

Ciprofloxacin mechanism of action through the inhibition of bacterial gyrase, an enzyme involved in DNA replication, recombination and repair. By interfering with gyrase, ciprofloxacin arrest bacterial cell growth. Chitosan

mechanism of action that owing to the electrostatic interaction between cationic components of chitosan (NH_3^+ groups) and anionic residue of bacteria (-COO^- or PO_4^{-3} groups) by condensation amine group of chitosan and carbonyl group of ciprofloxacin, this can be explained on the basis that the **π**-electrons of imine groups (C=N) of Schiff bases enhance the lipophilicity of ciprofloxacin-chitosan Schiff bases which can facilitate penetration of the Schiff base molecules into the microbial cell membranes and then can disturb the respiration process of the cell and block the synthesis of proteins, which hinder the further growth of bacteria. On the other hand, molar ratio play a key role in change of the physicochemical properties and subsequently biological properties.

Conclusion:-

The antimicrobial activity of ciprofloxacin was enhanced by the condensation reaction with chitosan forming the corresponding Schiff base. The antimicrobial activity of ciprofloxacin was dependant on the degree of substitution of ciprofloxacin on chitosan. Schiff's base (SB5) which formed by the condensation reaction of ciprofloxacin with chitosan at ratio (1:1) exhibited the highest antibacterial activity especially against *Staphylococcus aureus* species at concentration 25 µg/ml compared with ciprofloxacin itself and all other Schiff bases. These results recommend the presented chemical route for modification of ciprofloxacin for medical applications.

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