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Bis–aldehyde based Chitosan Schiff base Polymer: Evaluation of Thermal, Cytotoxic, Antibacterial, Antioxidant, Anticancer and Wound Healing Properties

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ABSTRACT

Polymer composites based on natural polymer have received greater attention due to their biocompatible and biodegradable nature. Chitosan a natural polymer obtained from marine source extensively used for many biomedical applications. Chitosan is a bio-based polymer with comparable physico-chemical properties of cellulose. Hence, researchers have explored a number of chemical alterations in chitosan to enhance its characteristics and broaden its functional potential range. In this work, we report chitosan Schiff bases were formed by interaction of CS with bis-aldehyde at room temperature and explore its biological properties. The synthesized compounds were characterized by various analytical techniques. Chitosan and its derivatives are garnering a lot of interest in a variety of settings. Higher solubility as compared to unmodified significantly improved biological studies such as antimicrobial activity against *Klebsiella* and Methicillin-resistant *Staphylococcus aureus* (MRSA) and against yeasts (*Candida albicans*, *Trichoderma viridae*, and *Aspergillus niger*), paving the way for the potential use of these chemicals in the pharmaceutical industry as a means of preventing the spread of harmful microorganisms.

Keywords: Chitosan, Natural polymer, Chitosan-based Schiff base, Bio compatibility &Anti-microbial activity.

INTRODUCTION

Chitosan is a naturally occurring polycationic linear polysaccharide that is produced by partially deacetylating chitin. It is also called deacetylated chitin[1]. Chitin is a fundamental part present in the exoskeleton of insects, shells (especially crabs, prawns and squid), and fungal cell walls. Chitosan is a second most abundant natural polysaccharide after the cellulose. Biodegradable and produced from plants, chitosan has remarkable film-forming properties. One other thing: it's harmless, biodegradable, and compatible with living organisms. Aside from renewable materials found in nature, chitosan is also present in industrial processes[2]. Chitosan has a primary amine on position C-2, a primary hydroxyl on position C-3, and a secondary hydroxyl on position C-6, in that order[3]. The biological activity of chitosan is largely affected by the primary amine at C-2 of glutamine, one of three reactive groups responsible for this activity[4-5].

Chitosan and its derivatives have significant use in several fields, including as biotechnology, cosmetics, the natural sciences, agriculture, the creation of functional foods, wastewater treatment, environmental protection, and the preservation of our planet[6]. Antibiotic resistance research and the prevalence of bacteria, which may cause serious diseases, highlight the need to develop novel antibiotic compounds. The epidemiological and economic consequences of antibiotic resistance have made it the leading infectious disease concern at the present time[7]. Furthermore, Every thing from manufactured synthetic substances to establish removes, food handling side-effects to biofermentative cycles, and even municipal garbage might be considered as a potential therapeutic option to fight germs. All of these things have the makings of bioactive molecules that could have important effects, like antimicrobials or molecules that can dissolve biofilms or change how biofilms form. From this point of view, chitosan (CS) and other natural antimicrobial compounds are at the forefront of widespread interest in CS-related research around the world. Medicinal plants and natural substances have a long history of usage, and chitosan's many useful qualities make it a promising candidate for further research into potential novel pharmacological and antibacterial uses. Interactions with various forms of chitosan (solutions, films, and composites) have been investigated in both *in vivo* and *in vitro* settings as a potential antibacterial agent against a variety of species, including yeasts, fungus, bacteria, algae, and bacteria[8]. Research on chitosan and its derivatives have been booming because to the material's broad-spectrum antibacterial action, which was first suggested by Allan and Hardwiger[9], as well as its enormous economic potential. On considering the issues and findings in literature we account on the synthesis and characterization of bis-aldehyde based Schiff base Chitosan polymer and study its biological applications.

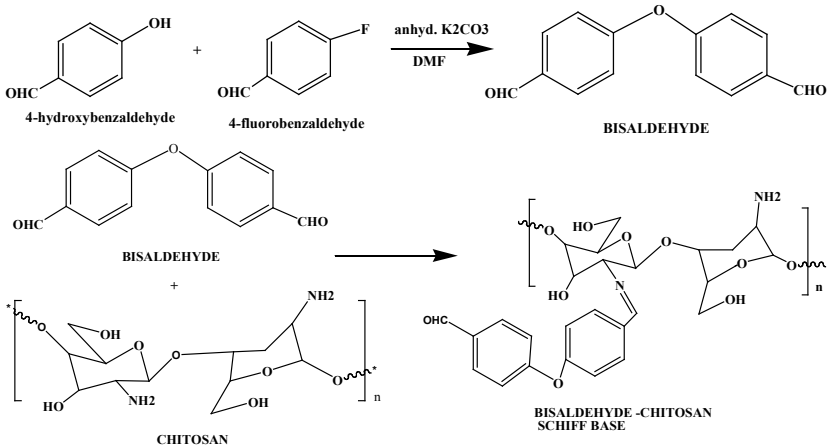
EXPERIMENTAL METHODS

Materials

A Rgrade4-fluorobenzaldehyde (98%), 4-hydroxybenzaldehyde (97%), anhydrous K_2CO_3. ethyl acetate, chloroform, and toluene purchased from Sigma Aldrich. Dimethyl sulfoxide and diethyl ether solvents from Merck. Also, the. Sigma Aldrich supplied the methanol, ethanol, and N, N-dimethylformamide.[10]

Methods

In a round bottom Flak, 50 mL of 2% was added slowly to 0.5 g of deacetylated Chitosab with stirring. The above chitosan solution was agitated in a stirrer for three hours at 50°C until a thick, yellow solution with a pH of 6.0 was formed[11,12]. Then, 0.35 g of Bis-aldehyde dissolved in 15 mL of ethanol was added slowly, and the blend was disturbed continually with a reflux condenser for about 12 h at 60°C. White crystalline solids with a yield of 94% (0.72 g) are the end product of the reaction. The precipitate formed is filtered, washed with acetone,



Scheme of Chitosan and bis-aldehyde Chitosan Schiff base

and then dried at 50°C for six hours.

Characterization Techniques

FT-IR spectrum for the compound obtained was studied using BURKER ALPHA-T ATR-FTIR spectrometer in between 4000–400 cm^{-1}. Surface morphology of samples was examined by scanning electron microscope (HR-SEM, S2600 HITACHI). The thermal properties were analyzed using Q500

HI-RES Thermogravimetric analyzer.

RESULTS AND DISCUSSION

FTIR spectroscopy

For both chitosan and BA-CSB, the FTIR spectrum was observed from 4000-400 cm^{-1} using a Perkin Elmer spectrometer. Absorption spectra for the BA-CSB sample, with the most important findings summarised in Fig. 1. In the case of BA-CSB samples, it is important to note the absence of absorption in the area about 1651 cm^{-1}. It has been observed that the absorption profiles of -OH and -NH_2 groups (specifically, OH and NH_2 stretching vibrations) and C-O-C and C-N groups (particularly, asymmetric stretching of C-O-C and C-N) exhibit higher values compared to the imine bond (C=N stretching, imine), which is present with sufficient intensity in the spectral region around 1640 cm^{-1}. It should be noted that there is complete spectral overlap at 1651 cm^{-1}, where the C=O bond absorbs light (C=O stretching, amide 1). Therefore, no absorption in the area of 1651 cm^{-1} in the absorption ranges of samples BA-CSB. A comparison of the CS, BA-CSB absorption spectra with data presented by

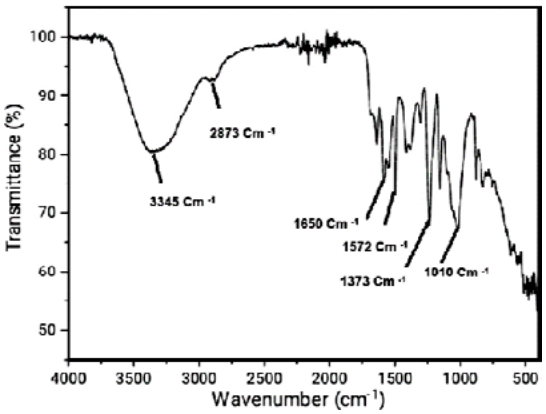


Fig. 1. IR Spectra for BA-CSB

other groups[12-16] shows agreement.

^{1}H NMR spectroscopy

The proton NMR spectra of BA-CSB was recorded at a temperature of 298.5K, employing a BRUKER NMR Spectrometer with a frequency of 400 MHz and utilizing DMSO-d6 as the solvent. Fig. 2 illustrates the ^{1}H NMR spectrum derived from BA-CSB. Fig. 2 reveals the values of δ1.8 and 2.5-3.4ppm due the presence of free hydrogen H_2–H_6 and -OCH_3 attributed to chitosanare characteristic of chitosan[17]. In particular, the chemical shift of the proton in aldehyde occurred at δ9.95 ppm in BA-CSB. Additionally, the hydrogen in imine of BA-CSB may be attributed to the new peak seen at 9.97 ppm and 9.92 ppm in chemical shift. The Chemical Shift

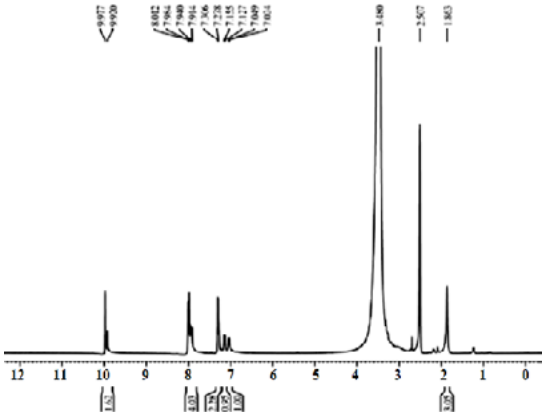


Fig. 2. C^{1}H-NMR of Schiff Bis-Aldehyde-Chitosan Base(BA-CSB)

δ7.1 and δ7.9 ppm indicates the Aromatic protons.

^{13}C NMR spectroscopy

^{13}C NMR. spectra of the Bis-Aldehyde and BA-CSB are shown in Fig. 3 and Table 1. In CS, the signals can be interpreted as δ = 190.64ppm, 160.99ppm, 132.57ppm, 119.57ppm, 77.54ppm and 76.69ppm, this is in accordance with the previous reports. In BA-CSB, there are some new Carbon peaks: δ = 39.05-40.72ppm, δ = 160.89pm, δ = 192.24ppm,

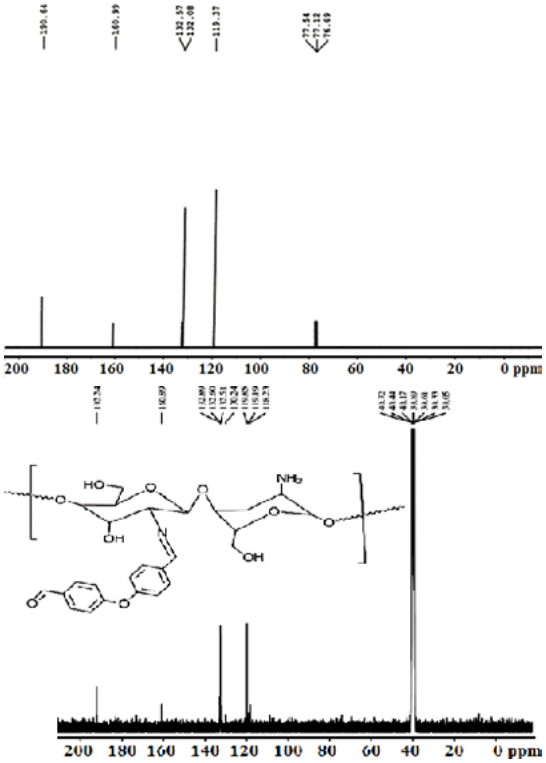


Fig. 3. ^{13}C-NMR spectrum of Bis-aldehyde and BA-CSB

Table 1: ^{13}C-NMR Spectral determination

Compound	δValue in ppm	Type of carbon
Bis Aldehyde	190.64	Aldehydic carbon
	119.37	Aromatic carbons
BA-CSB	192.24	Aldehydic Carbons
	119.85	Aromatic carbons
	160.89	Imine Carbon

δ = 160.89ppm, δ = 132.89ppm and δ = 130.24ppm.

Thermal analysis

Chitosan and its derivatives may have their thermal characteristics characterised with the use of DSC. At temperatures ranging from room temperature all the way up to 350°C, for the prepared Sampl are shown in Fig. 4.1, 4.2 & 4.3. Dehydration of water content may be associated with the large endothermic peak about 100°C seen in the DSC spectra of chitosan. The peaks at 247°C and 250°C, respectively, are exothermic peak due to chitosan and Schiff base (I), which correlate to the thermal breakdown of the pyranose ring. The samples were subjected to thermogravimetric analysis in between the temperature range of 20°C to 600°C in order to investigate their stability after high temperature treatment.[14-16].

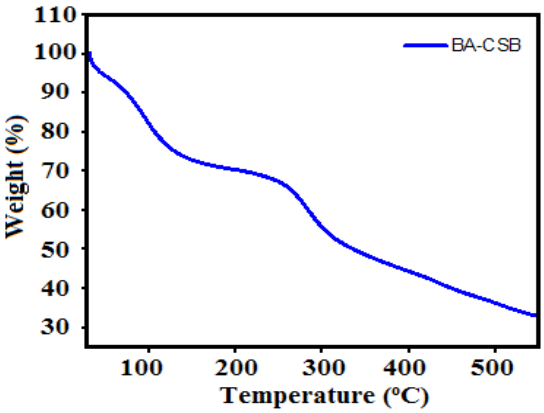


Fig. 4.1. TGA analysis of BA-CSB

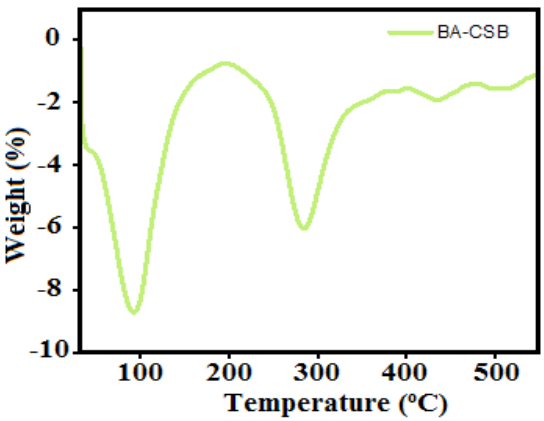


Fig. 4.2. DTA of BA-CSB

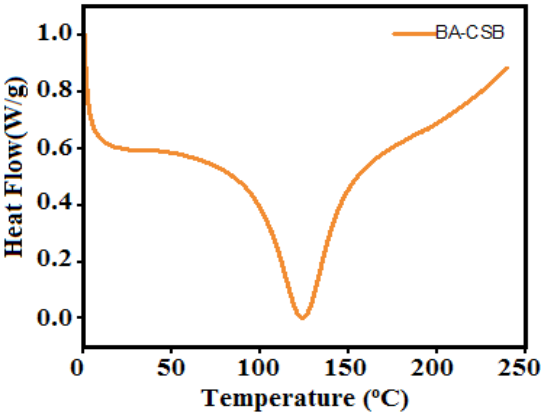


Fig.4.3. DSC of BA-CSB

Scanning Electron Microscopy

To find out how the samples looked on the surface, scanning electron microscopy was used. In addition, a cross-section was also taken to improve the material qualities of the films that were produced. The SEM pictures of Bisaldehyde-Chitosan (Fig. 6, 3–4) and All of the produced Chitosan Samples (Fig. 51–2) are included in the supplementary materials. Chitosan powder has a spherical appearance with rounded corners. In subsequent processes, chitosan was modified into flat-structured structures. In line with the studies in the literature, all of these findings indicate that the chitosan was

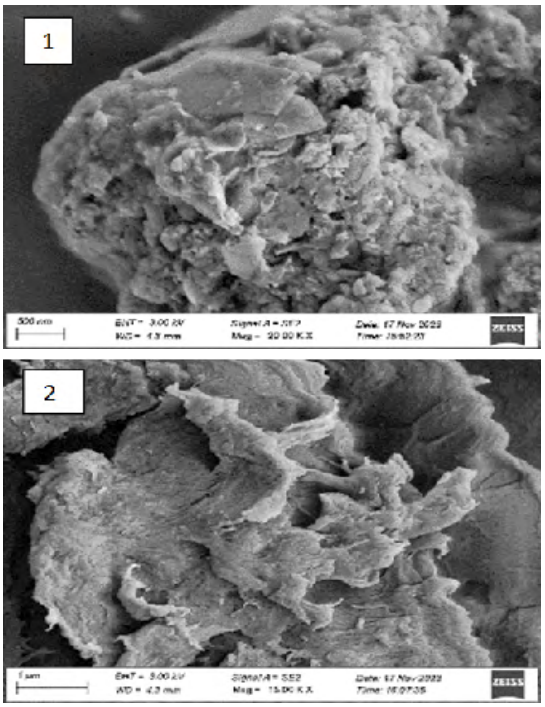


Fig. 5. 1 & 2. SEM Studies of Chitosan

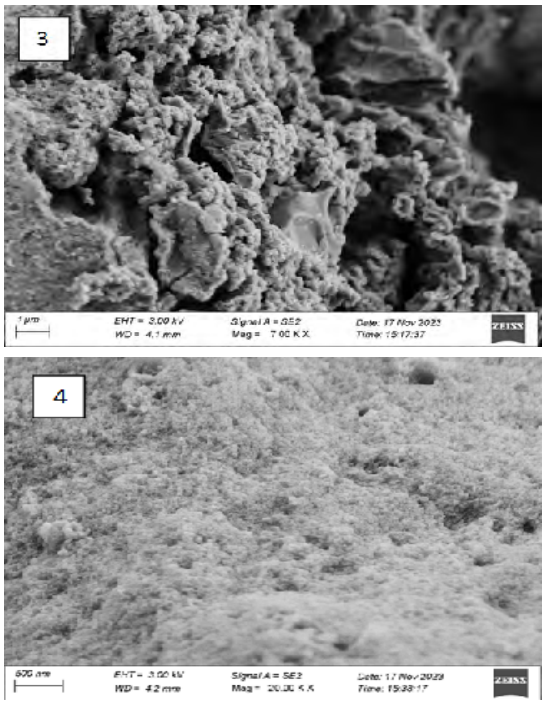


Fig. 6. 3 & 4. SEM studies of Bisaldehyde-Chitosan

effectively manufactured.[13]

Cytotoxicity Assay

To determine the viability of the synthesized materials for applications in the biomedical field, their cytotoxicity was assessed using the MTT assay. VERO cell lines obtained from and maintained by the National Centre for Cell Sciences (NCCS), Pune, India, were cultured in DMEM media, with 10% fetal bovine serum (FBS), penicillin (1000 U/mL), and streptomycin (100 ug/mL) in a humidified atmosphere of 5% CO_2 at 37°C. The cells (1×10well) were plated into a 24-well plate, and once they reached ~80% confluence, they were treated with test articles at various concentrations for 24 hours. After 24 h, the cells were washed with PBS or serum-free DMEM. Then 0.5% MTT solution (5 mg/mL) was added to the wells and then placed back into the incubator for another 4 hours. The formazan crystals formed were dissolved in DMSO (1 mL/well), and absorbance was read at 570nm using a UV/Vis spectrophotometer. Percent viability was calculated for each treatment and to determine the IC_{50} values to characterize the cytotoxicity.

%Cell viability = A570 of treated cells/A570 of control cells×100 (1)

Graphs are plotted which was shown in the Fig. 7. Each test includes a cell control and an example control to allow for a comprehensive comparison of cell viability evaluations (Figure 8).

Table 2: Study on the Sample's Cytotoxicity Against the VERO Cell Line

Sr. No	Concentration (µg/mL)	Absorbance (O.D)	Cell Viability (%)
1	1000	0.462	51.16
2	500	0.504	55.81
3	250	0.547	60.57
4	125	0.588	65.11
5	62.5	0.632	69.98
6	31.2	0.675	74.75
7	15.6	0.718	79.51
8	7.8	0.762	84.38
9	Cell control	0.903	100

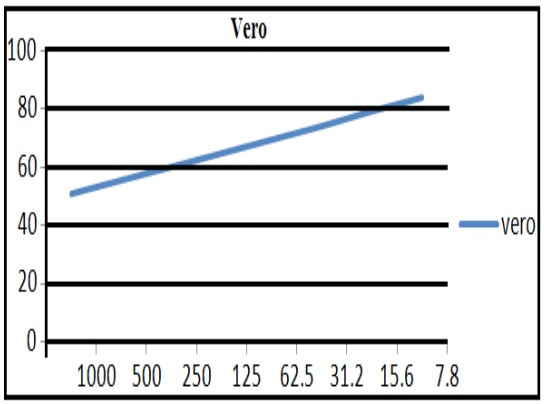


Fig. 7. Cytotoxicity Assay Graphical representation of BA-CSB

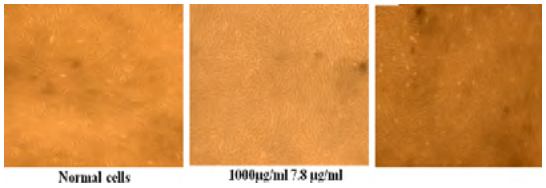


Fig. 8. Cytotoxicty Culture for BA-CSB

Wound healing assay

The wound healing assay is a commonly used *in vitro* assay for two-dimensional collective cell migration using VERO cell lines. The wound healing assay consists of applying controlled “wound” to a confluent monolayer of cells to assess how much they migrate into the wound. Cell migration is an important process for tissue repair and regeneration, as well as for cancer metastasis and development. VERO cells were seeded in six-well plates and allowed 24 h for formation of a monolayer. After confirming the confluence of the cells in the well, a scratch was performed in the well with a sterile pipette tip, while treating the wells with a 100 µg/mL concentration of the sample or positive controls. All plates were then incubated further for 12 h where cell migration into

the wound was viewed using microscopy (Fig. 3). The wound closure of 72% indicated that cell migration had occurred and demonstrated the properties of

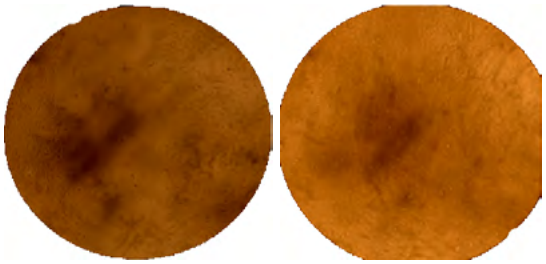


Fig. 9(a). Size of the wound 1.15. (b) Size of the wound 0.32

the material to induce wound healing (Figure 9).

$$\text{Wound Closure \%} = \frac{\text{Measurement at 0th hr} - \text{Measurement at 24th hr}}{\text{Measurement at 0th hr}} \times 100 \quad (2)$$

Anti-cancer activity

MCF-7 breast cancer cells from NCCS, Pune, were cultured in DMEM with 10% FBS, penicillin (1000 U/mL), and streptomycin (100 µg/mL) in a humidified 5% CO_2 incubator at 37°C. For cytotoxicity assessment, cells (1×10∂well) were seeded in 24-well plates and treated with different concentrations of the test sample after reaching confluence. After 24 h, wells were washed with PBS or serum-free DMEM, followed by the addition of MTT solution (0.5%, 5 mg/mL, 100 µL/well) and incubation for 4 hours. DMSO (1 mL) was added to dissolve formazan crystals, and absorbance was measured at 570nm. Cell viability and IC_{50} values were calculated to determine the cytotoxic effect.

%Cell viability = A570 of treated cells/A570 of control cells×100 (3)

Each test includes a cell control and a sample control to allow for a comprehensive comparison of cell viability evaluations (Figure 11

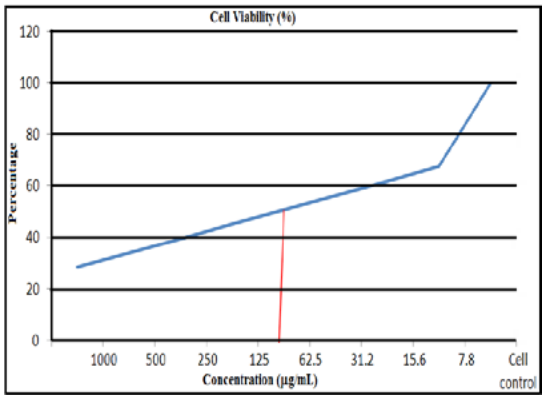


Fig. G1. Cell viability% of BA-CSB

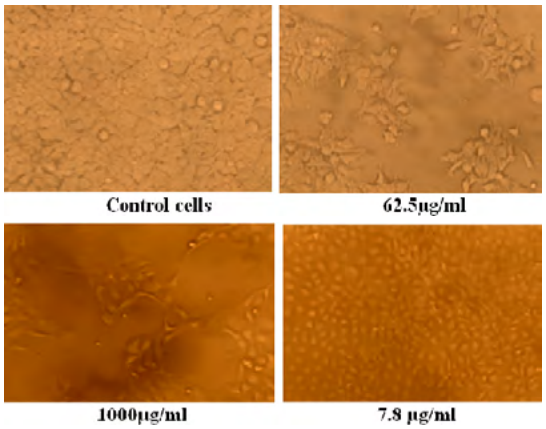


Fig. 11. MCF 7 cells

and Figure G1).

Antibacterial Activity

Stock cultures were maintained on nutrient agar slants at 4°C. For testing, a loopful of culture was transferred into nutrient broth and incubated at 37°C for 24 h to activate the culture. Antibacterial activity was assessed using the agar disc diffusion method on Mueller Hinton Agar (MHA). After the medium solidified in Petri dishes, bacterial inoculum was evenly spread using a sterile swab. Sterile discs loaded with 20 µL of the sample at concentrations of 1000 µg, 750 µg, and 500 µg were placed on the MHA plates and incubated at 37°C for 24 hours. The antibacterial effect was determined by measuring the diameter of the inhibition zones. Results are

Table 3: Zone of Inhibition

Sr. No	Organisms	Zone of Inhibition (mm)			
		Sample (µg/mL)			Standard
		1000	750	500	
1	*Klebsiella*	19	12	10	29
2	(MRSA) Methicillin-resistant *Staphylococcus aureus*	10	8	8	14
3	*Staphylococcus*	11	10	10	16
4	*Escherichia coli*	17	17	9	18
5	*Bacillus subtilis*	40	35	31	47
6	*Salmonella*	14	14	10	15

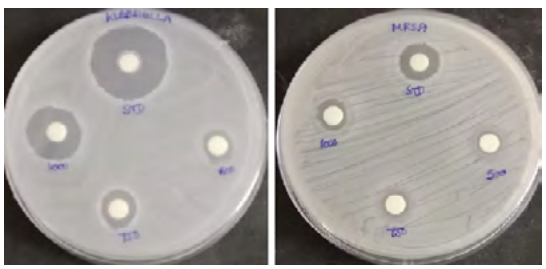


Fig. Z1. ZOI of Klebiella and MRS

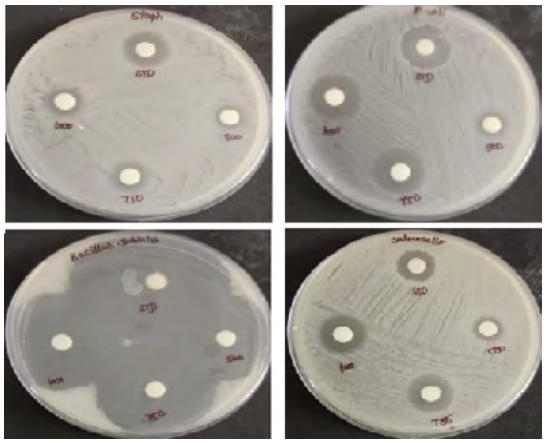


Fig. Z2. ZOI of *Staphylococcus*, *Escherichia coli*, *Bacillus subtilis* and *Salmonella*

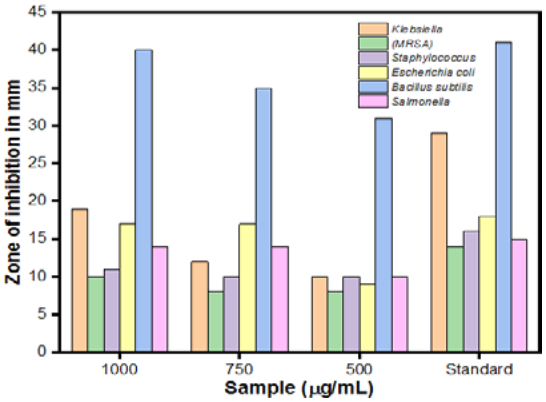


Fig. C1. ZOI vs concentration for BA-CSB

shown in Table 3 and Figure C1.

Minimum inhibitory concentration

Bacterial inoculums were prepared by suspending 18–24-h-old colonies in broth and adjusting turbidity to match the 0.5 McFarland standard (~1–2×10^8 CFU/mL) using a white background with black lines for comparison. The suspension was then diluted to achieve a final concentration of ~5×10^5 CFU/mL for testing. MIC was determined using the serial dilution method. Sterile LB broth (1 mL) was added to eight tubes and autoclaved. After cooling, 1 mL of the test sample was added to the first tube and serially diluted across the remaining tubes. Each tube received 100 µL of the prepared bacterial inoculum (including *Salmonella*, *S. aureus, B. subtilis*, and *E. coli*) and was incubated at 37°C for 24 hours. Turbidity was observed post-incubation, with the lowest concentration showing no visible growth recorded as the MIC. Results are depicted in

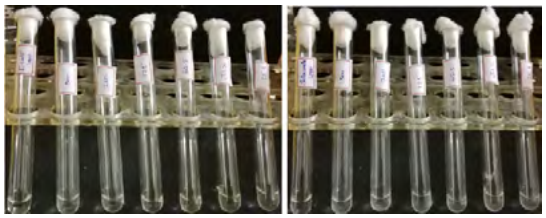


Fig. M1(a). *Salmonella* (b). *Escherichia coli*

Fig. M2(c). *Staphylococcus aureus.* (d) *Bacillus subtilis*

Figures M1 and M2.

Minimum Bactericidal concentration (MBC) determination

To determine the MBC, the Sample were drawn from(1000µg/mL, 125µg/mL, 15.6µg/mL) Tubes and plated enumerate the viable colonies by pour plate method. It was then incubated for 24 h, the outcomes for this period were shown (Figure M3,

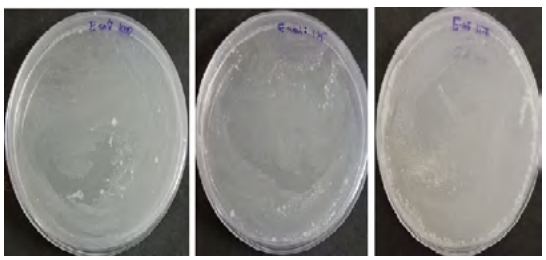


Fig. M3. MBC of *Escherichia coli*

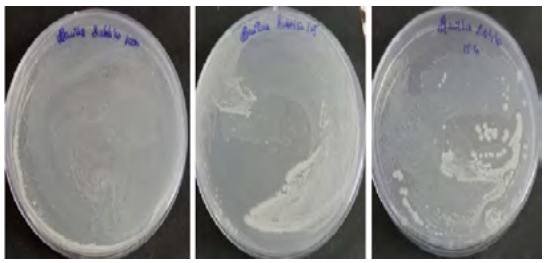


Fig. M4. MBC of *Bacillus subtilis*

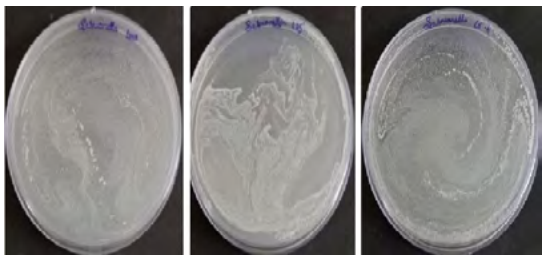


Fig. M5. MBC of *Salmonella*

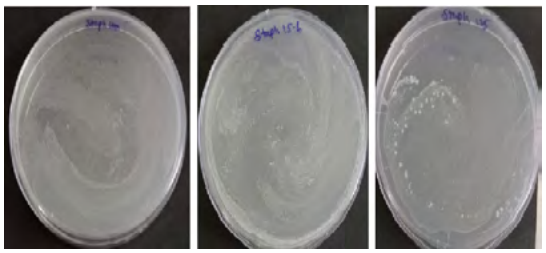


Fig. M6. MBC of *Staphylococcus aureus*

Table 4: MBC Determination

Organism	Concentration	OD value
Escherichia coli	1000	0.058
Total Number of Colonies:	500	0.079
$15X10^{-1}$, $33X10^{-4}$ and $112X10^{-7}$	250	0.106
	125	0.142
	62.5	0.211
	31.2	0.256
	15.6	0.321
Bacillus subtilis	1000	0.113
Total Number of Colonies:	500	0.167
$14X10^{-1}$, $48X10^{-4}$ and $96X10^{-7}$	250	0.219
	125	0.276
	62.5	0.354
	31.2	0.412
	15.6	0.498
Salmonella	1000	0.073
Total Number of colonies:	500	0.116
$32X10^{-1}$ and $54X10^{-4}$	250	0.185
Too Numerous to Count$X10^{-7}$	125	0.267
	62.5	0.308
	31.2	0.387
	15.6	0.453
Staphylococcus Aureus	1000	0.127
Total Number of Colonies:	500	0.168
$12X10^{-1}$, $46X10^{-4}$	250	0.249
Too Numerous to Count$X10^{-7}$	125	0.283
	62.5	0.376
	31.2	0.425
	15.6	0.498

M4, M5 & M6)(Table 4).

Antifungal evaluation

The antifungal activity of Chitosan bis Schiff base (BA-CSB) was assessed using the agar well and disc diffusion methods against *Candida albicans*, *Trichoderma viride*, and *Aspergillus niger*. Fungal cultures were maintained on Sabouraud Dextrose Agar (SDA) slants at 4°C and activated by culturing in Sabouraud Dextrose Broth at room temperature for 48 hours. For the well diffusion assay, 1 mL of BA-CSB and its complexes was added to wells in SDA plates inoculated with fungal suspensions. In the disc diffusion assay, sterile SDA plates were swabbed with fungal inoculum, and sterile discs loaded with 20 µL of the sample or amphotericin B (positive control) were placed on the surface. Plates were incubated at 28°C for 24 h[18]. Antifungal efficacy was determined by measuring the diameter of the zones of inhibition

Table 5: Antifungal study-Zone of Inhibition(mm)

Organism	Zone of inhibition (mm) Sample(µg/mL)			Standard
	1000	750	500	
Candida albicans	23	21	20	40
Trichoderma viride	19	12	10	38
Aspergillus niger	27	17	17	40

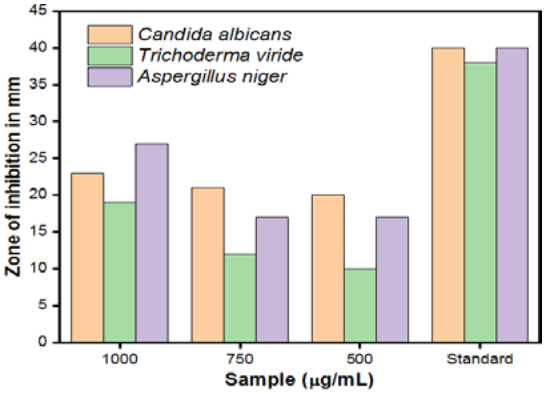


Fig. C2. ZOI VS concentration for BA-CSB

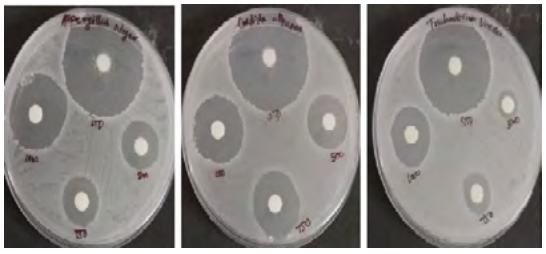
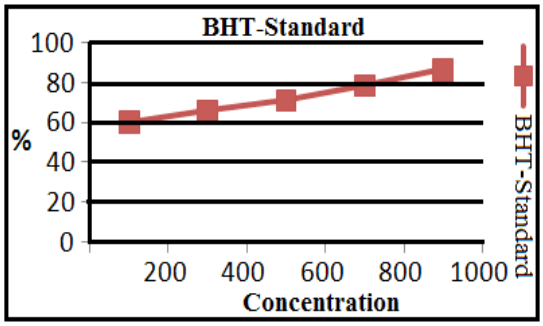


Fig. 12. Antifungal Screening of BA-CSB

(ZOI), as shown in Figure 12.(Table 5 & Figure C2).

Antioxidant assay

Antioxidants play a key role in neutralizing free radicals and preventing oxidative damage to cells and biomolecules[17-18]. Due to their effectiveness and affordability, synthetic antioxidants are gaining more attention over natural ones. In this study, the antioxidant potential of various Schiff bases and their metal complexes was evaluated using the DPPH assay[19]. The DPPH (1,1-diphenyl-2-picrylhydrazyl) assay is based on the ability of antioxidant compounds to donate hydrogen atoms to stabilize the DPPH free radical, which is deep violet in color and shows a strong absorbance at around 520nm. Upon reduction by an antioxidant (AH), DPPH loses its violet color, forming a light yellow reduced form[20]. The decrease in absorbance is measured spectrophotometrically to assess radical scavenging activity.



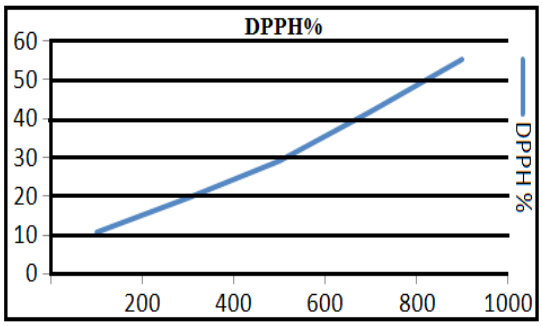


Fig. 13. BHT-Standard and DPPH%

Table: 6 Anti-oxidant DPPH

Sr.No	Concentration (µg/mL)	O.D Value			Average	DPPH%
1	200	0.582	0.585	0.580	0.582	10.87
2	400	0.524	0.526	0.522	0.524	19.75
3	600	0.461	0.463	0.459	0.461	29.40
4	800	0.378	0.380	0.376	0.378	42.11
5	1000	0.291	0.293	0.289	0.291	55.43

$$\%\text{Antioxidant activity} = \frac{(\text{Absorbance at blank}) - (\text{Absorbance at test}) \times 100}{(\text{Absorbance at blank})} \quad (5)$$

CONCLUSION

In conclusion, bis-aldehyde based chitosan Schiff base was successfully synthesized by simple one step rapid reaction. The functional characteristics of the synthsised polymer was confirmed by FTIR spectroscopy, thermal analysis and scanning electron microscopy (SEM). Additionally, the results of the cellular toxicity test indicated that Schiff base is non toxic. The studies revealed that the prepared bis-aldehydebased chitosan Schiff base are anti-oxidants, anti-cancer agent, and antibacterial agents. Also, it is observed that chitosanderivatives may preferentially enter cancer cells and exhibit pathways that work against cancer. The evidence of inhibitory zones against *Klebsiella*, MRSA (Methicillin-resistant *staphylococcus aureus*), *E. coli* shows the potential antibacterial characteristics. Hence, the results confirmed that the synthesized bis-aldehyde based chitosan Schiff base polymer is a potential biomaterial for medical applications.

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