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RESEARCH ARTICLE

Microwave assisted synthesis of chitosan epoxy asparagine hydroxamate (CE-AH) Characterization and Study of its antimicrobial activity

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ABSTRACT:
Green synthetic approaches are emerged as a remarkable technology for the production of biopolymer based novel derivatives. Microwave assisted synthesis method is considered as safe alternative to conventional physiochemical methods due to its environmental friendly nature.
Chitosan was derivatized with epichlorohydrin, asparagine using microwave. After this the product was regenerated from the mixture of water and methanol (1:1)then washed with ethanol, methanol. Solvents like DMSO, DMF are used while synthesis. The characteristics of newly formed derivative was studied by Fourier transform Infrared spectrum(FT-IR), Thermogravimetric analysis(TGA), Nuclear Magnetic Resonance(NMR), Mass spectra, Scanning Electron Microscopy(SEM), elemental analysis etc. Antimicrobial activity was studied on different bacteria and fungi by well diffusion method.

KEYWORDS: Microwave assisted synthesis, chitosan, asparagine, SEM, NMR, TGA antimicrobial activity.

INTRODUCTION:

Microwave assisted method is a green methodology in the synthesis of chitosan derivatives due to its unique characteristics of rapid volumetric heating, increased reaction rates, enhanced reaction selectivity, and energy saving, compared with conventional heating methods.[1-3]

Chitosan is a natural, cationic polysaccharide obtained from chitin. It is the second, after cellulose, most abundant natural polymer. This copolymer consist of -1, 4 linked N-acetyl-D-glucosamine and D-glucosamine units (Fig.1).[4] Depending on conditions of the deacetylation process (deacetylation of chitin provides chitosan), different forms of chitosan are available. Those forms vary in the deacetylation degree (DD) (indicating the amount of free amino groups) and the average molecular weight of the polymer. These features determine the physiochemical properties of the polymer and its application.[5]

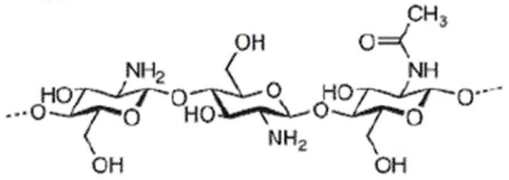

Fig1:- Chitosan

Asparagine, encoded by the codons **AAU** and **AAC** is an α-amino acid that is used in the biosynthesis of proteins. It contains an α-amino group (which is in the protonated −NH+3 form under biological conditions), an α-carboxylic acid group (which is in the deprotonated $-COO^-$ form under biological conditions), and a side chain carbetamide.[6-7] Fig 2

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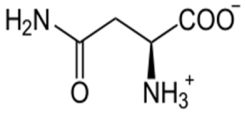


Fig 2. Asparagine

Antimicrobial polymers, also known as polymeric biocides that is polymer having the ability to inhibit the growth of micro-organisms such a bacteria and fungi.[8]

Chitosan and its derivatives have attracted considerable interest due to their antibacterial and antifungal activity.[9-12] The possibility of a biocide effect occurrence depends on the chitosan specimen form as well (solution, powder, membrane, etc.[13-15]

The antimicrobial activity of chitosan is influenced by no. of factors like the degree of polymerization and sale of its other physiochemical properties.[16-18] chitosan exhibits higher antibacterial activity against gram positive and gram negative bacteria.[19-20]

MATERIAL AND METHODS:

MATERIAL:

Chitosan was procured from local industry. All Analytical reagent grade chemicals used were procured from Sigma Aldrich, Lobe Chemicals and Aces Chemical Works. The antimicrobial strains (Bacterial and Fungal Strains) were obtained from Dr. S. N Medical College, Jodhpur (Rajasthan), India.

METHOD:

Synthesis of Chitosan epoxy asparagine hydroxamate (CE-AH):

The synthesis of CE-AH resin is carried out in two steps:

Preparation of epoxy ether of chitosan :-

1 mole of chitosan was slurried in DMSO solvent in a round bottom flask for 24 hours. Then 50% aqueous NaOH was added in the slurry to make the reaction mixture alkaline and it was constantly magnetically stirred at 45°C for 2 hours. Further 1 mole of epichlorohydrin was added gradually with continuous stirring and the pH was adjusted to 9-10 then the reaction mixture was subjected to microwave for 15 minutes. Later, the compound was filtered on vacuum pump with 80% aqueous methanol containing few drops of nitric acid to remove inorganic impurities of chloride ion and excess of alkali then the compound formed was oven dried and used for further derivatization.

Synthesis of chitosan epoxy ether of asparagine hydroxamate :

Chitosan epoxy was added to asparagine dissolved in DMSO and slurred for hour with constant magnetic stirring. Then the reaction mixture was subjected to microwave for 15 minutes. Asparagine derivative of chitosan epoxy formed was taken in round bottom flask excess methanol and thionyl chloride were added and temperature raised up to 40°C at rotavapour for two hour. Now methanolic solution of hydroxyl amine chloride was added to get the hydroxamate of the ester with pH was maintained at 9 to 9.5 by sodium bicarbonate solution. The chitosan derivative thus produced was filtered off and washed with double distilled water and finally dried. Fig 3

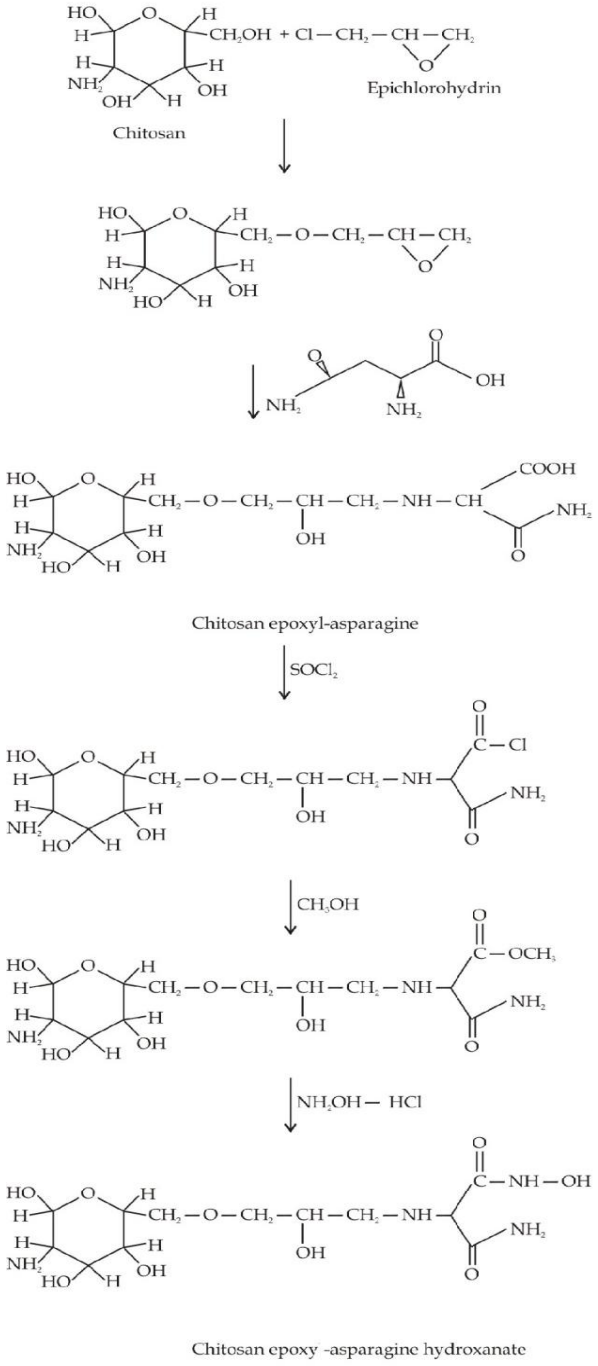


Fig 3:- synthesis of chitosan epoxy asparagine hydroxamate(CE-AH)

CHARACTERIZATION:
The newly formed derivative was characterized by FTIR spectroscopy, H^1 NMR Spectroscopy, Mass spectrometry, Scanning Electron Microscopy (SEM) and Elemental Analysis (EDX)

FT-IR Analysis:
IR Spectra was recorded with Bruker spectrophotometer.

H^1 NMR Analysis:
NMR Spectra was determined by Bruker AV-II 300 MHz FT-NMR Spectrometer. The compound was dissolved in DMSO.

Mass spectral Analysis:
DART-MS was recorded on a JEOL-AccuTOF JMS-T1OOLC Mass spectrometer having a DART **(***Direct analysis in real time***)** source. The compound was subjected as such in front of DART source. Dry Helium was used with 4 LPM flow rate for ionization at 350°C. The orifice 1 was set at 28 V.

Elemental Analysis:
The element determine by SEM EDX Method.

Antimicrobial Activity:
An anti-microbial is a substance that inhibits the growth of microorganisms such as bacteria, fungi. The minimum inhibitory concentration (MIC) of chitosan ranged from 0.005 to 0.1%depending on the species of bacteria, fungi and molecular weight of chitosan.[21]

The bacterial strains Escherichia coli, klebsiella pneumonia, Staphylococcus aureus, Pseudomona aeruginosa, staphylococcus (CONS). Funga strain Candida albicans, Candida parapsilosis, Candida tropicalis, Candida krusie, Rhodoturula. The pathogenic organisms were obtained from S.N. Medical College, Jodhpur. Ampicillin (antibacterial) and Fluconazole (anti fungal) were used as standard drugs.
Antimicrobial sensitivity test using Agar well diffusion method.

Preparation of innoculum:
The bacterium and fungi were inoculated in 1% peptone, 0.5%yeast extract and 1%NaCl. The inoculation was conducted at 37°C for 24h with shaking. The obtained suspension was diluted with the same peptone medium solution.

The plates were inoculated by dipping a sterile spread into inoculums. The sterile spread was streaked all over the surface of the medium. Finally the sterile spread was passed round the edge of the agar surface. The inoculum was dried for a few minutes, at room temperature, with the lid closed.

Antimicrobial susceptibility test:
Wells of approx 6 mm were dig on the sterile agar plate. Solution of compound was filled in well using micropipette. The plates were incubated in an upright position at 37°C for 24 h. The diameter of inhibition zones formed was measured in mm with transparent ruler and the results were recorded.

RESULTS AND DISCUSSION:
The newly formed derivative was characterized by FTIR spectroscopy, H^1 NMR Spectroscopy, Mass spectrometry, Scanning Electron Microscopy(SEM), Elemental analysis.

FT-IR Analysis:
FT-IR spectrum of the compound shows a peak at N–H in NHOH (3409.002), O–H in NHOH (3741.042), –CO (1697.78), Acid chloride R-CO-Cl (1743.08), Amide CO-NH_2 (1539.50-1647.64) Fig 4.

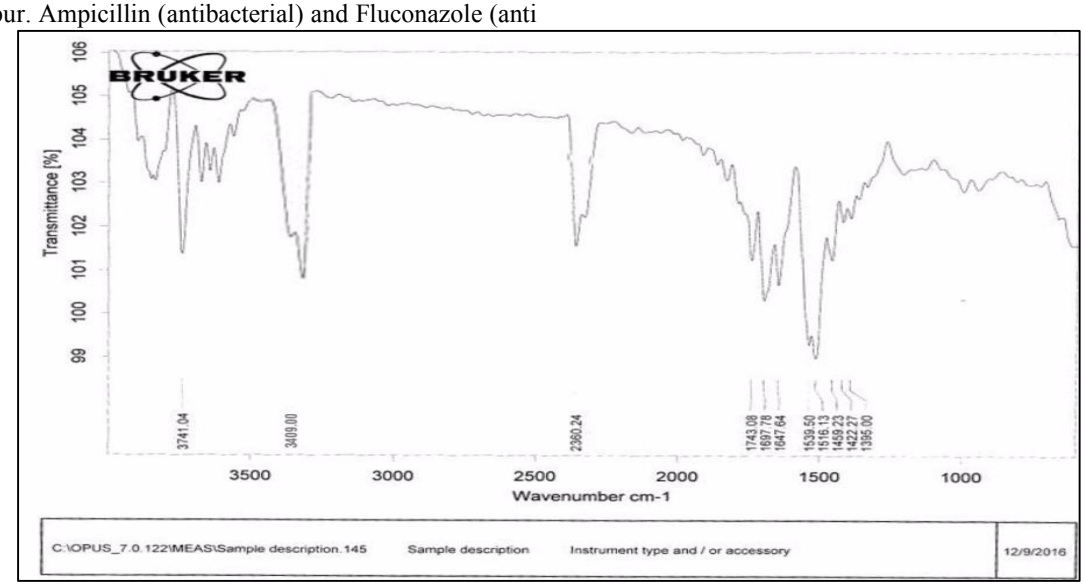


Fig 4 IR SPECTRA

H[1] NMR Analysis:
The compound was dissolved in DMSO. [1]H NMR peak interpretation shows peak at —NH—O **H** (δ 10.0-12.0,1H), —CO—N **H** —OH(δ 1.5-3.2), —Aromatic (Ar — H) (δ 7.3, 1H), -C H2 —O—R (δ 3.5-5.5, 2H), Fig 5

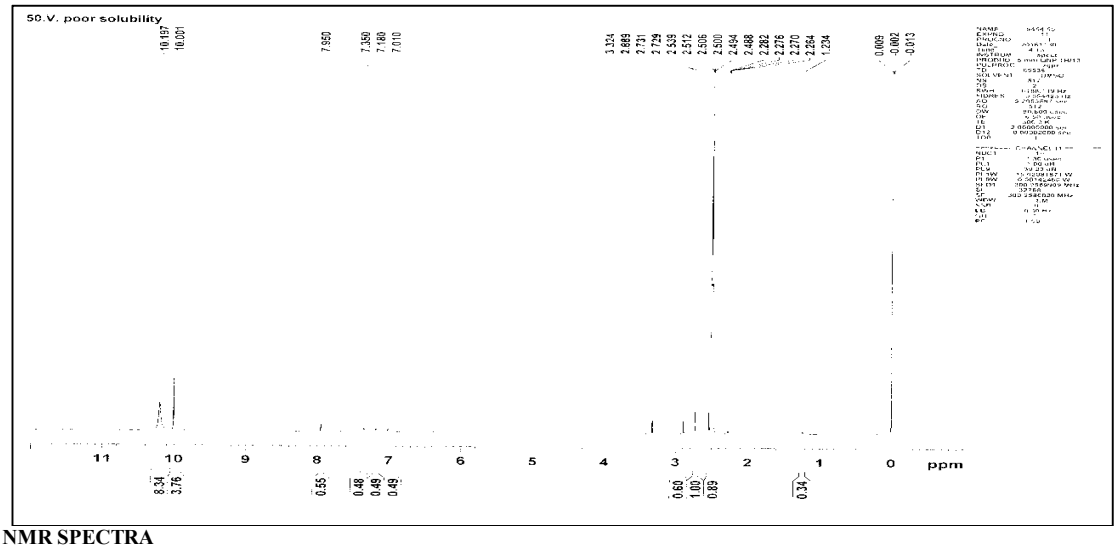


Fig 5 NMR SPECTRA

Mass spectral Analysis:
The base peak of CE-AH obtained at 391.38. Fig 6

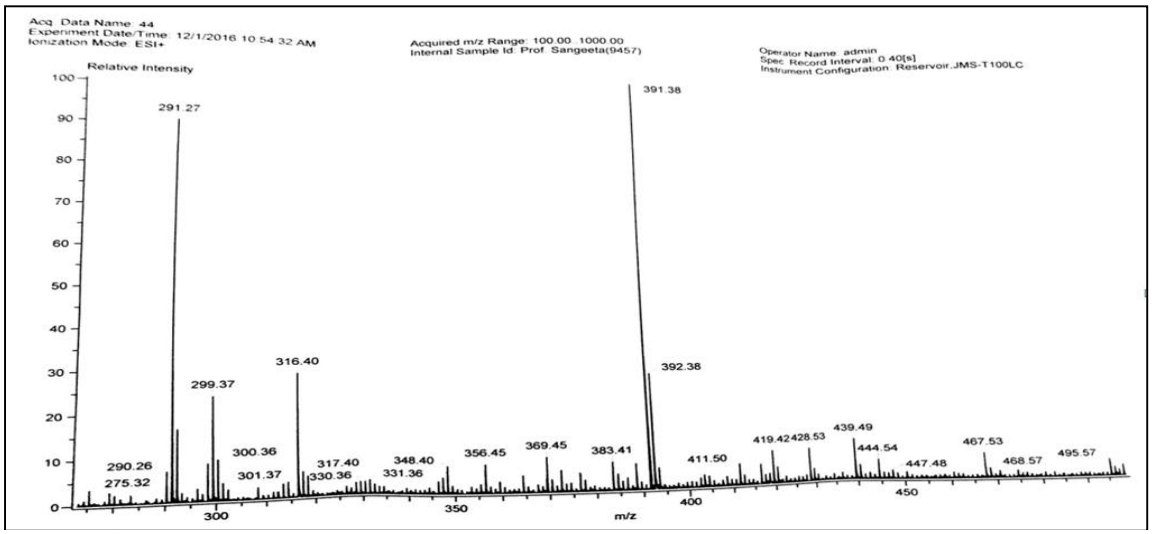


Fig 6 MASS SPECTRA

Thermogravimetric analysis:
TGA of the CE-AH was carried out using TGA Q 500 V 6.7 Build 203. The thermogram of the compound was analyzed for their thermal stability upto 600◦C under inert atmospheric conditions. It breaks at three points. First at 80c, here 20% weight loss occur mainly due to desorption of water. Second breakdown at 300◦C and 50% weight loss occur. Third breakdown at 500◦C. here maximum 80% weight loss occur due to break down of weaker bonds and loss of organics. it is clear that formation of chitosan derivative increases its thermal stability. Fig 7

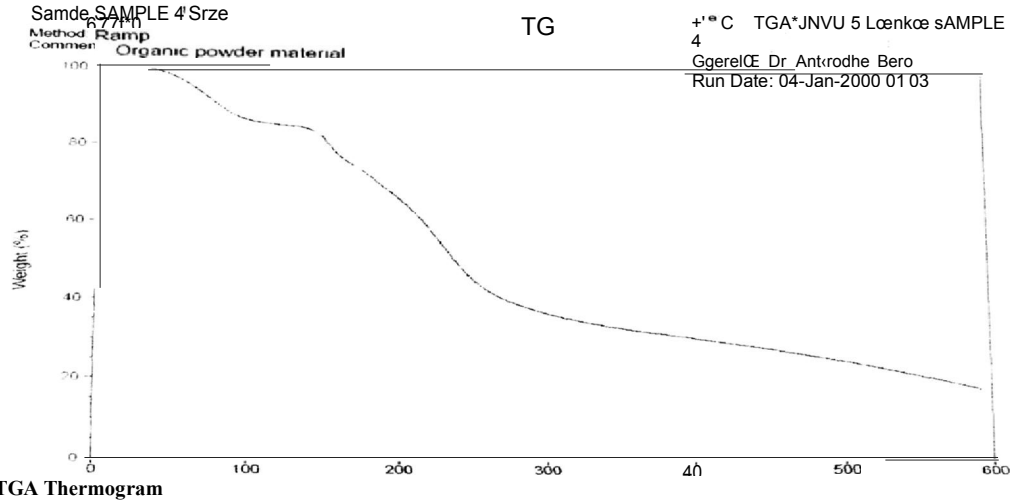


Fig7 :-TGA Thermogram

Scanning Electron Microscopy (SEM) and Elemental Analysis (EDX)

The SEM is used to generate high-resolution images of shapes of derivative and to show spatial variations in chemical compositions Thermogram of CE-AH show that the surface of derivative is smooth, porous and unequal size of particals. Fig 8-9

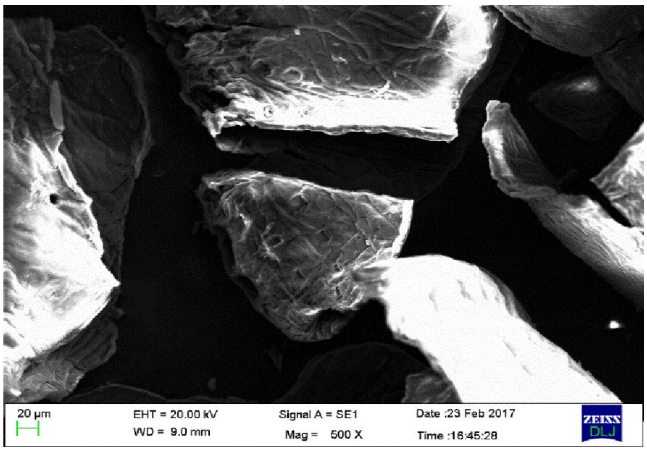


Fig 8 SEM IMAGE

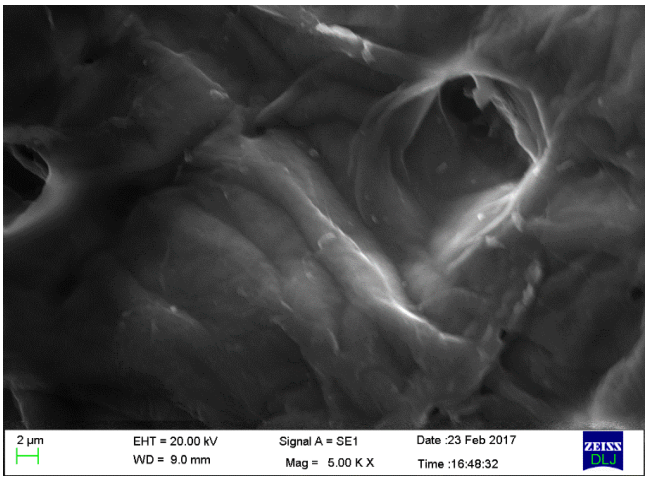


Fig 9 SEM IMAGE

ELEMENTAL ANALYSIS

Elemental analysis is a process where a sample of some material is analyzed for its elemental composition such as carbon, hydrogen, nitrogen and the major elements of the composite are carbon and Nitrogen. Composition of newly synthesized film is given in table 1.

Table 1 Elemental analysis

Element	Weight%	Atomic %
C	71.46	68.23
N	32.13	30.95

Antimicrobial activity:

The antimicrobial activity of newly chitosan derivatives against bacterial (gram negative and gram positive) and fungal strain are given table 2 and 3. It shows inhibition zone against *Klebsiella pneumonia, Staphylococcus aureus, Pseudomonas aeruginosa,* and no zone of inhibition against *Escherichia coli* funga strain *Candida albicans, Candida parapsilosis, Candida tropicalis, Candida cruise,* and no zone of inhibition against *Rhodoturula.* Fig 10-11

TABLE 2 :- Anti bacterial activity of CE-AH derivatives

S. no	Bacteria	Type	Inhibition Zone
1	*Pseudomonas aeruginosa*	Gram Positive	+ve
2	*klebsiella pneumonia*	Gram Negative	+ve
3	*Staphylococcus aureus*	Gram Positive	+ve
4	*Escherichia coli*	Gram Negative	-ve

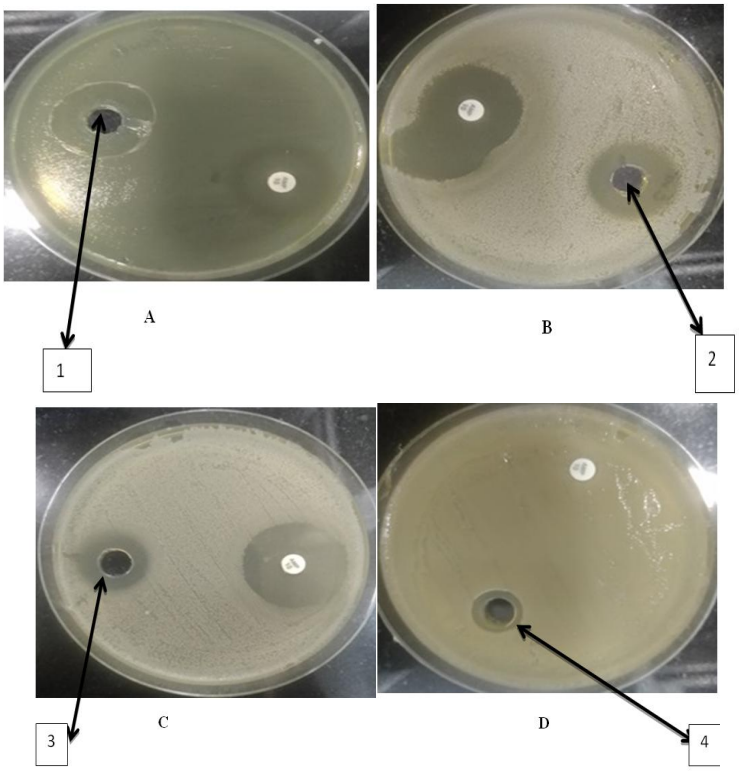
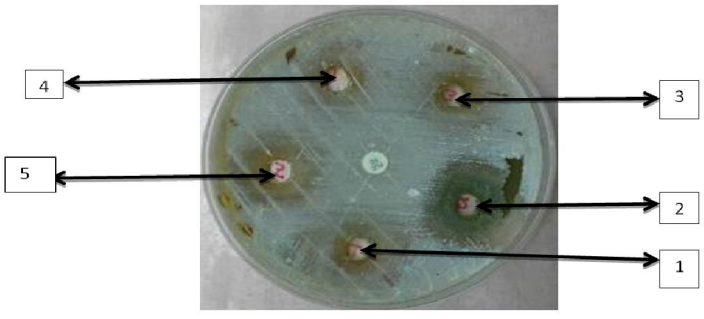


Fig10 Photographs showing Antibacterial activity [A],[B],[C],[D]



[E]

Fig11 Antifungal activity

TABLE 3 :- Anti fungal activity of CE-AH derivatives

S.no	Fungi	Inhibition Zone
1	*Rhodotorula*	-ve
2	*C. albicans*	+ve
3	*C.tropicalis*	+ve
2	*C.parapsilosis*	+ve
5	*C.krusei*	+ve

CONCLUSION:

In this study, synthesisof chitosan derivatives by an efficient microwave assisted method is described. ^{1}H NMR, IR, MASS analysis are used to confirm the polymer structure in much shorter time. The synthesized compound showed significant antimicrobial activity against bacteria and fungi. The mechanism of antibacterial activity of chitosan was that it could make the bacteria flocculate and kill it. The introducing of more amino groups directly on the back bone of chitosan, in addition to its original amine groups has succeeded in increasing its antibacterial activity and doesn't affect its cytotoxicity.

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