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Biogenic Synthesis Of Chitosan Silver Nanocomposites Using Mud Crab (Scylla Serrata) And Its Anti-Microbial And Anti-Cancer Studies

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Abstract

Chitosan is a marine polysaccharide commonly seen in the crustacean family, and the exoskeleton is covered by chitosan. In this study, shells of mud crabs were subjected to chitosan extraction which has been followed by demineralization, deproteination, and deacetylation. The chemical compositions of chitosans were characterized using Fourier transform infrared spectroscopy (FTIR). It exhibits antimicrobial activity against pathogens - E.coli, Staphylococcus, Klebsiella pneumonia, Bacillus cereus, and Salmonella typhi. It has therefore received attention as a potential food preservative. Transform Infrared Spectroscopy (FTIR) also spots out chemical bonds in a molecule by creating rather an infrared absorption spectrum. FTIR is an effective instrument for analysis and for also detecting the functional groups as well as characterizing covalent bonding information. FTIR spectroscopy finds out the functional molecules involved in the synthesis of nanoparticles and also, provides accuracy and reproducibility, which helps to detect small absorbance changes in the sample. The chemical compositions of chitosans were characterized further using FESEM.The FESEM pattern performs or indicates the silver nanoparticles to be embedded in a matrix of CS. The size of the particles is seen as with their uniformity. Not only this, but also the FESEM image of Ag nanoparticles shows somewhat the particles of spherical shape. Also, by Transmission Electron Microscopy (TEM) technique, the chemical compositions of chitosans were characterized. It exhibits that synthesized chitosan/silver nanocomposites have indeed a shape such as spherical with their uniformity as to their dispersion. Further, the chemical compositions of chitosans were characterized using Electron dispersive spectroscopy (EDS). The EDS histograms reveal rather successfully the percentage weight ratios of the elements found on the chitosan surface and the nanosilver concentrations in this study. In addition, by XRD analysis the chemical compositions of chitosans were characterized. It recognizes the multifarious crystalline phases present in the material so that the information touching the chemical compositions are revealed. In this study, mcf-7 cell line is chosen. Mcf-7 is a commonly used breast cancer cell line. For evaluation of

	anticancer study against mcf-7, the mcf-7 cell line is chosen for the study. In this study, anti-cancer study was very specially subjected to research and the chemical compositions of chitosans were characterized very seriously by FESEM, TEM, EDS, FTIR, and XRD for such anticancer study and therapy.
CC License CC-BY-NC-SA 4.0	***Keywords: Mud crab, Chitosan, Antibacterial activity, Antimicrobial activity, FTIR, FESEM, TEM, EDS, XRD, mcf-7 cell, Anti-Cancer study.***

1. INTRODUCTION

Chitosan is a marine polysaccharide commonly seen in the crustacean family, and the exoskeleton is covered by chitosan. Chitosan (CS) is a natural, hydrophilic, positively charged polymer with easy fabrication, good biocompatibility, and similarity of flexibility to natural tissue [2,6,11,13](1).CSNPs have been extensively used for bioactive compounds because of their high physicochemical stability, ability to enhance the bioavailability, non-toxicity, and potential targeted [16](2).Chitosan (deacetylated chitin) is a biopolymer prepared from shellfish and has non-toxic, biodegradable, and biocompatible advantages. It exhibits antimicrobial activity and has therefore received attention as a potential food preservative of natural origin. Moreover, chitosan displayed excellent antibacterial activity but it was ineffective in preventing oxidative rancidity (Badawy and Rabea 2017; Marei et al. 2018; Rabea et al. 2009).

Deacetylated form of chitin is chitosan which consists of a polymer of N-acetyl D-glucosamine bonded through β-1, 4-glycosidic bond (3, 4). This chitosan has multiple functional properties and biological activities thus used in pharmaceutical, nanotechnology, and agricultural industries (5- 8). Its antimicrobial activities made them to be used in food preservation as coating agents on wrappers of various foods (8). Their non-toxicity and non-allergenicity made them to be used in biomedical applications and also used in treating wounds (9-12), (4). Chitosan-coated nanoparticles are used for heavy metal removal, nanocarrier synthesis, drug delivery etc. (13-18).

For the sake of the research as to the anticancer nature of chitosan or for the sake of an invitro evaluation of anticancer potential of chitosan and chitosan silver nanocomposites for evaluation of antimicrobial and anticancer studies against cells like mcf7 and Hepg2, collection and extraction of chitosan and chitosan silver nanocomposites, and how such process is to be done is explored in this study. The same process is explored as with mud crab. Cancer is rather such a major crisis of public health worldwide and is as the second leading cause of death in the USA (19). Almost about 9.6 million people are estimated to have died in 2017.Thus, from many different forms of cancer. It seems that every sixth death has got occurred in the world due to cancer, making it as the death's second leading cause and the first being the cardiovascular diseases (20).Further, thus, the total number of cancer deaths inevitably continues to increase. Hence only such extraction of chitosan and chitosan silver composites for evaluation of anticancer study against cells like mcf7 and Hepg2 has been explored in this study and research very meticulously. Biocomposites are nothing but biocompatible or eco-friendly composites. They contain a large number of both organic and inorganic components, such as natural polymers, synthetic polymers, poly- saccharides, proteins, sugars, ceramics, metals, as well as nanocarbons (21). Biocomposites synthesis of composites is thus also explored, in this study, for the sake of anticancer study against cancer cells. Recently, the prevalence of cancer has tended to increase at a fast track, while attempts have been made to reduce cancer risk factors. Chemotherapy has, to date, been scientifically recognized as one of the primary cancer treatment methods. Chemotherapy uses chemicals in order to kill or prevent cancer cell growth. Drugs used for chemotherapy also drastically attack healthy cells. This unwanted or unexpected attack has failed the mode of conventional chemotherapy. It is therefore essential to establish effective treatments that can overwhelm or defeat the above difficulties. Thus only this study attempts rather to extract chitosan and chitosan silver Nanocomposites (22).These chitosan silver nanocomposites are vital most biocomposites against the cancer and are vital to give a remedy to cancer. While Chemotherapy uses chemicals to kill or prevent the growth of cancer cells, we tend to use these vital most composites or biocomposites like chitosan and chitosan silver nanocomposites against cancer cell growth, and hence, the research as to such extraction of chitosan silver nanocomposites in this study. When it comes to a question of characterization, in this study, the assembled chitosan, assembled on silver nanoparticles are prepared and characterized by UV–visible, TEM, FTIR, XRD, SEM techniques. Such characterization is introduced, explored in this study by means of solution mixing method. Thus, the obtaining of Ch/AgNPs (chitosan silver nanocomposites) process has been explored in this study (23). Chitosan and some of its complexes have been found good for many biomedical applications indeed (24).Hence, the total attempt in this research or study is as towards extracting chitosan silver nanocomposites.The present day existing anticancer therapies such as chemo, radio, and hormone therapy are connected with a high load of reversible as well as irreversible adverse effects, limited therapeutic efficacy as along with low chances of quality survival. Hence, in order to minimize the side effects importantly, the improving therapeutic potency or promisingly targeted patient compliance cancer therapies are highly desirable (25) and in quest of such therapies, this attempt and new study such as this paper has been presented here. Thus, very meticulously an invitro evaluation of anticancer potential of chitosan silver nanocomposites for evaluation of antimicrobial and anticancer study against cancer cells has been attempted in this paper or study. Due to its (chitosan's) biocompatible and biodegradable nature, it is utilized in biomedical applications very much and thus, in scaffold engineering as an absorption enhancer, and it is also utilized for bioactive and controlled drug release. In cancer therapy, chitosan has multifaceted applications, such as assisting in gene delivery and as well in chemotherapeutic delivery, and as an immune adjuvant for vaccines: which is presented and highlighted in this study as recent applications of chitosan and chitosan derivatives like chitosan silver nanocomposites in cancer therapy (26).

Antibacterial and antimicrobial agents like chitosan as well as its derivatives like chitosan silver nanocomposites and chitosan's disinfected systems are becoming more and more necessitate or important every day or day by day. Hence only, they have been studied for possible use in a numerable healthcare environments, industries, laboratories, and even houses (Ali et al. 2015) (Hosseinnejad and Jafari 2016) (27). Most vitally, the use of these materials like Antibacterial and antimicrobial agents is as to sterilize medical environments and equipments so that thousands of deaths can be assuredly prevented and these deaths are due to hospital-acquired infections, such as linens and clothing where bacteria could grow and infect the human body (Yılmaz Atay and Çelik 2017) (28).Thus, as in quest of preventing the cancer deaths, this study has been made with utmost a meticulous research.

2 MATERIALS AND METHODS

2.1 Sample

Mud crab shells used in this study were obtained from Ashtamudi Lake, Kollam district, Kerala. The crab shells were thoroughly washed to remove impurities using water. The washed shells were air-dried for two weeks. Shell particles were grinding well for easy extraction. The fine sample was stored in an airtight container for further analysis.

Chemicals and reagents

The chemicals used in this study include Sodium hydroxide, Hydrochloric acid and such all were supplied by Nice Chemical industry, India. Distilled water also was used for the analysis.

2.2 Methods

Extraction of Chitosan from chitin using chemicals in three stages. Chitin and chitosan are both polysaccharides, and chemically these are similar to cellulose. Chitosan, which has received a considerable attention because of its properties, is indeed as a natural as well as a linear polysaccharide made from chitin by a chemical process involving deproteinization, demineralisation**,** decolouration and deacetylation(Bautista-Baňos et al. 2006)(29).

1) Deproteinization:

The deproteinization step is a difficult step due to the event of disruption of chemical bonds between chitin and proteins. But anyhow, deproteinization is performed heterogeneously by using the chemicals which also do depolymerize the biopolymer. The absolute removal process has to be done to remove the protein present for the end of biomedical applications, because the human population is allergic to the primary culprit, the protein component (30).

The mud crab shells were deproteinized by treating 2.5 g of the mud crab shells in a beaker containing 1.25M NaOH and 50 ml distilled water and allowing for two hours of shaking at 70 rpm and then after that(the shaken) being put in a water bath for 1 hour (at 70 degrees). The mixtures were allowed to settle and the excess NaOH was removed by decantation, followed by washing with tap water until pH neutral was obtained. Filtration was performed using Whatman filter paper No 1. The collected residues were dried in sunlight to obtain a deproteinized material.

2) Demineralization:

Demineralization follows the method of the removal of the minerals, especially such as calcium carbonate ($CaCO_3$) as by means of an acid treatment. And that acid will be or can be such as hydrochloric acid (HCl),

nitric acid (HNO3), sulfuric acid (H2SO4), or acetic acid (CH3COOH) conventionally. Such conventional demineralization process is usually carried out using dilute hydrochloric acid rather (31).
In the step of demineralization of the deproteinized crab shells with 2M of HCl, placed in a water bath (at 80 degrees) for 1 hour, the mixture was allowed to cool and settle. Excess acid was removed by washing with tap water to a neutral pH. Finally followed by filtration using Whatman filter paper No.1, chitin is obtained and further, the chitin obtained was dried in sunlight.

3) Deacetylation:
Chitin is an insoluble linear polymer of β-1, 4-N-acetyl-d-glucosamine (GlcNAc; A). It can all be converted to chitosan, which is a soluble heteropolymer of GlcNAc and d-glucosamine (GlcN; D) residues, by means of partial deacetylation (32).
Extracted chitin was treated with 50% concentrated NaOH to remove the acetyl groups of chitin. This process is called deacetylation. After deacetylation, the sample was filtered and washed several times with tap water until the pH was neutral.

Biogenic synthesis of Chitosan Silver nanoparticles
Synthesis of silver nanoparticles–chitosan composite particles sphere (AgNPs-chi-spheres) was rather characterized and was indeed fulfilled by UV–vis spectroscopy, Fourier transform infrared (FT-IR) spectroscopy, X-ray diffraction (XRD), and finally by scanning electron microscopy (SEM). Further also, it was characterised by zetasizer nano. UV–vis spectroscopy characterization performed or showed that AgNPs-chi-spheres gave maximum absorption at a wavelength of 410 nm. Also, the XRD spectra showed that the structure of AgNPs-chi-spheres were crystalline and spherical.

UV- VIS study
UV–vis is a common mode or technique to characterize nanoparticles like chitosan nanoparticles. This technique allows to confirm the formation of the nanoparticles by means of sheer measuring the Surface Plasmon Resonance (SPR).Preparation and characterization of Chitosan nanoparticles (CHNP) is presented with the aim of determining the particle size indeed and morphology, surface chemistry, thermal stability through a simple and cost effective process of production preparation.
CHNP were prepared through drop wise addition of sodium tripolyphosphate solution to chitosan solutions in the ratio of 1: 1 under stirring. Chitosan gave a yield of 61% from the crab shell and a degree of deacetylation (DOD) of 75%. The low DOD could be responsible for the high yield of chitosan which is due to the incompletely removed acetyl groups in chitin. The final weight of the chitosan was observed to have increased after the conversion to nanoparticles, and this could be as a result of the crosslinking of the chitosan and the TPP.
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In this study, UV – Vis study was employed to exhibit surface Plasmon resonance at the wavelengths of 300 – 600 nm using a Labtronics LT291 spectrophotometer. UV-Vis spectroscopy is a reliable technique for the primary characterization of synthesized nanoparticles.

XRD analysis or X-Ray Diffraction

X-Ray Diffraction is an analytical technique that is as non-destructive one and frequently engaged as to analyse nanomaterials for their crystalline structures indeed. This XRD technique often helps to recognize the multifarious crystalline phases present in the material so that the information touching the chemical compositions are revealed.

By using different concentrations of silver nitrate, the X-ray diffractogram of synthesized chitosan-silver nanocomposites can be done (34).

In this study, carboxymethyl chitosan–nanosilver hybrids or silver nanocomposites were prepared at 80 °C using different concentrations of silver nitrate and characterised.

FTIR

Characterization of Chitosan

FTIR spectroscopy is used to find out the functional molecules involved in the synthesis of nanoparticles. FTIR provides accuracy and reproducibility, which helps to detect small absorbance changes in the sample.

Using the Shimadzu instrument from 4000cm-1 to 400cm-1, scanning was done and finalised the compounds. Vibration patterns that are reached or obtained from the spectrum of FTIR perform or point out the functional groups present, which are as carbon, nitrogen, and oxygen. The FTIR spectra, of course, formed bands such as characteristic bands in the frequency range between 4000 and 400 cm–1.The mud crab shell waste was gotten from the carotenoid extraction process and then, consecutively it was meticulously treated with 2.0 % of potassium hydroxide (KOH) solution and KOH was with such a ratio as of ground shell to the solution of 1:20 (w/v), with a constant stirring mode for 2 h at 90 °C to remove the protein indeed. Fourier Transform Infrared Spectroscopy (FTIR) spots out chemical bonds in a molecule by creating rather an infrared absorption spectrum. FTIR is an effective instrument for analysis and for also detecting the functional groups as well as characterizing covalent bonding information.

In this study, FTIR spectroscopy is used to find out the functional molecules involved in the synthesis of nanoparticles.

FTIR provides accuracy and reproducibility, which helps to detect small absorbance changes in the sample. Using the Shimadzu instrument from 4000cm-1 to 400cm-1, scanning was done and finalised the compounds.

FESEM

The FESEM pattern performed or indicated the silver nanoparticles to be embedded in a matrix of CS. The shift such as in the peaks of CS caught under observation in the FTIR spectrum points out the formation of CS–Ag composite.

FESEM technique was used here in this study and with the FESEM image of CS performs (shows) bundle form of particles with a morphology of a leaf. Not only this, but also the FESEM image of Ag nanoparticles shows somewhat the particles of spherical shape. The size of the particles is seen as with their uniformity and the mixture of CS and Ag is seen as is shown by the FESEM image of CS–Ag nanocomposite (35).

SEM is a surface imaging method, fully capable of resolving particle sizes, nanomaterial shapes, and the surface morphology of the synthesized particles at the micro and nanoscales. ZEISS SEM instrument was used here to identify the size and shape of the synthesized nanoparticle.

EDS

Electron dispersive spectroscopy (EDS) is such a technique which has been used to examine the elements' presence as to and through the wavelength amplitudes of the x-rays which stand as emitted after their electrons have been hit by some other electron beam (Swapp, 2012) (36).The EDS of chitosan, nanosilver and chitosan–silver nanocomposites were respectively prepared in this study. The presence such as of nanosilver on the chitosan surface assured absolutely the preparation of the chitosan-silver nanocomposites successfully. The EDS histograms revealed rather successfully the percentage weight ratios of the elements found on the chitosan surface and the nanosilver concentrations in this study.

TEM

Transmission Electron Microscopy (TEM) is a technique. It is such a technique in which a beam of high-energy electrons transmitted through the solid is used and it is a technique by imaging the internal structure of solids. Transmission Electron Microscopy (TEM) technique is such an arrangement that can be compared to the basic optical microscope. TEM image pointed out or performed that synthesized chitosan/silver nanocomposites have indeed a shape such as spherical with their uniformity as to their dispersion and with their average particle size of about 20-30 nm (37).

Antibacterial study

Silver nanoparticles (Ag NPs)'s antibacterial efficacy was studied especially with the association of chitosan silver nanocomposites in this study. Layers were deposited alternatively as layer by layer (LBL) on chitosan polymer in a thin film shape over a quartz plate and stainless steel strip. In this study, we synthesised the eco-friendly green synthesis of hybrid chitosan-silver nanoparticles (CS-AgNPs) and for that it was studied as to the use of Lactobacillus reuteri ATCC 55730 or the cell free extracellular biomass of L. reuteri as a reducing as well as capping agent for the synthesis of CS-AgNPs (38).

Cytotoxicity analysis against MCF7 Cell line

Chitosan (Cs) and its silver nanocomposites are as a biocompatible, biodegradable cationic polymer having a huge potential or the ability of targeted drug delivery and these also showed, in our study, higher antioxidant activity than their base materials. Further, their anti-cancer efficacy was observed rampantly against MCF-7 breast cancer cells in this study (40).

3. RESULTS AND DISCUSSION

FTIR

Characterization of Chitosan

Vibration patterns that are reached or obtained from the spectrum of FTIR perform or point out the functional groups present, which are as carbon, nitrogen, and oxygen. The FTIR spectra, of course, formed bands such as characteristic bands in the frequency range between 4000 and 400 cm–1.The mud crab shell waste was gotten from the carotenoid extraction process and then, consecutively it was meticulously treated with 2.0 % of potassium hydroxide (KOH) solution and KOH was with such a ratio as of ground shell to the solution of 1:20 (w/v), with a constant stirring mode for 2 h at 90 °C to remove the protein indeed. Fourier Transform Infrared Spectroscopy (FTIR) spots out chemical bonds in a molecule by creating rather an infrared absorption spectrum. FTIR is an effective al instrument for analysis and for also detecting the functional groups as well as characterizing covalent bonding information.

In this study, FTIR spectroscopy is used to find out the functional molecules involved in the synthesis of nanoparticles. FTIR provides accuracy and reproducibility, which helps to detect small absorbance changes in the sample. Using the Shimadzu instrument from 4000cm-1 to 400cm-1 scanning was done and finalised the compounds. The results are as shown in the following fig.1. The result can be confirmed as: The FTIR spectra formed characteristic bands in the frequency range between 4000 and 400 cm–1.
The spectra were also minutely and meticulously compared with the standard chitosan and resultant correlations were observed in the spectra in the following fig.1. The spectra were also minutely and meticulously compared with the standard chitosan and resultant correlations were observed in the spectra in the following fig.1.

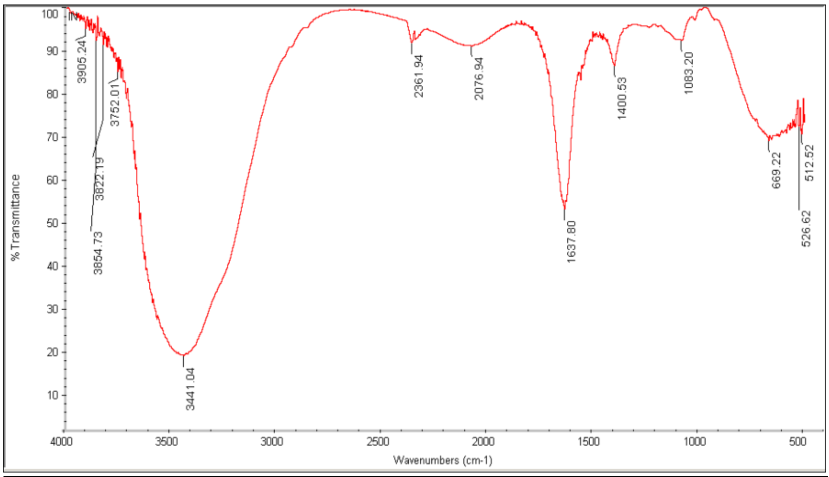


Fig.1. FTIR spectra of the chitosan silver nanocomposites extract.

EDS

Electron dispersive spectroscopy (EDS) is such a technique which has been used to examine the elements' presence as to and through the wavelength amplitudes of the x-rays which stand as emitted after their electrons have been hit by some other electron beam (Swapp, 2012)(36).The EDS of chitosan, nanosilver and chitosan-silver nanocomposites were respectively prepared in this study. The presence such as of nanosilver on the chitosan surface assured absolutely the preparation of the chitosan-silver nanocomposites successfully. The EDS histograms revealed rather successfully the percentage weight ratios of the elements found on the chitosan surface and the nanosilver concentrations in this study. The results are as shown in the following fig.2.

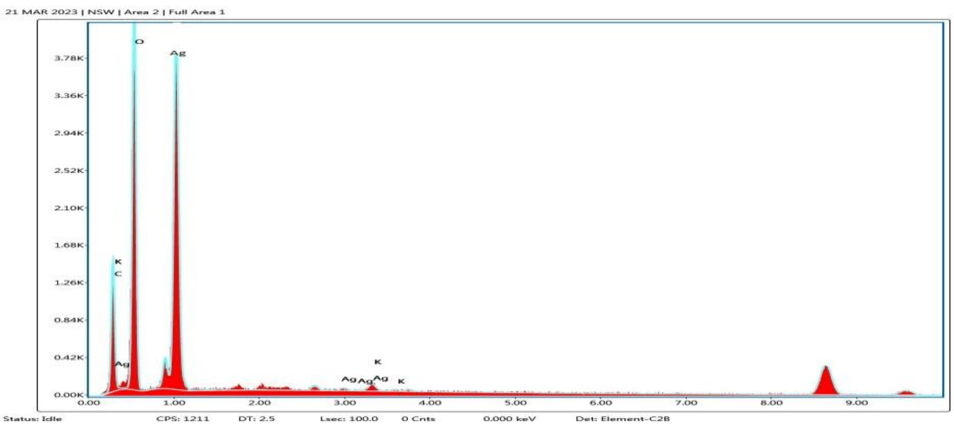
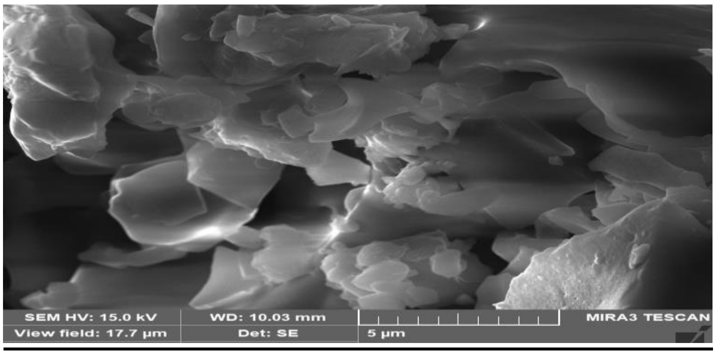


Fig.2. EDS characterisation for the synthesis of chitosan silver nanocomposites.

SEM

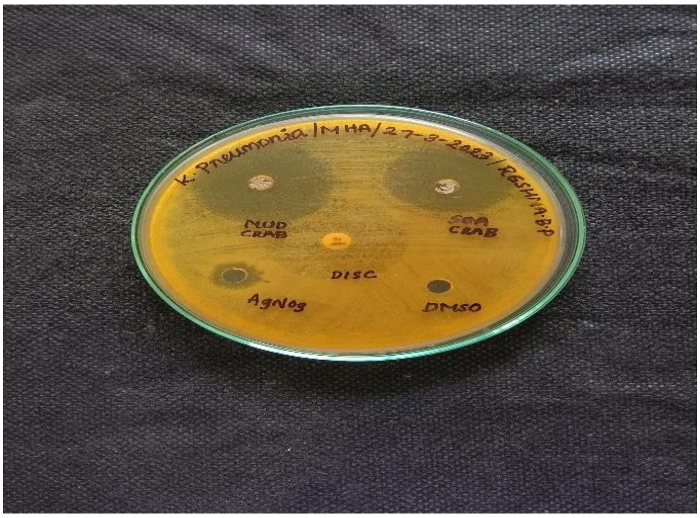
SEM is a surface imaging method, fully capable of resolving particle sizes, nanomaterial shapes, and the surface morphology of the synthesized particles at the micro and nanoscales. ZEISS SEM instrument was used here to identify the size and shape of the synthesized nanoparticle. The image obtained was as following. SEM images of silver based chitosan nanocomposite was viewed in different magnification as following. In order to determine the morphology of Chitosan silver nanocomposites, SEM images were recorded. One of the recorded images is as follows.

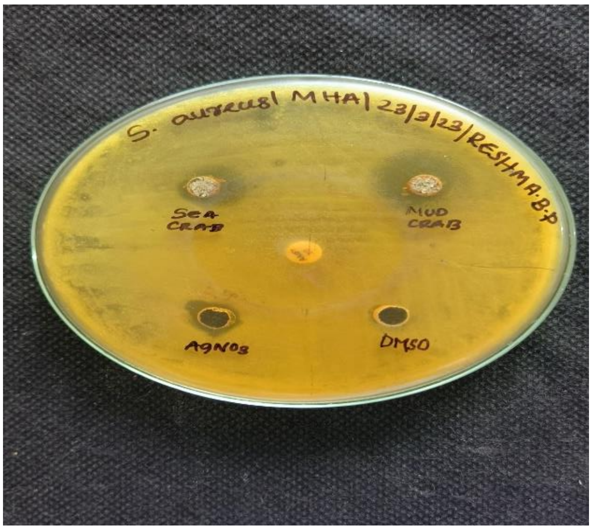


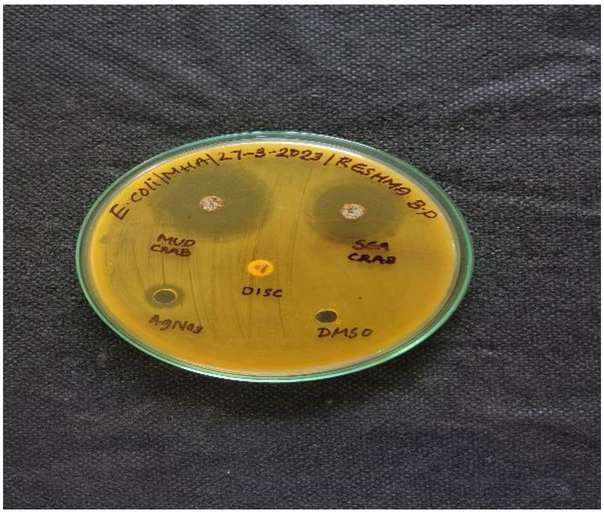
The findings for the antibacterial activity were as follows comparatively: comparing mud crab's, sea crab's antibacterial activity as follows.

ANTIBACTERIAL ACTIVITY

BACTERIA	SAMPLE USED				
	MUD CRAB	**SEA CRAB**	**AGNO3**	**DISC**	**DMSO (-)**
E-coli	20 mm	16 mm	4 mm	--	--
Staphylococcus aureus	8 mm	4 mm	2 mm	--	--
Klebsiella pneumonia	26 mm	18 mm	4 mm	--	--
Bacillus cereus	4 mm	20 mm	4 mm	--	--
Salmonella typhi	18 mm	6 mm	4 mm	--	--







The following anticancer reading was obtained as the result of the study and the experiment comparatively: and herein, the comparison between the concentration results of both mud crab and sea crab is given:

ANTICANCER READING –ELISA

SAMPLE	CONCENTRATION				
	5	**10**	**15**	**20**	**25**
MUD CRAB	0.563	0.508	0.482	0.433	0.351
SEA CRAB	0.612	0.582	0.514	0.474	0.426

Control = 0.765

$$\% \text{ of cell death} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} * 100$$

$$= \frac{0.765 - 0.563 * 100}{0.765}$$

$$= 26.40$$

SAMPLE	CONCENTRATION				
	5	10	15	20	25
MUD CRAB	26.40	33.59	36.99	43.39	54.11
SEA CRAB	20	23.92	32.81	38.03	44.31

Conclusion

In this study, crab shells were used for deriving chitosan. FTIR was used for making chitosan silver nanocomposites. The derived chitosan silver nanocomposites were characterized using FTIR, EDS and SEM very specially to determine as to the antimicrobial activity and anticancer activity. Chitosan Silver nanocomposite exhibited excellent antimicrobial and anticancer activity against cancer cells like mcf 7 and Hepg2 cells. The chitosan silver nano composites effectively controlled indeed the biofilm growth of cancer cells. No such cytotoxic effects of chitosan silver nanocomposite were observed against murine macrophages, and it was successfully confirmed that chitosan silver nanocomposites could be safely used in therapeutic purposes.

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