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Synthesis, Characterization and Antibacterial Activity of Ternary Nanochitosan / Iron Oxide Nanoparticles / Egg Shell Blend

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Abstract

The current research work reports the synthesis, characterization and evaluation of antimicrobial behavior of ternary nanochitosan/iron oxide nanoparticles/egg shell blend prepared in 1:1:1 ratio. The prepared ternary blend samples were characterized using FT-IR and XRD studies respectively. FT-IR results of the prepared ternary nanochitosan/iron oxide nanoparticles/egg shell blend clearly indicate the formation of hydrogen bonding and in addition the appearance of new peaks suggested that the nanochitosan gets binded effectively with the iron nanoparticles and egg shell. The change in crystallinity was elucidated from XRD analysis and also the potentiality of antimicrobial behavior of the synthesized ternary nanochitosan/iron oxide nanoparticles/egg shell blend against four bacterial species namely *Staphylococcus aureus, E. coli*, *pseudomonas aureginosa, shigella* and against three fungal species namely *Aspergillus niger, Rhizopus and mucor* were tested and evaluated. These observed results of bactericidal and fungicidal action of the synthesized ternary nanochitosan/iron oxide nanoparticles/egg shell blend reveals that the prepared ternary blend has the greater potential to kill the microorganisms to a greater extent and hence this synthesized ternary nanochitosan/iron oxide nanoparticles/egg shell blend was found to be suggested as the promising candidate for biomedical applications.

Keywords

Nanochitosan, iron oxide nanoparticles, egg shell, antibacterial, antifungal.

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INTRODUCTION:

Nano-sized composites (nano particles, nano materials) are able to attach more copies of microbial molecules and cells which are found to be effective against a variety of organisms. [1,2] They are expected to be more effective in penetrating and disrupting bacterial cell membranes. Based on this regard, increasing investigation has been given to chitosan and its derivatives by many researchers. The molecular structure of chitosan is prerequisite for its antimicrobial activity and is widely recognized for its potent antimicrobial activity against a wide range of target organisms like algae, bacteria, yeasts and fungi in experiments involving in vivo and in vitro with high killing rate but low toxicity toward mammalian cells. [3,4,5]

Especially from a pharmaceutical viewpoint, the modification of chitosan nanoparticle has received much attention as polymeric platforms in relation to their potential application due to its non-toxic property, biocompatibility, biodegradability, high permeability, cost-effectiveness and an excellent film forming ability.[6,7] Small size and quantum size effect makes chitosan nanoparticles to exhibit superior activities.

In rural areas of many developing countries, the natural products were utilized as medicines since it is cheaper, more effective and impart least side effects as compared to synthetic medicines.[8,9] Because of the apparent low toxic effects on human cells, the usage of natural products for the development of antimicrobials has become attractive. Among the various natural products, the eggshell is chosen in this work since it is a complex, multifunctional biomineral material with an organic phase of lipids and proteins made up of polysaccharides, hydroxyapatite crystals, lipids and glycoprotein with antimicrobial principles.[10]

In recent years, research groups worldwide are focusing on NPs with antimicrobial properties as a promising tool towards controlling microbial adhesion. The recent developments of nanotechnology in synthesizing biocompatible and functionalized magnetic nanoparticle have numerous applications in various fields. Due to outstanding magnetic, physico-chemical, thermal and mechanical properties, the iron oxide magnetic NPs can be utilized in various fields. Based on the literature survey, the main aim of this work was to evaluate the antibacterial activity of the ternary nanochitosan/iron oxide nanoparticles/egg shell blend prepared in 1:1:1 ratio and the obtained results were investigated in detail.

MATERIALS AND METHODS:

Materials:

The egg shells were collected from local market of Vellore, India. Chitosan biopolymer was procured from Indian Sea Foods, Cochin, Kerala and certain chemicals namely sodium tripolyphoshate, glacial acetic acid, oxalic acid, ferrous sulphate and polyvinyl alcohol was purchased from Nice Chemicals Pvt Ltd, Thomas Bakers Pvt Limited, Sisco Research Laboratories Pvt Ltd. All the chemicals used in the present research work were of analytical grade.

Preparation of nanochitosan:

By the interaction of oppositely charged macromolecules (positively charged chitosan macromolecule with the anionic tripolyphosphate), the nanochitosan can be prepared in this work through ionotropic gelation method. Initially the homogeneous chitosan solution was synthesized by dissolving 1g of chitosan in 200 ml of 2% acetic acid solution prepared in deionized water. Followed by this dissolution, the complete stirring of the chitosan solution was done for about 15 minutes. After this stirring process is over, the sodium tripolyphosphate solution (0.8g of TPP (sodium tri poly phosphate) dissolved in 107ml of deionized water) is added to the above prepared homogeneous chitosan solution and this solution mixture was then allowed to stir well using magnetic stirrer for a period of 30 minutes. A milky emulsion like appearance of nanochitosan obtained was then allowed to settle for 24 hours, mother liquor is decanted and the milky suspension like substance settled at the bottom (NCS) of beaker is kept in a freezer and stored for further use.

Preparation of egg shell:

The egg shell collected from local market was washed with double distilled water. The washed egg shells were left to air-dry and then placed in glass jars in the oven at 100°C for 24 h to dry to constant weight. The dried eggshells were crushed in a clean sterile porcelain mortar and further ground in a Warren blender into fine powder. This finely powdered egg shells were preserved in different tightly packed containers for subsequent use. The photograph of the prepared egg shell powder was shown below (Figure-A).

Figure-(A): Egg shell powder

Preparation of iron oxide nanoparticles by hydrothermal method:

The iron oxide nanoparticles were synthesized using low temperature combustion method by employing iron oxalate as precursor. By utilizing the hydrothermal method reported by Priscilla Prabhavathi and her coworkers [11], the iron oxide nanoparticles can be prepared in two stages as follows:

Stage: 1- Synthesis of Iron oxalate Precursor

Equimolar amounts of ferrous sulphate and oxalic acid (each 0.25g) taken in a beaker was dissolved in minimum volume of water and stirred for 15 minutes using a magnetic stirrer. This will lead to the formation of iron oxalate dehydrate (yellow precipitate). It was then filtered, washed with distilled water and finally dried with acetone. The photograph of the prepared iron oxalate precursor was represented below (Figure-B).

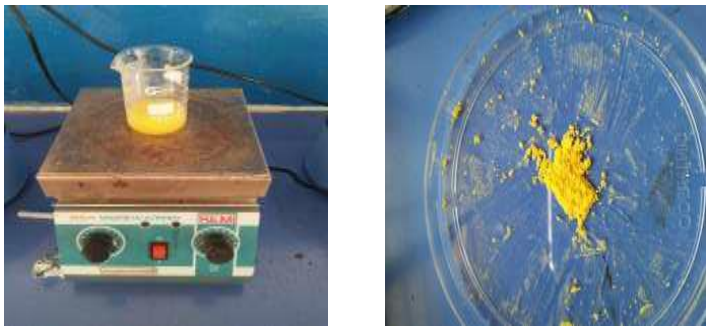


Figure-B: Photograph of Iron oxalate

Stage: 2- Synthesis of iron oxide nanoparticles

The above prepared iron oxalate precursor was mixed with polyvinyl alcohol in the weight ratio of 1:5. The resultant mixture was then powdered well in a mortar, mixed in a silica crucible and ignited in an electric furnace and while heating, the temperature should not exceed 300°C. During the heating process, initially the PVA is melted, then frothed and finally undergoes complete ignition process leading to give iron oxide as residue. The obtained sample (iron oxide residue) is then calcinated at 110°C for 4 hours to remove impurities and the iron oxide nanoparticles was then cooled, dried and powdered for further use. The photograph of the prepared iron oxide nanoparticles was represented below (Figure-C)

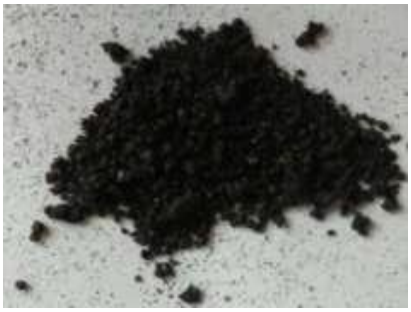


Figure-C: Photograph of iron oxide nanoparticles

Preparation of nanochitosan/iron nanoparticles/egg shell blend:

About 1g of the above prepared nanochitosan, 1g of iron oxide nanoparticles and 1g of egg shell powder taken in a beaker were dissolved in minimum amount (10 ml) of distilled water and this solution mixture was then stirred well completely (30 minutes) by using magnetic stirrer. Followed by this, the above prepared ternary blended mixture was then poured into petri plates, allowed to dry and then stored in air tight box for further use as biosorbent.

CHARACTERIZATION:

FT-IR Spectral analysis

With the help of 200 FT-IR spectrophotometer, the FT-IR spectrum of prepared samples were recorded in the wave number range from 400-4000cm^{-1} with the resolution of 2 cm^{-1} at 25°C during 64 scans.

X-ray diffraction (XRD) Analysis

To know whether the prepared nanochitosan/iron nanoparticles/egg shell blend is crystalline or amorphous, X-ray diffraction study was done. The X-ray diffractogram of the prepared samples was tested by an X-ray scattering SHIMADUZ XD Diffractometer using Ni filter Cu Kα radiation source (λ=0.154nm), set a scan rate = 10/min, using a voltage of 40kV and a current of 30 mA.

RESULTS AND DISCUSSION:

Fourier Transform IR Spectroscopy

The FTIR spectrum of nanochitosan/iron nanoparticles/egg shell blend was taken for structural characterization. FTIR has been used to investigate the possible chemical interactions via functional groups between nanochitosan and added iron nanoparticles and egg shell. Also, this FT-IR technique helps in directly monitoring the vibrations of the functional groups which characterize molecular structure and can provide certain structural clues to the overall structure

of the unknown substance. Figure-1 represents the FT-IR spectral details of nanochitosan prepared from chitosan by ionotropic gelation method.

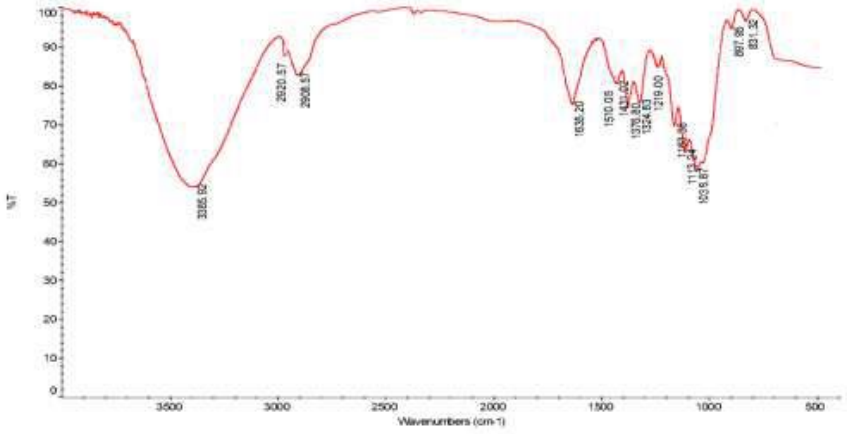


Figure-1: FT-IR spectrum of nanochitosan

The strong and wide broad peak obtained in the wave number range between 3500-3300 cm^{-1} in case of nanochitosan (Figure-1) is attributed predominantly to the hydrogen-bonded O-H stretching vibration (3385.92 cm^{-1}) arising due to physical interactions with TPP. Peaks of N-H stretching from primary amine have been overlapped in the same region [12]. Certain absorption bands appeared at around 2920.57 cm^{-1}, 2908.57 cm^{-1}, 1635.23 cm^{-1}, 1510.05 cm^{-1} and 1376.80 cm^{-1} represents the asymmetrical and symmetrical stretching in CH_2 group, NH_3^+ stretching, C=O stretching in amides, NH bending and OH in plane bending in alcohols respectively. [13]

Appearance of new absorption band at around 1219.00 cm^{-1} attributed to P=O linkage indicate that the cross links were developed between the ammonium ion in chitosan molecule and phosphate ion present in ionic cross linking agent (TPP).[14] These observed results concluded that the tripolyphosphoric groups of TPP gets cross linked effectively with the ammonium groups of chitosan resulting in the formation of nanochitosan with various inter and intra-molecular linkages.[15]

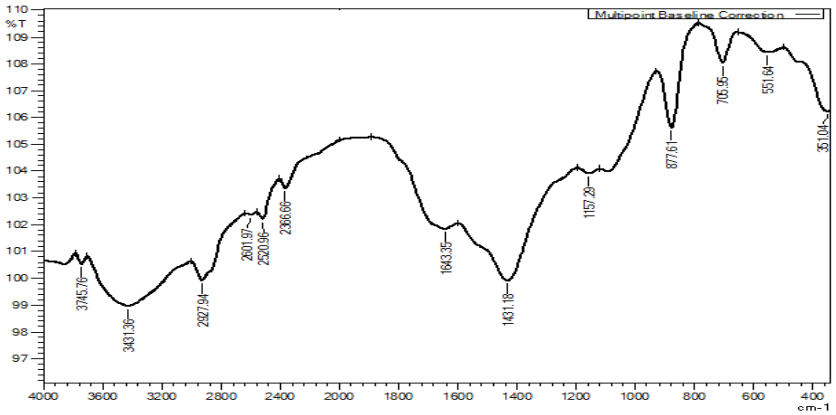


Figure-2: FT-IR spectrum of Nanochitosan/Iron oxide nanoparticles/egg shell ternary blend

The FT-IR spectral details of nanochitosan/iron oxide nanoparticles/egg shell ternary blend represented in **Figure-2** showing the prominent broad band at 3431.36 cm^{-1}, 2601.97, 2520.96cm^{-1} was assigned to the intermolecular hydrogen bonded OH stretching in alcohols and OH stretching in acids, calcium carbonate(carbonate ion)[16] present in egg shell respectively. Due to the presence of aliphatic C-H stretching in methylenic group a peak was appeared at 2971.94 cm^{-1} and certain strong bands observed at 2366.60 cm^{-1}, 1643.35cm^{-1}, 1431.18cm^{-1}, 1157.29 cm^{-1}, 877.61 cm^{-1}, 705.95 cm^{-1} and 551.64 cm^{-1} corresponds to NH_3^+stretching, C=O stretching in acids, C-N Stretching, P=O stretching, Fe-O stretching (magnetite linkage) [17] and NH wagging respectively.

The comparison of FT-IR spectral details of prepared ternary blend with nanochitosan, reveals that certain main bands observed due to carbonyl stretching, O-H

stretching in nanochitosan were shifted to lower wavenumbers in case of ternary blend. These observed shifting of peaks to the various wave numbers indicate that the nanochitosan, egg shell and iron oxide nanoparticles were blended effectively in the ternary blend formation.Also it was identified that the broadening of band appeared at around 3435.22 cm^{-1} in case of ternary blend suggests that the intermolecular hydrogen bonding had taken place effectively between the added components.In addition, the effective blending process can also be concluded from the appearances of new additional peaks in the case of ternary blends due to the presence of acids, calcium carbonate, vibrations of the Fe-O bonds when compared to the nanochitosan. Hence from these observed additional peaks it was evident that the nanochitosan gets blended effectively with iron nanoparticles and egg shell respectively.

X-ray diffraction (XRD) studies

X-Ray Diffraction (XRD) is a fast-analytical method which is mainly utilized for the identification of a crystalline material. The analyzed material, which is finely ground, homogenized and average bulk composition was tested via XRD. Besides, this XRD technique is also able to provide information on unit cell. [18] The X-ray diffractogram details of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend prepared in 1:1:1 ratio was shown in **Figure-(3).**

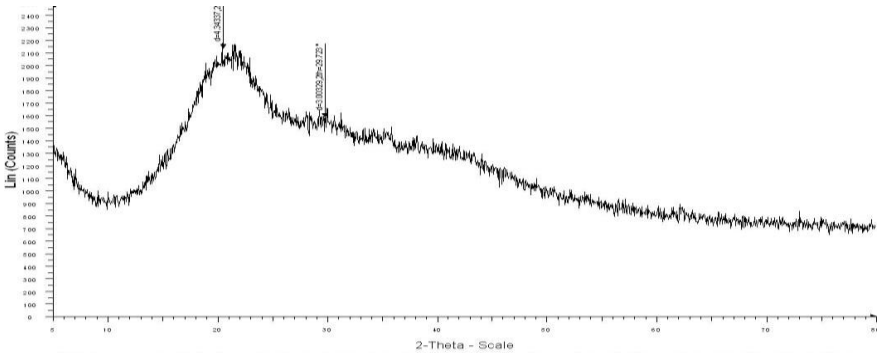


Figure-3: X-ray diffractogram of nanochitosan/iron oxide nanoparticles/egg shell ternary blend

The X-ray diffractogram of nanochitosan/iron oxide nanoparticles/egg shell ternary blend represented in figure-3 shows a broad peak at around 2Θ :10° - 40°. The broad region ranging from approximately 2Θ :10° - 40° is related to the predominant amorphous phase. Peak broadening observed is consistent with the small particle size.[19] The observed results reveals that the prepared sample shows a broader peak with lesser degree of crystallinity value suitable for various applications.

Antibacterial activity

The development of new antibacterial agents with novel and more efficient mechanisms of action is definitely an urgent medical need. [20] In the present research work, by utilizing the well diffusion method, the antibacterial activity of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend were tested against four bacterial species namely *Pseudomonas aureginosa, E.coli, Shigella and Staphylococcus aureus.* The drug ampicillin which is an effective antibacterial agent towards the selected four bacteria was used as a reference antibacterial agent in order to compare the results.

The photograph of the antibacterial activities of the prepared sample against four selected bacterial species was represented in **Figure-(i)-(iv)**. The zone of inhibition values grown around the prepared samples against the growth of the selected bacteria measured in mm using ampicillin used as standard and the graphical representation of the antibacterial activities of the prepared sample was shown in **Table-1** and **Graph-1**

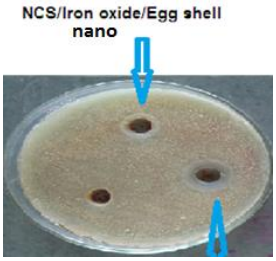


Figure-(i): Photograph of antibacterial activity of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend against *E.coli*

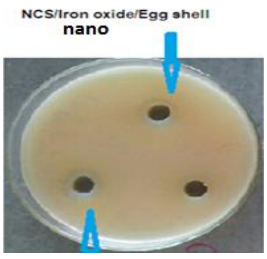


Figure-(ii): Photograph of antibacterial activity of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend against *pseudomonas aureginosa*

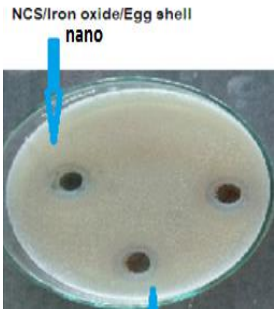


Figure-(iii): Photograph of antibacterial activity of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend against *staphylococcus aureus*

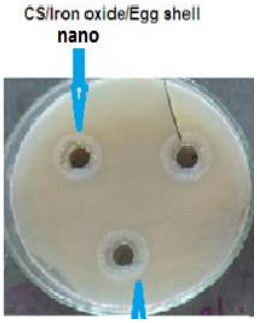
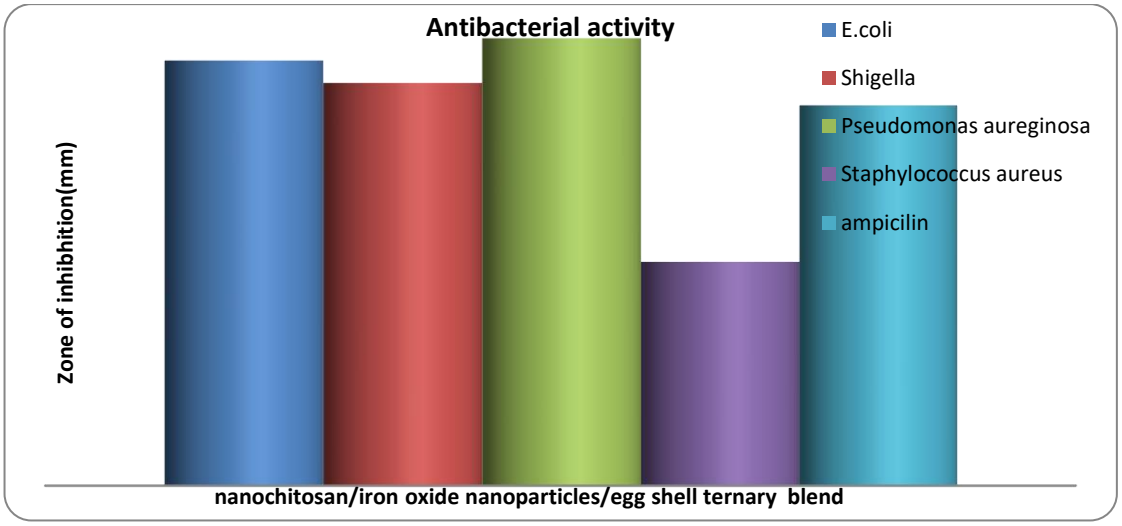


Figure-(iv): Photograph of antibacterial activity of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend against *shigella*

Table-1: Antibacterial activities of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend

Sample	Zone of inhibhition (mm)				Ampicilin (Standard)
	E.coli	*Shigella*	*Pseudomonas aureginosa*	*Staphylococcus aureus*	
Nanochitosan / iron oxide nanoparticles/egg shell ternary blend	19	18	20	17	17

Graph-1: Antibacterial activity of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend against *E.coli, Staphylococcus aureus*, *Pseudomonas aureginosa and Shigella*



From the observed results presented in the **Table-1, Figure- (i), (ii), (iii) , (iv),** and **Graph-1** it was evident that the prepared nanochitosan/iron oxide nanoparticles/ egg shell ternary blend showed very good antibacterial activity against all the four species namely *E.coli, Staphylococcus aureus*, *Pseudomonas aureginosa and Shigella*.

Antifungal activity

The antifungal activities of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend were tested against *Aspergillus Niger, Rhizopus* and *Mucor* by well diffusion method. The drug Polymyxin B sulphate which is an effective antifungal agent towards the fungal species selected was used as a reference antifungal agent.

The photograph of the antifungal activity of the prepared sample against three selected fungal species was represented in **Figure-(v), (vi) and (vii).**The zone of inhibition values grown around the prepared samples against the growth of the selected fungi measured in mm using polymyxin B sulphate used as standard was shown in **Table-2** and the results of screening of antifungal activities of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend was shown in **Graph-2.**

Figure-(v): Photograph of antifungal activity of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend against *Aspergillus niger*

Figure-(vi): Photograph of antifungal activity of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend against *Rhizopus nigricans*

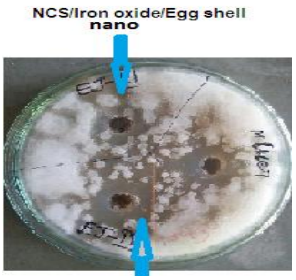
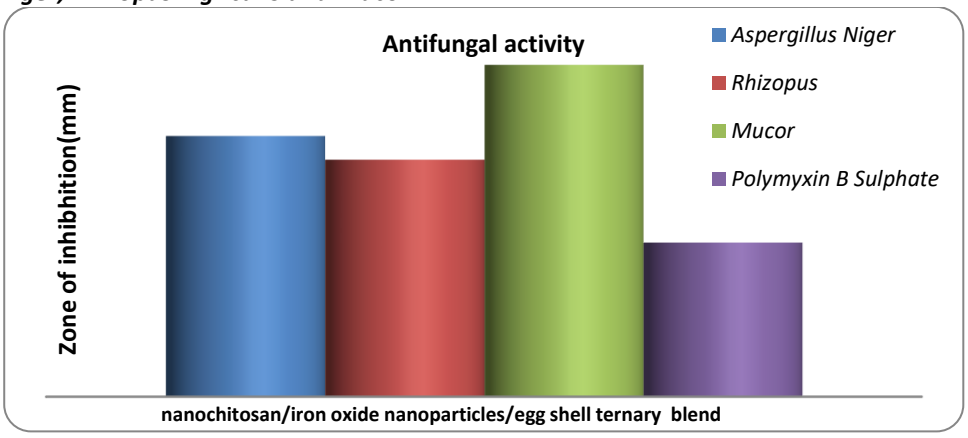


Figure-(vii): Photograph of antifungal activity of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend against *Mucor*

Table-2: Antifungal activities of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend

Sample	Zone of inhibhition (mm)			Polymyxin B Sulphate(standard)
	AspergillusNiger	*Rhizopus*	*Mucor*	
nanochitosan/iron oxide nanoparticles/egg shell ternary blend	22	20	28	13

Graph-2: Antifungal activity of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend against *Aspergillus Niger, Rhizopus nigricans and Mucor*



The results presented **Table-2, Figure- (v), (vi), (vii) and Graph-2** indicate that the above prepared nanochitosan/iron oxide nanoparticles/ egg shell ternary blend shows higher antifungal activity against *Aspergillus Niger, Rhizopus nigricans and Mucor*.

CONCLUSION:

This study investigated the synthesis of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend in 1:1:1 ratio. Certain FT-IR absorption bands were observed due to the various functional groups (OH, COO-, Fe-O) present in three added polymeric components and these observed peaks conclude that the added three components were blended effectively. The highly amorphous behavior of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend was elucidated from the XRD studies. Results of nanochitosan/iron oxide nanoparticles/ egg shell ternary blend against all the four bacterial species *E.coli, Staphylococcus aureus*, *Pseudomonas*

aureginosa and Shigella and three fungal species *Aspergillus Niger, Rhizopus nigricans and Mucor* showed that the prepared sample possesses potent anti-microbial effect and hence this prepared nanochitosan/iron oxide nanoparticles/ egg shell ternary blend can be used for future application in antimicrobial therapy.

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