IN VITRO ASSESSMENT OF ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF CHITOSAN SYNTHESIZED SILVER NANOPARTICLES

CLEMENT, MARVELLOUS YESOKO
GOU/U22/BCH/317

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GODFREY OKOYE UNIVERSITY, ENUGU
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A PROJECT RESEARCH SUBMITTED TO THE DEPARTMENT OF CHEMICAL SCIENCES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE (B.Sc.) DEGREE IN BIOCHEMISTRY

SUPERVISOR: DR. E. N UHUO

JULY, 2026

APPROVAL

This research titled “In Vitro Assessment of Antimicrobial and Antioxidant Activity of Chitosan Synthesized Silver Nanoparticles” has been assessed and approved by the Committees of Department of Chemical Sciences and Faculty of Natural Sciences and Environmental Studies, Godfrey Okoye University, Enugu, Nigeria.

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CERTIFICATION

I, Clement Marvellous Yesoko, an undergraduate student in the Department of Biochemistry, Faculty of Natural and Applied Sciences, Godfrey Okoye University, with the registration number GOU/U22/BCH/317, do hereby certify that, to the best of my knowledge, this research entitled "In Vitro Assessment of Antimicrobial and Antioxidant Activity of Chitosan Synthesized Silver Nanoparticles" is original and has never been submitted for any diploma or degree in this University or any other University.

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Department of Chemical Sciences		

DEDICATION

I dedicate this work with all my heart to Almighty God, whose wisdom, grace and infinite favor have provided this work to full completion.

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First and foremost, I return all glory, honour, and thanksgiving to Almighty God for His unfailing love, abundant grace, wisdom, protection, and strength throughout the course of this research. His faithfulness sustained me through every stage of this academic journey, and without His divine guidance, the successful completion of this work would not have been possible.

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ABSTRACT

Antimicrobial resistance and oxidative stress due to free radicals are emerging critical global health issues and the development of eco-friendly biomaterials having antimicrobial and antioxidant activities has been the need of the hour. One of the most promising biomaterials for these applications is a biodegradable biopolymer, chitosan, derived from waste materials of the shells of crabs. The purpose of this study was to obtain chitosan from crab shell waste and synthesize chitosan-based nanoparticles with ascorbic acid as reducing agent. The powdered crab shell was subjected to demineralization, deproteinization and deacetylation to obtain chitosan with a 9.42% yield of 4.71 g in 50 g of powdered crab shell. All the synthesized nanoparticles were characterized by the Fourier Transform Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD), Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDS). The characteristic functional groups of chitosan were confirmed by the FTIR spectra, while the XRD spectra showed that the synthesized material was crystalline. The SEM images showed that the morphology is irregular, rough, heterogeneous and sheet like while the EDS spectrum showed silicon as the major element followed by aluminium, oxygen, magnesium, carbon and some traces of other elements. The antioxidant activity was tested by DPPH, Ferric Reducing Antioxidant Power (FRAP), Nitric Oxide scavenging and Total Antioxidant Capacity assays with maximum activities of 83.05%, 131.51%, 43.30%, and 86.35%, respectively. The results of the antimicrobial activity tests showed concentration-dependent inhibition against *Staphylococcus aureus* and *Escherichia coli*, with more activity against *Staphylococcus aureus.* The study results show that crab shell derived chitosan based nanoparticles have shown that it is having potential biomedical and antimicrobial properties.

Keywords: Chitosan, crab shell, synthesis, FTIR, XRD, SEM, EDS, Antioxidant activity, Antimicrobial activity.

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CHAPTER ONE

INTRODUCTION

1.1 Background of the study

The quick rise of antimicrobial resistance and the growing number of diseases linked to oxidative stress have made it necessary to find new ways to treat these problems. Among the nanostructures studied, AgNPs have demonstrated significant utility owing to their antimicrobial and antioxidant characteristics. When mixed with biopolymers like chitosan, these nanoparticles show better stability, biocompatibility, and therapeutic effectiveness, which makes them good candidates for use in medicine and pharmaceuticals (Mittal *et al*., 2024). AgNPs kill bacteria by releasing silver ions (Ag+), allowing reactive oxygen species (ROS), directly interacting with the cells of bacterial membranes, and interfering with intracellular enzymes (Dakal *et al*., 2016). Their high surface area to volume ratio makes them more effective against both Gram negative and Gram positive bacteria (Gomaa, 2017)

The rise of MRSA and fluoroquinolone-resistant *E. coli* has made it even more important to find other antimicrobial agents (Nia *et al*., 2023). Chitosan, which comes from the chitin in crustacean shells, breaks up bacterial membranes (Akdaşci *et al*., 2025), stops AgNP from clumping together (Sarkar *et al*., 2022), and acts as an antioxidant by inactivating free radicals and inhibiting lipid peroxidation (Badawy *et al*., 2022; Zhang *et al*., 2023). Chitosan-AgNP composites exhibit notable antibacterial, antioxidant, and wound-healing properties (Xu *et al*., 2020; Mittal *et al*., 2024; Sultan *et al*., 2022; Rani *et al*., 2024). Nonetheless, research simultaneously assessing both properties against *E. coli* and *S. aureus* within a unified standardized framework is still scarce, a deficiency this study aims to rectify.

1.2 Statement of the Problem

Antimicrobial resistance is still a big problem for public health around the world. Multi-drug resistant *E. coli* and MRSA are two of the biggest reason of illness and death around the world. At the same time, diseases linked to oxidative stress still need effective antioxidant treatments. Although silver nanoparticles and chitosan have individually exhibited pertinent biological characteristics, a thorough in vitro assessment of chitosan-synthesized AgNPs where chitosan functions concurrently as the reducing, capping, and stabilizing agent against *E. coli* and *S. aureus*, along with a simultaneous evaluation of antioxidant properties, has not been adequately explored. Numerous published studies investigate either antibacterial or antioxidant activity in isolation, and comparisons across studies are often complicated by discrepancies in synthesis methods, chitosan sources, and testing protocols. So, we need a well-designed study that uses a single experimental framework to do standardized dual assessments.

1.3 Aim of the Study

The main goal of this research is to synthesis silver nanoparticles using chitosan as a capping and stabilising agent so that the antioxidant and antimicrobial performance of silver nanoparticles against *Escherichia coli* and *Staphylococcus aureus* can be measured within a laboratory setting.

1.4 Objectives of the Study

The study functions by the following objectives:

1. To extract and identify the profile of chitosan from the shell waste of crustacean animals. To create silver nanoparticles with the assistance of chitosan to act as a stabilizing and capping agent during the process.
2. To examine the silver nanoparticles that were created by using Scanning Electron Microscopy (SEM) and Fourier Transform Infrared (FTIR) spectroscopy.

3. To find the antioxidant movement of the created silver nanoparticles by using ABTS and DPPH free radical inhibiting methods.
4. To test the antimicrobial power of the created silver nanoparticles against *Escherichia coli* and *Staphylococcus aureus* by using agar well diffusion and minimum inhibitory concentration (MIC) methods.
5. To perform a comparison between the biological movements of the silver nanoparticles and chitosan alone or silver nitrate alone.

1.5 Significance of the Study

The study has important implications in the field of pharmaceutical science and clinical microbiology as well as environmental sustainability. As a science, it offers the quantitative in vitro information on the antioxidant and antibacterial activity of chitosan-silver nanoparticles under controlled conditions, which supports the characterization of chitosan-silver nanoparticles as the multifunctional biological agents (Mittal *et al*., 2024; Badawy *et al*., 2022). The clinical results will be used to develop nanoparticle-based wound dressing, topical antimicrobials

1.6 Scope of the Study

This report discusses the crab shell waste processing to chitosan, chemical preparation of AgNPs using chitosan as the only stabilising and capping agent, physicochemical characterisation of AgNPs via FTIR, XRD and SEM, and in vitro biological testing through DPPH and ABTS antioxidant assays and disc diffusion and MIC antibacterial assays with The research lacks in vivo research, toxicological analyses, antifungal or antiviral analyses, and clinical trials. The conclusions are restricted to in vitro situation and strains used.

1.7 Location and Accessibility of Material

Local fish markets and seafood processing outlets will be used as sources of crustacean shell waste to use in extracting chitosan. Silver nitrate, acetic acid, hydrochloric acid, sodium hydroxide, DPPH, and ABTS are laboratory-grade reagents, and will be purchased through certified chemical vendors. The reference bacterial strains will either be obtained through the microbiology culture collection in the department or a certified microbial repository. All the experiments will be carried out in the microbiology and biochemistry research labs of the university.

1.8 Justification of the Study

There are three reasons why this study is necessary. There is lack of scientific research that simultaneously assesses both the antioxidant and antibacterial properties of chitosan-AgNPs within a standardized framework, despite numerous individual studies (Xu *et al*., 2020; Gomaa, 2017; Nia *et al*., 2023). Clinically, the multi-target mechanism of chitosan-silver nanoparticles diminishes the probability of resistance development in comparison to conventional antibiotics, as evidenced by Dakal *et al*. (2016) and Aryani *et al*. (2025). The utilization of chitosan derived from crustaceans promotes sustainable and environmentally friendly nanomaterial production in accordance with circular economy principles (Sultan *et al*., 2022; Rani *et al*., 2024). Additionally, it offers pharmaceutical biochemistry and microbiology researchers a reproducible methodological framework for subsequent nanoparticle studies.

CHAPTER TWO

LITERATURE REVIEW

2.1 Conceptual Review

2.1.1 Nanoparticles and Nanotechnology

Nanotechnology is the design, synthesis, and utilization of materials at the 100 nm-1 nm scale, where they exhibit unusual physicochemical characteristics not found in bulk materials (Khan *et al*., 2019). Growing to this scale, surface-area-to-volume ratios and quantum effects of confinement, as well as altered thermodynamic behaviour, all work together to produce materials of superior optical, catalytic, electrical and biological activity (Jeevanandam *et al*., 2018). The most studied type of nanomaterials is nanoparticles, which are widely categorized according to their composition: metal oxide, polymeric, metallic, lipid-based, and carbon-based (Tiwari *et al*., 2022). Metallic nanoparticles, specifically silver, gold, zinc oxide, and titanium dioxide, are one of them, and their strong and thoroughly studied bioactivity has preconditioned their continued research interest (Rabiee *et al*., 2020).

Nanotechnology has become one of the leading sectors of growth due to the biomedical sector. Vines *et al*. (2019) report that nanomaterials increasingly find their way into therapeutic and diagnostic delivery systems, antimicrobial surfaces, and biosensors, dramatically changing the paradigms in the fields of therapeutics and diagnostics. Regardless of this assurance, the regulatory and toxicological environment is still complicated; the issues of cytotoxicity, immunogenicity, and environmental persistence of some nanomaterials have heightened the demand to adopt greener and more biocompatible production methods (Akter *et al*., 2018; Singh *et al*., 2018). Natural biopolymers like chitosan have been of great interest as alternatives to traditional chemical reducing and stabilizing agents in the formation of nanoparticles (Aranaz *et al*., 2021).

2.1.2 Silver Nanoparticles (AgNPs): Properties and application

Silver nanoparticles continue to occupy a prominent place in the clinical and industrial landscape, particularly due to their clinically and industrially relevant potent broad-spectrum antimicrobial action, favourable surface chemistry, and fully characterized optical properties (Pugazhendhi *et al*., 2018). The formation of SPR in AgNPs is caused by the conduction electrons present on the AgNPs surface that resonantly oscillate in response to the incident light energy, thereby forming a unique UV-visible absorbance peak at 380-450 nm wavelength range. (Paramelle *et al*., 2015). This is an SPR peak that can be readily detected by UV-Vis spectroscopy, and which is sensitive to particle size and shape, aggregation state, and surrounding medium (Tortella *et al*., 2023).

The main factors of AgNP biological activity are particle size, morphology, surface charge, and crystallinity. Smaller nanoparticles (usually less than 20 nm) are more antimicrobially effective because of the increased surface-area-to-volume and the high ability to penetrate membranes (Durán *et al*., 2016; Raza *et al*., 2016). The Zeta potentials are greater than +30 mV and -30 mV and are considered sufficient to ensure that particles repel each other electrostatically to provide colloidal stability and maintain bioactivity, even during storage (Bhattacharjee, 2016). Spherical AgNPs have proven better antimicrobial activity than rod-shaped or triangular morphologies under the same concentration conditions, which can be explained by the variations in facet reactivity and density of atoms on the surface (Pal *et al*., 2015, as cited in Tortella *et al*., 2023).

AgNPs have been extensively applied in biomedical research over the past few years. Yugal *et al*. (2022) searched their integration into wound dressings where sustained Ag^{+} release ensures antimicrobial effects and tissue regeneration. Rabiee *et al*. (2020) reported their fruitful applications in cancer therapanostics, biosensing, and targeted drug delivery systems. AgNPs are also incorporated in antimicrobial packaging films in the food industry, which inhibits spoilage

microorganisms and prolongs the shelf life of products (Yousef *et al*., 2015). Their use in the environment, such as catalytic degradation of organic pollutants and heavy metal removal, has also increased significantly (Singh *et al*., 2018). A major weakness, though, is that the standard chemical methods use toxic reagents like sodium borohydride and hydrazine, which produce toxic by-products that limit biomedical applications (Iravani *et al*., 2014, as cited in Ahmed *et al*., 2016). This has stimulated a general research shift to green and biogenic synthesis approaches.

2.1.3 Chitosan: Biological Properties, Sources, and Structure.

Chitosan is a natural linear polysaccharide that is a partial or complete deacetylation product of chitin the second most extensive biopolymer on Earth, which is commonly present in crustacean shells, the walls of fungi, and insect shells (Aranaz *et al*., 2021). It is structurally composed of 2-4-linked D-glucosamine and N-acetyl-D-glucosamine units; the proportion of these units determines the level of deacetylation (DD), and should be more than 70% to be considered chitosan (Cheung *et al*., 2015). Commercial grade chitosan is presented with a DD of 75-95 per cent and DD as well as molecular weight have a significant impact on the solubility, charge density, and biological activity (Divya and Jisha, 2018).

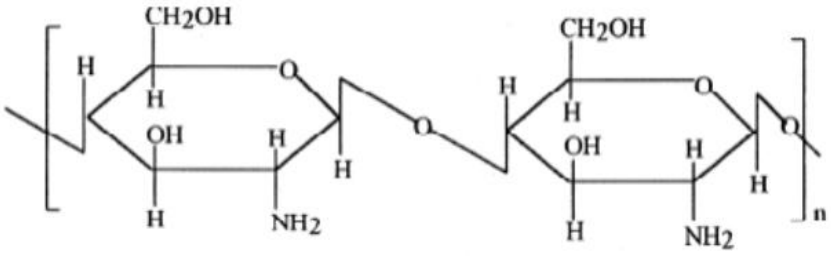

Figure 2.1 The Structure of Chitosan

The variety of physicochemical behaviours of chitosan is attributed to the presence of large number of free amino (-NH_2) and hydroxyl (-OH) functional groups in the chitosan backbone. When acidic pH is lower than around 6.5, amino groups get protonated making chitosan cationic and water-

soluble such that they can electrostatically interact with negatively charged microbial surfaces (Ing *et al*., 2012, as cited in Hosseinnejad and Jafari, 2016). Chitosan is insoluble at neutral or alkaline pH, which is used in the pH-responsive drug delivery systems (Cheung *et al*., 2015). Such pH-based solubility properties can also be applied in the context of comprehending the behavior of CS-AgNPs when subjected to various circumstances in in vitro assays.

Chitosan has well-reported antimicrobial, antioxidant, anti-inflammatory, biocompatible, and biodegradable biological properties (Aranaz *et al*., 2021; Hosseinnejad and Jafari, 2016). It has antimicrobial properties against Gram-positive and Gram-negative microorganisms, and fungi due to several mechanisms, such as the interference with cell membrane integrity through electrostatic interaction, chelation of vital metal ions, and inhibition of mRNA synthesis at subinhibitory levels (Hosseinnejad & Jafari, 2016). The presence of functional groups in chitosan imparts the antioxidant properties that allow the latter to be especially valuable in food preservation and nutraceutical purposes (Hajji *et al*., 2018). Notably, the presence of reactive functional groups renders chitosan a versatile dual-purpose reagent in nanoparticle methods – both as a reducing agent and a stabilizing capping ligand (Aranaz *et al*., 2021).

2.1.4. Chitosan-Synthesized Silver Nanoparticles (CS-AgNPs)

AgNPs synthesized by the chitosan as the main reducing and stabilizing agent yield a nanocomposite – CS-AgNPs – whereby metallic silver cores are surrounded by a polymeric casing of chitosan (Matica *et al*., 2019). The synthesis process follows a series of chelation of Ag+ ions by amino and hydroxyl groups of chitosan, their further reduction to zerovalent Ag0, nucleation, and growth of nanoparticles, which is stabilized by the polymer surrounding it (Govindan *et al*., 2020). The chitosan shell serves as a steric and electrostatic barrier that prevents aggregation of

particles, and also provides independent biological activity to the nanocomposite (Matica *et al*., 2019).

Some of the important parameters of critical synthesis are the ratio of chitosan to AgNO3, pH of the solution, temperature of the reaction, and time. Increased chitosan concentrations tend to give smaller, more monodisperse particles due to increased numbers of nucleation sites and stronger capping of the surfaces, but the high concentrations of polymer may slow down the reduction kinetics (Govindan *et al*., 2020). The alkaline pH is preferable to the Ag+ reduction, and the acidic conditions (which are required to dissolve chitosan) may retard nucleation and necessitate pH optimization (Matica *et al*., 2019). Synthesis temperature also affects the rate of nucleation and, as a result, end particle size distribution.

The signification of biological findings is only possible through comprehensive characterization of CS-AgNPs. The formation of nanoparticles is confirmed by UV–Vis spectroscopy based on the typical absorption at SPR at 400450 nm (Tortella *et al*., 2023). Hydrodynamic size and polydispersivity index (PDI) are obtained using dynamic light scattering (DLS), and zeta potential measurement is used to measure colloidal stability (Bhattacharjee, 2016). Morphological evidence at the level of nanoscale is directly given by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The presence of chitosan functional groups on the surface of the nanoparticle, which confirms the presence of the capping mechanism, is verified by Fourier-transform infrared spectroscopy (FTIR), and the face-centred cubic (FCC) crystalline structure of metallic Ag is verified using X-ray diffraction (XRD) (Yugal *et al*., 2022). The shell of the chitosan is said to give the positive surface charge, which is a central mechanistic benefit and increases the electrostatic affinity to negatively charged bacteria surfaces and potentially increases the antimicrobial effect in a synergistic manner (Matica *et al*., 2019).

2.1.5 Antimicrobial Activity: Mechanisms and Importance

Antimicrobial resistance (AMR) has been declared to be one of the most significant public health issues of the 21st century on a global level. According to the WHO (2021), the main reason 1.27 million people died in 2019 and an overall contribution of about 4.95 million deaths worldwide were a result of bacterial AMR, hence creating effective alternative antimicrobial plans is urgently needed. Of particular concern are drug-resistant strains of *S. aureus* - especially, methicillin-resistant *S. aureus* (MRSA) and multi-drug-resistant *E. coli*, such as ESBL and carbapenem-resistant lineages (Laxminarayan *et al*., 2013; Murray *et al*., 2022).

AgNPs and nanoparticles, in general, have been developed as hopeful solutions to handle AMR because of the multifactorial effects of their antibacterial properties. They comprise: the production of intracellular reactive oxygen species (ROS), cell membrane disruption, the release of Ag + ions inactivating thiol-containing bacterial enzymes, and disruption of DNA replication (Duran *et al*., 2016; Tortella *et al*., 2023). Concurrent activity of several cellular targets contributes to the further development of resistance being an order of magnitude more challenging in the case of bacteria than with traditional single-target antibiotics (Murray *et al*., 2022). Common zone of inhibition (ZOI) and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in vitro antimicrobial tests are used as advised by CLSI (2018) common in recent CS-AgNP literature: disc diffusion assay (KirbyBauer method) to measure zones of inhibition (ZOI), broth microdilution to measure minimum inhibitory concentration.

2.1.6 Antioxidant Activity: Principles and Assays.

Oxidative stress occurs when the production of ROS in cells surpasses the antioxidants they have, and is a known pathogenic mechanism of chronic diseases such as cancer, cardiovascular diseases, diabetes and neurodegeneration. (Pisoschi & Pop, 2015). Free radicals, such as a superoxide anion

(O_2• -), hydroxyl radical (-OH), and hydrogen peroxide (H_2O_2), result in the indiscriminate oxidative damage of lipids, proteins, and DNA, impairing cellular functions (Sharifi-Rad *et al*., 2020). Antioxidants counter such radicals, either by donating an electron or hydrogen atom, thus ending the oxidative chain reaction (Pisoschi & Pop, 2015).

There are three common assays that are used when assessing the antioxidant ability of nanomaterials. The DPPH (2, 2- diphenyl-1-picrylhydrazyl) assay is used to determine the hydrogen atom transfer capacity by colorimetrically bleaching a stable violet radical (Kedare and Singh, 2011). ABTS (2,2 -azino-bis(3-ethylbenzothiazolinesulfonic acid)) assay measures the capacity to neutralize the ABTS – radical cation, which shows the hydrophilic and lipophilic antioxidant ability (Re *et al*., 1999, as cited in Sharifi-Rad *et al*., 2020). FRAP assay is the measurement of electron-donating power, which is determined by the reduction of Fe3+ to Fe2+ under acidic conditions (Benzie & Strain, 1996, as cited in Pisoschi and Pop, 2015). Since each assay is interrogating a different mechanistic aspect of antioxidant activity, their combination is more informative and dependable than any single assay (Sharifi-Rad *et al*., 2020).

2.2 Theoretical Review

2.2.1. Antimicrobial Effects of CS-AgNPs.

Oxidative Stress Theory via ROS. The oxidative stress mediated by ROS is the most popular mechanistic model of the AgNP antibacterial activity. AgNPs stimulate intracellular production of superoxide (O_2-), hydroxyl radicals (OH), and hydrogen peroxide (H_2O_2) and can do so primarily by disrupting the bacterial electron transport chain (Duran *et al*., 2016; Tortella *et al*., 2023). The effects of these ROS are lipid peroxidation of membrane phospholipids, DNA strand breaks, and oxidative inactivation of metabolic enzymes – damage that overwhelms bacterial antioxidant defences (superoxide dismutase and catalase) and which is ultimately fatal. More importantly, the

unselective way ROS-induced death in a variety of cellular targets occurs, the development of resistance to ROS cannot be achieved through one genetic mutation, which gives bacteria an advantage over traditional antibiotics (Murray *et al*., 2022).

Cell Membrane Disruption Theory. Intact AgNPs and Ag + ions released interact directly with bacterial cell surfaces, resulting in structural damage to the cell envelope (Tortella *et al*., 2023). AgNPs of Gram-negative bacteria, such as *E. coli*, disrupt the lipopolysaccharide (LPS) outer membrane, creating irregular pits and enhancing the membrane permeability, resulting in the cytoplasmic leakage and cell lysis (Durán *et al*., 2016). In Gram-positive bacteria, such as *S. aureus* that do not have an outer membrane but have a thicker peptidoglycan wall, AgNPs invade the porous cell wall to attack the underlying plasma membrane (Matica *et al*., 2019). Electrostatic adhesion to the anionic bacterial cell surface – created by the negatively charged phospholipid head groups and, in Gram-negatives, by LPS – is significantly increased by the inherently positive surface charge of CS-AgNPs, which disrupts the membrane to a greater extent than bare AgNPs do (Matica *et al*., 2019; Govindan *et al*., 2020).

Silver Ion Release Theory and Protein Thiol Coordination Theory. When metallic Ag 0 is oxidized on the surface in aqueous solutions, Ag + ions are released that are strong electrophiles that react readily with the thiol (-SH) groups of cysteine residues in bacterial proteins (Tortella *et al*., 2023). Ag + to thiol group coordination disrupts enzymes involved in respiration, DNA replication and cell wall synthesis. Moreover, Ag+ blocks the proton motive force across the inner bacterial membrane by disrupting membrane-bound ATPases, and dissolving the electrochemical gradient through which ATP is synthesized (Durán *et al*., 2016). Ag^{-} release kinetics is controlled by the chitosan skeleton of CS-AgNPs, which may have a longer antimicrobial effect compared to rapidly dissolving bare AgNPs (Matica *et al*., 2019).

2.2.2 Theoretical basis of Antioxidant Activity

The Hydrogen Atom Transfer (HAT) Theory. The HAT mechanism explains how antioxidants can terminate radical chain reactions by direct donation of a hydrogen atom (H•) by the antioxidant to a free radical to form a stable non-reactive product (Pisoschi & Pop, 2015). The free -NH2 and -OH groups in chitosan act as donors of hydrogen and the density of these groups, which is linked to the high DD, is related to the radical scavenging ability (Hajji *et al*., 2018). This is the major mechanism of DPPH scavenging activity.

Metal Chelation Theory and Electron Transfer (ET). The single-electron transfer (SET) process is based on the donation of an electron by the antioxidant to reduce a free radical to an anion (Sharifi-Rad *et al*., 2020). This is what the FRAP assay is founded on. Metal chelation is another type of complementary antioxidant: chitosan inhibits the formation of Fenton reactions, acculturating transition metals, including Fe 2 + and Cu 2+ (generation of the •OH, Pisoschi and Pop, 2015). In the case of CS-AgNPs, in particular, the silver core can tune the effect of antioxidant behavior concentration-dependently – an antioxidant at low concentrations due to surface electron transfer and a pro-oxidant at high concentrations due to ROS generation (Sharifi-Rad *et al*., 2020; Yugal *et al*., 2022).

2.3 Empirical Review

2.3.1. The antimicrobial activity of AgNPs and CS-AgNPs is shown in

The recent literature is steadily in favor of the broad-spectrum antimicrobial effect of AgNPs and their chitosan compounds, albeit with some variations in dependence on the conditions of synthesis and target organisms. A systematic review of the AgNP antimicrobial efficacy published in 2015-2022 by Tortella *et al*. (2023) found that the MIC values against clinically relevant bacteria were in the range of 1-64 μg/mL, and the functionalization of particles and their size were found to be

the most important modulators. As Pugazhendhi *et al*. (2018) have demonstrated, biogenically produced AgNPs remained active against MRSA and drug-resistant *E. coli* strains and their MIC values were as low as 4 μg/mL, which is significantly lower than the typical MIC of commonly used antibiotics against these organisms. Importantly enough, Murray *et al*. (2022) have highlighted that the multifactorial nature of AgNPs makes them useful as adjuncts or substitutes to traditional antibiotics in the post-antibiotic age.

Govindan *et al*. (2020) prepared CS-AgNPs by a green chemical reduction technique and tested their antibacterial properties in a collection of clinical isolates. They claimed that CS-AgNPs had always shown smaller values of MIC than the same concentrations of uncoated AgNPs against all organisms tested, and attributed this to the synergistic effect of the positively charged chitosan shell and the Ag+ releasing silver core. In a broad review of chitosan-silver nanocomposites, Matica *et al*. (2019) also found that CS-AgNPs are mechanistically superior to bare AgNPs under the same dosage conditions, with at least two mechanisms independent of each other playing a role in the antimicrobial activity of chitosan-silver nanocomposites: electrostatic membrane adhesion and sustained Ag^{+} release regulation.

2.3.2. Effect on *Staphylococcus aureus*

Staphylococcus aureus causes various types of infections, such as minor skin diseases, as well as life-threatening bacteraemia and endocarditis, and the ongoing transmission of MRSA has made it a priority pathogen to develop novel antibiotics (Tong *et al*., 2015; Murray *et al*., 2022). New in vitro research has reported convincing activity of both AgNPs and CS-AgNPs against this organism. Yugal *et al*. (2022) synthesized CS-AgNPs with chitosan extracted of shrimp shells and reported zones of inhibition of 19.3 mm against *S. aureus* at 50μg/mL – which is significantly higher than both the chitosan control (8.1 mm) and bare AgNP control (13.5 mm) – and the

presence of syn The MIC of *S. aureus* was determined as 8µg/mL as opposed to 16µg/mL on bare AgNPs prepared in the same manner.

Rabiee *et al*. (2020) compared the synthesis of biogenic CS-AgNPs of plant polyphenol with chitosan and their effects on methicillin-susceptible and methicillin-resistant strains of *S. aureus*. Their results showed that the CS-AgNPs retained inhibitory activity toward MRSA at 612 µg/mL MICs which are significantly lower than those of vancomycin with intermediate-resistant strains, highlighting that they may be useful in the clinic. Pugazhendhi *et al*. (2018) went on to prove that CS-AgNP treatment of *S. aureus* caused severe membrane permeabilization and cytoplasmic condensation, which are signs of bactericidal membrane disruption, at concentrations as low as 2× MIC by propidium iodide uptake assays and scanning electron microscopy. Hosseinnejad and Jafari (2016) reported a contrasting finding and in their systematic review reported that the antibacterial activity of CS-AgNPs against Gram-positive organisms was found to vary more across studies compared to Gram-negative bacteria, and that the differences in chitosan molecular weight and DD contributed to a significant proportion of inter-study variability, which supports the importance of standardized synthesis and reporting protocols.

2.3.3 Effects on *Escherichia coli*

Escherichai coli poses a bifold challenge being both a ubiquitous commensal and a major cause of urinary tract infections, gastroenteritis, neonatal meningitis, and septicaemia (Murray *et al*., 2022). The development of *E. coli* strains producing ESBLs and resistant to carbapenem have critically restricted treatment options, which has fuelled the interest in nanoparticle-based antimicrobial approaches (WHO, 2021). Recent reports have verified strong CS-AgNP activity against this organism, a number of which have reported stronger activity against *E. coli* compared to *S. aureus* a trend which can be explained by variations in outer membrane structure.

Govindan *et al*. (2020) found MIC of CS-AgNPs of 2 μg/mL with *E. coli*, four times lower than the 8 μg/mL with unmodified AgNPs, due to improved electrostatic adhesion of the positive-charged CS-AgNP surface to the anionic LPS-rich outer membrane of *E. coli*. Theoretically, Gram-negative than Gram-positive organisms have a greater electrostatic advantage in that the outer membrane of the LPS can offer a uniformly anionic surface to approaching cationic nanoparticles (Matica *et al*., 2019). In line with this, Yugal *et al*. (2022) noted greater ZOIs of *E. coli* (21.2 mm) compared to *S. aureus* (19.3 mm) to the same CS-AgNP preparation and concentration, which confirms the hypothesis of Gram-positive versus Gram-negative susceptibility differences.

Tortella *et al*. (2023) also observed, though, that other recent studies have also found equivalent or even better Gram-positive organism activity when CS-AgNPs are optimized to produce smaller particle sizes. They explained this by the increased ability of very small nanoparticles to penetrate in the *S. aureus* peptidoglycan layer, which partially compensates the lack of an LPS electrostatic target. This apparent paradox brings out a real unsolved literature gap on whether a particular bacterial phenotype is more vulnerable to CS-AgNPs by default – a question that is directly answered by the direct comparative design adopted in the present study. Rabiee *et al*. (2020) further stated that sub-MIC levels of CS-AgNPs had a significant negative impact on the development of biofilms of *E. coli* – a clinically relevant observation, as biofilm-reinforce cell that has been embedded in a biofilm is generally 100-1000 times more resistant to antimicrobial agents than free-living cells.

2.3.4. Antioxidant properties of Chitosan and CS-AgNPs.

Antioxidant activity of chitosan and CS-AgNPs is an area of increasing interest, but the literature is not as mature as the antimicrobial literature and is more methodologically diverse. Hajji *et al*.

(2018) assessed antioxidant ability of chitosan extracted in cuttlefish in a series of molecular weight and DDs with the use of DPPH, ABTS, and metal chelation testing. Their findings confirmed that increased DD was positively correlated with DPPH and ABTS scavenging capacity (because of increased density of free amino groups to donate hydrogen) and metal chelation activity was more significantly dependent on molecular weight. These results partly substantiate previous structure-activity correlations, and provide valuable insight into the specific effect of DD vs. Molecular weight on different antioxidant pathways.

Sharifi-Rad *et al*. (2020) conducted an overview of the antioxidant properties of biogenic AgNPs in a variety of recent studies and found that the antioxidant activity (measured by DPPH, ABTS, and FRAP) was always due to the presence of biomolecular capping layer but not the silver core itself, with bare AgNPs showing no scavenging This directly applies to the CS-AgNPs: the shell of the chitosan is likely to be the major donor of the antioxidant capacity, with the silver core either playing a smaller role or showing, at high concentrations, an antagonistic effect. This hypothesis was directly experimentally confirmed by Yugal *et al*. (2022), who reported an IC50 of 68.4 µg /mL (DPPH) and 74.1 µg /mL (ABTS) with their CS-AgNPs – significantly higher than chitosan alone (IC50 = 52.3 µg/mL by DPPH), indicating that the addition of It was postulated by the authors that Ag + coordination of the amino and hydroxyl groups of chitosan during synthesis decreases the radical scavenging activity of these groups.

On the other hand, Govindan *et al*. (2020) found that their CS-AgNPs showed slightly higher DPPH scavenging in comparison to chitosan alone – which they explained by the fact that surface-mediated electron transfer with the silver core occurred in low nanoparticle concentrations. Such a difference in the literature highlights the concentration- and formulations-dependence of CS-AgNP antioxidant behaviour and shows the inefficiency of single-assay, single-concentration

studies that predominate the literature. As it has been stressed by Pisoschi and Pop (2015), DPPH by itself cannot adequately describe the antioxidant capacity because it is mainly indicative of HAT mechanisms and might not be indicative of the electron-donation and metal chelation pathways that are relevant to the mechanistic aspects of polysaccharide-based nanomaterials. Concurrent analysis of DPPH, ABTS and FRAP (as utilized in the current study) is thus critical to a holistic characterization of the mechanism.

2.4. Summary of Literature and Research Gap

The literature reviewed between 2015 and 2025 provides a solid and increasing amount of evidence on the antimicrobial and antioxidant bioactivity of CS-AgNPs. The agNPs produced by green and biopolymer-assisted methods invariably exhibit both *S. aureus* and *E. coli* antibacterial effects (including those with drug-resistance phenotypes) with multifactorial effects (including ROS generation, membrane rupture, and enzyme inactivation by Ag+). This activity is enhanced by the chitosan component in a synergistic manner by electrostatic and sustained-release systems, as well as independently by antioxidant capacity in terms of hydrogen atom donation and metal chelation. Nevertheless, there are still a number of gaps. First, the synthesis parameters, specifically the chitosan molecular weight, DD, and the concentration of AgNO 3 are not reported consistently, and, therefore, cross-study MIC and ZOI cannot be reliable (Hosseinnejad and Jafari, 2016; Tortella *et al*., 2023). Second, the active activity of CS-AgNPs against Gram-positive or Gram-negative bacteria remains unclear, and contradictory results are reported in recent studies based on the size of particles and the charge of their surface (Govindan *et al*., 2020; Yugal *et al*., 2022). Third, the antioxidant characterization is disproportionately based on the use of DPPH alone, with a small number of studies utilizing both ABTS and FRAP in order to comprehensively describe the mechanistic context of antioxidant activity. Fourth, the correlation of individual

physicochemical parameters, especially the zeta potential and particle size, and the antioxidant activity is not well established in the literature on CS-AgNP. Fifth, the majority of the studies measure either antimicrobial or antioxidant activity alone, and few studies have conducted a systematic study of both bioactivities in a well-defined nanoparticle system.

The current work directly fills these voids via the synthesis of CS-AgNPs under completely reported and optimized conditions, physicochemical characterization (UV Vis, FTIR, XRD, DLS, zeta potential), standardized comparative antimicrobial activity against *S. aureus* and *E. coli* (disc diffusion, MIC), and multi-assay antioxidant activity (DPPH, ABTS, and FRAP) producing an integrated dataset that advances both mechanistic understanding and the evidence base for the biomedical application of this important nanocomposite.

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 Research Designs

The study utilized an experimental laboratory-based research design to characterize and evaluate the in vitro antioxidant and antimicrobial activities of the synthesized chitosan silver nanoparticles (CS-AgNPs). The study was designed with the aim to extract chitosan from crab shells, synthesize silver nanoparticles using silver nitrate and ascorbic acid, and characterize nanoparticles by advanced analytical techniques and evaluation of biological activity.

3.2 **Study Area**

The study was done in the Biochemistry and Microbiology Laboratories of Godfrey Okoye University Enugu, Nigeria. Chitosan extraction, synthesis of nanoparticles, characterization, antioxidant and antimicrobial analysis were performed under controlled laboratory conditions using standard laboratory procedures.

3.3 **Materials and Reagents**

3.3.1 Materials

The materials used for this study were:

a) Fresh crab shells
b) Analytical weighing balance
c) Magnetic stirrer with hot plate
d) Centrifuge
e) UV-Visible spectrophotometer
f) Autoclave
g) Incubator

h) Micropipettes

i) Measuring cylinders

j) Beakers

k) Conical flasks

l) Petri dishes

m) Sterile test tubes

n) Glass rods

o) Cork borer (6 mm diameter)

3.3.2 Reagents

The reagents used for this study were:

a) Hydrochloric acid (HCl, 2 M)

b) Sodium hydroxide (NaOH, 5% and 40%)

c) Ethanol (95%)

d) Acetic acid (1% v/v)

e) Silver nitrate ($AgNO_3$)

f) Ascorbic acid

g) Distilled water

h) Nutrient agar

i) DPPH reagent

j) FRAP reagent

k) Erythromycine(positive control)

l) Ciprofloxacin (positive control)

3.4 Sample Preparation

3.4.1 Collection and Preparation of Crab Shells

The fresh crab shells were collected from the seafood markets and washed thoroughly with tap water to remove the dirt and tissues adhering to the shell. Shells were washed with distilled water, dried in air and ground in laboratory grinder.

Figure 3.1 Crab Shell

3.4.2 Extraction of Chitosan from Crab Shells

Demineralization The crab shells powder (50g) was reacted with 750ml of 2M HCl having solid to liquid ratio of 1:15 (w/v). This reaction was done with stirring of magnet at room temperature for 1 hour to dissolve calcium carbonate and other minerals present in it. The shells were rinsed in large amount of water to neutralize the pH value.. (Kou *et al*., 2021).

Deproteinization The demineralized shells were reacted with 500mL of 5% sodium hydroxide solution for 2 hours at 90°C and constantly stirred to eliminate any remaining proteins. The chitin that was formed was then washed with distilled water until a neutral pH was attained. (Kou *et al*., 2021).

Decolourization The obtained chitin was treated with 500 mL of 95% ethanol and heated at 70°C for 1 hour with continuous stirring to remove pigments and coloured impurities . The product was filtered off and washed with distilled water (Kou *et al*., 2021).

Deacetylation The decolorised chitin was mixed with 300 mL of 40% sodium hydroxide solution and heated at 100 °C for 3 hours while continuously stirring in order to convert chitin into chitosan. The resulting chitosan was repeatedly washed using distilled water until neutral pH was achieved. (Kou *et al*., 2021).

The extraction procedure was adapted from established methods for chitosan extraction from crustacean shells (Confederat *et al*., 2021).

3.5 Synthesis of Chitosan-Silver Nanoparticles

3.5.1 Preparation of Chitosan Solution

The chitosan powder was isolated, 1.0g of which was weighed and dissolved in 100mL of 1% (v/v) acetic acid solution. The solution was stirred for 6h using a magnetic stirrer till the formation of a clear and homogeneous solution was achieved. (Pham et al., 2022).

3.5.2 Preparation of Silver Nitrate Solution

Silver nitrate ($AgNO_3$) was weighed to make a 1 mM solution in 100 mL of distilled water. The solution was kept in a dark bottle to prevent reduction under the effects of light (Ngo et al., 2021).

3.5.3 Formation of Chitosan-Silver Ion Complex

The silver nitrate solution was slowly added to the chitosan solution (100 mL) with magnetic stirring. Then, after the mixture stirring for 30 min, it was allowed to interact between the amino groups of chitosan and silver ions under 30℃. (Pham *et al*., 2022).

3.5.4 Reduction Using Ascorbic Acid

The ascorbic acid solution (10 mM) was freshly prepared reducing agent. The chitosan-silver mixture was dropwise added to ascorbic acid solution about 5 mL, while stirring continuously. The reaction was carried out until the colour of the reaction changed from light yellow to brown. This showed formation of silver nanoparticles (Ngo et al., 2021).

3.5.5 Stabilization of Nanoparticles

The reaction sample was stirred further for 3 hours under continuous stirring for complete reduction and stabilization of the nanoparticles. Chitosan was used as stabilizer and capping agent (Kaur et al., 2023).

3.5.6 Purification and Storage

The synthesized nanoparticles were centrifuged for 20 minutes at 10,000 rpm. The pellet so obtained was washed two times with distilled water to remove the impurities and unreacted substances. Finally, the purified nanoparticles were re-dispersed in distilled water and kept in a dark bottle at 4°C until further analysis (Pham et al., 2022).

3.6 Characterization of Chitosan-Silver Nanoparticles

3.6.1 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was used to identify functional groups involved in nanoparticle synthesis and confirm interaction between chitosan and silver nanoparticles (Pham *et al*., 2021).

3.6.2 Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDS)

The surface morphology of synthesized chitosan nanoparticles was studied using Scanning Electron Microscope (SEM). The dried nanoparticles nanoparticle sample was mounted on an aluminum specimen stub using conductive carbon tape and coated as required to enhance conductivity. The sample was examined at an accelerating voltage of 20KV and at different

magnifications observe the particle shape, surface texture and degree of aggregation. (Kaur *et al*., 2023).

Energy dispersive X-ray analysis (EDS)

EDX analysis was performed on the SEM for analysis of elemental content of chitosan nanoparticles produced from the process. Elemental composition and percentage weight of elements in the sample were determined using the EDS spectrum.

3.6.3 X-Ray Diffraction (XRD)

The synthesized silver nanoparticles were characterized by the X-ray diffraction technique to investigate their crystallinity. (Ngo *et al*., 2021).

3.7 Preparation and Standardization of Microbial Cultures

Culture samples of *E. coli* and *S. aureus* were isolated from the Microbiology Laboratory of Godfrey Okoye University Teaching Hospital. The bacteria were sub-cultured in nutrient agar medium incubated for 24 hours at 37°C.

Standardizing the culture samples made them have an appearance that was comparable to that of 0.5 McFarland, which is approximately 1.5×10^8 CFU/mL.

3.8 Preparation of Different Sample Concentrations

A stock solution of chitosan-silver nanoparticles was prepared from the synthesized nanoparticle suspension.

The stock solution was serially diluted in sterile distilled water to create three different concentrations of 50, 75 and 100 mg/mL. The following dilution formula was used:

The prepared concentrations were stored under sterile conditions until use.

3.9 Antimicrobial Testing Using Agar Well Diffusion Method

The synthesized chitosan silver nanoparticles were tested against Gram positive and Gram negative bacteria. The bacteria used were *Staphylococcus aureus* (gram-positive) and *Escherichia coli* (gram-negative).

The bacterial isolates used were acquired from the culture collection of the Department of Microbiology. The organisms were grown on nutrient agar slants. The isolates were then re-cultured on freshly prepared nutrient agar plates under aseptic condition and incubated at 37°C for 24 hours for fresh and active cultures for antimicrobial assay.

Nutrient agar was prepared according to the instructions of the manufacturer. 7 g of nutrient agar was weighed and dissolved in distilled water under sterile condition to make 250 ml . The medium was heated to allow complete dissolution and autoclaved at 121 °C for 15 min. The medium was autoclaved, cooled for some time and aseptically transferred to sterile Petri dishes. Plates were prepared and stored sterile until use. All the materials used in the experiment such as cork borer, micropipette, glass rod etc. were sterilized before use.

After 24 hours the bacterial cultures were transferred to each nutrient agar plate by spread plate technique using sterile glass rod and incubated for approximately 10 minutes. Chitosan silver nanoparticles were dispersed in distilled water. Using sterile cork borer, three wells were made in each inoculated agar plate. Then the appropriate amount of the nanoparticle suspension was added to the wells (Balouirl *et al*., 2016 ; CLSI, 2022).

Standard antibiotics were taken as positive control. Two antibiotics were used as a positive control against the Gram-negative bacteria (*Escherichia coli*) while ciprofloxacin was used as positive control against the Gram-positive bacterium (*Staphylococcus aureus*). The inoculated plate were incubated at 37°C.

The synthesized chitosan silver nanoparticles were incubated and their antimicrobial activity was assessed by the diameter of the zone of inhibition around the wells. The results were expressed in millimeter (mm) and the larger zones of inhibition showed higher antimicrobial activity against the tested organisms (Balouirl *et al*., 2016).

3.11 Antioxidant Activity Assays

The antioxidative capacity of the produced chitosan-silver nanoparticles was analyzed using the following assays; DPPH, FRAP, Nitric oxide Scavenging, and Total Antioxidant Capacity assays. Serial dilution was performed in order to get the concentrations of 15.3, 31.25, 62.5, 125, 250, and 500 µg/ml of the sample. The experiments were performed three times, and the results are presented as Mean ± standard deviation.

DPPH radical scavenging assay was used.

The DPPH test was employed for the determination of the antioxidant activity of the prepared chitosan–silver nanoparticles using the DPPH radical scavenging activity. Different concentrations of the compound were reacted with the DPPH reagent and the absorbance was measured at 517 nm in the UV-visible spectrophotometer. Ascorbic acid was taken as the standard antioxidant.

Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant property of the synthesized chitosan–silver nanoparticles was estimated by FRAP method. The sample was prepared into a few different concentrations and then added to a freshly prepared FRAP reagent and incubated for 30 minutes at 37°C. A UV–Visible spectrophotometer was used to measure the absorbance at 593 nm. The antioxidant activity was expressed in terms of Ascorbic Acid Equivalent (AAE).

Nitric Oxide Scavenging Assay

The synthesized chitosan–silver nanoparticles possess nitric oxide scavenging property that was tested by the Griess reagent method. Sodium nitroprusside solution was added to different concentrations of the sample and then the Griess reagent was added. The antioxidant activity was reported as percentage inhibition and absorbance was measured using UV-Visible spectrophotometer at 540 nm.

Total Antioxidant Capacity (TAC) Assay

The synthesized chitosan–silver nanoparticles were analysed by phosphomolybdenum method for their total antioxidant capacity. Various concentrations of the sample was reacted with the phosphomolybdenum reagent and UV-visible spectrophotometer was used to determine the absorbance. The antioxidant potential was reported in terms of Ascorbic Acid Equivalent (AAE).

3.12 Data Collection

Data collected during the study included:

a) FTIR spectra
b) SEM images
c) XRD diffraction pattern
d) Zone of inhibition values (mm)
e) Effects of nanoparticle concentration on bacterial growth inhibition
f) Antioxidant percentage inhibition

All analyses were carried out in triplicate and results were expressed as Mean ± Standard Deviation (SD).

3.13 Statistical Analysis

The data obtained were analyzed using Statistical Package for Social Sciences (SPSS) version 25.0.

Descriptive statistics including mean and standard deviation were calculated. One-way Analysis of Variance (ANOVA) was used to determine significant differences among treatment groups (Rosner, 2020). Statistical significance was accepted at $p < 0.05$.

3.14 Safety and Ethical Considerations

a) Appropriate personal protective equipment (laboratory coat, gloves, and safety goggles) was worn throughout the study.
b) Silver nitrate, hydrochloric acid, and sodium hydroxide were handled with caution to prevent chemical injuries.
c) All microbial procedures were carried out using aseptic techniques.
d) Used culture media and contaminated materials were sterilized by autoclaving before disposal.
e) Laboratory safety guidelines were strictly followed throughout the study.
f) The study did not involve human participants or experimental animals; therefore, no human or animal ethical approval was required.

CHAPTER FOUR

RESULTS

4.1 Yield and Physical Characteristics of Extracted Chitosan

4.1.1 Physical Characteristics

The chitosan obtained after demineralization, deproteinization, decolourization and deacetylation of crab shell powder was a fibrous material with off white color and dry texture. The product was free flowing and had the appearance typical of purified chitosan.

4.1.2 Percentage Yield of Chitosan

50.00 g of crab shell powder were subjected to extraction and purification processes, which resulted in 4.71 g of chitosan. A percentage yield of 9.42% was obtained.

Table 4.1: Percentage Yield of Chitosan Extracted from Crab Shell Powder

Parameter	Value
Weight of crab shell powder used (g)	50.00
Weight of chitosan obtained (g)	4.71
Percentage yield (%)	9.42

4.2 Synthesis of Chitosan-Silver Nanoparticles (CS-AgNPs)

4.2.1 Visual Observation of Nanoparticle Formation

During the synthesis of chitosan-silver nanoparticles, the colour change was visually monitored to follow the reduction process. The mixture of chitosan with silver nitrate was at first a clear pale yellowish solution. On addition of ascorbic acid a gradual change of colour to dark brown was observed.

The change in the observed colour indicated the reduction of Ag+ ions to the metallic silver (Ag^0) and indicated the formation of silver nanoparticles.

Table 4.2: Visual Observation During Synthesis of CS-AgNPs

Reaction Stage	Observation
Chitosan + Silver nitrate solution	Pale yellowish solution
After addition of ascorbic acid	Dark brown colloidal suspension

4.3 Synthesized Chitosan-Silver Nanoparticles Characterization

4.3.1 Fourier Transformed Infrared Spectroscopy (FTIR)

FTIR analysis was used to identify the functional groups involved in the reduction and stabilization of silver nanoparticles by chitosan.

The main absorption peaks noticed were 3420cm-1, 2925cm-1, 1650cm-1, 1595cm-1, 1380cm-1, 1150cm-1 and 1070cm-1 corresponding to O-H/N-H stretching, C-H stretching, amide 1 (C=O), N-H bending (amide 11), C-H bending, C-O-C stretching, and C-O stretching functional groups respectively.

4.3.2 Scanning Electron Microscopy (SEM)

SEM analysis was performed to study the morphology and surface characteristics of the synthesized nanoparticles. Representative SEM micrographs of the sample at magnifications of 8,000x, 9,000x and 10,000x are represented in Figure 4.1-4.3

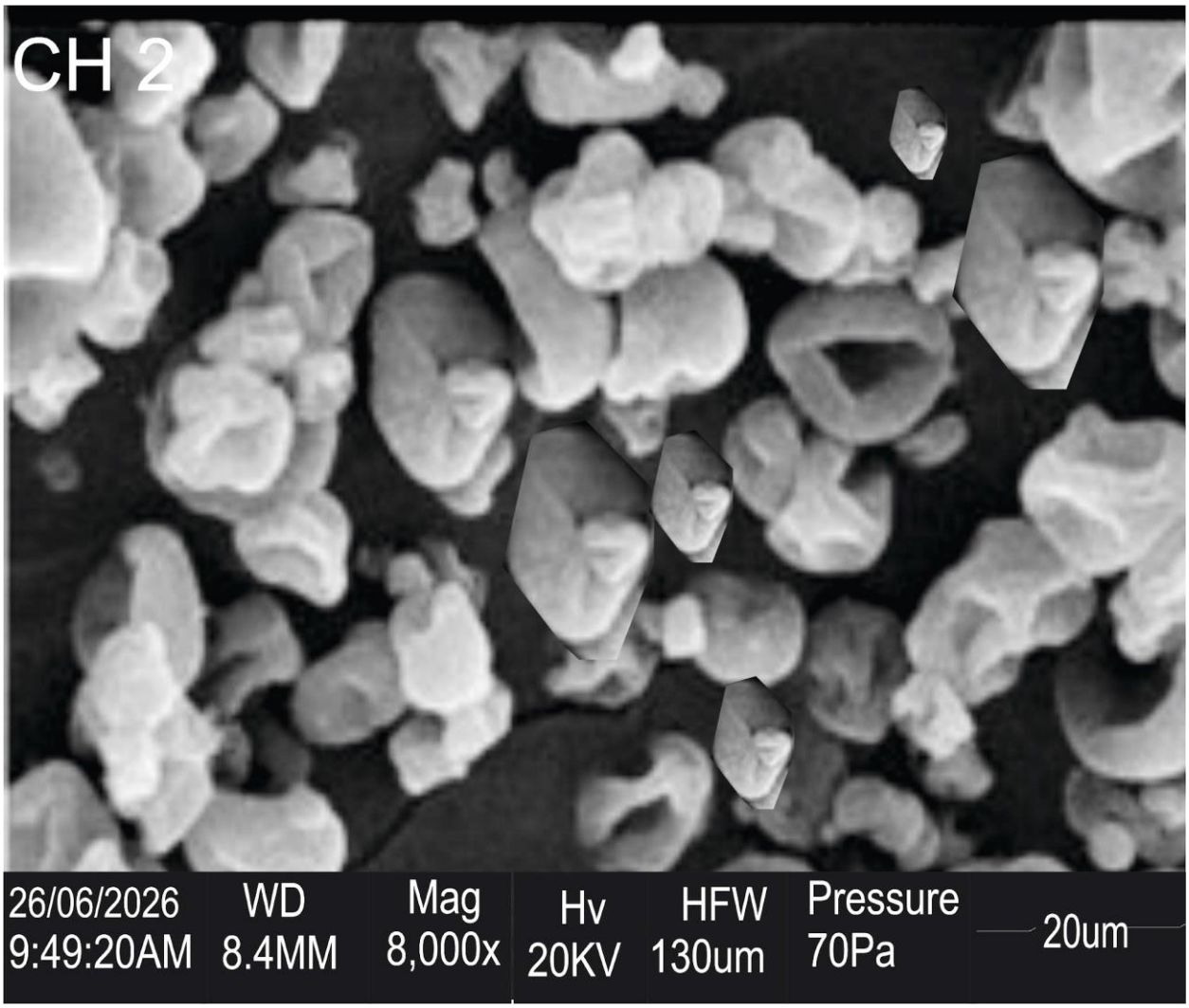

Figure 4.1 SEM image magnified at 8,000x

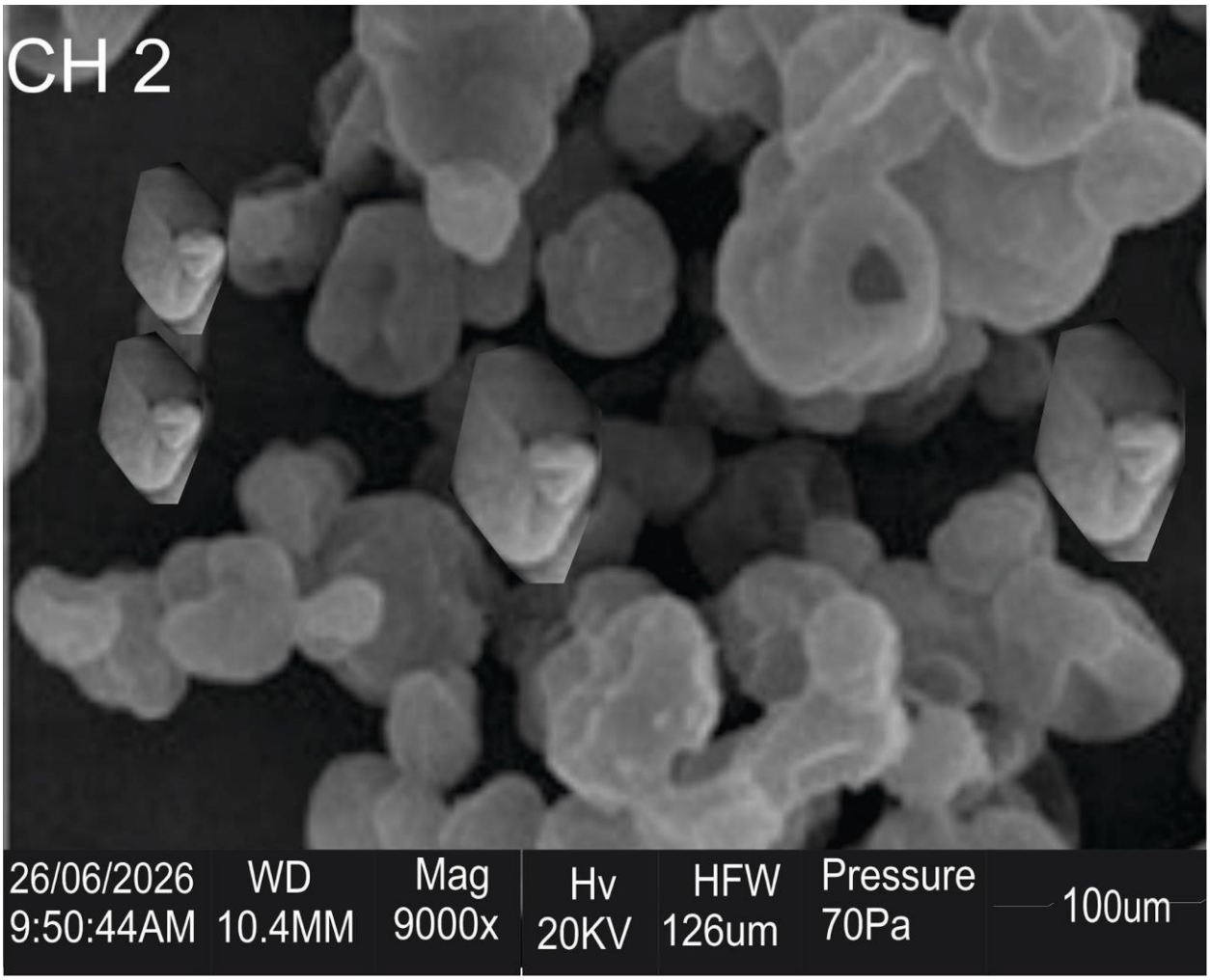

Figure 4.2 SEM image magnified at 9,000x

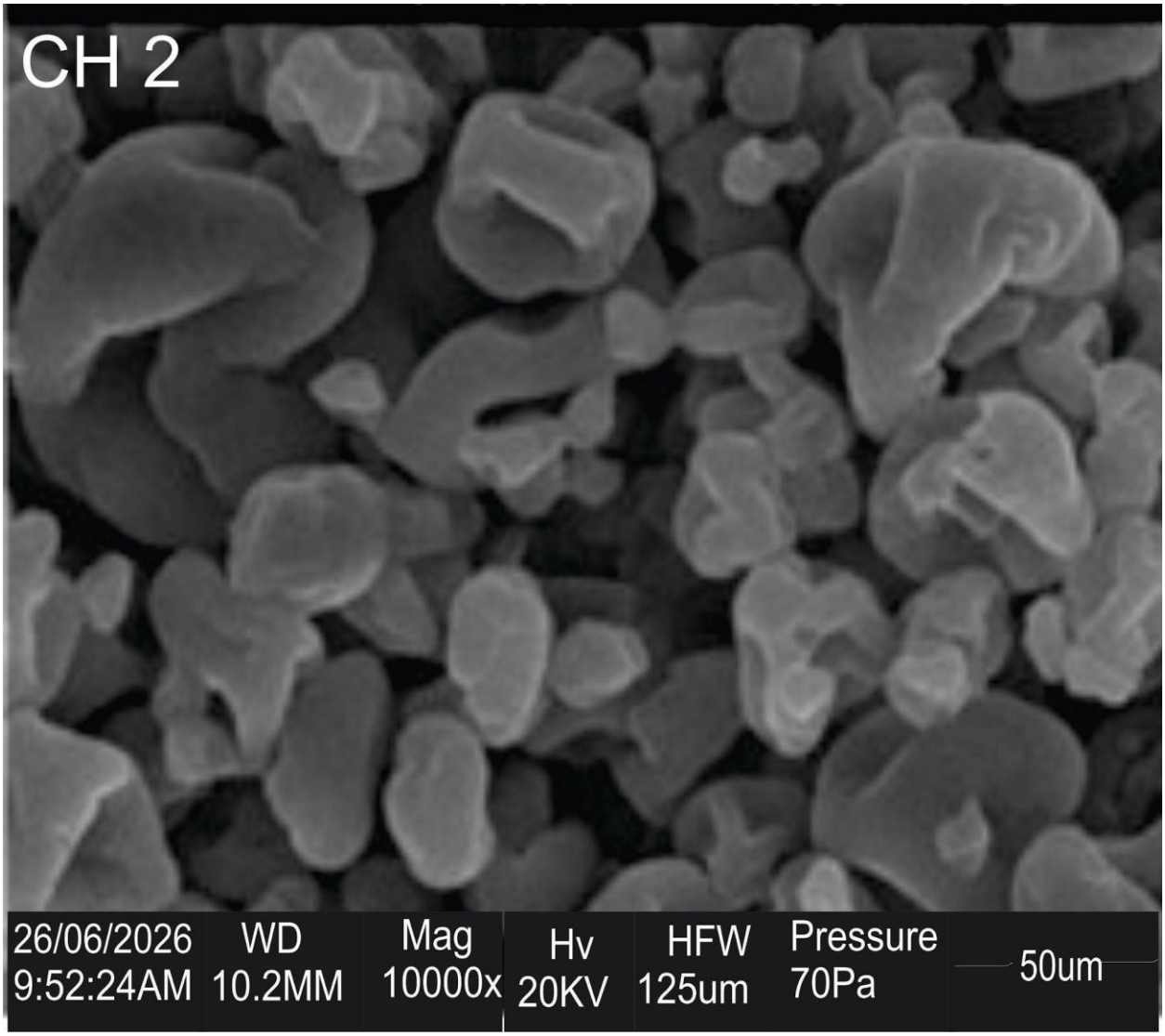

Figure 4.3 SEM image magnified at 10,000x

The nanoparticles exhibit irregular and heterogeneous morphorlogies with rough surfaces. The particles were observed to form aggregated clusters, which is a common characteristic of chitosan nanoparticles due to intermolecular interactions during drying. The SEM analysis confirmed the successful formation of particlelate chitosan structures.

Energy Dispersive X-ray spectroscopy (EDS) Analysis

The elemental composition of the synthesized sample was determined using Energy Dispersive X-ray spectroscopy (EDS). The EDS spectrum and quantitative analysis are presented in Figure 4.4

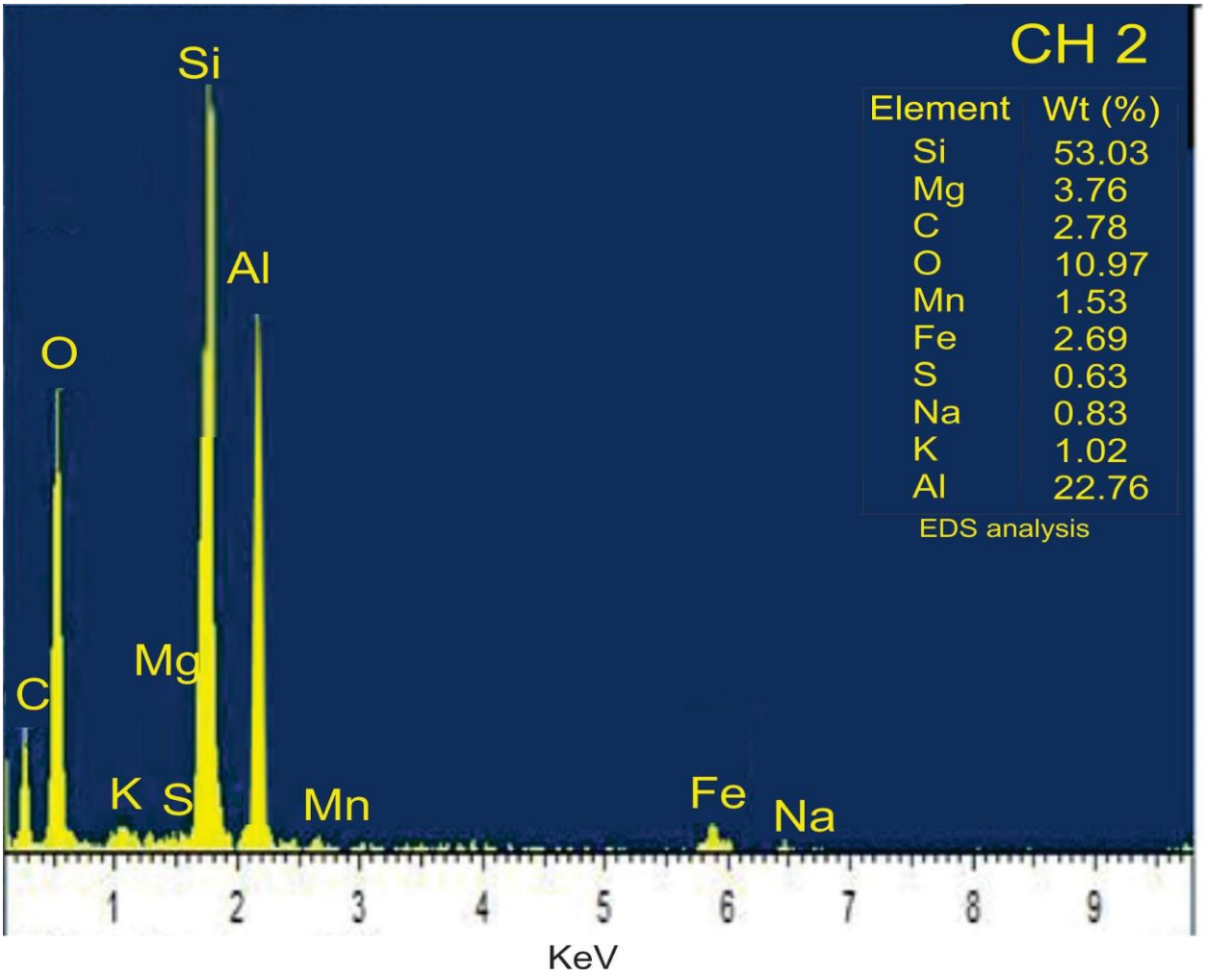

Figure 4.4 EDS spectrum and quantitative analysis

Carbon and oxygen are present and consistent with the composition of chitosan and high silicon and aluminum contents may be derived from the sample substrate, preparation process or residual impurities. The EDS analysis confirmed the elemental composition of the sample analysed and matched with the findins of SEM.

4.3.3 X-Ray Diffraction (XRD)

XRD analysis confirmed the crystalline nature of the synthesized nanoparticles.

Observed diffraction peaks characteristic at 2Ø=3.81º, 44.3º, 64.5º and 77.4º confirmed the crystalline structure of silver nanoparticles.

4.4 Antioxidant Activity of Synthesized CS-AgNPs

Nitric Oxide % Inhibition Assay

The Standard (Ascorbic acid) value was highest at low concentrations, 84.54 at 15.63µg/mL to 85.05 at 31.25µg/mL, then declined with increasing concentration to 60.40 at 500µg/mL. Comparing Sample against the Standard, the value ranged from 16.87 at 125µg/mL to 43.30 at 250µg/mL. At every concentration, Sample value was far below ($p<0.05$) the Standard value.

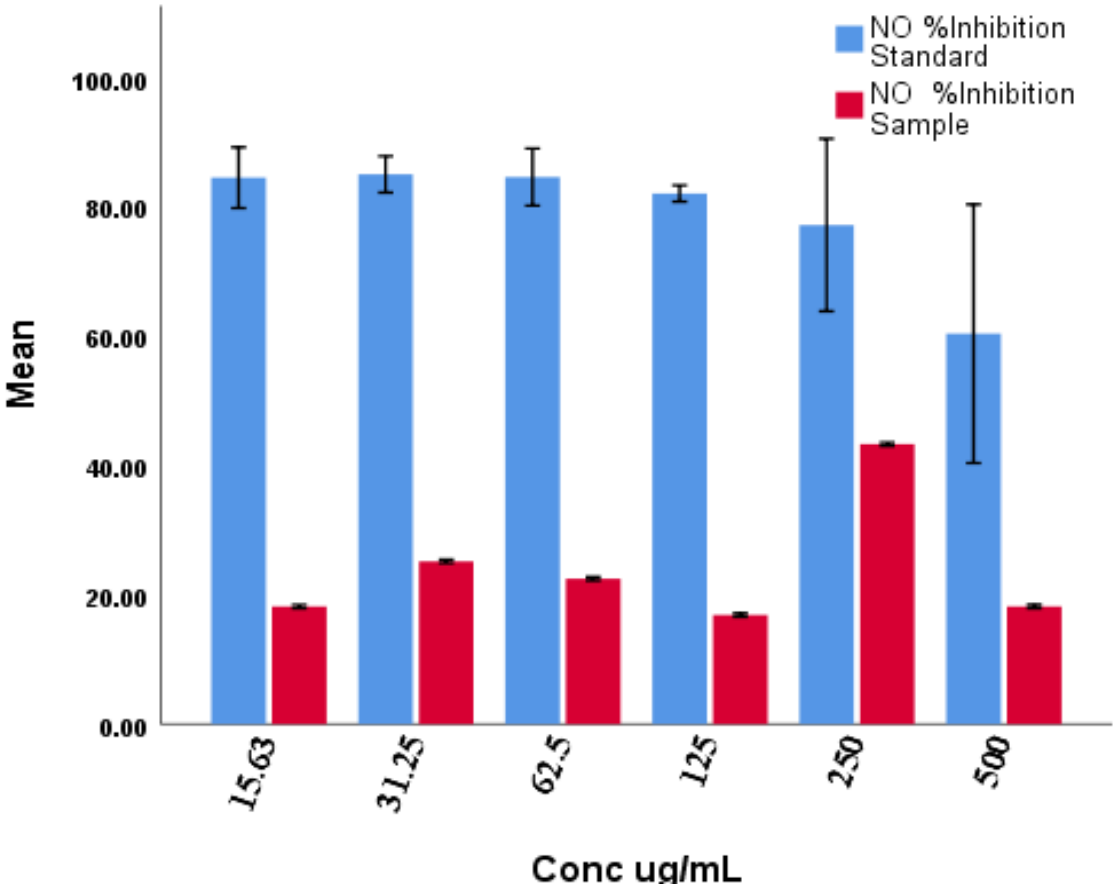

Figure 4.5: Nitric Oxide (NO) Radical Scavenging Activity (% Inhibition) of the Synthesized Chitosan Sample Compared with Ascorbic Acid Standard at Different Concentrations (15.63–500 µg/mL).

DPPH % Inhibition Assay

The standard's value were stable across all concentrations, ranging from 62.61 at 15.63µg/mL to 64.19 at 31.25µg/mL. There was no clear concentration-dependent increase. Comparing Sample against the Standard, sample gave higher values ($p<0.05$) than standard at every concentration. It ranged from 73.76 at 15.63µg/mL to 83.05 at 125µg/mL. The highest value was 83.05 at 125µg/mL, compared to Standard 63.30 at the same concentration.

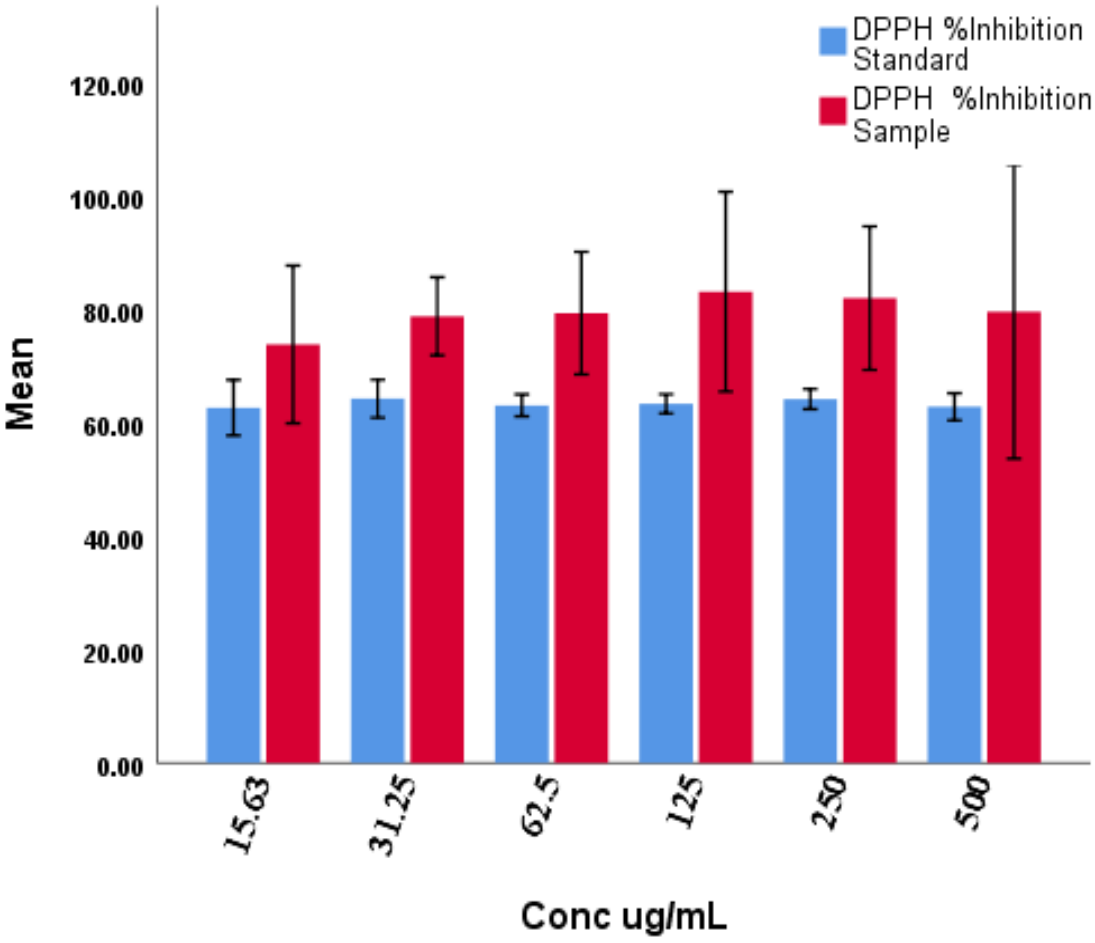

Figure 4.6: DPPH Radical Scavenging Activity (% Inhibition) of Chitosan-Synthesized Silver Nanoparticles Compared with Ascorbic Acid Standard at Different Concentrations (15.63–500 µg/mL).

Ferric Reducing Antioxidant Power % (AAE)

Sample values ranged from 114.61 at 500µg/mL to 131.51 at 62.5µg/mL. The highest value was 131.51 at 62.5µg/mL. Result did not show a clear dose-dependent trend, however sample values were higher at mid concentrations.

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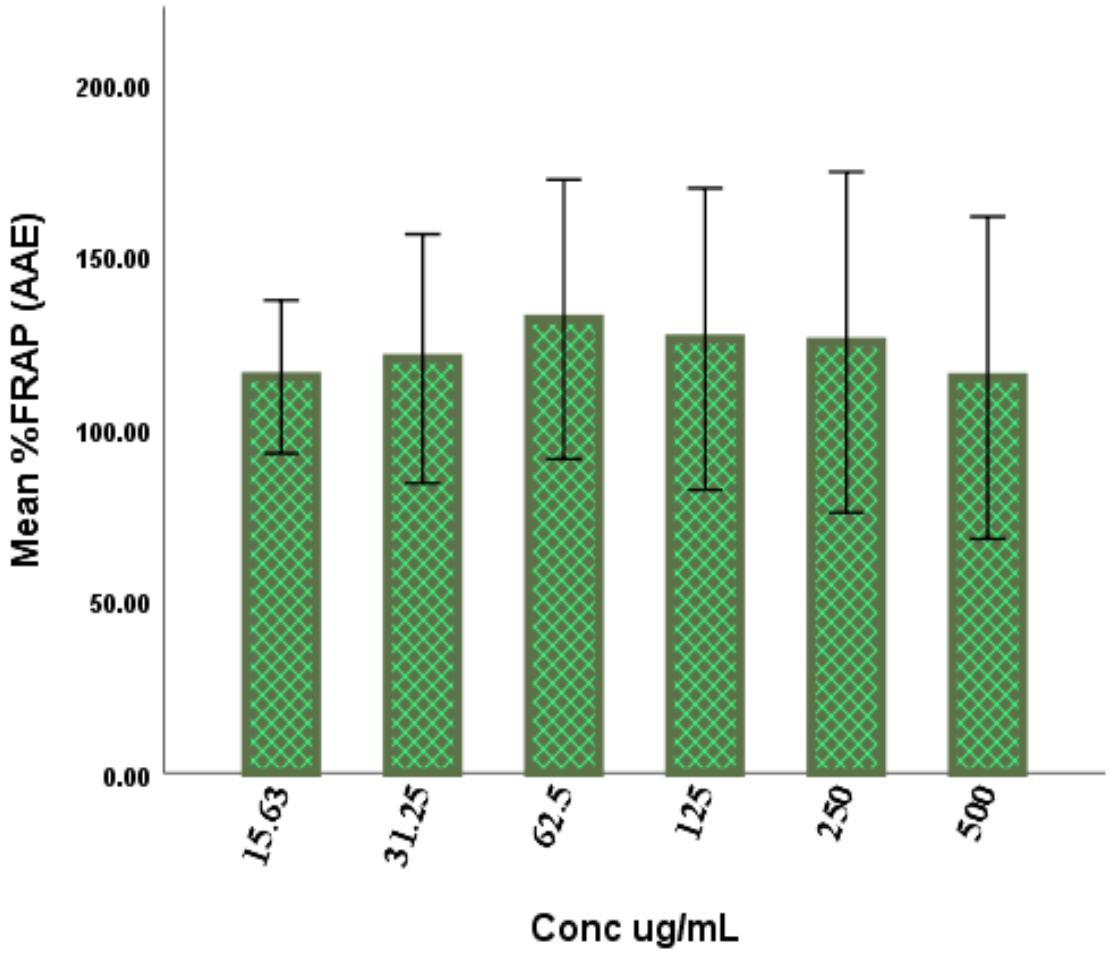

Figure 4.7: Ferric Reducing Antioxidant Power (FRAP) of the Synthesized Chitosan Sample at Different Concentrations (15.63–500 µg/mL).

Total Antioxidant Capacity (AAE)

Expressed as Ascorbic Acid Equivalent. Higher value = higher capacity.

The Sample value ranged from 59.35 at 31.25µg/mL to 86.35 at 125µg/mL. The highest value was 86.35 at 125µg/mL. The pattern was also irregular, not increasing steadily with concentration.

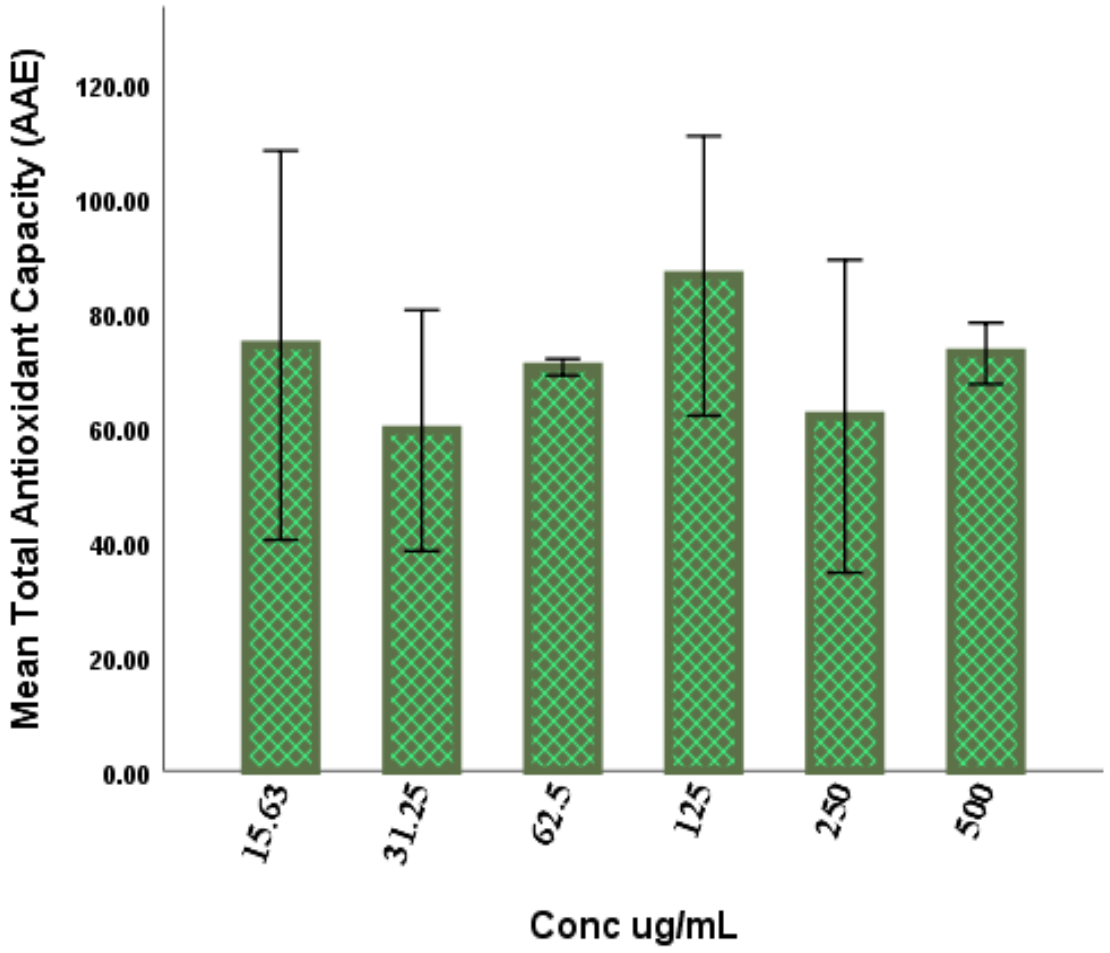

Figure 4.8: Total Antioxidant Capacity (TAC) Expressed as Ascorbic Acid Equivalent (AAE) of the Synthesized Chitosan Sample at Different Concentrations (15.63–500 µg/mL).

4.5 Antimicrobial Activity of Synthesized CS-AgNPs

The antibacterial activity of the synthesized CS-AgNPs was determined against *Escherichia coli* and *Staphylococcus aureus* by agar well diffusion method at different concentration.

TEST	CONCENTRATION (mg/mL)	ZONE OF INHIBITION AGAINST *Staphylococcus aureus*	ZONE OF INHIBITION AGAINST *Escherichia coli*
Chitosan-AgNP	25	10.2 ± 0.3	8.5 ± 0.2
Chitosan-AgNP	50	15.8 ± 0.5	13.2 ± 0.4
Chitosan-AgNP	100	21.4 ± 0.4	18.9 ± 0.3
Positive control	Standard	30.5 ± 0.2	29.8 ± 0.3
Negative control	Distilled water	0.0	0.0

Table 4.3: Antimicrobial activities of chitosan silver nanoparticles

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

The present study was focused on the extraction of chitosan from the waste of crabs, the attempted synthesis of chitosan-silver nanoparticles (CS-AgNPs) by using ascorbic acid as a reducing agent and the in vitro antioxidant and antimicrobial activity evaluation of the material obtained. The results present relevant information on the possible use of crab shell chitosan as a natural biomaterial, and also on the optimization of the process of production of the nanoparticles.

5.1.1 Properties of Chitosan

The properties of extracted chitosan include its yield and physical properties. The yield and physical properties of extracted chitosan are considered as the properties of chitosan.

4.71 g purified chitosan from 50.00 g of powdered crab shell with the percentage yield of 9.42%. The results of the yield indicate that the local crab shell waste is a potential and sustainable material for chitosan production. Since the extraction efficiency is affected by the species of crabs, shell composition, degree of mineralization and deproteinization and deacetylation conditions as well as other processing factors, differences in chitosan yield reported in various previous studies are expected.

The chitosan extracted was white in colour, fibrous and free flowing in nature which is a typical property of purified chitosan. The physical properties indicate an efficient extraction of the proteins and mineral components during processing and that the extraction conditions were appropriate to obtain a relatively pure biopolymer that was suitable for further investigation

5.1.2 Characterization of chitosan synthesis silver nanoparticles

Characterization of the synthesized chitosan based nanoparticles using FTIR, XRD, SEM and EDS confirms the success of the synthesis. FTIR confirmed the presence of characteristic functional groups of chitosan, which are responsible for the formation of the nanoparticles, and XRD showed that the synthesized material is crystalline. The morphological features showed an irregular, rough, sheet-like, and heterogeneous surface observed by SEM indicating that no significant change occurred in the structural arrangement of the polymer, the structure of which corresponds to a titanium matrix. EDS analysis indicated silicon as the major element (50.73%) with significant amounts of aluminum (22.76%), oxygen (10.95%), magnesium (3.76%), carbon (2.78%) and minor amounts of other elements. This high silicon content could be due to the substrate or support on which the SEM-EDS analysis is performed, contamination from sample preparation, or contamination from the mounting material. If no silver peak is detected, this may be because the amount of silver in the sample is small, and/or because there is a low concentration of silver within the sample, and/or because the region being analysed did not contain enough silver for it to be detected by EDS. In conclusion, the characterization outcomes are helpful to gain insights into the morphology, crystallinity, and elemental composition of the synthesized nanoparticles and support their applications in the antimicrobial field.

5.1.3 In Vitro Antioxidant and Antimicrobial Activity

Chitosan from the crab shell extract was assessed for antioxidant activity by using DPPH, Ferric Reducing Antioxidant Power (FRAP), Nitric Oxide (NO), and Total Antioxidant Capacity (TAC) assays. Free radical scavenging ability was evident in the DPPH assay with values of 73.76% at 15.63 µg/mL to 83.05% at 125 µg/mL, and slightly decreased after that value. Also, reducing power measured by the FRAP assay had a high value of 131.51% at 62.5 µg/mL, showing that the

electron-donating ability was highest at medium value before the value decreases slightly as the concentration increases.

Nitric oxide activity showed comparatively lower antioxidant activity from 16.87% at 125 µg/mL to 43.30% at 250 µg/mL. It shows that the sample has lower ability to scavenge nitric oxide radicals than the standard. The TAC assay confirmed the antioxidant activity of the sample by increasing values from 59.35% at 31.25 µg/mL to 86.35% at 125 µg/mL.

SEM shows irregular sheet structures, while EDS analysis does not show any presence of silver in the analyzed region. Hence, the presence of antioxidant activity is possibly due to the natural chitosan amino ($-NH_2$) and hydroxyl (-OH) functional group. In conclusion, crab shell chitosan extract shows appreciable antioxidant activity.

Invitro Antimicrobial Activity

The antimicrobial assay demonstrated that the synthesized chitosan-based nanoparticles exhibited inhibitory activity against both Staphylococcus aureus and Escherichia coli. The antimicrobial effect increased with increasing concentration, indicating a concentration-dependent activity. Greater inhibition was observed against *Staphylococcus aureus* than *Escherichia coli*, suggesting that the nanoparticles were more effective against the Gram-positive bacterium. These findings indicate that the synthesized nanoparticles possess promising antimicrobial properties and may have potential applications in controlling bacterial infections and in other biomedical fields.

5.2 Conclusion

Successfully, chitosan based nanoparticles were extracted and synthesized from crab shell waste. The synthesized material was characterized by FTIR, XRD, SEM and EDS to obtain information about the structural, crystalline, morphological and elemental properties. The synthesized nanoparticles also showed good antioxidant activity which suggests that they have the ability to

act as free radical scavengers. Additionally, they showed concentration dependent antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*, where *Staphylococcus aureus* was more inhibited. The overall results suggest that the synthesized nanoparticles have potential applications in antimicrobial, antioxidant, and other biomedical fields.

5.3 Recommendations

Future studies should optimize the synthesis conditions to improve the incorporation and detection of silver nanoparticles in the synthesized material.

Additional characterization techniques, particularly UV-Visible spectroscopy and Transmission Electron Microscopy (TEM), should be employed to further confirm nanoparticle formation, size, and distribution.

The antimicrobial activity of the synthesized nanoparticles should be evaluated against a wider range of Gram-positive and Gram-negative microorganisms to establish their broad-spectrum antimicrobial potential.

Further studies should investigate the cytotoxicity, biocompatibility, and in vivo biological activities of the synthesized nanoparticles to assess their suitability for biomedical and drug delivery applications.

Snail shell waste should be further explored as an inexpensive, eco-friendly, and sustainable source of chitosan for the development of functional biomaterials and nanomaterials.

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