GREEN SYNTHESIS OF Ag_2S NANOPARTICLES: EFFECT OF pH AND CAPPING AGENT ON SIZE AND SHAPE OF NPs AND THEIR ANTIBACTERIAL ACTIVITY

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This work reports on the synthesis of silver sulfide nanoparticles capped with either chitosan, green tea, *Combtretum molle* or black wattle extracts. The nanoparticles were synthesized in an aqueous solution in the presence of capping agent as stabilizer and reducing agents. Silver nitrate and thiourea were used as silver and sulfur precursor respectively. The formation of nanoparticles was confirmed using ultraviolet-visible spectroscopy, which showed silver sulfide peaks between 343-402 nm. The Transmission Electron Microscope images also confirmed the formation of silver sulfide nanoparticles with a spherical shape depending on the capping agent used. X-ray diffraction analysis revealed that the nanoparticles have the acanthite phase. Fourier transform infrared spectroscopy confirmed that the nanoparticles successfully capped. The nanoparticles were also tested for their antibacterial activity and they were found to be active against *Staphylococcus aureus* and *Escherichia coli*. The minimum inhibitory concentrations for the synthesized nanoparticles were between 0.15 and 0.31 mg/mL.

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1. Introduction

Silver sulfide (Ag_2S) is a direct band gap (0.9-1.05 eV) semiconductor with good optical limiting as well as high absorption coefficient and chemical stability properties. Ag_2S nanoparticles are major materials as photocatalysts, among all semiconductors [1]. There is a growing interest in the use of nanomaterials in biosciences due to their unique properties. The conjugation of semiconductor nanoparticles with biomolecules through surface modification of the nanoparticles added a new dimension to the possible application of nanoparticles. The biofunctionality of the nanoparticles permits selective interaction of the nanoparticles with the biochemical species thus increase the area of applications to biological systems. It is very important that the nanoparticles be in a water-dispersible form for their use in any biological applications. Water-soluble nanoparticles are produced through surface modification of organic soluble nanoparticles with biocompatible materials. The surface modification of nanoparticles increases their luminescent quantum yields, expand their stability, prevent their aggregation and also make them available for interaction with target analytes [2].

Also, the bactericidal effect of nanoparticles depends not only on the size and shape of nanoparticles but also on the functionalization of their surface. The main benefit of using plant extracts for nanoparticles synthesis is that they are easily available, nontoxic, economical and have a wide variety of metabolites that can assist in the reduction of metal ions. Also, plants mediated nanomaterial synthesis has great reducing power to rapidly reduce the wide range of metals [3, 4]. *Combretum molle* plant is widely used to treat constipation, leprosy, headaches, stomach pains, fever, dysentery, swelling, chest complaints, stomach ailments and to treat wounds. The black

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wattle plant leaves have a high protein content, while the bark is the most widely used tannin material in the world. Due to the OH^- groups in the ortho position on the aromatic rings, tannins are capable of forming chelates with metal ions [5, 6]. Phytochemical tests showed that the *Combretum molle* and black wattle plants contains tannins, proteins, flavonoids, phenols and glycosides, which justify their use as antibacterial by the population [7, 8]. Chitosan, a polymer that occurs naturally, is an antibacterial and bioactive polymer with large amounts of amine and hydroxyl groups [9]. Green tea, a product made from the *Camellia sinensis* plant, contains polyphenols which form complexes with metal ions in solution thus reduce them to the corresponding metals [10]. Silver ions and silver-based compounds are well known to be highly toxic to microorganisms [3].

Nanoparticles have been synthesized by chemical, physical and biological methods, where chemical reduction is the widely used method. Lately, biosynthesis using non-toxic, eco-friendly and convenient biological materials such as fungi, bacteria, biomolecules and plant extracts is under investigation [3]. This is done in order to replace the harmful capping and reducing agents that have been used in the synthesis of nanoparticles, which then hinders them to be applied in biological systems. Due to the increasing concerns about bacterial infections as a result of their antibiotic resistance, there is a growing need to develop new antibacterial agents. Therefore, this work focuses on the biosynthesis of Ag_2S nanoparticles using an environmental friendly method. The synthesis was done at room temperature, using environmental friendly as well as biocompatible capping/reducing agents (*Combretum molle*, black wattle, green tea and chitosan) and water as a solvent. The effects of pH on the synthesis of nanoparticles have been investigated due to its ability to affect the particle size. To the best of our knowledge, no work has been published on the synthesis and antibacterial activity of Ag_2S nanoparticles capped with *Combretum molle* and black wattle plant.

2. Methodology

2.1 Chemicals

Silver nitrate ($AgNO_3$) (99.8%), thiurea (H_2NCSNH_2) (99.9 %), ammonium hydroxide (NH_4OH) (99.9%) reagent grade were purchased from Merk (Germany). Acetone (99.8%), chitosan reagent grades was purchased from Sigma Aldrich (South Africa). Muller-Hinton broth and Muller-Hinton agar were purchased from Neogen (Michigan). *Escherichia coli* and *Staphylococcus aureus* bacterial cells were bought from Anatech (South Africa). All chemicals were used as purchased, without any further purification.

2.2 Synthesis of Ag_2S nanoparticles

Silver sulfide nanoparticles were prepared at room temperature by adding 0.1 M silver nitrate solution to 1.0 % (w/v)) of either green tea, chitosan, *Combretum molle* plant leaves, black wattle plant leaves in a round-bottom flask. The mixture was stirred for two hours under inert atmosphere. The resultant solution was centrifuged and extracted with acetone to obtain Ag_2S nanoparticles. The precipitated nanoparticles were then washed with excess acetone and dried at room temperature.

2.3 Effect of pH and capping agent

The effect of pH was investigated by adjusting the pH from 3-11 using ammonium hydroxide. The effect of pH was studied since it plays role in the synthesis of nanoparticles because it affects the size and shape of the nanoparticles. This is due to the fact that the pH affects the distribution of the functional group responsible for capping the nanoparticles. The effect of the capping agent was investigated by using 1.0 % (w/v)) of different capping agents, i.e., green tea, chitosan, *Combretum molle*, black wattle. The effect of capping agent was investigated since it plays a role in controlling the growth process and thus manipulates the size and shape of the nanoparticles. The samples were then characterized with Ultraviolet Visible spectroscopy (UV-Vis), Fourier transform infrared spectroscopy (FT-IR), Thermal gravimetric analysis (TGA), Transmission Electron Microscope (TEM) and X-ray diffraction (XRD) techniques.

2.4 Characterization

The optical analysis was carried out using UV-Vis spectrophotometer (Perkin Elmer Lamda 25) at room temperature across a 200-1100 nm wavelength range. To take the measurements, the samples were placed in quartz cuvettes cell of 1 cm path length, a beam was then passed through and the spectrum was recorded. The structural analysis was done using a Perkin Elmer spectrum 400 FT-IR-NIR Spectrometer equipped with a universal ATR sampling accessory. A JEOL JEM-2100 transmission electron microscope operating at 200 kV was used to study the morphology of the nanoparticles. The TEM was coupled with an energy dispersive X-ray (EDX) detector which was used to determine the elemental composition of the synthesized nanoparticles. For the analysis, the samples were prepared by placing an aliquot solution of the water soluble nanocrystalline material onto a carbon substrate supported on a copper grid and then the solvent was allowed to dry at room temperature. A Bruker D2 diffractometer at 40 kV and 50 mA and a secondary graphite monochromated Co K alpha radiation (l = 1.7902 A˚) was used to record the X-ray diffraction patterns. The measurements were taken at angle 2θ in a range of 5˚–90˚ with a scan rate of 0.01° 2θ s^{-1}. The thermal analysis was done using Perkin Elmer Thermogravimetric Analyzer (TGA 4000) with a heating range of 25 and 900 °C at a heating rate of 5°/min under nitrogen gas.

2.5 Antibacterial Activity and Minimum Inhibitory Concentration (MIC)

The antibacterial activity of Ag_2S nanoparticles was investigated using the disc diffusion method. About 20 mL of sterile molten Mueller Hinton agar was poured into sterile petri dishes. Triplicate plates were swabbed with the overnight culture (10^5cells/mL) of *Escherichia coli* and *Staphylococcus aureus* bacteria. A concentration of 5 mg/mL of Ag_2S nanoparticle samples capped with different capping agents (green tea, chitosan, *Combretum molle*, black wattle) were added into the discs, placed in the petri dishes and incubated for 24 hours at a temperature of 37 ± 2°C. The zone of inhibition, expressed as millimeter in diameter, was measured after 24 hours of incubation. The minimum inhibitory concentration (MIC) of the nanoparticles was investigated using 96 well Microtitre Dilution Assay method by Cchilla et al. [11]. The MIC was investigated using (2.5, 1.25, 0.62, 0.31, 0.15, 0.078, 0.039, 0.019 mg/mL) concentrations of nanoparticles. The experiments were performed in triplicates.

3. Results and Discussion

3.1 Optical and structural analysis

UV-Vis spectroscopy was used to study the effect of pH and capping agent on the optical properties of the Ag_2S nanoparticles. Fig. 1 shows the UV-Vis spectra of Ag_2S nanoparticles capped with green tea, *Combretum molle*, black wattle plant and chitosan under acidic and basic conditions. Green tea capped Ag_2S nanoparticles showed a peak at 387 nm under basic and 402 nm under acidic conditions. Chitosan capped nanoparticles showed a peak at 343 nm under basic and 350 nm under acidic conditions. *Combretum molle* capped nanoparticles showed a peak at 360 nm under basic and 365 nm under acidic conditions. Black wattle capped nanoparticles showed a peak at 352 nm under basic and 354 nm under acidic conditions. All capping agents gave broader peaks and at higher wavelengths under acidic conditions which could mean that the synthesized nanoparticles are either bigger or agglomerated under acidic condition. This could be due to the enhancement of the distribution of the hydroxyl groups of the capping agents under basic conditions, which resulted in the effective capping and thus smaller nanoparticle size.[12] Chitosan showed a peak at lower wavelength, which is an indication that it gives nanoparticles with smaller nanoparticles compared to the other capping agents. These findings indicate that pH has an effect on the optical properties of Ag_2S nanoparticles. Therefore basic conditions were chosen as optimum for the synthesis of Ag_2S nanoparticles using these capping agents.

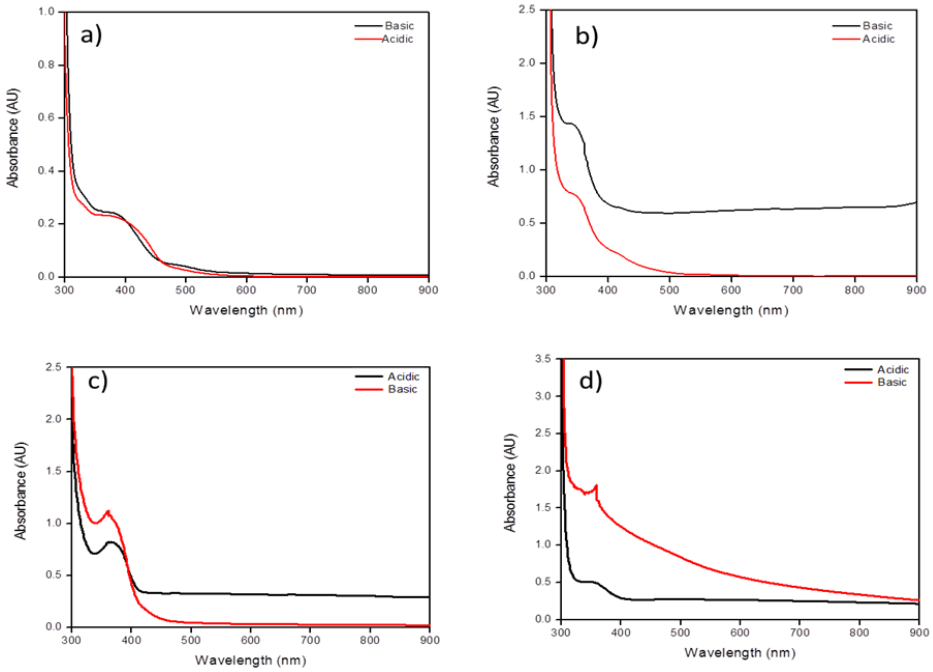

Fig. 1 : Uv-Vis absorption spectra of Ag_2S nanoparticles capped with a) greet tea, b) chitosan, c) Combretum molle, d) black wattle under basic and acidic conditions.

FTIR (Fig. 2) analysis was conducted in order to investigate the molecular interactions between Ag_2S nanoparticles and the capping agent. The spectrum for the pure black wattle plant showed a peak due to phenolic O-H at 3319 which shifted to 3279 cm^{-1} after incorporation of Ag_2S nanoparticles, indicating the reaction of the nanoparticles with the capping molecule. The peak at 1600 and 1450 cm^{-1} which are due to the characteristic of aromatic compounds, while the peaks between 600-1300 cm^{-1} are the substituted benzene rings [6]. The *Combretum molle* plant showed an O-H peak at 3306 cm^{-1} which shifted to 3279 cm^{-1} after the reaction with the nanoparticles, while the peak due to C=O observed at 1660 cm^{-1} shifted to 1615 cm^{-1}. The spectrum of pure chitosan showed transmissions at 3364 and 3299 cm^{-1} assigned to the overlap of O-H and N-H stretching vibrations, respectively. The band at 1650 and 1590 cm^{-1} corresponds to C=O and -NH_2 bending, and 1028 cm^{-1} to -C-O skeletal stretching. This trend was also observed in the chitosan capped Ag_2S-NPs spectrum. A shift in bands was noticed (from 3364 to 3312, 3299 to 3288, 1648 to 1641, and 1028 to 1021 cm^{-1}) [13,14]. Also, the broadness of peaks was observed in the chitosan capped nanoparticles. The presence of hydroxyl and amine groups indicated the interaction between the Ag_2S-NPs surface and the chitosan, suggesting that the NPs were capped by the chitosan. The spectrum of pure green tea showed O-H stretch at 3339 cm^{-1}, C=C stretch at 1623 cm^{-1}, C-O-C stretch at 1370 cm^{-1} and C-O stretch at 1052 cm^{-1}. These peaks shifted after the reaction with Ag_2S nanoparticles to 3350 cm^{-1}, 1639 cm^{-1}, 1377 cm^{-1} and 1044 cm^{-1} respectively. The existence of these functional groups on green tea capped Ag_2S indicated a successful capping of the nanoparticles by green tea [15]. The O-H peak obtained under basic conditions was observed to be longer and broader than in acidic conditions. This could be an indication that the association between the functional groups of the capping agent through H-bonding is more prominent under acidic pH, thus the structures stabilizes leading in the ineffectiveness utilization of the capping agent in the reduction process [16].

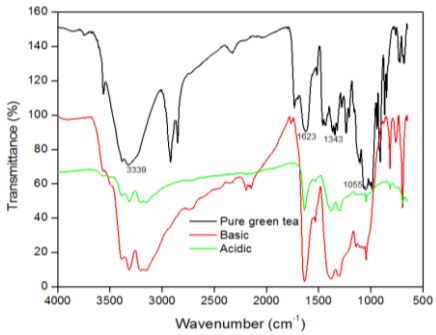

Fig. 2: FTIR spectra of Ag_2S nanoparticles capped with green tea under basic and acidic conditions.

3.2 Morphological analysis

TEM was used to investigate the effect of capping agents under acidic and basic conditions on the size and shape of Ag_2S nanoparticles. Fig. 3 shows the TEM images of green tea, black wattle, *Combretum molle* and chitosan capped Ag_2S nanoparticles. Green tea, chitosan, black wattle and *Combretum molle* capped Ag_2S nanoparticles gave spherical shape nanoparticles. All capping agents gave more agglomerated nanoparticles under acid conditions than under the basic conditions. Also, the nanoparticle sizes was reduced under basic than acidic conditions. These findings indicated that the basic conditions were able to activate the hydroxyl groups which are responsible for the interaction of the nanoparticles with these capping agents and thus increased their capping ability [17]. The reduction of the size of Ag-starch nanoparticles with an increase in pH as a result of the enhancement of the starch hydroxyl groups has been reported [12, 18]. Chitosan capped nanoparticles had the smallest particle size, which could be due to the large quantity of free amine groups on the chitosan, its reactivity and solubility dependent on pH as well as its good ability to chelate with transition metal ions. Also, the nanoparticles were well separated from each other which indicate that they are effectively stabilized by chitosan [19]. Chitosan capped Ag_2S nanoparticles have been synthesized by Hashmi [19], and a spherical shape was obtained. Black wattle tannins have been used as stabiliser for Pt nanoparticles reduced with sodium borohydride and small particle size with good dispersity were obtained which is due to the stabilization effect of black wattle tannins as a result of its unique molecular structure [17]. Ag-aloe extract nanoparticles have been prepared by Zhang and spherical nanoparticles with an average diameter of 20 nm were obtained [20].

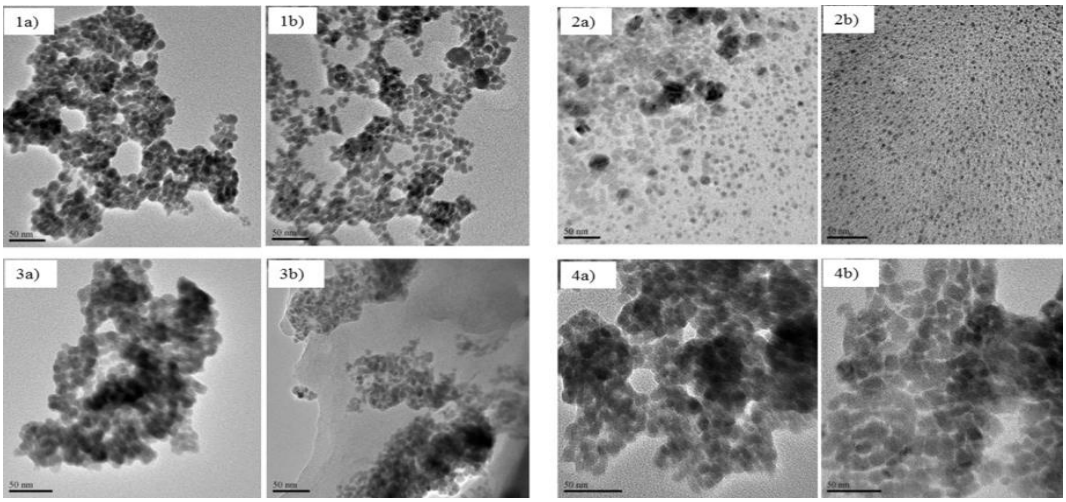

Fig. 3: TEM image of Ag_2S nanoparticles capped with 1) green tea, 2) chitosan, 3) Combretum molle and 4) black wattle under acidic (a) and basic (b) conditions.

Fig. 4a shows XRD patterns of green tea capped Ag_2S nanoparticles, and Fig. 4b the TGA thermographs of Ag_2S nanoparticles capped with green tea, chitosan, *Combretum molle* and black wattle. XRD patterns showed that the particles have acanthite phase of Ag_2S. The TGA thermographs showed two decomposition steps for all capping agents. The first degradation step was observed at around 100 °C and was due to water desorption from the nanoparticles. The second decomposition with weight loss was observed at 262 °C (16%) for green tea, 387 (2%) °C for chitosan, 221 °C (15%) for *Combretum molle* and 228 °C (11%) for black wattle and were due to the degradation of the capping agents. These small percentage weight losses with all capping agents indicate the good stability of the synthesized nanoparticles. *Combretum molle* was found to be the most stable capping agent with 2% weight loss.

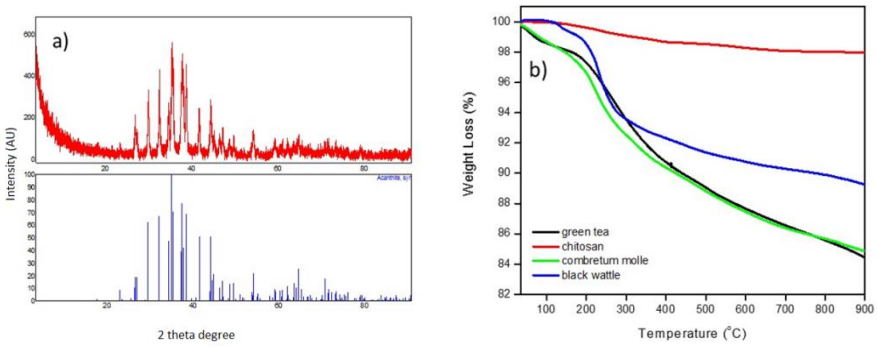

Fig. 4: a) XRD patterns of green tea capped Ag_2S nanoparticles b) TGA thermograph of Ag_2S nanoparticles capped with green tea, Combretum molle, black wattle and chitosan.

3.3 Antibacterial activity

The antibacterial activity Ag_2S nanoparticles capped with chitosan, green tea, *Combretum molle* and black wattle against *Escherichia coli* and *Staphylococcus aureus* was investigated. The nanoparticles showed to be susceptible towards both Gram negative and Gram positive bacteria species (Table 1). Ag_2S nanoparticles showed maximum inhibition zone of 12 ± 0.14 mm diameter with *Combretum molle* against *Escherichia coli* and 11 ± 0.16 mm diameter with black wattle against *Staphylococus aureus*. The minimum inhibitory concentration (MIC) was then further investigated at (0.37–0.023 mg/mL) and the results are shown in Table 2. Ag_2S nanoparticles capped with *Combretum molle* and chitosan showed the lower MIC values (0.15 mg/mL) for both bacteria species. The reason could be that they both gave smaller nanoparticles size than the black wattle and green tea. The reason why the Ag_2S nanoparticles showed the antimicrobial activity was due to that their surface could easily form a layer of water, thus silver sulfide ions could be released into the water. This was due to the water soluble capping agents used for their synthesis, which enhanced their water solubility in the biological system [20]. The bacterial cell membrane is negatively charged as it contains negatively charged phospholipid molecules, hence the positively charged silver ions were able to bind to bacterial cell membrane quickly resulting to the structures of bacteria to be changed and denatured. Also, Ag ions could also be strongly attracted to the thiol group (SH) of bacterial enzyme, resulting in the inactivation and even death enzymes [20].

The antibacterial activity of Ag-aloe extracts against *Escherichia coli* and *Staphylococcus aureus* have been investigated by Zhang and the results obtained for the aloe leaves extract alone were lower than those incorporated with silver nanoparticles [20]. The inhibition zone of 6.7 mm for the extract alone and 8 mm for Ag-aloe extract against *Escherichia coli* were obtained. Also, an inhibition zone of 6.1 mm for the extract alone and 7.5 mm for Ag-aloe extract against *Staphylococcus aureus* were obtained. The Cu-chitosan nanoparticles showed antibacterial activity against both gram negative and gram positive bacteria [21]. These finding are in agreement with the results obtained in this work. The highest antibacterial activity of *Combretum molle* extract in acetone against the Gram negative organisms *Escherichia coli* and *Shigella spp*. with an MIC value of 50 mg/mL has been reported by Asres [22]. The leaf and bark extracts of *Combretum molle* have used as antibacterial agents against *Staphylococcus aureus* bacteria, while the leaf and

bark parts showed antibacterial activity at 3 mg/mL, the seed and peeled stem parts had minimal activity only at 100 mg/mL [23]. The sensitivity of *Escherichia coli* to *Combretum fragrans*, *Combretum micranthum* and *Combretum molle* extracts has been investigated and the MIC found were 0.625 for *Combretum fragrans*, and *Combretum molle* and 2.50 mg/mL for *Combretum micranthum* [8]. In comparison with literature, the antibacterial activity results of the *Combretum molle* and black wattle leaves extract against Gram negative *Escherichia coli* and Gram positive *Staphylococcus aureus* obtained in this work showed lower MIC values (0.15-0.31 mg/mL). This indicates that the incorporation of the Ag_2S nanoparticles to plant extracts enhanced the antibacterial activity of the plants, which is in agreement with the results reported by Zhang [20].

Table 1: Antibacterial activity of nanoparticles against bacteria.

Nanoparticles	Zone inhibition (mm)	
	Escherichia. Coli	*Staphylococcus aureus*
Ag_2S-Green tea	9 ± 0.21	9 ± 0.24
Ag_2S-Chitosan	9 ± 0.16	10 ± 0.20
Ag_2S-Combtretum molle	12 ± 0.14	10 ± 0.12
Ag_2S-Black wattle	8 ± 0.24	11 ± 0.16

Table 2: MIC of nanoparticles against bacteria.

Nanoparticles	Concentration (mg/mL)	
	Escherichia coli	*Staphylococcus aureus*
Ag_2S-*Combretum molle*	0.15	0.15
Ag_2S-Black wattle	0.31	0.15
Ag_2S-Green tea	0.31	0.31
Ag_2S-Chitosan	0.15	0.15

4. Conclusions

The biosynthesis of silver sulfide nanoparticles was successfully applied. The XRD results confirmed that the synthesized silver sulfide nanoparticles had the acanthite phase, while the TEM analysis showed that the nanoparticles were spherical in shape. The pH used was found to have an influence on the size and agglomeration of the nanoparticles as synthesis under basic pH resulted in the reduction of both the size and agglomeration of the nanoparticles. Also, the size of the nanoparticles showed to be dependent on the capping agent used as chitosan gave the smallest nanoparticles size than the other capping agents. The antibacterial activity showed that the prepared chitosan, green tea, *Combretum molle* and black wattle capped silver sulfide nanoparticles had the antibacterial activity towards *Staphylococcus aureus* and *Escherichia coli* bacterial species. These results indicate that these nanoparticles are promising candidates as new generation antimicrobials as they displayed potential antimicrobial activities. These nanoparticles can therefore be utilized to inhibit growth against the bacteria species studied. Also, they can be used in biological systems as they have been synthesized using a green synthesis method.

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