ANTIMICROBIAL ACTIVITY OF SILVER NANOPARTICLES SYNTHESIZED USING GOAT MILK AGAINST PATHOGENS OF SELECTED VEGETABLES

ABSTRACT

Aim: Determination of the antimicrobial activity of silver nanoparticles synthesized using goat milk against pathogens of selected vegetables

Study design: Synthesis and characterization of silver nanoparticles using goat milk, determination of the antimicrobial activity of silver nanoparticles synthesized using goat milk against pathogens of selected vegetables

Methodology: Synthesis of Silver nanoparticles was done using Goat milk, and characterized using Ultra Violet-Visible absorption spectroscopy, Fourier-transform infrared spectroscopy (FTIR), X- ray diffraction (XRD) and Transmission Electron Microscopy (TEM).

Results: Maximum absorbance of Goat milk synthesized AgNPs was observed at 417 nm, with FTIR peaks at 3455 cm^{-1}, 1628 cm^{-1}, 1402 cm^{-1}, 1081 cm^{-1} and 517 cm^{-1}, indicating that proteins in Goat milk were the capping and stabilization molecules involved the synthesis of AgNPs. Transmission electron microscopy analysis showed that the biosynthesized particles were spherical in shape having a size of 10-100 nm, X- ray diffraction (XRD) pattern agreed with the crystalline nature and face-centered cubic phase of AgNPs. Evaluation of the antimicrobial activity of AgNPs synthesized using GM against the indicator strains (Sta*phylococcus aureus CIP* 9973, *Pectobacterium carotovorum* Pec1, *Enterobacter cloacae* AS10, *Klebsiella aerogenes* OFM28, *Proteus mirabilis* UPMSD3 and *Escherichia coli* 2013C-3342) isolated from selected vegetables, was carried out using the Agar diffusion assay at different concentrations of 25, 75 and 100 µl/ml.

Conclusion: The present study demonstrated that the AgNPs synthesized using Goat milk have potent biological activities, which can find applications in diverse areas.

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Key words: Characterization; Antimicrobial activity; silver nanoparticles; Goat milk; indicator strains

1. INTRODUCTION

To overcome the shortcomings of chemical methods of nanoparticle synthesis, biological methods have emerged as viable options (Mossallam *et al*., 2014). The major advantage of biological methods is the availability of amino acids (Kumari *et al.*, 2015), proteins or secondary metabolites present in the synthesis process, the elimination of the extra step required for the prevention of particle aggregation, and the use of biological molecules for the synthesis of AgNPs which is eco-friendly and pollution-free (Gurunathan *et al*., 2015). Biological methods provide controlled particle size and shape, which is an important factor for various biomedical applications (Shankar and Rim, 2015). Several studies have reported the synthesis of AgNPs using green, cost effective and biocompatible methods without the use of toxic chemicals among which includes the use of bacteria: *Pseudomonas stutzeri* AG259 (Klaus *et al*.,1999), *Lactobacillus* strains (Nair, 2002), *Bacillus safensis* LAU 13, (Lateef *et al*., 2015c), *Bacillus licheniformis* (Kalimuthu *et al*., 2008), *Escherichia coli* (Gurunathan *et al*., 2009), *Brevibacterium casei* (Kalishwaralal *et al*., 2010), fungi including *Fusarium oxysporum* (Shankar *et al*., 2003), *Ganoderma neo-japonicum* (Gurunathan *et al*., 2013), plant extracts such as *Allophylus cobbe* (Gurunathan *et al*., 2014), *Artemisia princeps* (Gurunathan *et al*., 2015), *Prunus persica* (Jayanta *et al*., 2016) and *Typha angustifolia* (Gurunathan, 2015) were utilized. In addition to these, several authors have also reported the use of biomolecules, such as biopolymers (Leung *et al*., 2010), starch (Kumar *et al*., 2014), fibrinolytic enzyme (Deepak *et al*., 2011), and amino acids (Shankar and Rhim, 2015) in the green chemistry approach synthesis of AgNPs. Lateef *et al*. (2015e) reported the Synthesis of AgNPs using paper wasp nest extract.

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About one third of milk production in developing countries come from Water buffalo, Goat, Sheep, Camel, Donkey, Horse, Reindeer and Yak (Wiley, 2010). Goat milk is a good source of protein, contains less sugar (lactose), 13% more calcium, 25% more vitamin B6, 47% more vitamin A, and 134% more potassium than regular cow's milk, it's easier for toddlers to digest and contains less allergenic proteins than cow's milk (Pluym *et al*., 1993). Milk is the most nutritionally complete food found in nature Mohammad *et al*. (2016). Milk protein contains all of the essential amino acids (leucine, isoleucine, valine, phenylalanine, tryptophan, histidine, threonine, methionine, and lysine). Milk protein also consists of approximately 82% casein and 18% whey (serum) proteins **(**Wiley, 2010). Mohammad *et al*. (2016) and Vivian**,** (2014) reported the synthesis of silver nanoparticles using sheep milk and the Amino acid tyrosine respectively.

Preservation and safety are presently two major challenges of the food industry because, huge economic losses are sustained yearly due to food spoilage. The antimicrobial potential of silver Nanoparticles has been exploited in several areas by the food industry: for the fabrication of food containers, refrigerator surfaces, storage bags, and chopping boards embedded with silver Nanoparticles, enabling food preservation for longer periods (Sharma *et al*., 2012). All of the above uses of silver in Nano form can be attributed to its antibacterial property. Biological methods for the synthesis of Ag (Silver) nanoparticles are gaining importance in the field of nanoparticle synthesis. In this research the antibacterial activity of silver nanoparticles synthesized using Goat milk was tested against pathogens of selected vegetables.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

Bacterial strains (Sta*phylococcus aureus CIP* 9973, *Pectobacterium carotovorum* Pec1, *Enterobacter cloacae* AS10, *Klebsiella aerogenes* OFM28, *Proteus mirabilis* UPMSD3 and

Escherichia coli 2013C-3342) used in this study were isolated from selected vegetable (Tomato (*Solanum lycopersicum*), Cucumber (*Cucumis sativus*), Cabbage (*Brassica oleracea*), Eggplant (*Solanum melongena*), Green Beans (*Phaseolus vulgaris*) and Pumpkin (*Telfairia occidentalis*) samples and were referred to as the indicator (test) strain.

2.2 Biogenic synthesis of Silver nanoparticles using Goat milk

Biogenic synthesis of ~~Silver~~ silver nanoparticles was carried out according to the method of Gholami *et al*. (2016) with modification. Milk extracted from Alpine Goat Breed at the Modern market, Benue state was sterilized using whiteman® membrane nylon filter (0.2 µm). Analytical grade ~~Silver~~ silver nitrate ($AgNO_3$) (Sigma-Aldrich (USA) was used. Synthesis of ~~Silver~~ silver nanoparticle using Goat milk (GM) was carried out under sterile conditions to maintain milk sterilization. Ten milliliter (10 ml) of GM was mixed with 90 ml of 1 mM $AgNO_3$ solution, and the resulting mixture incubated for 24 hours in a rotary shaker (200 rpm) under ambient conditions of room temperature (30 ± 2^{o}C). Reduction of silver ions in the reaction mixture was monitored by observing the color change of the reaction mixture. The reaction product was then separated by centrifugation at 10,000 rpm for 30 min and purified by re-dispersion of the pellet in sterile water. Biosynthesized ~~Silver~~ silver nanoparticles were finally collected by centrifugation at 10,000 × g for 30 minutes, washed twice with distilled water and unbound proteins removed by treating with 80% (v/v) ethanol. The purified nanoparticles were then freeze dried at −70 °C and used for characterization. Autoclaved Goat milk was used as control.

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2.2 Characterization of ~~Silver~~ silver nanoparticle synthesized using Goat milk (GSNPs)

2.2.1 UV–vis absorption spectrum of GSNPs

Formation of ~~Silver~~ silver ~~Nano~~ nano particles was confirmed visually. The conical flask was observed periodically for change in color over a 24 hours incubation time. Primary

characterization and ~~conformation~~ confirmation of stability of biosynthesized silver nanoparticles (AgNPs) was further carried out using Ultraviolet–visible spectroscopy (UV-2450 (Shimadzu).

2.2.2 Fourier transform infrared (FTIR) spectroscopy of GSNPs

Fourier transform infrared (FTIR) spectroscopy analysis was carried out using the method of Mohammad *et al*. (2016). The pellet of silver nanoparticles was redispersed into 1 ml of deionized water. Thereafter, the purified suspension was hot air dried in the oven (LVS 201T, Ilmvac GmbH, Germany, UK) to make powder for optical measurements, after which the dried nanoparticles were analyzed using FTIR spectrophotometer (Spectrum One, PerkinElmer, Inc, Waltham, MA, USA).

2.2.3 X-Ray Diffraction

An X'Pert Pro x-ray diffractometer (PAN analytical BV, the Netherlands) with Cu Kα radiation in a θ- 2θ configuration was used to determine the crystalline nature of ~~Biosynthesized~~ biosynthesized ~~Nanoparticles~~nanoparticles. The Silver nanoparticles synthesized using goat milk was dried in the oven at 60°C and the dried powder was further analyzed for the identification of their phase structure. The crystallite domain size was calculated from the width of the XRD peaks, and it was assumed that they were free from non-uniform strains, using the Scherrer formula: $D = 0.94 \lambda / \beta \cos \theta$, Where, D is the average crystallite domain size perpendicular to the reflecting planes, λ is the X-ray wavelength, β is the full width at half maximum (FWHM), and θ is the center ~~angel~~ angle of the peak in radian.

2.2.4 Transmission Electron Microscopic (TEM) analysis of GSNPs

Transmission electron microscopy (TEM) micrograph was obtained as follows. Drops of silver nanoparticles in suspension was placed on a 200 mesh hexagonal carbon films supported

by copper grids. Excess liquid was flicked off using a filter paper and the grids were then air dried before viewing. Micrograph was obtained using a JEM-1400 (JEOL, USA) operating at 200 kV.

2.3 Determination of the Antibacterial activity of silver nanoparticles synthesized using Goat milk

Antibacterial activity of biosynthesized AgNPs against the indicator strains (*Escherichia coli* 2013C-3342, *Staphylococcus aureus* CIP 9973, *Pectobacterium carotovorum* Pec1, *Enterobacter cloacae* AS10, *Klebsiella aerogenes* OFM28 and *Proteus mirabilis* UPMSD3) isolated from the selected vegetable samples (Tomato (*Solanum lycopersicum*), Cucumber (*Cucumis sativus*), Cabbage (*Brassica oleracea*), Eggplant (*Solanum melongena*), Green Beans (*Phaseolus vulgaris*) and Pumpkin (*Telfairia occidentalis*) was carried out using the agar well diffusion method (AWD). Turbidity of inoculums (indicator strain) was matched with 0.5 McFarland turbidity standard. Inoculum (2 ml) was then spread over Muller-Hinton agar (Hi Media, India) plate using sterile cotton swab in order to get uniform microbial growth**.** Wells of 8 mm diameter were bored on the plates with the aid of a sterile cork borer and the wells were loaded with different concentrations (50, 75 and100 µl) of AgNPs and then incubated for 18 hours at 37^{o}C. The antibacterial activity was then evaluated by measuring the diameter of inhibition zones. The Experiment was carried out in triplicate and the average diameter of the zones of inhibition recorded.

3. Results and discussion

3.1 Biosynthesis and ~~Characterization~~ characterization of ~~Silver~~ silver nanoparticles

Preliminary confirmation of the reduction of ~~metallic~~ silver(I) (Ag^{+}) ~~(Silver)~~ to Ag nanoparticle was observed visually by a change in color of the reaction solution to light reddish brown after 30 minutes of incubation. Autoclaved goat milk mediated the synthesis of AgNPs

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within 30 minutes of reaction under ambient conditions of room temperature (30 ± 2°C) with characteristic light reddish brown color, which further intensified with time and stabilized within 24 hours (figure 1). Krishnaraj *et al*. (2010) also reported the formation of AgNPs at 30 minutes of incubation time. Lateef *et al*. (2015c) reported that variations in the color of AgNPs colloidal solutions could be due to the composition of biomolecules responsible for the synthesis of the nanoparticles. Lateef *et al*. (2015c and 2015e) reported the formation of dark brown AgNPs using crude extracellular keratinase of *B. safensis* LAU 13 and Spider cobweb. Gholami *et al*. (2016) reported the formation of brownish red color of Platinum silver nanoparticles using sheep milk, Lee *et al*. (2013) earlier synthesized AgNPs using cow milk and also reported same color formation. Vivian, (2014) reported the formation of reddish colouration solution when silver nanoparticles was synthesized using Tyrosine which is an amino acid found in milk. Jayanta *et al*. (2016) also reported the formation of reddish colouration of solution when silver chloride nanoparticles was synthesized using the outer peel extract of Peach (*Prunu persic*).

Figure 1: Biosynthesis of Silver nanoparticles (AgNPs) using Goat milk

A: Silver nitrate solution;

B: Goat milk;

AB: Reaction mixture at time 0;

C: Biosynthesized Silver nanoparticles from Goat milk.

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The Ultra Violet -Visible Spectroscopy of Ag nanoparticles synthesized using goat milk reveals the surface plasmon resonance (SPR) features of synthesized nanostructures that are in agreement with those from previous studies (Wiley, 2010, Vivian, 2014) with SPR feature at 417 nm (figure 2) indicating monodispersity of the sample without any anisotropic features. Vivian, (2014), explained that the optical absorption spectra of metal nanoparticles are dominated by Surface plasmon resonance (SPR), which shifts to longer wavelengths with increasing particle size.

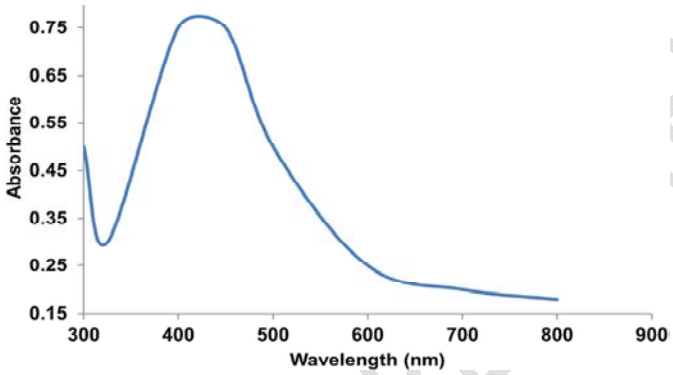

Figure 2: UV–vis absorption spectrum of Silver nanoparticles (AgNPs) synthesized using goat milk

Fourier transform infrared (FTIR) spectroscopy is frequently used to find out whether biomolecules are involved in the synthesis of nanoparticles, FTIR analysis is also beneficial as it provides evidence on the surface chemistry of these particles (Xi-Feng *et al*., 2016). FTIR absorption spectrum showed clear peaks at 3455 cm−1, 1628 cm−1, 1402 cm−1, 1081 cm−1 and 517 cm−1 (figure 3). Bands at 3455 cm−1 and 1628 cm−1 refer to the bonding vibration of the amide (N-H group) of proteins and the deformation atoms, respectively, indicating that the functional groups in ~~Goat~~ goat milk proteins such as amines, alcohols, alkenes and phenols were the capping and stabilization and biomolecules in the synthesis of AgNPs (Shankar *et al*., 2003).

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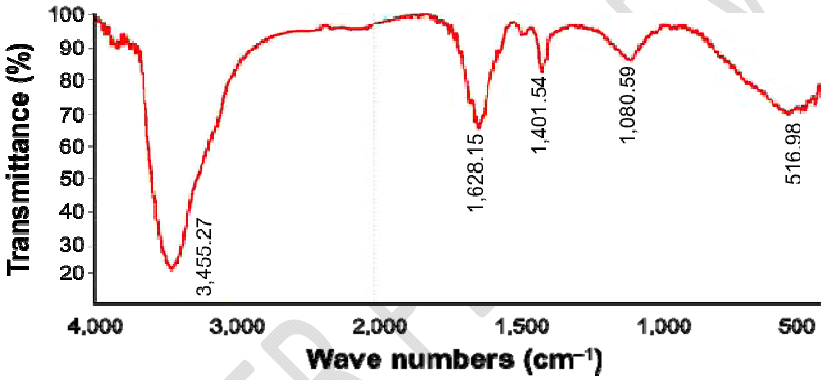

Figure 3: Fourier transform infrared (FTIR) spectroscopy spectrum of Silver nanoparticles synthesized using goat milk

X-ray diffraction (XRD) is a popular analytical technique which has been used for the analysis of both molecular and crystal structures, qualitative identification of various compounds, quantitative resolution of chemical species (Sreedevil *et al*., 2015), measuring the degree of crystallinity, isomorphous substitutions and particle sizes (Xi-Feng *et al*., 2016). The crystalline nature of AgNPs synthesized using GM was investigated by XRD and the corresponding XRD diffractogram observed. The pattern clearly shows main peaks at (2θ) 36.1°,

44.55° and 67.74° corresponding to the 111, 200 and 220, planes for silver respectively. By comparing JCPDS (file no: 89-3722), the typical pattern of green-synthesized AgNPs was found to possess a face centered cubic (FCC) structure (figure 4).

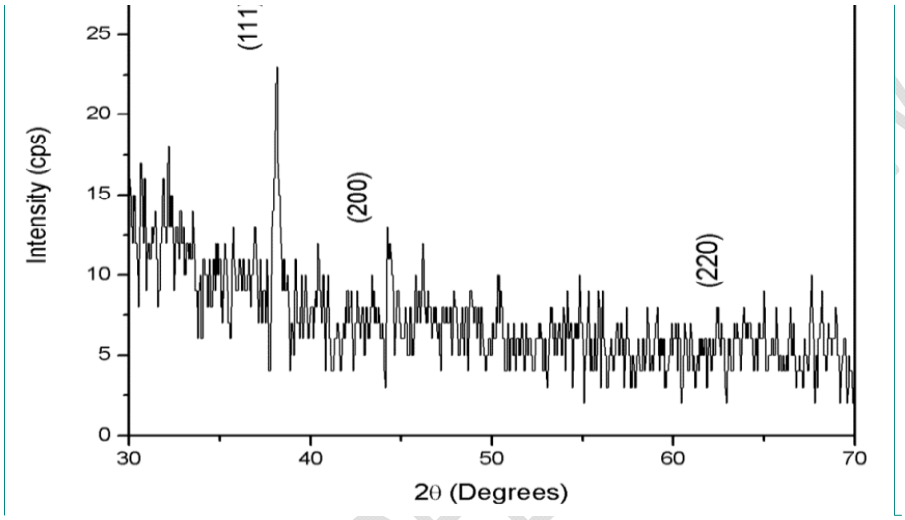

Figure 4: XRD pattern of biosynthesized Silver nanoparticles (AgNPs)

Transmission electron microscopy analysis is a valuable, frequently used and important technique for the characterization of nanomaterials, used to obtain quantitative measure of particle or grain size, size distribution, and morphology (Zanchet *et al*., 2001). TEM analysis of the (GM) bio-reduced AgNPs confirmed that the size of the metal particles was in the nano range and were roughly spherical in shape with sizes ranging from 10 – 100 nm (figure 5). The magnification of TEM is mainly determined by the ratio of the distance between the objective lens and the specimen and the distance between objective lens and its image plane (Joshi and Bhattacharyya, 2008). The size of the particles agreed with the noted SPR band. TEM images of

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Ag nanostructures used in this study showed the well-formed Ag nanoparticles (Lateef *et al*., 2015c), the AgNPs particles were single and pure.

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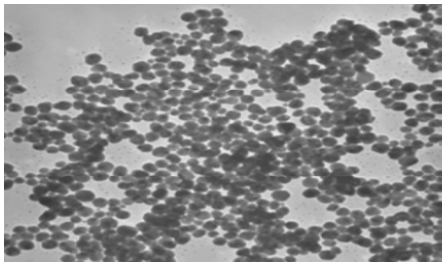

Figure 5: Transmission electron microscopy of silver nanoparticles synthesized using goat milk

3.2 Antimicrobial activity of ~~Silver~~ silver nanoparticles synthesized using goat milk against the indicator strains

Silver nanoparticles (AgNPs) at concentration of 50, 75 and 100 µl was used to inhibit the growth of the indicator strains (*Escherichia coli* 2013C-3342, *Staphylococcus aureus* CIP 9973, *Pectobacterium carotovorum* Pec1, *Enterobacter cloacae* AS10, *Klebsiella aerogenes* OFM28 and *Proteus mirabilis* UPMSD3). At 50 µl concentration, Silver Nano particle exhibited maximum activity of 14.2 ± 1.23 mm and 14.1 ± 0.68 mm against *E. coli* 2013C-3342 and *P. mirabilis* UPMSD3 respectively while Inhibitory Activity of 13 ± 0.53 mm, 13 ±1.86 mm and 11 ± 1.17 mm was recorded against *Klebsiella aerogenes, Staphylococcus aureus* and *P. carotovorum* respectively (table 1). Lowest inhibitory activity of 8.5 ± 0.44 mm was demonstrated against *E. cloacae* AS10). Higher inhibitory activity of silver nanoparticles was observed with 75 and 100 µl concentration as compared to 50 µl concentration with maximum inhibition activity of 17 ± 0.29 mm, 16 ± 0.15 mm, and 14.99 ± .2 mm as observed against *Staphylococcus aureus, Proteus mirabilis, Klebsiella aerogenes and Escherichia coli* respectively on the other hand maximum inhibitory activity of 19 ± 0.73 mm, 18 ± 0.92 mm and 18 ± 0.82mm was observed against *S. aureus* CIP 9973, *P. mirabilis* UPMSD3 and *K. aerogenes*

OFM28 respectively at concentration of 100 µl (~~table1~~ Table1) . Inhibitory activity of AgNPs against *Escherichia coli* 2013C-3342 and *P. carotovorum* Pec1 was measured at 17 ± 0.64 mm and 14.05 ± 2.44 mm respectively while lowest inhibitory activity (12 ± 0.73 mm) of AgNPs at concentration of 100 µl was observed against *Enterobacter cloacae* AS10. Control treatment made up of silver nitrate (100 µl) recorded lowest over all inhibitory activity of 5 ± 2.76 mm across all strains, with 50, 75 and 100 µl concentration of AgNPs recording over all inhibitory activity of 12.3 ± 2.25 mm, 14.5 ± 2.29 mm and 16.34 ± 2.76 mm respectively across strains. Results from statistical analysis indicate that difference in inhibitory activities due to AgNPs concentration is significant ($p<0.001$) (table 1). The present study has shown that Goat milk synthesized Silver nanoparticles can also be used in as a potent antimicrobial against pathogens and food spoiling organisms.

Table 1: **Antimicrobial activity of Silver nanoparticles (synthesized using goat milk) against the indicator strain**

AgNPs (µl/ml)	Diameters of Inhibition Zone (mm) Indicator strains						
	E.cloacae	***E. coli***	***K.aerogenes***	***P.carotovorum***	***P.mirabilis***	***S. aureus***	**Overall**
50	8 ± 0.44^{B}	14 ± 1.23^{B}	13 ± 0.53^{B}	11 ± 1.17^{B}	14 ± 0.68^{B}	13 ± 1.86^{B}	12 ± 2.25^{B}
75	11 ± 0.14^{C}	14 ± 0.2^{BC}	16 ± 0.5^{C}	12 ± 0.02^{B}	16 ± 0.15^{BC}	17 ± 0.29^{C}	14 ± 2.2^{C}
100	12 ± 0.73^{C}	17 ± 0.64^{C}	18 ± 0.82^{D}	14 ± 2.4^{B}	18 ± 0.92^{C}	19 ± 0.73^{C}	16 ± 2.7^{D}
Control	4 ± 0.44^{A}	10 ± 1.17^{A}	6 ± 0.92^{A}	3 ± 0.73^{A}	5 ± 1.23^{A}	2 ± 0.44^{A}	5 ± 2.76^{A}

Means within a column by the same letter(s) are not significantly different according to ANOVA with $p < 0.05$ followed by TukeyHSD post-hoc test at 5% level of significance

$^{A, B, C, D}$ Result are means ± Standard deviation of the independent experiments.

Legend:

AgNPs = Biosynthesized Silver nanoparticles;

E. cloacae = *Enterobacter cloacae* AS10;

E. coli = *Escherichia coli* 2013C-3342;

K. aerogenes = *Klebsiella aerogenes* OFM28;

P. carotovorum = *Pectobacterium carotovorum* Pec1;

P. mirabilis = *Proteus mirabilis* UPMSD3;

S. aureus = *Staphylococcus aureus* CIP 9973;

Control = Silver nitrate

Conclusion

This study examined the use of Goat milk in the synthesis of ecologically friendly AgNPs. The particles were spherical in shape with size range of 10–100 nm. The particles were crystalline in nature, showing the characteristic face centered cubic structure. The biologically synthesized AgNPs showed excellent antimicrobial activities against the strains of pathogenic bacteria (*Escherichia coli* 2013C-3342, *Staphylococcus aureus* CIP 9973, *Pectobacterium carotovorum* Pec1, *Enterobacter cloacae* AS10, *Klebsiella aerogenes* OFM28 and *Proteus mirabilis* UPMSD3) isolated from selected vegetable samples. The use of Goat milk synthesized Silver nanoparticles is therefore recommended in the control of pathogenic organisms of Vegetable samples. Extensive toxicological and acceptability tests should further be carried out on the product.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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