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In vitro studies on the antidiabetic and antibacterial activity of biosynthesized silver nanoparticles with aqueous extract of *Moringa oleifera* leaf

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Abstract
Silver nanoparticles having applications in the field of medicine and biology are shown to have tremendous health benefits. Diabetes is still on rampage and antibacterial resistance is currently a global health challenge. This study focuses on the *in-vitro* antidiabetic and antibacterial activity of biosynthesized silver nanoparticles with aqueous extract of *Moringa oleifera* leaf. An aliquot (5 mL) of extract sample was added to 50 mL of 1 mM aqueous $AgNO_3$. Reaction mixtures were heated and maintained at 70^0C for 10 minutes. Colour change to dark brown solution and a UV-vis spectrum peak at 400 nm confirmed silver nanoparticle synthesis. The antidiabetic activity of the nanoparticle was studied using an *in vitro* alpha-amylase inhibition assay**.** Acarbose was used as antidiabetic control drug. Disc diffusion method was used for antibacterial susceptibility testing on Mueller-Hinton agar medium with ampiclox as control antibiotic. Results of the analysis showed significant inhibition of alpha amylase that resembled the activity of acarbose. The highest percentage inhibition of alpha amylase by AgNPs was observed at 65.625% while that of acarbose was 90.357%. Antibacterial inhibition assay revealed that 100% biosynthesized silver nanoparticles had significant ($p<0.05$) inhibitory effects on *Eschericia coli* and S*treptococcus pyogenes.*

Keywords: Antibacterial; Antidiabetic; Acarbose; Biosynthesized; Nanoparticles

INTRODUCTION

Nanoparticle can be defined as a particle of matter that ranges from 1 to 100 nanometers (nm) in diameter [1]. Designing molecules at nanoscale inputs modified characteristics like electronic, catalytic, optoelectronic and magnetism. Intrinsic properties of nanoparticles are greatly influenced by their shape, size and surface morphology [2]. Nanoparticle creation can be made of any solid or liquid material. Metals, dielectrics and semiconductors inclusive. Nanotechnology manipulates molecules or atoms at sub-microscopic levels to modulate their functionality [3]. Over the years, nanoparticles synthesis has become an upcoming area of research in the field of material science because of their wide variety of applications in the field of chemical and bio-sensing, catalysis, photonics, medicine etc. [4].

Nanoparticle synthesis can be driven from a wide variety of materials including Gold [5], Copper [6] Platinum [7], Zinc Oxide [8], etc. but in comparison to these silver

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nanoparticles remain the widely accepted option because of its relative nontoxicity to humans [9].

Silver nanoparticles are nanoparticles synthesized from silver and have sizes ranging from 1 to 100 nm. Commonly used silver nanoparticles are spherical, but thin sheets, diamond and octagonal shapes are also common [10]. Their extremely large surface area allows the efficient binding of large number of ligands [2].

Silver nanoparticles are being employed in the fields of medicine and biology. They possess extraordinary antimicrobial properties [11]. For example, silver nanoparticle biosynthesis with leaf extract of *Artemisia nilagirica* had potent bactericidal effect on *Bacillus subtilis, Escherichia coli, Proteus subtilis and Staphylococcus aureus* [12]. Similarly, silver nanoparticle biosynthesized with apple extract had antibacterial effects on *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* [13]. These antimicrobial attributes of silver nanoparticles have prompted the fabrication of nano-silver products like wound dressings, contraceptives, implants and nano-silver coated surgical equipment. It is also utilized for the formulation of water treatment, antimicrobial paint coatings, food cans and textiles [14].

Nanoparticle used in the field of medicine especially anti diabetic and bacterial application has continued to find relevance because of its large surface area to volume ratio, which provides it with the capability to interact with any kind of membrane and enzymes in a more enhanced manner. Silver nanoparticles (Ag NPs) synthesized with lemongrass demonstrated anti-diabetic potential *in vitro* [15]. *Moringa oleifera* showed optimum potential for biosynthesis of silver nanoparticles by rapidly reducing silver ions (Ag^+ to Ag^0) [16]. Flavonoids, terpenoids and polysaccharides predominant in *Moringa oleifera* are basically responsible for reducing and capping AgNPs [17].

Silver nanoparticles have been biosynthesized with different biological and chemical methods [4]. They are considered bio-compatible but the chemical synthetic methods often lead to the formation toxic chemicals that may have adverse effects in medical applications [18]. Thus, the pressing need for green methods for synthesis of silver and other metal nanoparticles that are free from the formation of harmful chemicals. Therefore, this research work, the *in vitro* studies on the antidiabetic and antibacterial activity of biosynthesized silver nanoparticles with *Moringa oleifera* leaf was designed.

EXPERIMENTAL METHODS

Materials. Prior to further preparations, fresh *Moringa oleifera* leaves were harvested from the tree and taken for identification and authentication at the herbarium unit of the Botany Department, University of Calabar. Human saliva α-amylase, silver nitrate ($AgNO_3$) pellets, chemicals for the synthesis of 3, 5-dinitrosalicylic acid (DNSA) reagent (sodium hydroxide (NaOH) pellets, potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$), Phosphate buffer (Na_2HPO_4-$7H_2O$, $NaH_2PO_4H_2O$ and HCl) were purchased from Sigma-Aldrich, USA.

All absorbance readings were done with "ultraviolet-visible spectrophotometer" (Drawell, model: Du-8600). All sterilizations for bacterial inhibition assessment were done with the "vertical cylindrical pressure steam sterilizer" (Triup International Corp. model: TR-S50L), while incubations were done with "Electrothermal Incubator" (Searchtech. Instruments, Model: DNP-9052)

Preparation of aqueous extract of *Moringa oleifera* leaf. Fresh leaf samples (20 g) were weighed out, washed and rinsed with distilled water and kept in a beaker. Distilled water (100 mL) was added to it and boiled on a hot plate

for five minutes for active components to leach out into solution. Extract was used as a reducing/stabilizing agent for the synthesis of silver nanoparticles.

Synthesis of silver nanoparticles. Silver nanoparticles was synthesized using the method of Moodley et al. [17]. In 50 mL of 1 mM aqueous $AgNO_3$, an aliquot (5 ml) of aqueous leaf extract sample was added. The reaction mixtures were heated and maintained at 70^0C for 10 minutes to drive nanoparticle formation. Colour change was observed at one minute. and intensified within the reaction time of 10 minutes. Nanoparticle formation was determined by observing the colour shift of the reaction mixtures from a clear white to dark brown colour. To avoid nanoparticle agglomeration, the vessels were withdrawn from the water bath, wrapped in foil, and preserved in the dark at room temperature until the colour intensities of the solutions reached their limit. As a negative control, a 50 mL aliquot of $AgNO_3$ consisting of 5 mL distilled water was treated as mentioned above. UV–vis spectral analysis was used to confirm the formation of silver nanoparticles by the reduction of Ag+ from $AgNO_3$. The nanoparticle solutions were diluted 1:2 in distilled water, and distilled water was used as a control. A UV–vis spectrometer was used to screen nanoparticle solutions and the control from 250–600 nm.

Alpha amylase inhibition assay. The basic theory used was the quantification of the sum of maltose liberated using the process [19]
process. Different volumes of silver nanoparticles (20 µL, 40 µL, 60 µL, 80 µL, 100 µL) were pre-incubated for 30 minutes at room temperature with 100 µL of α-amylase solution (0.1mg/ml). A further 100 microliter of starch solution (1 percent w/v) was applied to the mixture, which was then incubated at room temperature for 10 minutes. To quench the reaction, the DNSA (3, 5-dinitrosalicylic acid) reagent (100 µL of 96 mM solution) was added, and the solution was heated in a water bath for 5 minutes. An equivalent amount of enzyme in each case was substituted with sodium phosphate buffer that was held at a pH of 6.8 as a control. At 540 nm, the absorbance was measured with a spectrophotometer. The experiment was repeated two more times. As a positive control, acarbose (25 mg tablet dissolved in 0.5 ml distilled water) was used. The following formulae were used to quantify percentage inhibition:

$$\% \text{ Inhibition} = c - \frac{T}{C} \times 100$$

Where:
C= optical density of control
T= optical density of test sample.

Preparation of alpha Amylase. The human saliva alpha amylase came as a 0.53 mg lyophilized powder. The powder was dissolved in the already prepared phosphate buffer saline (PBS, pH 6.8, 0.02 mol/L) at 0.1 mg/mL of phosphate buffer prior to use [19].

Preparation of microbial culture. Clinical isolates were obtained from the Microbiology unit of the University of Calabar Teaching Hospital (UCTH). Isolates had been subjected to biochemical analytic testing [gram staining, oxidase test, motility test, indole test, carbohydrate utilization, methyl red Voges – Proskauer (VP) test] to confirm the organism. Test results were compared with the Bergey's manual of determinative bacteriology and supplementary reference works. Sub-culturing was carried out using Mueller Hinton Agar. The agar medium was sterilized at 121^0C with the use of an autoclave at a pressure of 15 PSI for 15 minutes. Agar medium was allowed to cool to 45^0C then poured in Petri dishes. Quadrat streaking method was used to inoculate the plates. Inoculum was incubated in an inverted position for 24 hours at 37^0C. All inoculations were carried out in a sterile inoculating chamber. Broth culturing was done

in peptone solution for 24 hours. The peptone powder (15 g) was dissolved in distilled water (1litre), then sterilized at 121^0C in an autoclave at a pressure of 15 PSI for 15 minutes before use.

Antimicrobial susceptibility testing. Disc diffusion method was used. Discs used were adequately sterilized. Test organisms (0.1 ml) were introduced unto the surface of the solidified agar plate (Mueller-Hinton Agar). Inoculate were evenly spread on the agar surface with the use of a sterile glass spread. Ampiclox[$C_{35}H_{36}CIN_6NaO_9S_2$] (500mg dissolved in 1.5ml of distilled water) was used as control. Sterile discs were soaked in respective extracts (silver nanoparticles and Moringa extract) of undiluted and 50% diluted were placed on the different inoculated agar plates and left in an incubator at 37^0C for 24 hours. The above step was done in triplicate to establish consistency. Same procedure was repeated for the control (ampiclox). After the 24-hour. period, the plates were observed for zones of clearance (or zones of inhibition). Zones of clearance were measured and recorded in millimetre.

Statistical analysis. The data was analysed using analysis of variance and expressed as Mean $\pm$ SEM . The significance level was set at $P<0.05$. The data was analysed using the SPSS computer program version 22.0.

RESULTS

Colour change and UV-Vis Spectroscopy. When $AgNO_3$ was added to *Moringa oleifera* leaf extract, the colour changed from a greenish yellow solution to a dark brown solution in 5 minutes at temperatures ranging from 60 to 70^0 C as demonstrated in Figure 1. The surface plasmon resonance of AgNPs showed a rise starting at 450 nm and having its peak at 400 nm as demonstrated in Figure 2. Figure 3 shows the percentage inhibition of human saliva alpha amylase by the biosynthesized silver nanoparticles with *Moringa oleifera* leaf extract in comparison with the standard antidiabetic drug, Acarbose. From the figure we observe that the percentage inhibition of the alpha amylase by the biosynthesized silver nanoparticles increased with higher concentrations of silver nanoparticle, like the effects exhibited by the antidiabetic drug, Acarbose.

Figure 1. Colour change signifying AgNps synthesis. (A) is clear $AgNO_3$ solution, (B) is *Moringa aqueous* extract and (C) is synthesized silver nanoparticle solution

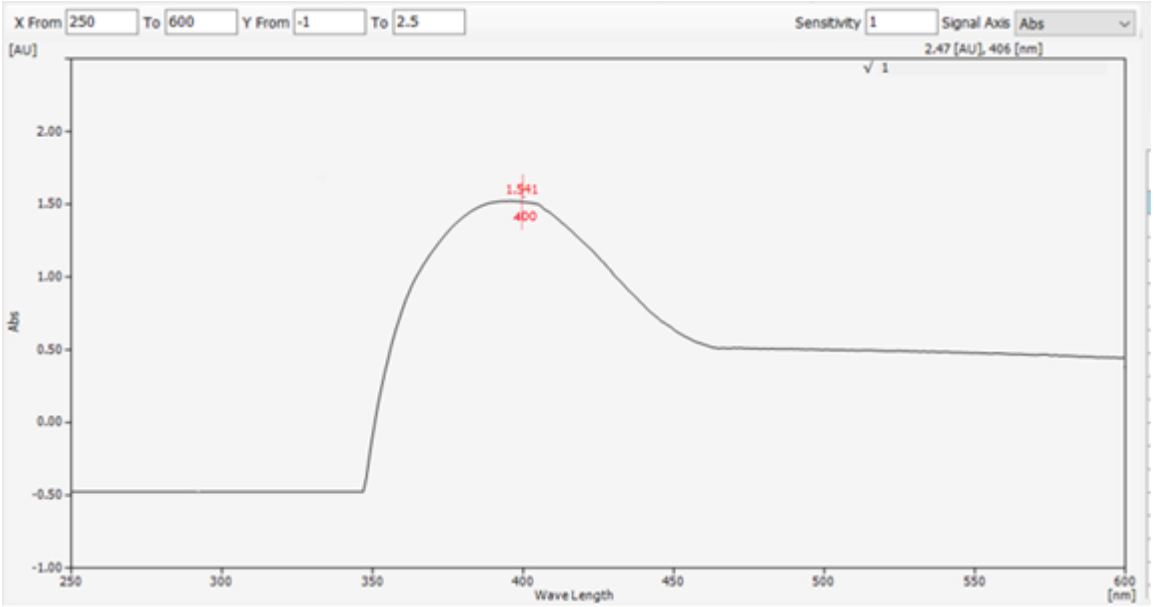

Figure 2. UV-VIS Spectrum of silver nanoparticles synthesized with*Moringa oleifera*leaf extract

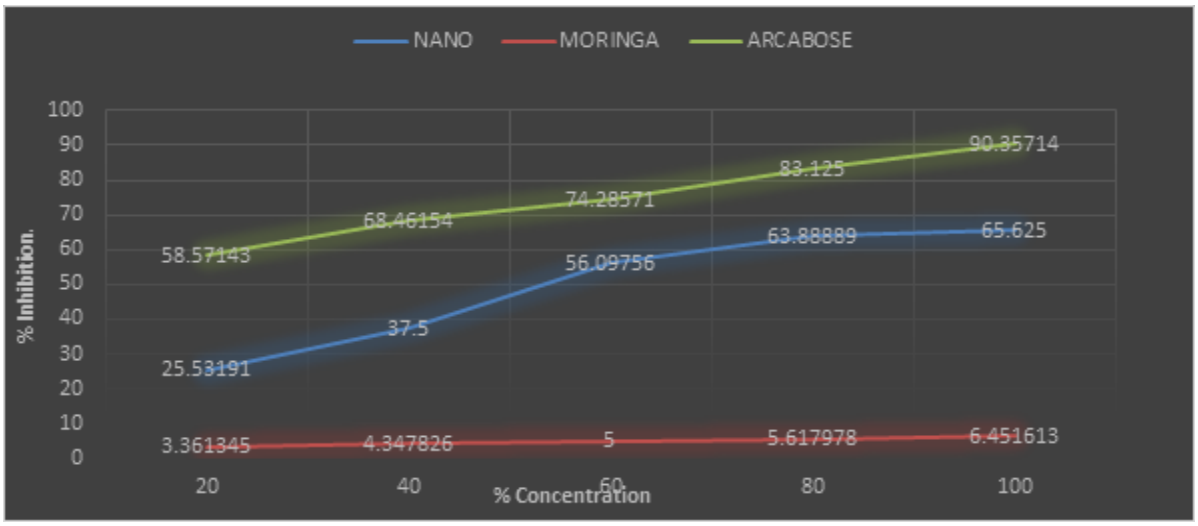

Figure 3. Chart showing percentage alpha amylase inhibition

Table 1: Microbial Inhibitory efficacies of the different extracts

ORGANISM	AMPICLOX 500 mg	M1-100 (mM)	M1-50 (mM)	M2-100 (mM)	M2-50 (mM)
Staphylococci	40.0±0.71^{a}	10.0±0.66^{b}	10.0±1.06^{b}	12.0±0.87^{b}	12.0±0.70^{b}
Klebsiella spp.	30.0±1.31^{b}	10.0±1.03^{a}	10.0±0.72^{a}	11.0±0.94^{a}	10.0±0.73^{a}
Eshericia coli	40.0±0.92^{b}	11.0±1.07^{a}	11.0±0.91^{a}	14.0±1.08^{c}	11.0±0.80^{a}
Streptococci	50.0±0.86^{a}	40.0±1.42^{b}	10.0±0.71^{c}	23.0±0.83^{e}	12.0±0.71^{c}

Values are expressed as mean±S.D of triplicate determinations. Means with same superscript letter across rows were not significantly different at $p<0.05$

Ampiclox = $C_{35}H_{36}ClN_6NaO_9S_2$; M1-100 = 100% *Moringa oleifera* leaf extract; M1-50 = 50% *Moringa oleifera* leaf extract; M2-100 = 100 % Biosynthesized Silver nanoparticles; M2-50 = 50 % Biosynthesized Silver nanoparticles

Table 1 shows the microbial inhibitory efficacies of both moringa leaf extract and the biosynthesized silver nanoparticle in two concentrations: 50% and 100%. From the table we observe that none of the preparations had any significant inhibitory effects on

Staphylococcus aureus and *Klebsiella pneumonia*. On the contrary, 100% biosynthesized silver nanoparticles had significant inhibitory effects on *Escherichia coli* and *Streptococcus pyogenes* respectively at $p<0.05$.

DISCUSSION

The green approach to silver nanoparticles synthesis remains the safest option when compared to conventional methods owing to the resultant toxic end products [17]. This research demonstrates a significant success in the concentration dependent inhibition of human saliva alpha amylase in comparison with the standard antidiabetic drug, Acarbose. Maximum inhibition of the alpha amylase enzyme by the highest concentration of the silver nanoparticle preparation (100%) is a 65.6% inhibition while acarbose inhibition at same concentration is a 90% inhibition. Similarly, biosynthesis of AgNps with *Cymbopogon citratus* (lemon grass) presented significant alpha amylase inhibition activity [15]. There are numerous natural products that function as alpha-glucosidase inhibitors [20]. The culinary mushroom maitake (*Grifola frondosa*), for example, has been shown to have a hypoglycemic effect in studies [21] Since the mushroom naturally produces an alpha glucosidase inhibitor, hence, Maitake reduces blood sugar [22]. The study of the hypoglycemic activity of the *Cuminum nigrum* seeds (0.5 and 1.5g/kg) in normal and diabetic rabbits induced with alloxan showed that *Cuminum nigrum* possessed significant hypoglycemic activity [23]. Reference [24] reported significant alpha amylase inhibition by silver AgNps synthesized with crude methanol extract of *Moringa oleifera* leaf *when* compared to contemporary methanol extract of moringa leaf but did not however discuss the mechanism of inhibition. Silver nanoparticles clearly do not resemble sugar molecules structurally therefore, alpha amylase inhibition is believed to occur through an active site that is different from substrate binding site. Hence, this kind of inhibition is a noncompetitive inhibition [25]. It was also observed that as silver nanoparticles concentrations were increased above 60% and approaching 100%, a plateau was gradually formed indicating that saturating the enzyme with AgNps may not produce any further inhibitions than already established. In this light, biosynthesized silver nanoparticles could be formulated accordingly in view of being incorporated as potential diabetic treatment option.

Silver nanoparticles synthesized with *Moringa oleifera* leaf showed broad spectrum antimicrobial activity on both fungi and bacteria [17]. Similarly, biosynthesized AgNps with apple extract demonstrated bactericidal activities against both gram-positive and gram-negative bacteria [13]. Microbes used for this research were majorly bacteria namely: *Klebsiella Pneumoniae, Escherichia coli* (gram-negative) and *Staphylococcus aureus, Streptococcus pyogenes* (gram-positive). In a bid to regulate bacterial growth rates by means of inhibition and consequently control the spread of infectious diseases, silver nanoparticles synthesized with *Moringa oleifera* leaf extracts as capping/reducing agent was assayed for its antibacterial activity. Standard antibiotic Ampiclox was used as control while *Moringa oleifera* leaf extract was used alongside for comparative purposes. 100% biosynthesized silver nanoparticles had significant inhibitory effects on *E coli* and *Streptococcus pyogenes* respectively at $p<0.05$ even though *Staphylococcus aureus* and *Klebsiella pneumoniae* were not significantly inhibited by any of the preparations. It is believed that biosynthesized silver nanoparticles contain active ingredients that caused the bacterial growth inhibitions [16]. This growth inhibition activity was brought about by the production of reactive oxygen species and accumulation of nanoparticles in

bacteria cytoplasm or outer membrane [26]. *E coli* and *Streptococcus pyogenes* are gram-negative and gram-positive bacteria respectively inferring that silver nanoparticles have efficacies in inhibiting some species of both gram-negative and gram-positive bacteria.

Conclusion. This study demonstrates the anti-diabetic and bacterial inhibition potential of biosynthesized silver nanoparticles of *Moringa oleifera* leaf (in-vitro). From the study it was observed that silver nanoparticles can inhibit alpha amylase enzyme activity and would be clinically beneficial if the procedures are modified towards the management of hyperglycemia in type two diabetes. On the other hand, silver nanoparticle is seen to inhibit both gram-positive and negative bacteria including *Streptococcus pyogenes* and *Escherichia coli* respectively meaning that it can be considered as possible antibiotic substitution.

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