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IN VITRO ANTIOXIDANT AND ANTIDIABETIC PROPERTIES OF SILVER NANOPARTICLE SYNTHESIZED BY *Serratia* spp.

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Abstract

Diabetes mellitus has been identified as a chronic disease that requires massive attention around the world. It is a metabolic disease linked to hyperglycemia and associated with oxidative stress. The development of diabetes mellitus treatment utilizing nanoparticles (NPs) is highly desired as the rate of morbidity increase annually. Silver nanoparticles (AgNPs) are widely used due to their unique characteristic and promising potential in the biomedical field. The production of AgNPs incorporated the green synthesis method, rendering it an eco-friendly and low-cost approach compared to physical and chemical means of synthesis. The biosynthesis of AgNPs mediated by bacteria is preferable since it can be produced on a large scale in minimal time besides the size and shape of the well-defined nanoparticles. Even though being used widely both as antimicrobial agent and as a potential drug carrier in the treatment of cancer, the study regarding biosynthesis of AgNPs with antioxidant and antidiabetic activity mediated by marine bacteria is scarce and yet to be explored. Thus, in the current study, AgNPs were synthesized by *Serratia* sp. using two formulations, NT27-AgNPs and NT39-AgNPs, via the green synthesis method. In addition, the potential antioxidant and antidiabetic activity of AgNPs mediated by marine bacteria were determined by various antioxidant assays, i.e., DPPH, ABTS, and FRAP assays. Furthermore, the inhibition against α-amylase and α-glucosidase (antidiabetic assay) and toxicity assessment upon exposure to AgNPs by cell viability assay was also performed. The prepared AgNPs were further characterized using UV-Visible spectrophotometer, which exhibits the absorption spectra at 380 nm indicates the existence of AgNPs. The diameters of the colloidal suspension for NT27-AgNPs and NT39-AgNPs were 236.50 and 157.30 nm, with charges of −6.05 and −7.65, analyzed using dynamic light scattering (DLS) spectroscopic technique. Despite being highly effective in inhibiting both α-amylase and α-glucosidase activity, both formulations showed low antioxidant activity. Meanwhile, the formulations showed undesirable cytotoxicity towards the growth of 3T3-L1 preadipocyte cells in the cell viability assay. Based on the current data, these compounds can be antidiabetic agents with low compatibility with diabetic cells.

INTRODUCTION

In recent years, nanotechnology-based systems have attracted considerable attention in the pharmacology, nutrition, and energy sectors [1]. Nanoparticles (NPs) associate the biological and physical sciences in the structural, chemical, mechanical, magnetic, electrical, and biological aspects. Their small size (<100 nm) allows them to move freely in the body than bigger materials, overcoming several biological barriers. Furthermore, the small-sized nanoparticles with their large surface-to-volume ratio render their exploitation as delivery agents drug encapsulation and site-specific delivery, increasing drug efficacy and reducing toxicity [2,3].

Silver nanoparticles (AgNPs) are gaining major attention due to their effectiveness as an antioxidant involved in scavenging free radicals besides antidiabetic properties by inhibiting α-amylase and α-glucosidase enzymes [4,5].

Diabetes mellitus (DM), or diabetes, is a metabolic abnormality signified by a high blood glucose level that affects over 100 million people worldwide [6, 7]. There are two major types of DM, Type I insulin-dependent diabetes mellitus (T1DM) and type II non-insulin-dependent diabetes mellitus (T2DM). T1DM is mainly caused by the destruction of β cells of the pancreas, resulting in impaired insulin secretion (Devendra et al., 2004). In comparison, T2DM is the most prevalent type of diabetes that resulted from insulin resistance and comprised approximately 90%–95% of all diabetic cases [8]. This led to the urgency of developing nanomedicine for the effective treatment of DM, such as AgNPs. The metallic NPs could significantly inhibit α-amylase and α-glucosidase, i.e., highly specific carbohydrate-hydrolyzing enzymes, and achieve maximal therapeutic efficacy. Inhibition of these enzymes causes post-prandial anti-hyperglycemic effects by reducing glucose absorption rate and extent [9].

It is known that antioxidant is fundamental in DM prevention since the accumulation of excess free radicals contributes to the pathogenesis of this chronic disease [10]. Excessive free radicals reduce the body's antioxidative defence system and cause the rise of oxidative load, leading to oxidative stress [11]. Kositsawat and Freeman [12] reported that individuals with low antioxidant levels have a higher risk of severe DM complications. Akhtar et al. [13] stated that AgNPs could probably scavenge reactive oxygen species (ROS) due to their high surface-to-volume ratio, electronic nature, catalytic, redox properties, and oxygen vacancy defects. Because of these properties, the AgNPs will primarily affect the cell redox environment, either by stimulation or inhibition of ROS generation, assisting in T2DM treatment. However, with all the considerable antioxidant activities *in vitro*, the clinical significance is yet to be determined [14-15]. Therefore, it is crucial to find environmentally mild bioresources to synthesize AgNP for the improvement of the antioxidant and antidiabetic properties [16].

The synthesis of metallic nanoparticles like silver nanoparticles can be done through the chemical, physical, or biogenic synthesis methods, involving bacteria, fungi, yeast, and plant extract [17,18,19,20]. However, the synthesis of AgNPs using chemical and physical methods is detrimental since it introduces toxicity to the environment and is economically expensive [21]. Meanwhile, the biogenic synthesis method that uses biological organisms is preferable due to its simple method, non-toxic, reliable, rapid, and produces well-defined size and shape nanoparticles under optimum conditions [22, 23].

A previous study stated that few bacteria can synthesis AgNPs intracellularly, whereby their intracellular components serve as the reducing and stabilizing agents [24]. The green synthesis of AgNPs with naturally occurring reduction agents may be a viable way of replacing more complex physiochemical syntheses. AgNPs synthesis by bacteria resulted in a stable and preferable biosynthesis as it can rapidly produce NPs on a large scale. Kalishwaralal et al. [25] reported when aqueous silver ions incubated with the culture supernatant of a nonpathogenic bacterium, *Bacillus licheniformis*, it could synthesize highly stable silver nanoparticles (40 nm) through bioreduction. Similarly, in this study bioreduction being employed for this study but using different bacteria namely *Serratia* spp which going to give rise to different size and feature of AgNPs. Most bacteria will use nitrate as the source of nitrogen, whereby nitrate reductase is crucial for converting nitrate to nitrite to utilize the reducing power of used nicotinamide adenine dinucleotide (NADH). This NADPH can act as the reducing agent of silver nitrate salt to form AgNPs.

The current study aimed to develop a cost-effective green synthesis AgNPs using *Serratia* spp. The synthesized AgNPs were characterized for both size and charge using the dynamic light scattering (DLS) spectroscopic techniques. The *in vitro* free radical-scavenging potency and antidiabetic potential of the AgNPs were also evaluated. Furthermore, the compatibility of AgNPs against a preadipocyte cell line (3T3-L1) based on their ability to inhibit cell viability was also investigated. This study reports the synthesis of silver NPs mediated by *Serratia* spp. and the evaluation of their various biological properties for the first time.

MATERIALS AND METHODS

Materials

1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich (USA), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (Sigma-Aldrich), 2,2,-azinobis-3-ethulbenzoline-6-sulfonic acid (ABTS) (Thermo Fisher Scientific), 3,5-Dinitrosalicylic acid (Sigma-Aldrich (USA), Acarbose (Cayman Chemical Company), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) tetrazolium bromide (Sigma Aldrich. Co), ascorbic acid (R&M Chemicals (UK), Acetic acid (Chemiz (Malaysia), amylase (Sigma-Aldrich (USA), dimethylsulfoxide (DMSO)

(Nacalai Tesque Inc (Japan), dipotassium hydrogen phosphate (Merck (Germany), Dulbecco's Modified Eagle medium (Gibco (USA), fetal bovine serum (Gibco (USA), ferric chloride hexahydrate (R&M Chemicals (UK), ferrous sulphate heptahydrate (R&M Chemicals (UK), glucosidase (Sigma-Aldrich (USA), hydrochloric acid (Fisher Scientific (USA), methanol (Chemiz (Malaysia), nutrient agar (Hi-Media (India), nutrient broth (Hi-Media (India), p-Nitrophenyl-a-D-glucopyranoside (PNPG) (Sigma-Aldrich (USA), phosphate buffer saline pH 7.4 Gibco (USA), Potassium dihydrogen phosphate (R&M Chemicals (UK), silver nitrate (Systerm (Malaysia), sodium bicarbonate (Merck (Germany), sodium hydroxide ((R&M Chemicals (UK) and starch (Sigma-Aldrich (USA).

Isolation of Marine Bacteria

Two isolated and identified marine bacteria that were previously reported, i.e., AQ5-NT27 and AQ5-NT39 were acquired from the Eco-Remtech Laboratory [26]. The marine bacteria were grown in 10 mL nutrient broth at 28°C in an orbital shaker set at 150 rpm for 24 h.

Biosynthesis of Silver Nanoparticles

The bacteria were cultured in 10 mL of nutrient broth for 24 h and centrifuged using the ultracentrifuge at 7500 rpm for 10 min. The supernatant was collected, and the pellet was discarded. Then, 10 mL of 0.5 M $AgNO_3$ solution was added to the supernatant, and the tube was covered with aluminum foil and left for 24 h in the dark. Colloidal AgNPs produced were freeze-dried into powdered form to ensure stability of the AgNPS and increase the shelf-life. One of the Falcon tubes that were not added with $AgNO_3$ served as the control.

Characterization of Silver Nanoparticles

The newly synthesized AgNPs solution was characterized using the UV-Vis spectrophotometer. The absorbance spectra of the samples were recorded between 340 to 700 nm using 2 mL of colloidal AgNPs. Next, the hydrodynamic diameter, polydispersity index, and zeta-potential of AgNPs were characterized using the DLS principle.

Determination of Antioxidant Activities

1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

The free radical scavenging properties of AgNPs were determined using the 1,1–diphenyl-2-picrylhydrazyl (DPPH) free radical assay, as stated by Bekir et al. [27] with slight modification. The DPPH reagent was prepared by dissolving 39.4 mg of DPPH in methanol/ethanol. Various concentrations of the AgNPs were prepared (0–100 µg/mL) by dilution with deionized water. Then, 20 µL of each AgNPs concentration was allowed to react with 180 µL of 0.1 mM DPPH in 70% methanol in a 96-well plate wrapped with aluminum foil. All test tubes were incubated in the dark at room temperature for 30 min, and the absorbance (A) was obtained at 517 nm using the 96-well microplate reader. The percentage inhibition of free radical scavenging activity was calculated using the formula:

$$\text{Percentage of inhibition} = \frac{\text{Abs of negative control} - \text{Abs of sample}}{\text{Abs of negative control}} \times 100\%$$

2,2,-azinobis-3-ethylbenzoline-6-sulfonic acid (ABTS) Scavenging Activity

The free radical scavenging activity of AgNPs was determined by an assay modified from Ulloa et al. [28]. The 2,2,-azinobis-3-ethulbenzoline-6-sulfonic acid (ABTS) solution was prepared by mixing 5 mL of 7 mM ABTS solution with 5 mL of 2.45 mM potassium persulfate ($K_2S_2O_8$). Then, the solution was incubated at 37 °C for 16 h. The ABTS scavenging activity was determined by mixing 180 µL of ABTS solution with 20 µL of AgNPs in a 1.5% DMSO solution. The mixture was incubated for 16 h at room temperature in the dark until the reaction was stable, and the absorbance (A) was read at 734 nm for various concentrations (0–100 µg/mL) using the 96-well microplate reader. Ascorbic acid was the positive control for this assay and as a comparison whether the sample tested having better antioxidant capacity, while distilled water was used as the blank. The negative control only comprised reagent without sample, and the percentage inhibition of scavenging activity was calculated using the formula:

$$\text{Percentage of inhibition} = \frac{\text{Abs of negative control} - \text{Abs of sample}}{\text{Abs of negative control}} \text{ x } 100\%$$

Ferric Reducing Antioxidant Power Assay (FRAP)

The ferric reducing antioxidant power (FRAP) assay was performed as previously described by Clarke et al. [29], with slight modification. Firstly, the FRAP reagent was prepared freshly by mixing 300 mM of acetate buffer (pH 3.6), 10 mM of 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl, and 20 mM of $FeCl_3.6H_2O$ in the volume ratio of 10:1:1. Approximately 180 µL of fresh FRAP reagent was allowed to react with 20 µL of AgNP at various concentrations (0–100 µg/mL) in deionized water in test tubes. The tubes were incubated in the dark at room temperature for 30 min, and the absorbance was assessed at 595 nm using a microplate reader. $FeSO_4.7H_2O$ with concentrations of 0 – 80 µg/mL was used as the standard to construct the calibration curve, while ascorbic acid was the positive control. The assay was performed in triplicate and expressed in $\mu mole\ FE/mg$ unit.

The FRAP value was expressed as µmole ferric equivalent (FE) per microgram of the sample, defined as the antioxidant concentration with a ferric-TPTZ reducing ability equivalent to the $FeSO_4.7H_2O$.

$$\text{FRAP Value (µmole FE/mg)} = \frac{\text{x µg/mL} \times 0.02\text{ mL} \times 278.02\text{ mol/g}}{\text{Weight of sample (mg)}}$$

Where x = Concentration of antioxidant equivalent to $FeSO_4.7H_2O$ obtained from standard curve

Determination of Antidiabetic Activities

***In vitro* α-Amylase Inhibition Assay**

The assay was conducted using starch solution as the substrate, α-amylase as the enzyme, and AgNPs as the inhibitor, according to the method by McCue and Shetty [30] , with slight modification. A mixture of 0.50 µL AgNP samples was added to 50 µL 0.02 M sodium phosphate solution (pH 6–9 with 0.06 M sodium chloride) containing the α-amylase solution (0.5 mg/mL, EC 3.2.1.1). The solution was incubated at 37 °C for 10 min. After pre-incubation, 50 µL of 1% of soluble starch solution was added to each tube at specific intervals. The reaction mixture was incubated at 37 °C for 10 min, followed by the addition of 100 µL of dinitrosalicyclic acid (DNSA) color reagent. The test tubes were placed in a boiling water bath (80 °C for 10 min) to stop the reaction and cooled at room temperature. Next, the reaction mixture was diluted with 1 mL distilled water, and the absorbance was read at 540 nm using the UV/visible spectrophotometer. In this experiment, acarbose (purity > 95%) was used as the control and deionized water as the blank. The α-amylase inhibitory (AI) activity was calculated and expressed in % using the following formula by Shengule et al. (2018).

$$\text{AI (\%)} = \frac{(\text{Ac}-\text{As})}{\text{Ac}} \text{ x } 100$$

Where AI represents α-amylase inhibitory activity in percentage, A_c represents the absorbance value of the control reaction, and A_s represents the absorbance value of the phenolic acid sample or the standard. Acarbose was used as the standard for the test compound.

***In vitro* α-Glucosidase Inhibition Assay**

The α-glucosidase inhibition assay was determined according to Balan et al. (2016) [31], with slight modification. The assay mixture, which comprised 150 µL of 0.1 M sodium phosphate buffer (pH 6.9), 50 µL of α-glucosidase enzyme solution (1 U/mL), and 60 µL of AgNPs, was added into a 96 well-plate and incubated at 37 °C for 20 min. Then, 50 µL of 2 mM para-nitrophenyl-a-D-glucopyranoside (PNPG) in sodium phosphate buffer was added to the mixture and incubated at 37 °C for 20 min to allow the enzymatic reaction. About 140 µL of 0.1 M sodium carbonate solution was added to each plate to terminate the reaction, and the absorbance was measured at 405 nm using the 96-well microplate reader. The percentage of inhibition of AgNP against α-glucosidase activity was calculated using this formula:

$$\text{\% Inhibition} = \frac{(\text{A}_\text{c} - \text{A}_\text{s})}{\text{A}_\text{c}} \times 100$$

Where A_c represents the absorbance value of the control reaction and A_s represents the absorbance value of AgNPs at 405 nm. The assay was performed in triplicate, and the obtained data were averaged.

Cell Viability Assay (MTT Assay)

Preadipocyte cells (3T3 L1) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mM nonessential amino acid, 1 mM sodium pyruvate, and 1% antibiotic (100 U/mL of penicillin and 100 µg/mL of streptomycin). The cells were maintained in a 96-well plate at 37 °C in a 5% CO_2 incubator after adjusting to 3×10^4 cells/well. After overnight incubation period, different concentrations of AgNPs were added to the cells and incubated further for 24 h. The plate was incubated in a humidified atmosphere at 37 °C in the presence of 5% CO_2. After 24 h, the media was discarded, and the wells were washed with phosphate-buffered saline (PBS). A volume of 100 µL fresh media was added to each well with 10 µL of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent. The insoluble purple-colored particles were solubilized with a 10% sodium dodecyl sulfate solution and 50% N,N-dimethyl formamide. The solutions were incubated for 4 h. The media was discarded, and 100 µL DMSO was added into each well with gentle swirling and incubated for 30 min. After 30 min of incubation, the optical density was determined at 570 nm using a microplate reader. The viable cells were determined in all the experimental conditions and compared to the control (untreated cell). The experiment was repeated in triplicate for three independent studies. The percentage of cell viability was calculated using the formula:

$$\text{\% Cell viability} = \frac{(\text{A}_\text{S} - \text{A}_\text{B})}{(\text{A}_\text{c} - \text{A}_\text{B})} \times 100$$

Where A_S represent the absorbance value of the sample reaction containing cells and AgNP, A_c represent the absorbance value of the control reaction comprising only the cells, and A_B represents the absorbance value of the blank

containing 1% DMSO solution. The assay was performed in triplicate, and the data obtained were averaged.

Statistical Analysis

The statistical analyses were conducted using a one-way analysis of variance (ANOVA). The data presented were the means of the triplicate experiment, along with the standard error of the mean. The means were compared using GraphPad Prism that generates figures and graphs, and the difference at $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSIONS

Biological agents like microbes are ideal for nanoparticle synthesis. The biogenic nanoparticles are economically viable, easy to synthesize, and provide a green approach to synthesizing matter. This study attempted to synthesize AgNPs by employing marine bacterial isolates for their antioxidant and antidiabetic activities. Two marine bacterial samples, AQ5-NT27 and AQ5-NT39 isolated from Pantai Purnama and Bagan Lalang, were acquired from the Eco-Remtech laboratory, UPM. The samples were identified and designated as *Serratia* sp. [26]

The biosynthesis of AgNPs from these marine isolates used the same method as Noor et al. [26]. Figure 1 shows the biosynthesis of AgNPs by AQ5-NT27 and AQ5-NT39, with peaks at 420 and 340 nm. The spectra of the AgNPs showed a characteristic absorbance band for AgNPs as observed in previous studies [32].

The change of color from milky-white to brown with the addition of 0.5 M of $AgNO_3$ solution proved that the extracellular component of the supernatant had reduced Ag^+ into Ag^0 within 24 h incubation at room temperature, in the dark, and upon agitation (Figure 2). The changes of color from yellow to brown signifies the formation of AgNPs [33].

In this study, we found the colloidal suspension of AgNPs as observed in Figure 2 (c), which formed after 24 h of incubation along with sediment. A colloidal suspension of AgNPs is widely used due to its monodispersed properties and reported to be more stable than the sediment form of which reside polydispersed AgNPs. This biosynthesis of these AgNP colloids are strongly associated with the enzyme released by the bacteria itself, which is nitrate reductase [34]. Briefly, the nitrate ion of silver nitrate induced nitrate reductase, which gained an electron from NADH and oxidized into NAD^+, and finally undergoes oxidation to reduce the silver ions to AgNPs. Brayner et al. [35] stated that the concentration of cellular nitrogenase; which is an enzyme that catalyst the production if AgNPs would determine the NP size. Furthermore, the higher concentration of cellular nitrogenase led to the rapid formation of large AgNPs next to the cell wall, and the intermediate content forms small and non-aggregated nanoparticle colloids.

The DLS analysis was conducted to uncover the size and surface charge of the synthesized AgNPs. Figure 3 shows the average diameter of NT27-AgNP and NT39-AgNP colloidal suspension, i.e., around 236.5 and 157.3 nm, and the zeta potential of −6.05 and −7.65.

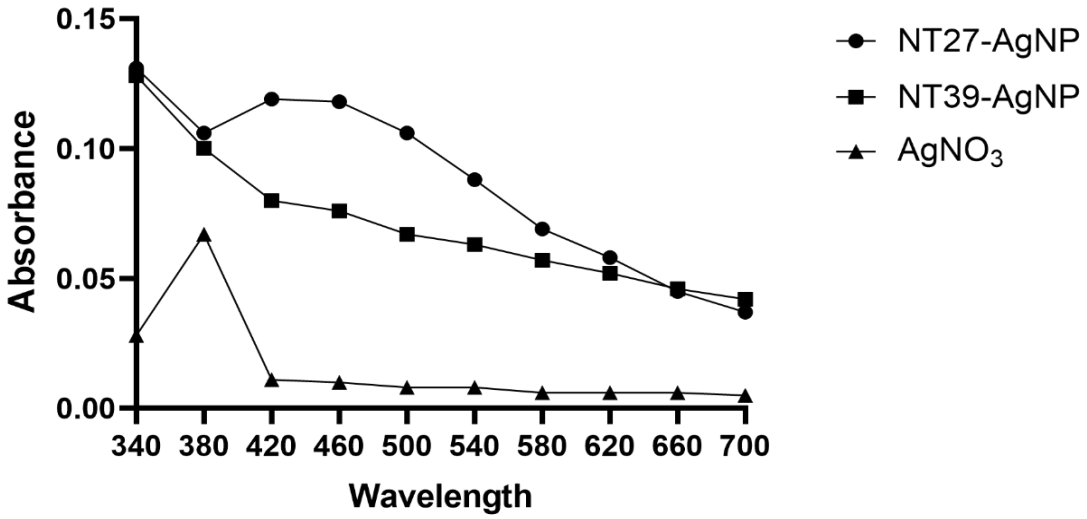


Figure 1: UV-Vis spectra of AgNPs. AgNPs synthesized by different bacteria namely, AQ5-NT27 and AQ5-NT39 with $AgNO_3$ as control

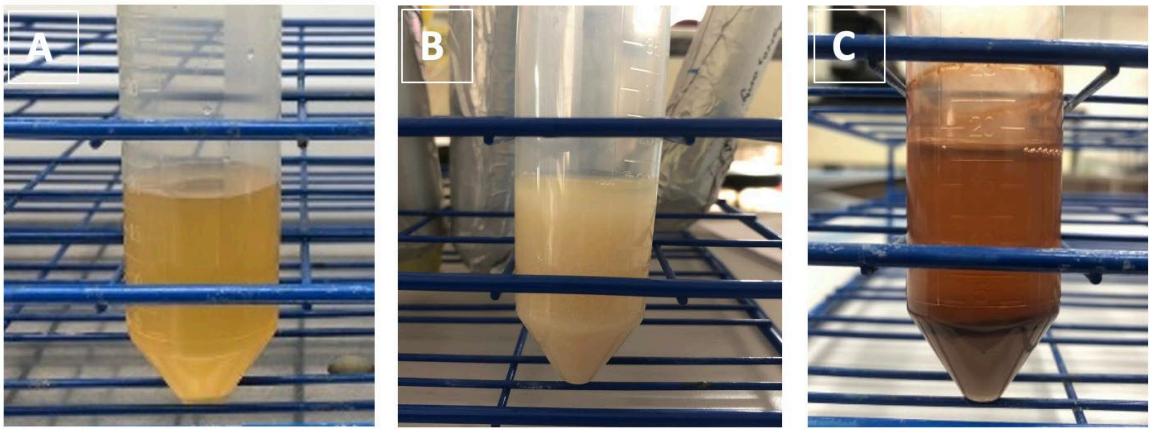


Figure 2: The color changes of solution (A) cell supernatant of isolated marine bacteria, (B) addition of $AgNO_3$, and (C) AgNPs colloid after 24 h for AQ5-NT27

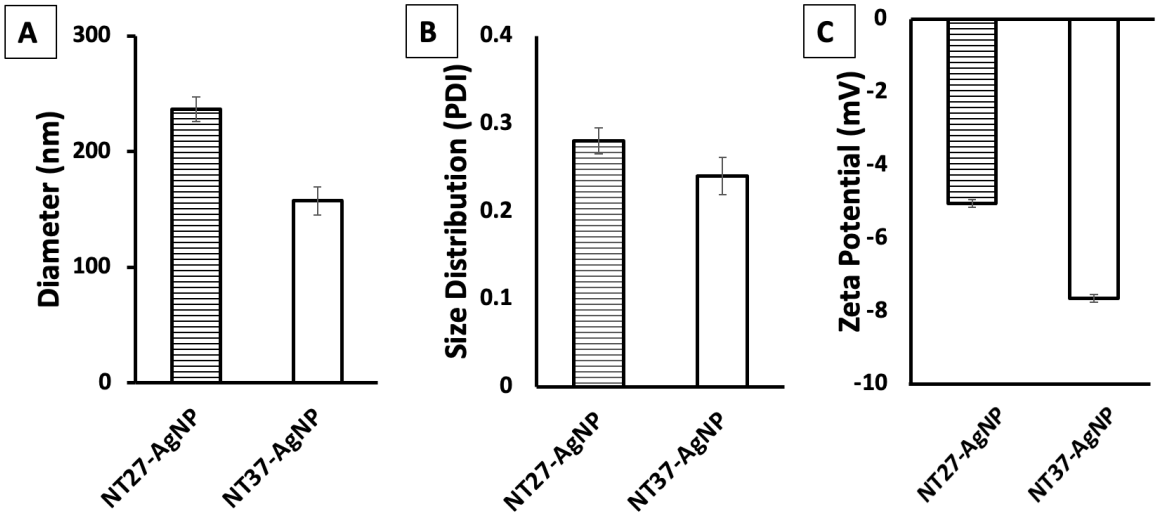


Figure 3: A) Hydrodynamic Diameter, (B) Size Distribution, and (C) Zeta Potential of different formulation AQ5-NT27 and AQ5-NT39 **(**Data represent mean±SD (n=3)

found to be biocompatible and eco-friendly [37]. It not only confer biocompatible characteristics to the nanoparticles but also act as stabilizer for AgNPs production by microbes [38].

Numerous studies demonstrated the antioxidant activity of plant-mediated AgNPs. However, the information on antioxidant activity by bacteria-mediated AgNPs is still scarce. Hence, this study tends to explore the potential antioxidant activity from bacteria-mediated AgNPs. The radical scavenging properties were determined using DPPH, ABTS, and FRAP assays for the AgNPs synthesized by different bacteria, AQ5-NT27 and AQ5-NT39.

DPPH is commonly used in scavenging free radical activity due to its ability to donate hydrogen [39]. This radical can be easily dissolved in methanol and, once reacted with sample with antioxidant properties namely AgNPs, will yield a faint yellow color at 517 nm. The color change is due to the donation of hydrogen atoms by the AgNPs to the stable DPPH molecule [40]. NT27-AgNPs and NT39-AgNPs exhibit a significantly lower inhibition from 20 µg/mL up to 100 µg/mL than the positive control, ascorbic acid (Figure

4). However, the inhibition of free radicals is not dose-dependent. At the highest concentration tested (100 µg/mL), the inhibition percentage is 10.14 ± 2.51% and 11.83 ± 1.38% for NT27-AgNPs and NT39-AgNPs.

Compared to the DPPH radical, which is naturally stable, ABTS needs hydrogen ions from the tested samples to become stable, causing it to lose color. Moreover, a strong antioxidant activity is indicated by the ability of samples to eliminate the hydroxyl radicals (OH^-) through oxidation or other methods [41]. The scavenging activity of ABTS is illustrated in Figure 5, which shows an overall low OH^- radical scavenging properties at all concentrations tested. It is noted that both formulations of AgNPs did not show any significant increase in scavenging ability at all tested concentrations. Both of the DPPH and ABTS low inhibition results may due to the non-existent of the hydrogen ion in the AgNP formulation. At 100 µg/mL, which is the highest concentration tested, both NT27-AgNPs and NT39-AgNPs exhibit 12.19 ± 0.42% and 13.27 ± 0.58% inhibition percentage. This values significantly low compared to the studies done by Shanmugam et al., (2016) when their formulation tested to have about 64-70 % inhibitions.

The FRAP assay is based on the ability of a sample to reduce Fe^{3+} to Fe^{2+} and produce a blue color at 593 nm. In this study, the reducing capabilities of NT27-AgNPs and NT39-AgNPs are demonstrated in Figure 6. Both AgNPs exhibit significantly lower ferum inhibition ability than the positive control, ascorbic acid, which exhibits the highest reducing ability at 100 µg/mL (57.88 ± 0.21 µmole [FE]/mg sample). Simultaneously, these AgNPs did not show any significant difference among each other along the concentration range. The FRAP values between 0.04 to 0.58 µmole (FE)/mg of the sample indicate that both formulations possessed a weak electron-donating property.

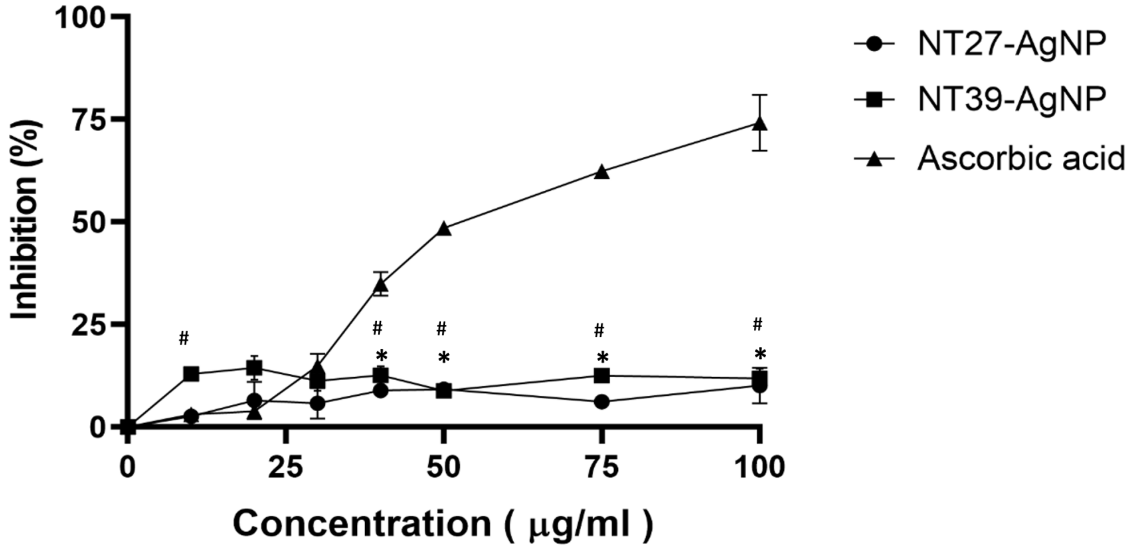


Figure 4: Antioxidant activities of NT27-AgNPs and NT39-AgNPs using DPPH assay. Results are expressed as means ± SEM (n=3). A one-way ANOVA followed by Tukey's post-test was performed. Values are significantly different at $p < 0.05$. Ascorbic acid acts as the positive control. Significant difference of NT27-AgNPs and NT39-AgNPs compared to ascorbic acid was denoted by * and #

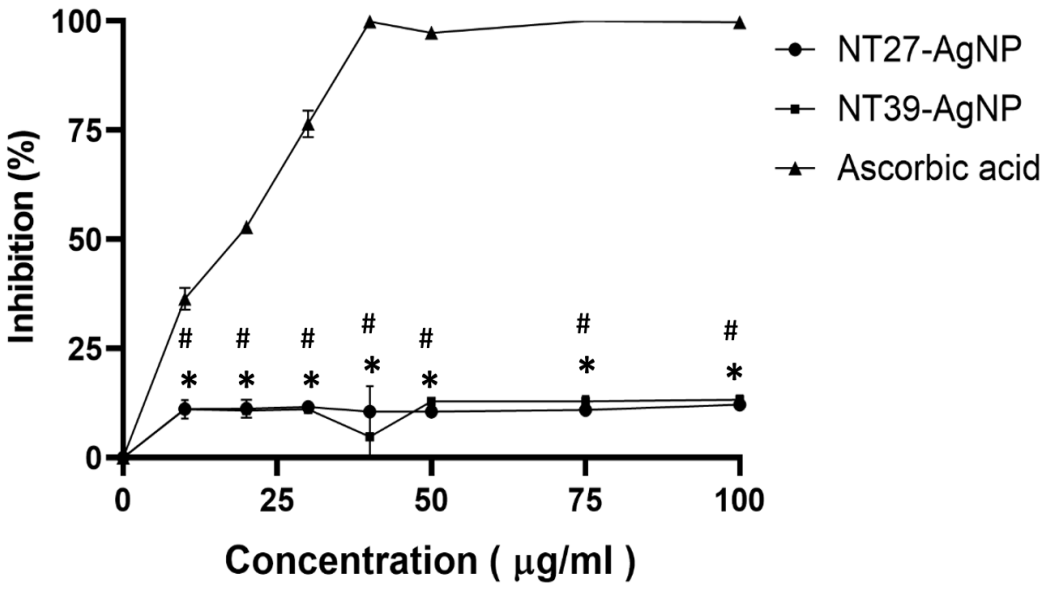
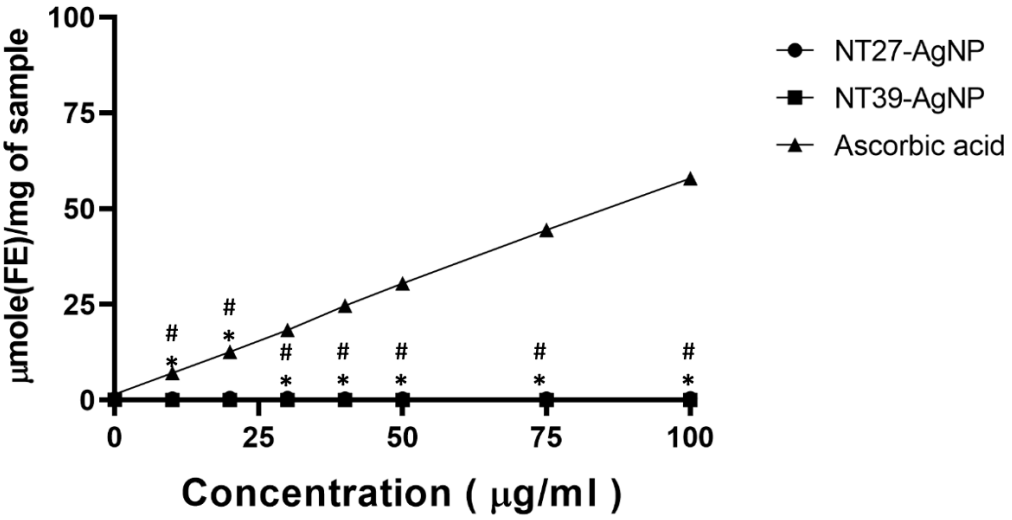


Figure 5: Antioxidant activities of NT27-AgNP and NT39-AgNP using ABTS assay. Results are expressed as means ± SEM (n=3). A one-way ANOVA followed by Tukey's post-test was performed. Values are significantly different at $p < 0.05$. Ascorbic acid acts as the positive control. Significant difference of NT27-AgNPs and NT39-AgNPs compared to ascorbic acid was denoted by * and #



Significant difference of NT27-AgNPs and NT39-AgNPs compared to ascorbic acid was denoted by * and #

Overall, both AgNPs possessed lower radical scavenging activity than those reported in previous studies due to their poor reducing ability. The antioxidant activity of AgNPs could be attributed to the EPS attached to their surface. In addition, the attachment of EPS on AgNPs is expected due to the EPS help to donates hydrogen ions that reacted with the radical DPPH, resulting in a low level of antioxidant properties [42]. Previously, Kodali and Sen [43] proved that EPS from a probiotic bacterium showed 45% inhibition towards DPPH free radicals. Since EPS from *Serratia* sp. has not been fully studied, it may cause the synergistic antioxidant property in both AgNPs. Meanwhile, the results of the scavenging activity in ABTS might be attributed to the presence of hydroxyl groups from EPS attached to the surface of AgNPs. Thus, it can be assumed that the EPS from *Serratia* sp. assists in reducing free radicals, particularly OH^- of ABTS by both AgNPs.

The α-glucosidase and α-amylase inhibitors are commonly used as an oral antidiabetic drug to treat T2DM [44]. The mechanism used to treat DM is via inhibiting the activity of α-amylase or α-glucosidase, carbohydrate-hydrolyzing enzymes in the gastrointestinal glucose absorption [45]. The inhibition of these enzymes allows the control of glucose levels in the body, i.e., by regulating the amount of glucose uptake to be absorbed into the bloodstream [46]. Therefore, apart from the antioxidant activity of AgNPs, their potential as an antidiabetic agent is also studied. In the current study, AgNPs are used to inhibit α-amylase and α-glucosidase since the inhibition of these enzymes could be one of the strategies in managing hyperglycemia strongly linked to diabetes development.

Delaying the digestion of carbohydrates could help in reduced postprandial hyperglycemia by inhibiting α-amylase. The current study used two formulations of AgNPs that act as an inhibitor to the enzyme, as observed in the inhibition activity in Figure 7. It can be seen that the highest inhibition of the enzyme activity by NT27-AgNPs is 52.55 ± 4.08%, indicating no significant difference compared to acarbose (positive control) in this experiment. Meanwhile, NT39-AgNPs show a lower inhibition, approximately 36.09 ± 5.59%, at the lowest concentration tested. It indicates that both AgNPs possess a strong α-amylase inhibition effect at 10 µg/mL. However, the percentage of inhibition for both AgNPs is not dependent on the concentration, as the percentage decreases significantly when the tested concentration increases.

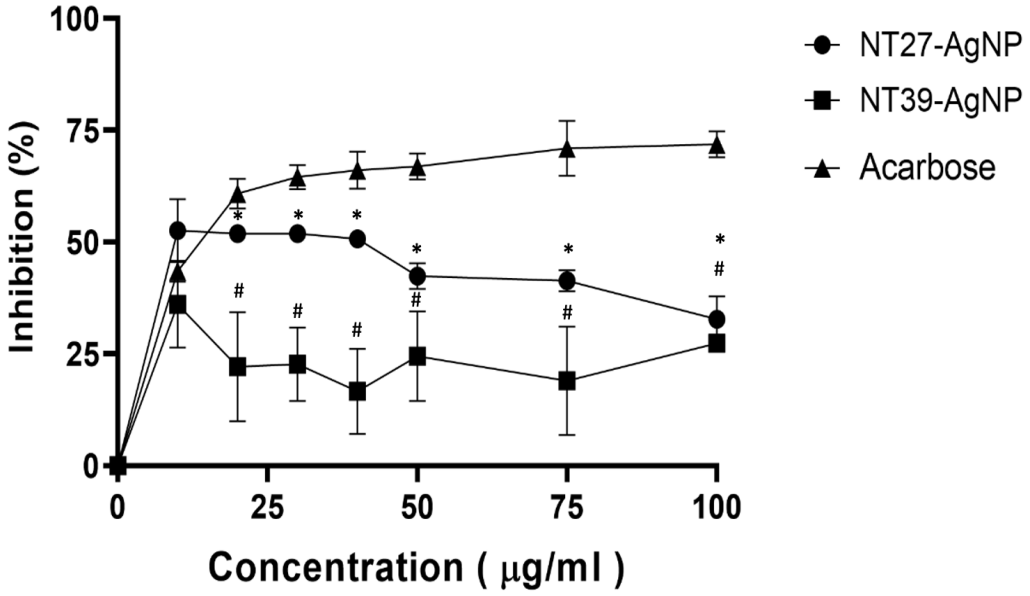


Figure 7: Antidiabetic activities of NT27-AgNPs and NT39-AgNPs using α-amylase inhibition assay. Results are expressed as means ± SEM (n=3). A one-way ANOVA followed by Tukey's post-test was performed. Values are significantly different at $p < 0.05$. Ascorbic acid acts as the positive control. Significant difference of NT27-AgNPs and NT39-AgNPs compared to acarbose was denoted by * and #

In general, NT27-AgNPs exhibit significantly higher inhibition of α-amylase activity than NT39-AgNPs. It is known that AgNPs capped with plant extract showed a high inhibition (75.55% at 50 µg/mL) of amylase enzyme due to the presence of phytochemicals [47]. However, as both of the AgNPS did not contain any phytochemicals and their inhibition of amylase activities rely solely on the conformational aspect of the NPs., The mechanism of action for bacteria-mediated AgNPs in inhibiting amylase enzyme is suggested due to the altered conformation of α-amylase caused by the binding of AgNPs to the enzyme and is known as a non-competitive mode of inhibition [48]. Moreover, the

inhibition of enzymes could also be due to the presence of EPS that blocked the active site of both enzymes [49]. PS attached to AgNPs and inhibits the enzyme is bound to the catalytic group and acts as a transition-state mimic for the cleavage of the α-1,4 glycosidic linkages, resulting in inhibition [50].

In the current study, NT27-AgNPs exhibit a greater inhibition of α-amylase than NT39-AgNPs. The possible reason is that NT27-AgNPs could be more active and possesses a high affinity towards amylase; thus, increasing inhibition and resulting in greater inhibition activity.

The inhibition of α-glucosidase by NT27-AgNPs and NT39-AgNPs is 26.99 ± 3.08% and 44.98 ± 7.42% at 10 µg/mL (Figure 8). In general, as the concentration increases, the inhibitory activity also increases, indicating concentration-dependent inhibitions for both AgNPs. This study reveals that NT27-AgNPs exhibit a high inhibition towards α-glucosidase activity than NT39-AgNPs and it directly related to the size of the NPs [48].

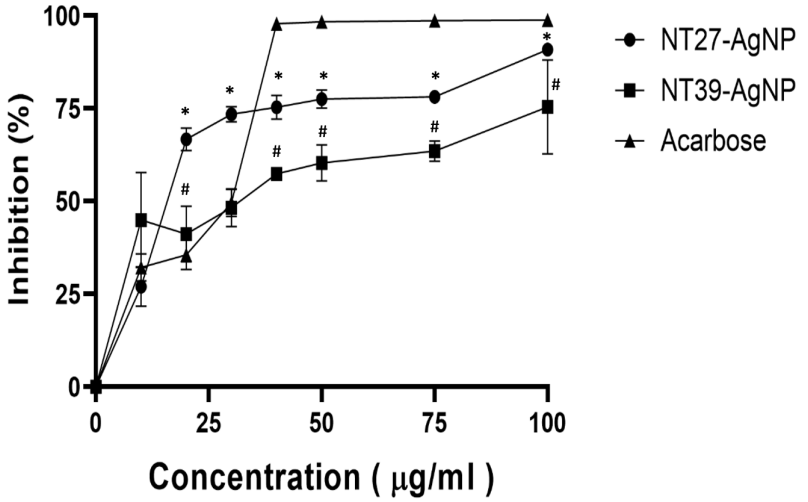


Figure 8: Antidiabetic activities of NT27-AgNPs and NT39-AgNPs using α-glucosidase inhibition assay. Results are expressed as means ± SEM (n=3). A one-way ANOVA followed by Tukey's post-test was performed. Significant difference of NT27-AgNPs and NT39-AgNPs compared to acarbose was denoted by * and #

NT27-AgNPs are more efficient in inhibiting α-glucosidase than NT39-AgNPs since they showed a high inhibition at 100 µg/mL, 90.86 ± 0.49% and 75.10 ± 7.30%. The theorized mechanisms of actions of increasing inhibitory activity of both AgNPs could be due to their high surface area. In addition, it is associated with the bigger hydrodynamic size (>100 nm) and small negative value of the charge (<10 mV) of AgNPs of NT27 in this study, i.e., more preferable by the enzyme than the smaller size and bigger negative value. The mechanism of high inhibition exhibited by the AgNPs could be due to the competition at the binding site of the enzyme between the AgNPs and starch, reducing the enzyme affinity to hydrolyze the glycosidic linkage in starch and suppressing postprandial hyperglycemia [51]. To sum up, AgNPs are proven to decrease the level of enzymes, which is crucial in hydrolyzing complex carbohydrates. Therefore, they could be proposed to be used directly as an inhibitor for α-glucosidase in diabetes treatment due to the high inhibition of amylase.

The cell viability assay was performed to determine the percentage of viable 3T3-L1 cells after treatment with AgNPs, i.e., the cytotoxicity of AgNPs towards the cells. Cell viability involved the MTT reduction as a direct assay of mitochondrial activity where the mitochondrial succinate dehydrogenase of viable cells can reduce MTT to form formazan. It is used to determine the mitochondrial dehydrogenase activities in living cells. MTT is reduced to purple formazan by nicotinamide adenine dinucleotide phosphate (NADH). The number of viable cells can be seen with the decrease in the intensity of the purple-coloured product in the wells. This indicated that when the cells die, they lose the ability to convert MTT into purple-coloured formazan product and accumulated near the cell surface and in the culture medium [52.] Eight different concentrations (0, 10, 20, 30, 40, 50, 75, and 100 µg/mL) of AgNPs were tested to determine the viability of 3T3-L1 cells.

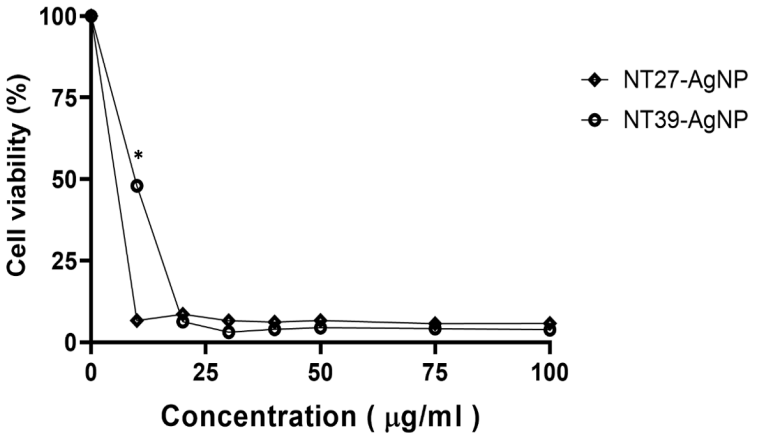


Figure 9: The effect of NT27-AgNPs and NT39-AgNPs on 3T3-L1 cell viability. Results are expressed as mean ± SEM (n=3). Significant difference of NT27-AgNPs compared to NT39- was denoted by *

The exposure of both NT27-AgNP and NT39-AgNP to the 3T3-L1 cells caused a significant reduction ($p < 0.05$) of the viable cells, around ~90% and ~60% (Figure 9). Figure 9 shows that AgNPs synthesized by *Serratia* sp. exhibit high toxicity towards 3T3-L1 cells. Apart from the concentration of NT39-AgNPs at 10 μg/mL that showed 47.98 ± 0.07%, which is considered moderately toxic, the rest are considered strongly toxic towards the adipocyte cells. It proved that both AgNPs are toxic to the cells, indicating the possibilities of induced oxidative stress in response to the low antioxidant activity.

The current study reveals that the IC_{50} values obtained are 9.93 ± 0.03 and 7.22 ± 0.02 μg/mL for NT27-AgNP and NT39-AgNP, considered strongly toxic towards the 3T3-L1 cells. The International Organization for Standardization [53] stated that treatment with substances that cause the percentage of viable cells of 80%, 60%º–80%, 40%–60%, and below 40% indicated non-toxic, weak, moderate, and strong toxicity. Throughout this finding, the current AgNPs have low compatibility towards 3T3-L1 cells and are not suitable as exposure to the high concentration of AgNPs caused cell death.

The strong toxicity might be due to the hydrodynamic diameter of both NT27-AgNP and NT39-AgNP, which are considered small even though not below 100 nm. Smaller hydrodiynamic-sized nanoparticles have a greater surface area by providing a huge proportion of their atom to be displayed on the surface [54]. It allows the inert materials to attach and exhibit toxicity towards the cells. Another possibility contributed to such toxicity is due to the absence of biological capping agents in AgNPs. Wen et al. [55] reported considerable small size of AgNPs (200 nm) are found toxic to 3T3-LI cell. To reduce the toxicity, the incorporation of plant extract acting as a capping agent to the AgNPs during the synthesis is needed instead of focusing solely only on the AgNPs . Wen et al. [55] also mentioned that exposure of human fibroblast cells towards AgNPs resulted in decreased cell viability due to the toxicity of AgNPs towards the mammalian cell line primarily mediated by released ions. When the colloidal AgNPs tested in an *in vivo* system using rodent, no toxicity can be observed at 47 mg of silver/kg of bw/day and cytokines response can be observed [56, 57]. However, since the current study was tested on higher range of concentration than Leino et al., [57], therefore the toxicity effect can be observed potentially toxic due to their small size and Ag^{+}-release capabilities. The full mechanism of colloidal AgNPs are requires extensive research to be conducted.

In addition, the nanosized AgNPs tend to slow down the fission mechanism of particles during the growth period. A similar finding reported that cell viability decreased as the concentration of AgNPs increased, indicating cell morphology changes [58]. Thus, the decrease of cell viability is due to the interruption of fission during cell growth *in vitro* by AgNPs.

Figure 10 shows that cell death is more prominent with increase AgNPs concentration. This is because the AgNPs became highly toxic as the concentration increases. The microscopic image of the confluent (80%) 3T3-L1 adipocyte cells in Figure 10 (A) depicts spindle-shaped cells differentiated from 3T3 cells. Treatment with AgNPs at 100 μg/mL caused morphological changes at the margins of the cells, e.g., changes of shape and rounded cells, indicating cell death, as can be observed in figure 10 D. The spherical cells are detached from the container and formed clusters. The cells treated with AgNPs have reduced volume with shrunken appearance, indicating dead bodies due to apoptosis, compared to the control cells (0 μg/mL) which can be observed in figure 10 (B), (C), and (D). These findings are supported by previous studies on the exposure of A549 lung cells with the suspension of AgNPs that resulted in a significant amount of early apoptotic, late apoptotic, and necrotic cells [59]. The slight increase in early apoptotic cells is due to AgNPs-induced ROS in A549 lung cells [60].

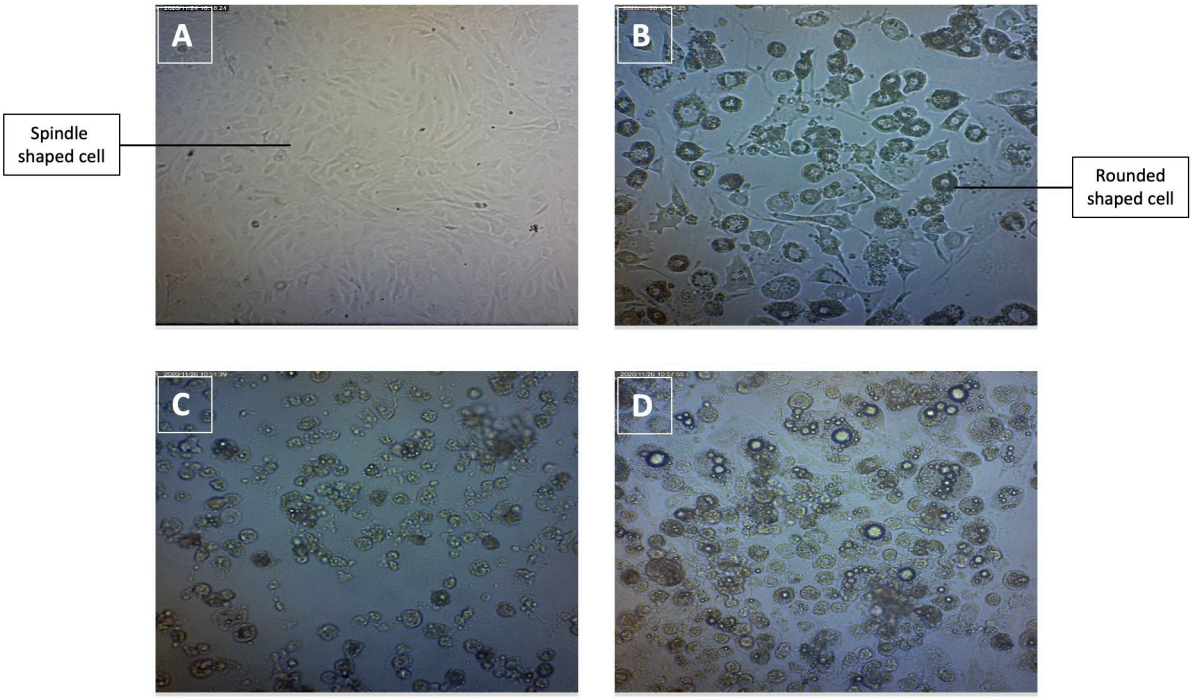


Figure 10: Microscopic image of cell culture (a) 3T3-L1 adipocytes, (b) 0 µg/mL after 24 h of treatment, (c) 10 µg/mL after 24 h of treatment and (d) 100 µg/mL of AgNPs after 24 h of treatment (microscope magnification: 10 ×)

CONCLUSION

Overall, AgNPs synthesized through a green route using marine bacteria, *Serratia* spp., are easy, cost-efficient, and eco-friendly. It gave rise to well-defined nanoparticles. Not only that there is no requirement of toxic chemicals in the process of AgNPs biosynthesize, the employment of bacteria can be done throughout the year for a large-scale approach. The presence of AgNPs was proven through the peaks in the characterization of a colloidal suspension of AgNPs using UV-Vis spectrophotometry. While, the hydrodynamic size and charge was determined via dynamic light scattering (DLS) technique, in the future the crystalline form of newly prepared AgNPs to further prove that this formulation is nanoparticles. In term of purity, the crystallinity study using XRD should also be carried out.

The development of diabetes can be intervened by inhibition of oxidative stress at which bacteria-mediated AgNPs serve as an antioxidant and an inhibitor of α-amylase and α-glucosidase enzymes. Despite their good inhibitory properties towards α-amylase and α-glucosidase, the bacteria-mediated AgNPs in the current study exhibit low radical scavenging properties. The treatment did not help in cytotoxicity aspect when tested in 3T3-L1 cells. In summary, the present study indicates the potential of AgNP acquired from *Serratia* spp. for antidiabetic purpose, but further study is warranted in order to understand and identify the presence of exopolysaccharides and characterize it as it can create a synergistic effect for the effectiveness of AgNPs towards antioxidant and antidiabetic activity.

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