Synthesis and Characterization of Nanoparticles from Extracts of Fruits of *Annona Muricata*: A Green Nanobiotechnology Approach

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Abstract

Green synthesis of nanoparticles from plant materials opens a new opportunity in nanobiotechnology and discourages use of expensive toxic chemicals. The aim of this study was to develop and optimise a method for synthesis of Silver Nanoparticles (AgNPs) from ethanolic extracts of fruits of Annona muricata as well as to characterise the green synthesized AgNPs. AgNPs were synthesized via $AgNO_3$ solution and characterized using spectroscopy and microscopy techniques. The formed AgNPs had an absorption maximum of 427 nm and were stable under different temperature, pH and storage conditions. Fourier Transform Infrared Resorption spectroscopy revealed the functional groups responsible for the synthesis and stabilization of the AgNPs. Scanning Electron Microscopy analysis revealed a spherical nature of the AgNPs while energy Dispersive X-Ray spectroscopy showed presence of Ag, Cl, Ca, and Si with Ag having the highest composition at 80%. X-Ray Diffraction and Dynamic Light Scattering revealed a crystalline nature of AgNPs with an average size of 60.12 nm and a polydispersity index of 0.1235 respectively. Transmission Electron Microscopy analysis further confirmed the crystalline and spherical nature of the AgNPs. This study reports an efficient, eco-friendly and low-cost method for synthesis and recovery of stable AgNPs using ethanolic extracts of Annona muricata fruits as both reducing and capping agents has been reported. The synthesized AgNPs could have many biomedical and clinical applications.

Keywords: Annona Muricata; Silver Nanoparticles (AgNPs); UV/VIS; FTIR; XRD; Fruit Extracts.

Introduction

Nanoparticles are materials that are small enough to fall within the nanometric range, with at least one of their dimensions being less than a few hundred nanometres. This reduction in size brings about significant changes in their physical properties with respect to those observed in bulk materials. A very interesting application of nanoparticles in the scope of life sciences is their use as 'smart' delivery systems where they are usually loaded with a drug or therapeutic agent (Gonzalez-Melendi et al., 2008). The various developed chemical and mechanical methods of producing nanoparticles include ball milling, thermal quenching, precipitation techniques, vapor deposition. However, these methods are often costly, and may result in toxic byproducts. Generally, nanoparticles are synthesized in three ways: physically by crushing larger particles, chemically by precipitation, and through gas condensation (Murphy et al., 2006; Wiley et al., 2007). The commercial significance of nanoparticles is limited by the nanoparticle synthesis process, which is generally energy intensive or requires toxic chemical solvents and is costly.

Annona muricata is known as Soursop (English), Graviola (Portuguese), Guanábana (Latin American Spanish), Omusitafeli / Ekitafeli (Uganda), and other local indigenous names as has been enlisted (Coria-Te´llez et al., 2018; Gavamukulya et al., 2017). The *Annona muricata* tree is about 5–10 m tall and 15–83 cm in diameter with low branches (Benavides et al., 2004; Orwa et al., 2009). It is widely distributed in the tropical regions of Central and South America, Western Africa, Central and Eastern Africa and Southeast Asia (Gavamukulya et al., 2015; Pinto et al., 2005) at altitudes below 1200 m above sea level, with temperatures between 25 and 28 °C, relative humidity between 60 and 80%, and annual rainfall above 1500 mm. The fruit is an edible collective ovoid berry, dark green in color.

The effectiveness of many species of medicinal plants depends on the supply of active compounds. It has therefore been widely proposed to combine herbal medicine with nanotechnology, because nanosystems can deliver the bioactive components at a sufficient concentration during the entire treatment period, directing them to the desired sites of action, and hence potentiating the action of the compounds, an aspect that conventional herbal treatments do not meet (Ansari et al., 2012; Bonifácio et al., 2014). Among several noble metal nanoparticles, silver nanoparticles have attained a special focus (Ahmed, Ahmad, et al., 2016a). Silver nanoparticles are of particular interest because of their antimicrobial, anticancer and cytotoxic activities. The aim of this study was therefore to develop and optimise a method for the synthesis of AgNPs from ethanolic extracts of fruits of *Annona muricata* as well as to characterise the green synthesized AgNPs.

Materials and Methods

Samples collection, authentication, preparation and extraction

Ripe fruits of *Annona muricata* were collected from the wild in Eastern Uganda in the districts of Kaliro, Iganga and Mbale during the month of January 2018. A sample of the plant was collected, pressed, dried and the plant was identified and authenticated in the Makerere University Botanical Herbarium (MHU) by Dr Namaganda Mary and a voucher specimen was deposited in the herbarium with the accession number MHU50860. The Fruits of *Annona muricata* were washed with clean water and then peeled to remove the fresh pulp. The pulp was then cut into small pieces and placed in a hot air oven to dry at 50^{o}C for a week. The dried pulp was then milled into a powder using an electric grater. 50 g of powdered fruits were extracted using 250 ml of absolute ethanol for three days by the plant tissue homogenization method as previously described (Gavamukulya et al., 2014). The light brown Ethanolic Extracts of *Annona muricata* fruits was then filtered and kept at 4^{o}C until use.

Synthesis of Silver Nanoparticles

AgNPs were synthesized by the following method. About 50 ml of the filtered fruits extract was mixed with about 450 ml of 1 mM $AgNO_3$ solution in a 500ml flask and mixed thoroughly, forming a uniform mixture. The mixture was then rested at room temperature in the dark storage cabinets for up to about 72 hours, with continuous monitoring. After about 3hours, the mixture was observed to start changing from light brown to yellowish brown. After about 72 hours, the mixture had completely changed colour to dark brown. This color change is visual evidence of formation of AgNPs or reduction of silver ions into AgNPs due to the excitation of surface plasmon vibration (Ezealisiji et al., 2017; P. & T., 2017; Santhosh et al., 2015).

Characterization of the AgNPs

The synthesis of AgNPs from the ethanolic extract of fruits of *Annona muricata* was further confirmed by ultraviolet - visible spectroscopy (UV/VIS) in the range of between 300nm to 650nm (Kumar et al., 2017; Santhosh et al., 2015) and ethanol was used as a blank.
About 10ml of the formed AgNPs suspension in boiling tubes were subjected to different temperature conditions by heating in a digital water bath for about 3 minutes each and measuring the absorbance spectra on the UV/VIS in a scan range of 350nm to 650nm (Ghoshal & Bhatnagar, 2017). The temperature tested included room temperature (25^{o}C), 35^{o}C, 45^{o}C, 55^{o}C, 65^{o}C, 75^{o}C, and 85^{o}C.

About 15ml of the formed AgNPs suspension was aliquoted into 5 test tubes each containing about 3 ml of the AgNPs suspension. The suspensions in the test tubes were then adjusted to and subjected to different pH conditions ranging from about pH 2 to about pH 11. The suspension in each test tube was subjected to a different pH condition. The specific pH conditions tested were pH 2, 4, 7, 9, and 11. The pH were adjusted by either adding drops of 1N NaOH or 1N HCl until the desired pH was achieved as

observed on the pH meter (Ghoshal & Bhatnagar, 2017; Verma & Mehata, 2016). The absorbance spectra of the suspensions were then measured on the UV/VIS in a scan range of 300nm to 650nm.

About 20ml of the formed AgNPs suspension was aliquoted into four 15ml universal tubes each containing about 5 ml of the AgNPs suspension. The suspensions in the tubes were then stored at different temperature conditions for a period of 3 months. The temperatures at which the storage was done included room temperature (which varied between at about 20^{o}C to 30^{o}C during the experimental period), 4^{o}C, -20^{o}C and -80^{o}C. At the end of the 3 months, the samples were retrieved from the different storage facilities allowed to thaw at room temperature and then their absorbance spectra were measured on the UV/VIS in a scan range of 300nm to 650nm.

Recovery of the synthesized AgNPs and further characterizations

About 400 ml of the AgNPs suspension were transferred into different plastic bottles of about 250ml capacity each and frozen in freezer at -80°C for a period of about 12 hours. The frozen suspension was then removed from the freezer and allowed to completely thaw at room temperature. Upon thawing, the AgNPs were visibly observed spread throughout the now much clear suspension. The suspension with the dispersed AgNPs were then recovered by transferring them into 50ml universal centrifuge tubes and centrifuging them at and RCF of 4025 g for a period of between about 20 minutes to about 45 minutes. After centrifugation, the supernatant in each of the tubes was poured off and the silver nanoparticles were retained as pellets at the bottom of the tubes. The pellets were then washed several times with distilled water (about 10ml of distilled water were added to each tube and then centrifuged afresh for about 5 minutes to wash and dissolve any water-soluble impurities). The now clean AgNPs were then lyophilized and kept in airtight tubes at 4°C until further use. A total of 1.2 g of AgNPs were recovered following lyophilization.

FTIR measurements were carried out to identify the promising biomolecules in the *Annona muricata* ethanolic extract accountable for the reduction of the silver ions and also the capping agents liable for the stability of the bio-reduced AgNPs as previously reported (Madivoli et al., 2018). The representative FTIR spectra of the recovered and dried AgNPs synthesized from ethanolic extracts of fruits of *Annona muricata* were recorded and the major and minor peaks were manifested and identified accordingly.

Scanning electron morphological analysis of Silver nanoparticles were performed using Scanning electron microscope FEI XL30 Sirion FEG (Oxford Instruments Plc, Abingdon, United Kingdom) operated at an accelerating voltage of 6 kV. The system was equipped with an Energy Dispersive X-ray Spectrometer (EDX) system from EDAX having a lithium doped silicon detector.

TEM was employed to characterize the size, shape and morphologies of formed biogenic synthesized AgNPs. A drop of AgNPs suspension was deposited on carbon coated copper grids and the film on grid was then dried. The TEM was operated and the measurements were performed at accelerating voltage of 100 KV.

XRD analysis was employed to determine the average crystalline size of the AgNPs formed. The XRD diffraction data was analyzed using the Match! Software (Crystal Impact, Bonn, Germany) and the average crystalline size of the AgNPs formed in the bio-reduction was determined using the Scherrer equation, with a constant of 0.94.

The hydrodynamic size distributions and polydispersity index (PDI) of the silver nanoparticles were analyzed by using dynamic light scattering (DLS) instrumentation. The average particle size, size distribution by intensity as well as PDI were determined by injecting 1:20 dilution of silver nanoparticle resuspension into the U-shaped glass

cuvette of the photon correlation microscope as previously reported (Danaei et al., 2018; Ezealisiji et al., 2017; Kumar et al., 2017).

Results

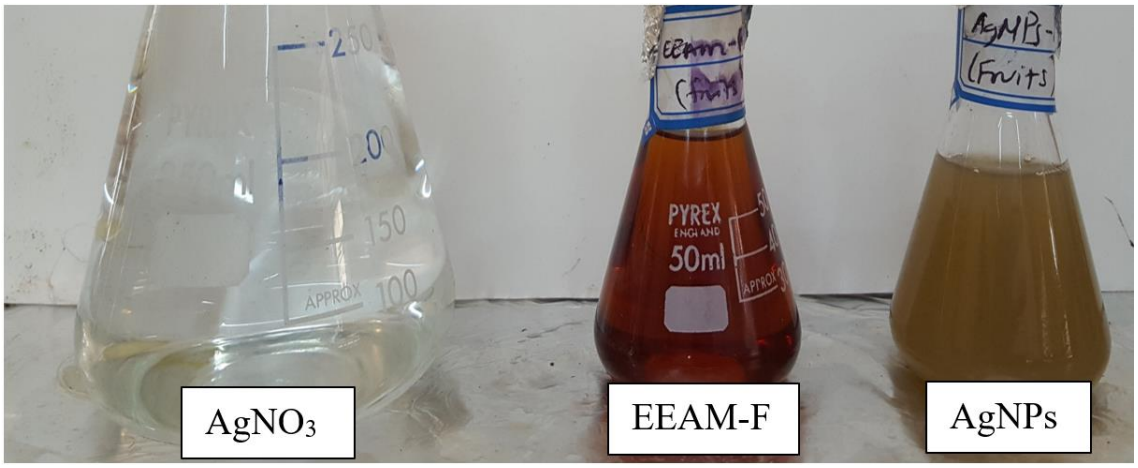

Figure 1: Photo showing colour of the green synthesized AgNPs relative to the Ethanolic extract of *Annona muricata* fruits (EEAM-F) and Silver Nitrate solution ($AgNO_3$).

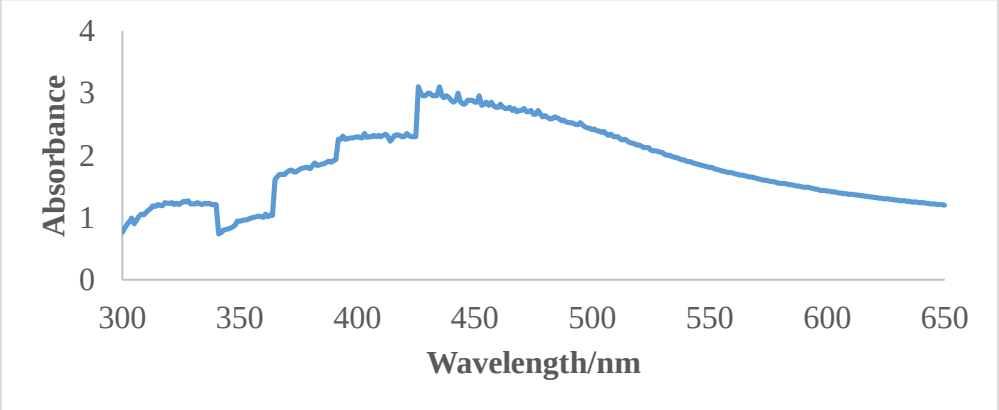

Figure 2: UV/VIS spectrum of fruits derived AgNPs at 72 hours of incubation.

The spectrum shown in figure 3 above has a maximum absorption peak at a wavelength of about 427 nm, which is in the range of the surface plasmon resonance for AgNPs which is reported to have an absorption maximum of between about 400nm to about 450nn.

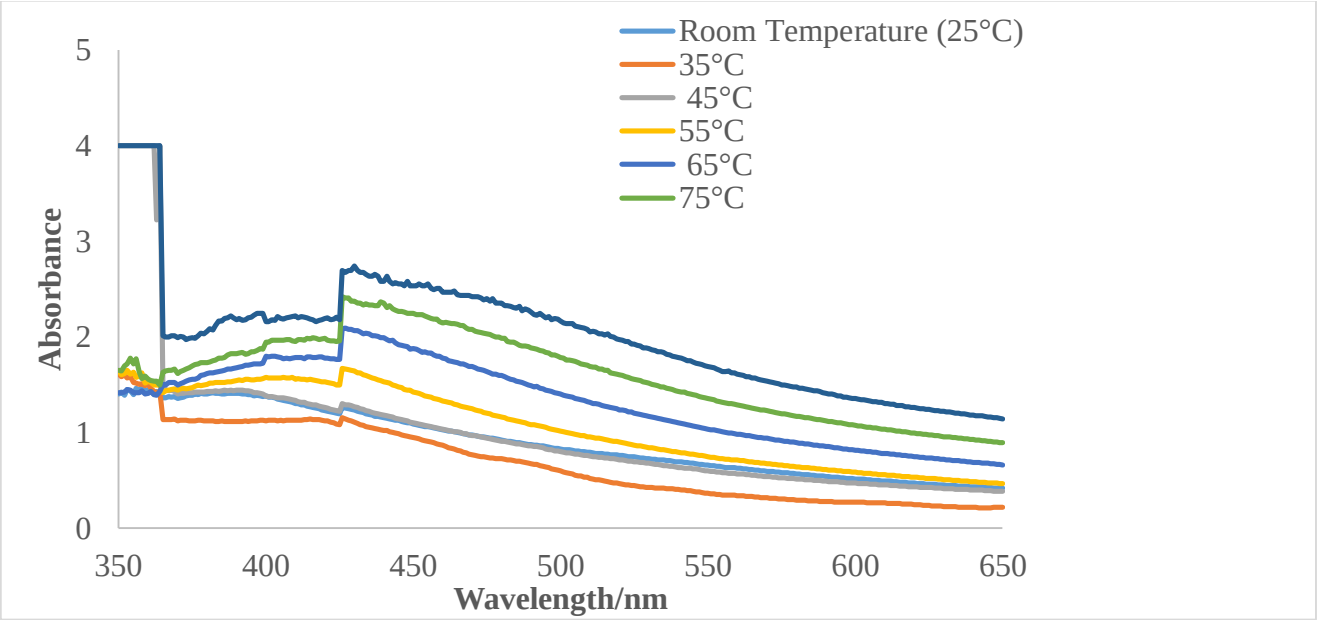

Figure 3: UV/VIS spectra showing temperature stability of AgNPs synthesized from fruits extract

From Figure 3 above it is evident that at all temperatures tested, the AgNPs remained stable maintaining a characteristic absorption maximum of about between 420nm to about 430nm which is within the AgNPs range.

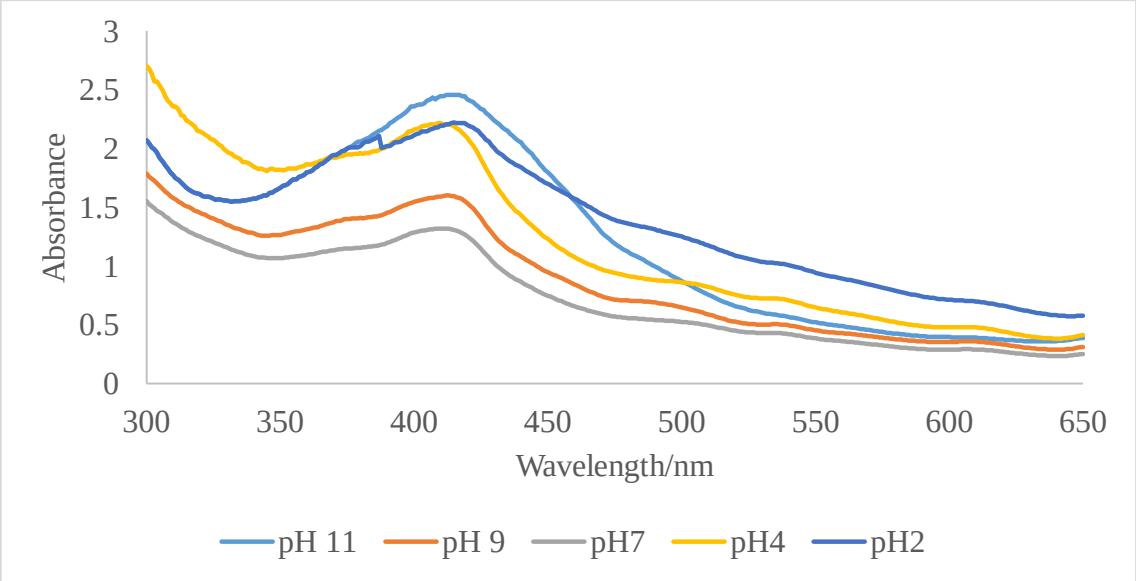

Figure 4: UV/VIS spectra showing pH stability of AgNPs synthesized from fruits extract

From Figure 4 above, it is evident that at all pH conditions tested, the AgNPs remained stable maintaining a characteristic absorption maximum of about between 410nm to about 420nm which is within the AgNPs range. There was a notable and strong relationship between AgNPs absorption spectra at extreme acidic and alkaline pH conditions of 2 and 11.

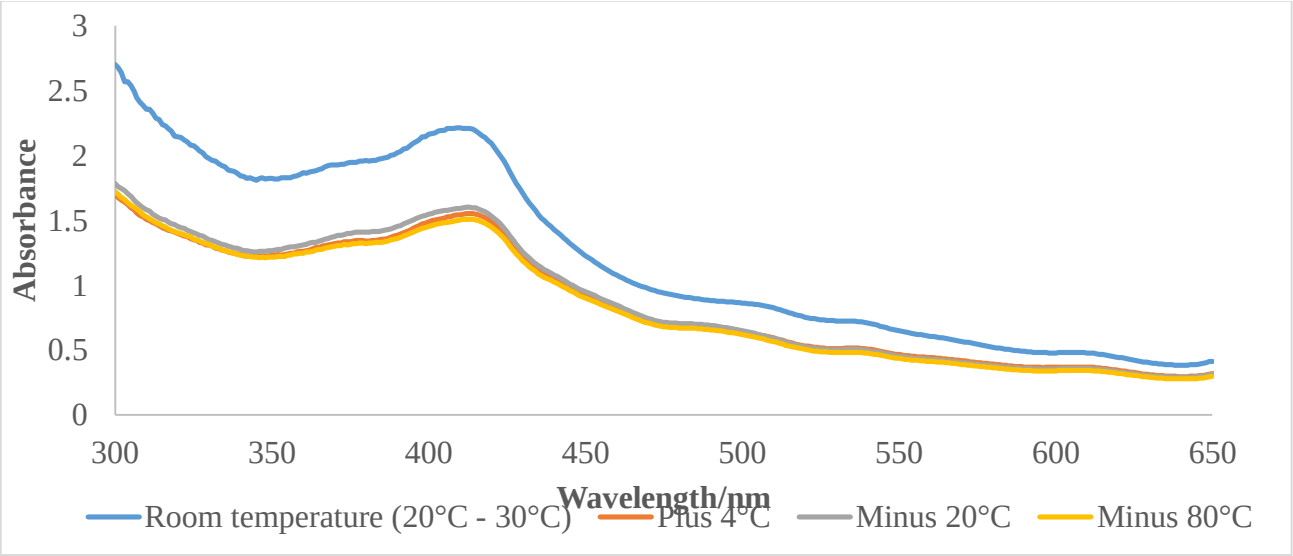

Figure 5: UV/VIS spectra showing storage stability of AgNPs synthesized from fruits extract

From Figure 5 above, it is evident that at all storage temperatures tested for the 3 months, the AgNPs remained stable maintaining a characteristic absorption maximum of about between 410nm to about 430nm which is within the AgNPs range. There was a notable increase in the absorption of the AgNPs at room temperature compared to other storage conditions, nevertheless, the absorption maximum was maintained in the AgNPs range.

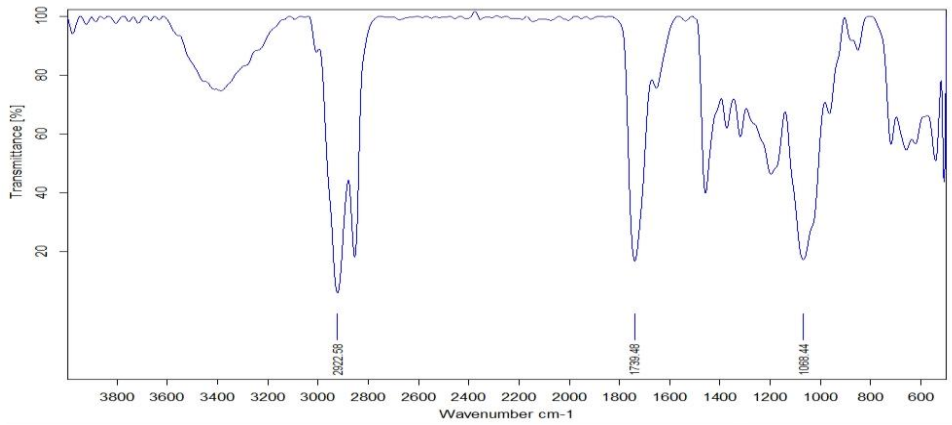

Figure 6: FTIR spectra of functional groups from the AgNPs synthesized from fruits extract

Table 1: FTIR functional group analysis of biosynthesized AgNPs from ethanolic extracts of Fruits of *Annona muricata*

Type of Peak	Frequency (cm−1)	Bond	Functional groups
Major	2922.58	C–H stretch	Alkanes and alkyls
	2850	C–H stretch	Alkanes and alkyls
	1739.48	C=O stretch	Aldehyde and Esters
	1500	N–O Stretch	Nitro group
	1068.44	C–O stretch	Alcohol group
Minor	3400	O–H Stretch	Carboxylic acids
	1650	C=O stretch	Amide
	1400	–C–H Bend	Alkane
	1200	C–O stretch	Acid
	900	=C–H bend	Alkenes
	700	C–Cl Stretch	Alkyl halide
	550	C–Br Stretch	Alkyl halide

As shown in Figure 6 and Table 1 above the functional groups responsible for the formation of the AgNPs included; Alkanes and alkyls, aldehydes and esters, nitro groups, alcohol groups, carboxylic acids, amides, alkenes, acids and alkyl halides.

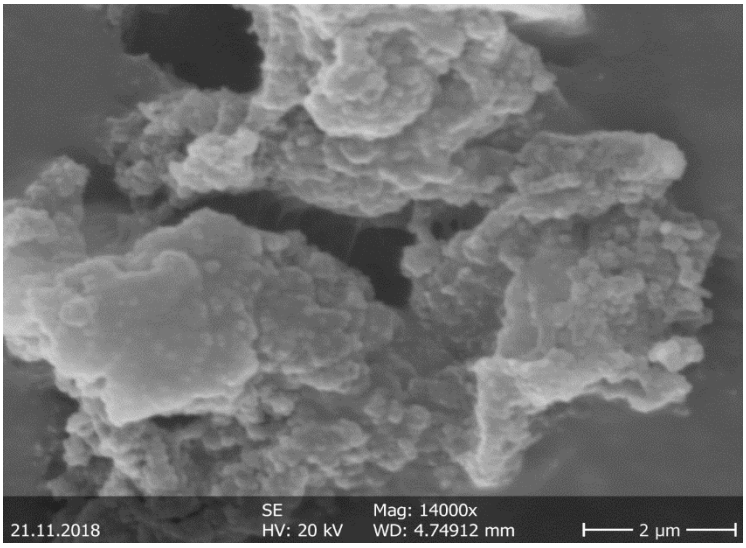

Figure 7: SEM micrograph showing the shape of AgNPs synthesized from fruits extract

As shown in Figure 7 above, the AgNPs were approximately spherical in shape with smooth surface. These results are in agreement with the shape of SPR band recognized from the UV- visible spectrum with absorption maximum at 427nm

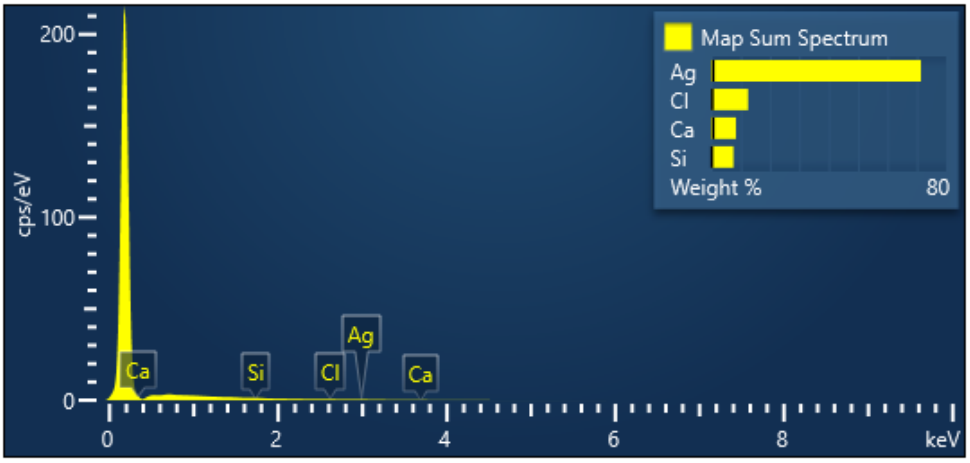

Figure 8: Energy Dispersive X-ray Spectrometer (EDX) spectra demonstrating the quantitative amounts of different elements present in the AgNPs synthesized from the fruits extract.

From Figure 8 above, the EDX spectra showed the presence of elements such as Ag, Cl, Ca, and Si. EDX quantitative analysis demonstrated that the highest concentration of a single element in the *Annona muricata* derived AgNPs was silver (Ag), at about 80%.

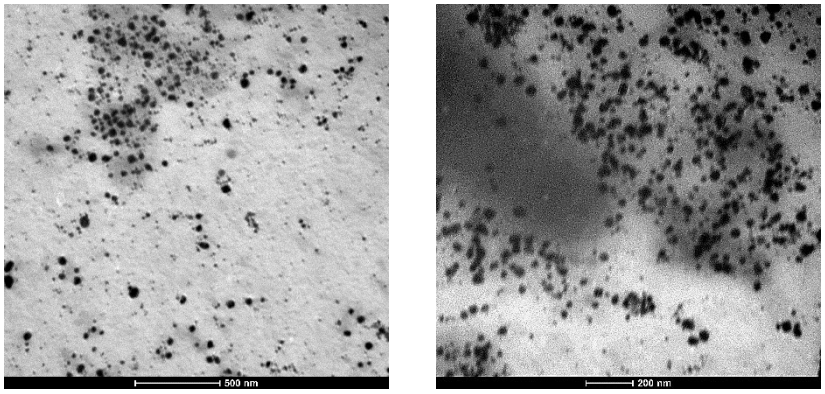

Figure 9: TEM Micrographs of the AgNPs at different resolutions

Figure 9 above shows the TEM micrographs of the AgNPs at different resolutions. The Micrographs reveal a spherical nature of the monodispersed AgNPs as well as a crystalline structure. Particle size analysis using the Image-J software further revealed the AgNPs having an average particle size of about 51 nm.

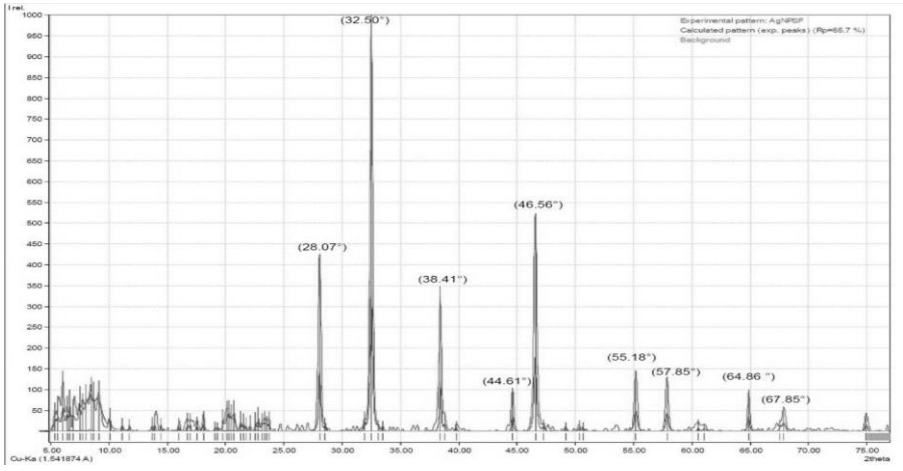

Figure 10: XRD diffraction pattern spectra of AgNPs synthesized from fruits extract

Figure 10 above shows the typical XRD pattern of biosynthesized AgNPs derived from ethanolic extracts of fruits of *Annona muricata*. Nine prominent diffraction peaks were observed at 28.07°, 32.50°, 38.41°, 44.61°, 46.56°, 55.18°, 57.85°, 64.86 °, and 67.85 °. The

average size of the AgNPs formed in the bio-reduction was determined using the Scherrer equation and is estimated as 60.12 nm.

Table 2: DLS Analysis results

Counts	Intensity (kCnt/s)	Attenuation Level (%)	Diameter (nm)	PD Index
1	1361	95.1	103.3	1.268e-01
2	1331	95.1	103.9	1.047e-01
3	1378	95.1	103.7	1.105e-01
4	1360	95.1	103.3	1.349e-01
5	1321	95.1	103.1	1.406e-01
Mean	**1350**	**95.1**	**103.5**	**1.235e-01**

Table 2 above shows the DLS analysis revealing the average particle size for the AgNPs as 103.5 nm with a polydispersity index of 0.1235.

Discussions and Conclusion

It has been known for a long time that silver nanoparticles exhibit a yellowish/ dark brown color in solution due to excitation of surface plasmon vibrations in AgNPs, and therefore reduction of the silver ion to AgNPs during exposure to the plant extracts could be followed by color change and thus UV/VIS spectroscopy (Ahmed, Saifullah, et al., 2016; Song & Beom, 2009). In the current study, the AgNPs formation was confirmed by the change in colour of the mixture from light brown to dark brown indicating the successful green synthesis process. The UV/VIS maximum absorption spectra of the synthesized AgNPs was recorded at 427nm which is in range with previously reported studies on synthesis on AgNPs from plant extracts. Various studies have reported

synthesis of AgNPs with UV/VIS absorption maxima at 435nm(Kumar et al., 2017), 430nm (Song & Beom, 2009), 420nm (Ezealisiji et al., 2017; S. B. Santhosh et al., 2015), 410nm (Otari et al., 2017) among others. The current results further provide, for the first time, a confirmation on the use of the *Annona muricata* fruits extracts in the green synthesis of AgNPs as a cheap and eco-friendly approach.

The importance and use of any substances greatly depend on its stability under different conditions. In the current study, the temperature and heat stability, pH and storage stability of the biosynthesized AgNPs was studied and results have been presented. From the results on temperature stability, it is evident that at all temperatures tested, the AgNPs remained stable maintaining a characteristic absorption maximum of about between 420nm to about 430nm which is within the AgNPs range (Kumar et al., 2017; Shah et al., 2015). This is very important implying that the AgNPs can be stable under various temperature/ heating conditions without losing their effectiveness.

In relation to pH stability, it is evident that at all pH conditions tested, the AgNPs remained stable maintaining a characteristic absorption maximum of about between 410nm to about 420nm which is within the AgNPs range (Malik et al., 2014). This is very important implying that the AgNPs can be stable under various pH conditions without losing their effectiveness. This property is very important especially of the AgNPs are going to be delivered via the gastrointestinal tract which has gradients of pH conditions. The reported stability plays a critical role in ensuring maintenance of effectiveness of the AgNPs and thus helps overcome one of the obstacles encountered by many conventional crude extracts from plants which lose effectiveness *in vivo* due to the changing pH gradients as previously reported (Bonifácio et al., 2014).

Storage stability is very important implying that the AgNPs can be stable under different storage temperature conditions without losing their effectiveness for long periods of time. The notable increase in the absorption of the AgNPs at room temperature compared to other storage conditions, could probably be attributed to the continuous exposure to the same conditions as those used in the synthesis process thereby allowing the process of formation of the AgNPs to continue throughout the storage period, albeit at very low rates.

Recovery of the biosynthesized AgNPs is of critical importance in the synthetic process. Various methods have been reported about the recovery of AgNPs (Shah et al., 2015). In the current study, we developed a blended method for quick and fast recovery of the AgNPs. We introduced a step where the AgNPs suspension is frozen for a period of 12-48 Hrs followed by thawing, centrifugation, washing and then drying. The freezing step allows for the particles to aggregate and thus easy sedimentation when the centrifugation step is conducted. This is the first study to report on such an optimization in the recovery of AgNPs.

FTIR results showed that the functional groups responsible for the formation of the AgNPs from ethanolic extracts of fruits of *Annona muricata* included; Alkanes and alkyls, aldehydes and esters, nitro groups, alcohol groups, carboxylic acids, amides, alkenes, acids and alkyl halides. These are probably due to the presence of most of the secondary metabolites reported much earlier in the plant (Ezealisiji et al., 2017; Gavamukulya et al., 2014 , 2017).

The AgNPs were approximately spherical in shape with smooth surface. These results are in agreement with the shape of SPR band recognized from the UV- visible spectrum with absorption maximum at 427nm. Many previous studies reported different shapes of AgNPs including spherical, conical, cuboidal, hexagonal, pentagonal among others (Ahmed, Ahmad, et al., 2016b; Malik et al., 2014). The spherical AgNPs synthesized in

the current study are therefore in line with the expected shapes for AgNPs. Similarly, EDX elemental analysis revealed that the AgNPs were composed of various elements as reported much earlier, with Ag taking the highest percentage composition at 80%. These results indicate the high purity of the AgNPs albeit with a few contaminants at the different subtle concentration which are probably due to the environmental conditions used during the synthesis process. Earlier studies on had also reported elemental compositions of AgNPs having Ag as the principle component (Nakkala et al., 2014; Otunola & Afolayan, 2018).

From the XRD diffraction patterns, the 2θ peaks observed at 38.41°, 44.61°, and 64.86° corresponds to (111), (200), and (220) reflection planes representing the face centered spherical structure of silver respectively (Kumar et al., 2016 , 2017). The extra peaks near to 28.07°, 32.50°, 46.56°, 55.18°, 57.85°, and 67.85 ° are due to the presence of bio-organic phase on the surface of particles. Generally, the broadening of peaks in the XRD patterns of solids signifies smaller particle size and reflects the effects of the experimental conditions on the nucleation and growth of the crystal nuclei (Kumar et al., 2017; Otunola & Afolayan, 2018; Umadevi et al., 2012). The average size of the AgNPs formed in the bio-reduction was estimated as 60.12 nm. TEM analysis further confirmed the crystalline and spherical nature of the monodispersed AgNPs. The average particle size as determined by TEM analysis was on average 51 nm, which is within range with that calculated using XRD.

Dynamic light scattering is a method that depends on the interaction of light with particles and the method can be used for measurements of narrow particle size distributions especially in the range of 2–500 nm (Tomaszewska et al., 2013). The AgNPs size was larger as presented by DLS (103.5 nm) as compared to XRD (60.12 nm) and TEM (51 nm). This difference could be explained by the fact that the size measured by DLS is based on a combination of the particles as well as the hydrodynamic radius which is not

a true size of the AgNPs due to the hydration layer around the particles as well as the presence of capping and stabilizing agents as previously explained (Danaei et al., 2018; Ezealisiji et al., 2017).

Polydispersity Index measures the homogeneous nature of nanoparticles, the smaller the PDI the more homogeneous nanoparticles. It is basically a representation of the distribution of size populations within a given sample. The numerical value of PDI ranges from 0.0 (for a perfectly uniform sample with respect to the particle size) to 1.0 (for a highly polydisperse sample with multiple particle size populations). Values of 0.2 and below are most commonly deemed acceptable in practice for polymer-based nanoparticle materials, while nanoparticles with PDI smaller than 0.3 is considered acceptable for drug delivery (Clarke, 2013; Danaei et al., 2018). The synthesized AgNPs had an average PDI of 0.1235, which is a great indication that they are highly homogenous and would be effectively used in various applications.

In conclusion, we have reported and optimized for the first time an efficient, eco-friendly and low-cost method for the synthesis and recovery of AgNPs using ethanolic extracts of fruits of *Annona muricata.* The synthesized AgNPs are stable under different temperature, pH and storage conditions. The method used resulted into formation and recovery of spherical crystalline monodispersed AgNPs with an average size of about 60.12 nm and a polydispersity index of 0.1235. With the successful synthesis of AgNPs in the current study, we do recommend further studies aimed at testing the synthesized AgNPs from this method for different biomedical and clinical bioactivities such as Antimicrobial, Anticancer, Anti-inflammatory, Antimalarial, Antidiabetic, Toxicities among others as a step towards the pharmaceutical utilization of these green synthesized AgNPs.

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