

ICONN 2015 [4<sup>th</sup> -6<sup>th</sup> Feb 2015]  
International Conference on Nanoscience and Nanotechnology-2015  
SRM University, Chennai, India

## A biogenic approach for green synthesis of silver nanoparticles using peel extract of *Citrus sinensis* and its application

T.Kokila<sup>1\*</sup>, P.S.Ramesh<sup>2</sup>, and D.Geetha<sup>1</sup>

<sup>1</sup>Dept of Physics, Annamalai University, Annamalai Nagar, India.  
<sup>2</sup>Physics wing DDE, Annamalai University, Annamalai Nagar, India.

**Abstract:** We present a simple and eco-friendly biosynthesis of silver nanoparticles (AgNPs) using *Citrus sinensis* peel extract (CSPE) as the reducing agent. The reaction process was simple for the formation of stable silver nanoparticles at room temperature by using the biowaste of the fruit peel. In the present study, synthesis of AgNPs through the reduction of aqueous silver nitrate (AgNO<sub>3</sub>) at room temperature by CSPE was presented. The synthesis of nanoparticles by CSPE has been kinetically monitored by UV-Visible spectroscopy (UV-Vis). Synthesized nanoparticles are characterized by X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), Atomic force microscopy (AFM) and Dynamic light scattering (DLS)/Zeta potential. The results confirmed that the CSPE is a very good bio-reductant for the synthesis of AgNPs and investigated. In addition in-vitro free radical scavenging activity of AgNPs are evaluated by two popular free radical scavenging assay 1,10-diphenyl-2-picrylhydrazyl (DPPH) and 2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)(ABTS). The nanoparticles are found to possess high antioxidant capacity and thus can be used as potential radical scavengers against deleterious damages caused by the free radicals.

**Keywords:** Biosynthesized AgNPs, Biosynthesis, Citrus sinensis, free radical scavenging activity.

### Introduction

Nanoparticles are being viewed as fundamental building blocks of nanotechnology. The most important and distinct property of nanoparticles is that they exhibit larger surface area to volume ratio. The most effectively studied nanoparticles today are those made from noble metals, in particular Ag, Pt, Au, and Pd. Silver nanoparticles are used in various applications such as biomedical devices, biosensors<sup>1</sup>, catalysis, electronics<sup>2</sup> and pharmaceuticals<sup>3</sup>. Many of these materials are not suitable for critical applications such as in medicine. Furthermore, they represent an environmental hazard. Eco-friendly methods for the synthesis of metal nanoparticles are needed to avoid or minimize such problems<sup>4</sup>. Nowadays, metal nanoparticles are synthesized using eco-friendly natural sources such as plant extracts, fruits, fungi, honey and microorganisms<sup>5</sup>.

The recent reports include the biosynthesis silver nanoparticles by plant parts like *Punica granatum* peel<sup>12</sup>, *Citrus sinensis* peel<sup>13</sup>, *Annona squamosa* peel<sup>14</sup>, Lemon Peel<sup>15</sup>, Banana peel<sup>16</sup> and Mango peel<sup>17</sup>. Silver nanoparticles prepared using biological materials have the properties of a high surface area, smaller in size and high dispersion.

Citrus fruits contain natural antioxidants, such as, flavonoids, alkaloids, coumarins, and phenolics<sup>18</sup>, and their peels, which represent a primary waste fraction, are used as sources of molasses, pectin, cold-pressed oils, and limonene<sup>19</sup>. The rich source of citric acid and ascorbic acid in the citrus fruit extract may possibly responsible for reduction of metal ions and efficient stabilization of synthesized nanoparticles. Since citrus fruits are a rich source of citric acid, *Citrus sinensis* was used as a bioreductant for the synthesis of silver nanoparticles.

For the last two three decades, extensive work has been done to develop new drugs from natural products because of the resistance of micro-organisms to the existing drugs. Nature has been an important source of a products currently being used in medical practice<sup>20</sup>. Varieties of synthetic methods have been employed for the synthesis of silver-based nanoparticles involving physical, chemical<sup>21</sup> and biochemical techniques<sup>22</sup>. Chemical synthesis methods employ toxic chemicals in the synthesis route which may have adverse effect in the medical applications and hazard to environment. Therefore, preparation of AgNPs by green synthesis approach has advantages over physical and chemical approaches as it is environmental friendly, cost effective and the most significant advantage is that the conditions of high temperature, pressure, energy and no toxic chemicals are required in this synthesis protocol<sup>23,24</sup>. In this study, we report the biogenic synthesis of AgNPs by using waste biomaterial CS peel extract, which was used as green reducing agent and stabilizer. The efficacy of the synthesized AgNPs as antioxidant property was reported.

## Experimental

### Materials

The peel of CS was collected from Agricultural farm, Annamalai University. Silver nitrate was purchased from Sigma–Aldrich and used as received. Double distilled water was used for the experiments. All glass wares were properly washed with distilled water and dried in oven.

### Preparation of CS extract

CS peel was used as a reducing agent for the development of AgNPs. The fresh peels of CS was washed repeatedly with distilled water to remove the dust and organic impurities present in it, and then dried on paper toweling. About 25g of peel were taken into the 100ml beaker containing 50ml double distilled water and then the peel was boiled at 80°C for 10min and filtered through Whatman No.1 filter paper twice. The resultant filtrate was stored at 4°C and used as reducing and stabilizing agent.

### Synthesis of silver nanoparticles

For the synthesis of silver nanoparticles the effect of extract quantity and concentration of metal ion solution were also evaluated to optimize the synthesis route producing the metal nanoparticles. The various concentrations (2.5ml-12.5ml) of aqueous peel extract were added into the 100ml aqueous solution of 1mM AgNO<sub>3</sub>. The reduction of silver ions takes place within 30min at room temperature. As a result brownish–orange color was formed, indicating the formation of silver nanoparticles.

### Characterization of Silver Nanoparticles

The nanoparticles were primarily characterized by UV-Visible spectroscopy, which has proved to be a very useful technique for the analysis of nanoparticles. Ultraviolet-Visible spectra were obtained using a Shimadzu UV-1650pc Spectrophotometer. The peel extract had the initial pH of 3.5. With the help of alkali (1M NaOH) and acid (1M HCL) the different pH viz., 2, 4, 6 and 8 were adjusted using the pH meter (Hanna – hi 2215 pH ORP Meter). XRD analysis was carried out on an X-Ray diffractometer (X'Pert-PRO). The high resolution on XRD patterns were measured at 3 KW with Cu target using a scintillation counter ( $\lambda=1.5406\text{\AA}$ ) at 40 kV and 40 mA were recorded in the range of  $2\theta=10^\circ-80^\circ$ . The changes in the surface chemical bondings and surface composition were characterized by using Fourier Transform Infrared (FT-IR) Spectroscopy (Nicolet Avatar series 330) ranging from 400 to 4000cm<sup>-1</sup>. Atomic Force Microscopy (AGILENT-N9410A series 5500)

was used to determine the size and morphology of AgNPs. The average size of AgNPs in aqueous medium and used to determine hydrodynamic diameter by DLS, The Zeta potential was measured with a Zeta sizer Nano ZS90 (Melfern International Ltd.) Instrument.

## 2.5. Determination of Antioxidant assays

### DPPH (1, 1-Diphenyl-2-Picryl Hydroxyl) radical scavenging assay:

DPPH scavenging activity was measured by the slightly modified spectrophotometric method of Brand-Williams<sup>25</sup>. DPPH is a stable free radical and accepts an electron, or hydrogen radical to become a stable diamagnetic molecule. DPPH reacts with an antioxidant compound that can donate hydrogen and gets reduced. The change in colour (from deep violet to blue) was measured. The intensity of the yellow colour developed was depends on the amount and nature of radical scavenger present in the sample.

#### Procedure

Different concentrations (50, 100, 150, 200, 250  $\mu$ l) of CS peel extract and biosynthesized AgNPs were added, in equal volume, to 0.1 mM methanolic DPPH solution. The reaction mixture was incubated for 30 min at room temperature under shaking condition and the absorbance was recorded at 517 nm. BHT was used as a standard.

$$\% \text{ scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

### 2,2'-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonic Acid)(ABTS+) Assay:

The total antioxidant activity of the samples was measured by (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)) ABTS+ radical cation decolorization assay according to the method of Re<sup>26</sup>.

#### Procedure

ABTS+ was produced by reacting 7mM ABTS+ aqueous solution with 2.4mM potassium persulfate in the dark for 12-16 hours at room temperature. The radical was stable in this form for more than two days when stored in the dark at room temperature. Then, 2ml of diluted ABTS+ solution was added to the sample varying concentrations of CS peel extract. The blank contained water in place of Seed extract. After 30 minutes of incubation at room temperature, the absorbance was recorded at 734nm and compared with standard BHT. Percentage of inhibition was calculated.

$$\% \text{ Scavenging} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

#### Statistical analysis

The experiments were carried out in triplicates and analysis were performed by excel sheet. Experimental results were expressed as mean  $\pm$  SD. Statistical analysis was performed using one way annova followed by ducan's multiple range test and a P < 0.05 was regarded to be significant.

## Results and discussion

### Phytoreduction of silver ions and Mechanism involved

Several factors influence the formulation of AgNPs such as plant source, organic compounds like alkaloids, polyphenols, proteins and even some pigments present in peel extracts. In the present work AgNPs were synthesized and characterized using CS peel extract. Figure 1 shows the vial (left corner) containing colloidal solution of 1mM AgNO<sub>3</sub> solution and the vial (Middle) contains the *Citrus sinensis* peel extract. The Right corner containing the mixture of 1mM AgNO<sub>3</sub> and 7.5 ml of *Citrus sinensis* peel extract after 30 min of reaction. A colorless solution of AgNO<sub>3</sub> solution is changes to orange color within 30 min in addition of 7.5 ml of *Citrus sinensis* peel extract and finally it reaches to the brownish orange color of colloid. The appearance of brownish orange color of the solution may indicate the formation of silver nanoparticles in the reaction mixture

and the color change of the solution is due to the excitation of surface Plasmon vibration in the silver nanoparticles.



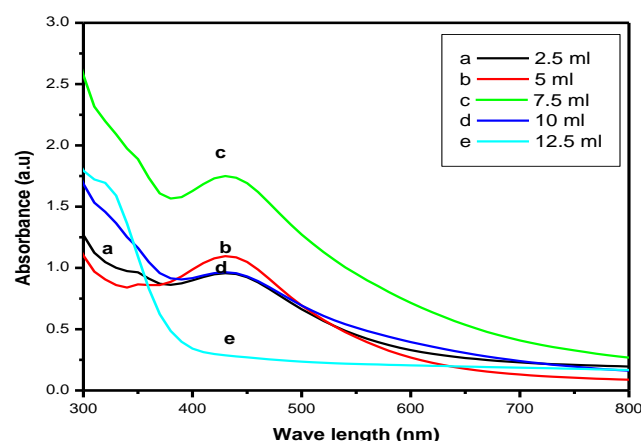
**Figure 1 (A) silver nitrate solution (B) *Citrus sinensis* peel extract and (C) brownish orange color indicating the formation of silver nanoparticles.**

### Mechanism:

The rich source of citric acid and ascorbic acid in the citrus fruit extract may possibly be responsible for the reduction of metal ions and efficient stabilization of synthesized nanoparticles. Fourier transform infrared (FTIR) spectra of CS peel extract were analyzed to explore the formation mechanism of silver nanoparticles. Comparison of FTIR spectra of CSPE before and after reaction demonstrated that flavonoids, alkaloids, coumarins, and phenolics in the CS extract may be mainly responsible for the reduction of silver ions. However, further studies are required to address the specific biomolecules involved in the synthesis of AgNPs.

### UV-Visible spectral analysis

SPR is a collective excitation of the electrons in the conduction band around the nanoparticle surface. Electrons conform to specific vibration modes by particle size and shape. Therefore, metallic NPs display characteristic optical absorption spectra in the UV-Visible region<sup>27</sup>. The absorption spectra of the biogenic AgNPs samples were characterized by UV-visible spectroscopy. UV-visible spectroscopy is one of the important techniques to ascertain the formation and stability of metal nanoparticles in aqueous solution.

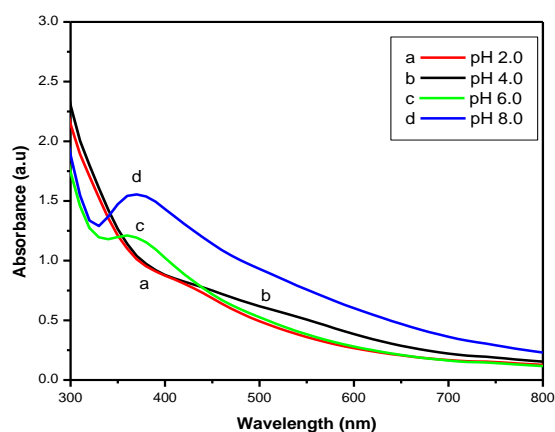


**Figure 2 (i) UV-Vis spectra for the AgNPs prepared with 1mM aqueous  $\text{AgNO}_3$  solution with various CS peel extract concentration ( (a)2.5 ml (b)5 ml (c)7.5 ml (d)10 ml and (e)12.5 ml) .**

Characteristic surface plasmon absorption band was observed at 425-440 nm for the brownish orange colored AgNPs synthesized from 1 mM silver nitrate with various peel extract concentration (2.5ml-12.5ml). It has been found that the optimum concentration for the synthesis of AgNPs is 7.5 ml extract of  $\text{Ag}^+$  ions. There is a small increase in the intensity of the SPR band from 2.5 to 7.5 ml. However, when the concentration is increased further, there is a decrease in the intensity of the SPR band. The increase in SPR band intensity from fig. 2(i)(a-c) is due to the formation of more AgNPs because of the high initial concentration of  $\text{Ag}^+$  ions. The regular decrease in SPR band intensity from curve (c-e) supports the formation of large sized AgNPs. The AgNPs prepared from 7.5 ml concentration of  $\text{Ag}^+$  are used for other characterizations.

## Effect of pH

To study the effect of pH on the formation and stability of AgNPs the reactions were carried out at different pH, ranging from 2 to 8 by adjusting with 0.1M hydrochloric acid or sodium hydroxide in acid and alkaline medium respectively at 7.5 ml CSPE and 1mM silver nitrate solution. At acidic pH the formation of AgNPs was less indicated by less intensity of SPR band which indicates the formation of large sized nanoparticles. With the increase of pH the rate of formation of AgNPs was quick and the intensity of SPR band steadily with the narrowing of the peak (Fig. 2(ii)). This may be due to the formation of monodispersed small sized AgNPs at high pH conditions.

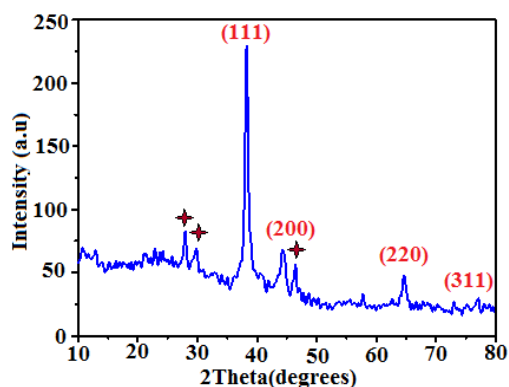


**Figure 2 (ii) UV-Vis spectra for the AgNPs prepared with 1mM aqueous AgNO<sub>3</sub> solution of 7.5ml CSPE at different pH values ((a)2 pH (b)4 pH (c)6pH and (d)8pH)**

The present investigation indicates alkaline pH is more suitable for synthesis of silver nanoparticles. A major influence of the reaction pH is its ability to change the electrical charges of biomolecules which might affect their capping and stabilizing abilities and subsequently the growth of the nanoparticles.

## XRD structural analysis

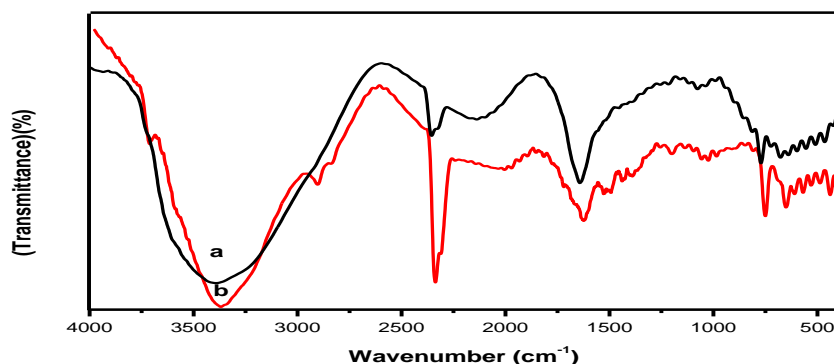
The structure of biologically synthesized Ag NPs was analyzed by XRD measurements. XRD spectrum of peel extract reduced silver nanoparticle shows four distinct diffraction peaks at 38.2°, 44.2°, 64.5° and 77.5°, the lattice plane value was observed which may indexed at (1 1 1), (2 0 0), (2 2 0) and (311) of the cubic silver (Fig. 3). The resultant data was matched with the database Joint Committee on Powder Diffraction Standards (JCPDS) file no. 01-087-0717. The resultant XRD spectrum clearly suggests that the silver nanoparticles synthesized from CSPE was crystalline in nature. It is interesting to note that other unassigned peaks (indexed by the star points) are also seen in the XRD pattern, in addition to the characteristic Bragg reflectance peaks for the silver nanoparticles. This, in turn, could be attributed to the bio-organic phase present in the extract, suggesting their possible crystallization on the surface of the silver nanoparticles. Similar results indicating the occurrence of unassigned peaks in the XRD pattern for the silver nanoparticles synthesized using different green procedures in support of the present work were also reported in recent years<sup>28,31</sup>.



**Figure 3 XRD spectra for the AgNPs prepared with 1mM aqueous AgNO<sub>3</sub> solution with 7.5 ml CS peel extract.**

The average crystalline size of silver nanoparticles was calculated using the Debye-Scherrer method, and the mean crystalline size (D) of the silver nanoparticles was found to be 20 nm.

### FTIR spectral analysis

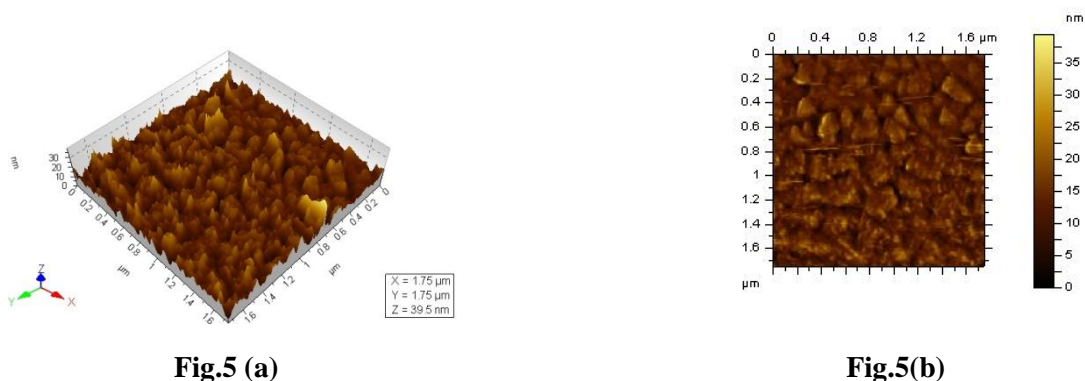


**Figure 4 FT-TR spectra for (a) CS peel extract (b) extract-reduced silver nanoparticles**

The FT-IR measurements were provided to describe and confirm the possible formation of bio reduction and efficient stabilization of green synthesized AgNPs by using CS peel extract. The IR spectrum of peel extract alone showed the distinct peak in the range of 3390, 2355, 1649, 771, 677, and 424  $\text{cm}^{-1}$  (Fig. 4a). FT-IR spectrum of biosynthesized AgNPs shows absorption peak at 3388, 2357, 1641, 769, 671 and 422  $\text{cm}^{-1}$  (Fig. 4b). Intense absorption is observed at 1643  $\text{cm}^{-1}$  and is characteristic of the C=C stretching aromatic ring. The peak at 3390 and 3388  $\text{cm}^{-1}$  reveals water and OH absorption frequency. The weak bands at 2355 and 2357  $\text{cm}^{-1}$  indicates carbonyl specific absorption. Particularly the peak at 1649  $\text{cm}^{-1}$  of extract changed to 1641  $\text{cm}^{-1}$  after synthesis reveals the reduction of silver ion to silver nanoparticles. The peak at 771 and 769  $\text{cm}^{-1}$  corresponds to C-H stretching of aromatic compounds. The peak at 677 and 671  $\text{cm}^{-1}$  could be assigned to the C=O stretching of carbonyl group. The broad peaks around 424 and 422  $\text{cm}^{-1}$  are related to AgNPs bonding with oxygen from hydroxyl groups. The comparison of FTIR spectrum between the leaf extract and AgNPs showed only minor changes in the position as well as the absorption bands. This indicates that AgNPs synthesized using the Cs peel extract are capped by proteins having functional groups of flavonoids, alkaloids, coumarins, and phenolics may be involved in reducing the silver salt to  $\text{Ag}^0$ .

### 3.5 Atomic force microscopy

The synthesized silver nanoparticles surface morphology and size of the nanoparticle were recorded by AFM. Fig.5 is 2D and 3D topography of biofunctionalized AgNPs.



**Fig.5 (a)**

**Fig.5(b)**

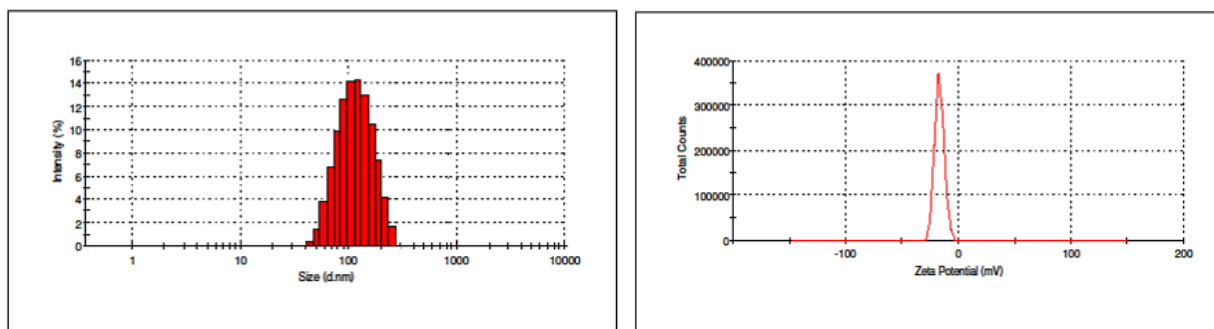
**Figure 5 Atomic Force Microscopy of AgNPs prepared with 1mM aqueous  $\text{AgNO}_3$  solution with 7.5 ml CS peel extract.**

The resultant silver nanoparticle images were observed as spherical in shape. Direct observation of the image revealed that the size of many of the AgNPs was in the order of 10 to 35 nm. The size distribution of the AgNPs was shown in Fig.5 (b), which clearly indicates the majority of the AgNPs are homogenous in size.



### DLS/Zeta Potential Analysis

The DLS instrument is known to measure the shell thickness of a capping or stabilizing agent enveloping the metallic particles along with the actual size of the metallic core. The size distribution vs. intensity graph has been shown in Fig. 6. The average size of the AgNPs was 110 nm and the particles size were larger as compared to the XRD and AFM results. The large size of particles observed by DLS is due to the fact that the measured size also includes the bio-organic compounds enveloping the core of the Au NPs.



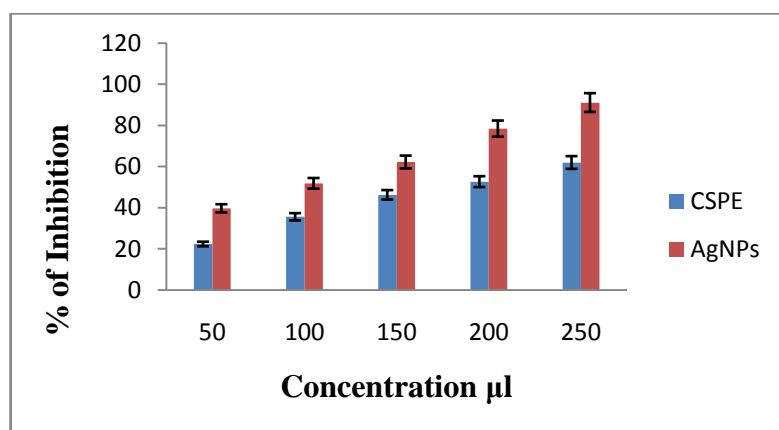
**Figure 6 DLS/Zeta potential for AgNPs prepared with 1mM aqueous AgNO<sub>3</sub> solution with 7.5 ml CS peel extract.**

Zeta potential values reveal information regarding the surface charge and stability of the synthesized silver nanoparticles. The zeta potential value of silver nanoparticles obtained from CSPE is -16.9mV (Fig. 6), indicating the stability of the synthesized nanoparticles. The rich source of citric acid and ascorbic acid in the citrus fruit extract may possibly responsible for reduction of metal ions and efficient stabilization of synthesized nanoparticles. The carbonyl groups from the citric acid and ascorbic acid may have a stronger ability to bind metal ion, so that these compounds could form a coat over the metal nanoparticle to prevent gathering of particles. The measurement of zeta potential is based on the direction of velocity of particles under the influence of known electric field.

### Antioxidant assays

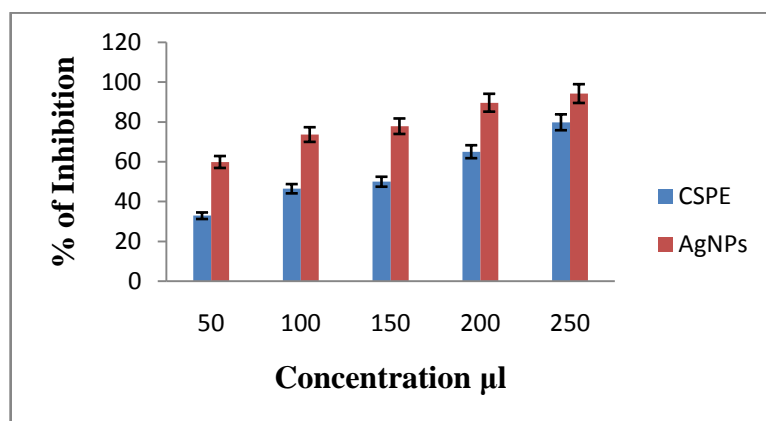
#### DPPH (1, 1-Diphenyl-2-Picryl Hydroxyl) radical scavenging assay

Silver exhibits the strong toxicity in various chemical forms to a wide range of micro-organism that is very well known and AgNPs have recently shown to be a promising antioxidant and antioxidant material. The antioxidant activity of CS peel extract and AgNPs was assessed by DPPH scavenging assay. DPPH was a stable compound and accepts hydrogen or electrons from silver nanoparticles. The results obtained in the DPPH assay showed effective free radical inhibition by both AgNPs and CSPE (Fig. 7(i)). The results obtained in the DPPH assay showed effective free radical inhibition by biosynthesized AgNPs. The average percentage inhibition of synthesized AgNPs was 64% as compared to that of CSPE (43%) at different concentrations used in this study and the activity increased with increasing concentrations of AgNPs.



**Figure 7 (i) DPPH free radical scavenging assay for CS peel extract and AgNPs**

### 3.7.2. 2,2'-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonic Acid)(ABTS)Assay



**Figure 7(ii)ABTS free radical scavenging assay for CS peel extract and AgNPs**

Biosynthesized nanoparticles show considerable potential in in-vitro trials for free radical scavenging activity. Both CS peel extract and Ag nanoparticles have potential for scavenging ABTS radical by surface reaction phenomenon in a dose dependent manner shown in fig.7 (ii). This study also demonstrates the high sensitivity of nanoparticles against ABTS radical as compare to peel extract. ABTS scavenging percentages of the nanoparticles increased from 54.83 % for peel extract and 79.03 % in case of AgNPs. The mechanism involved in this free radical scavenging feature of nanoparticles has significant impact in the sphere of Nanomedicine.

## Conclusion

Recycling of fruit waste is one of the most important means of utilizing it in a number of innovative ways yielding new products and meeting the requirements of essential products required in human, animal and plant nutrition as well as in the pharmaceutical industry. We describe the use of CS fruit waste material for the consistent and rapid synthesis of silver nanoparticles. These AgNPs have potential applications in the biomedical field and the straightforward procedure used has several advantages for a large-scale commercial production. The synthesized AgNPs using CS fruit peel extract proved excellent antioxidant activity. The antioxidant activity of AgNPs was well demonstrated by the inhibition. The power diffraction study showed that the FCC AgNPs were stable after 6 months in room temperature. AFM studies revealed spherical shaped AgNPs with size in the range of 10-35 nm. Over biological synthesis of AgNPs using plant waste material could be a conventional and eco-friendly method compared to chemical and physical synthesis. A fine tune of one or more experimental parameters is useful for the synthesis of AgNPs with particular size distribution and morphology.

## Acknowledgement

The authors gratefully convey their thanks to the Ministry of Science and Technology, Department of Science and Technology, New Delhi, for providing valuable INSPIRE fellowship.

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