Cytotoxic Effect on MG-63 Cell Line and Antimicrobial and Antioxidant Properties of Silver Nanoparticles Synthesized with Seed Extracts of Capsicum sp.

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Abstract: Applying the concept of ethnobotany, plant extract was taken into consideration as an alternative to chemicals synthesis of silver nanoparticle. The extracts from the chilli seeds were used to synthesize silver nanoparticles (AgNPs). In this study two species of chilli, Capsicum annuum and Capsicum frutescens, have been used to analyse the characteristics of the bio-active compounds found in their seeds. Analysis of the bioactive compound was performed by using Soxhlet extraction with solvents followed by Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and GC-MS. Furthermore, green synthesis of nanoparticles with chilli extracts was carried out using silver nitrate to detect its antimicrobial activity. The characterizations of both the nanoparticles were carried out using UV-Vis Spectroscopy, Atomic Force Microscopy (AFM), Fourier Transform Infrared Spectroscopy (FTIR), X-Ray Diffractometry (XRD), Scanning Electron Microscopy (SEM) and energy Dispersive X-Ray Spectroscopy (EDX). Antimicrobial activity against clinical pathogens and the antioxidant assay using DPPH and FRAP assays were performed. The cytotoxicity effects on osteosarcoma cell lines were also evaluated with the synthesized AgNPs.

Keywords: Ethnobotany; antimicrobial; silver nanoparticles; antioxidant assay; cytotoxicity assay.

1. Introduction

Synthesis of nanoparticles using biological materials has gained in recent years and among them, metallic nanoparticles are most promising as they contain remarkable antimicrobial activity [1]. Environmentally benign methods for biosynthesis of silver nanoparticles (Ag-NPs) using plant extracts has become a much sought after alternative to chemical and physical ones [2]. In comparison to the chemical methods, synthesis of nanoparticles using plant extract is much faster. Amongst the nanomaterials available for the biosynthesis of antimicrobials silver has emerged to be most effective in the field of nanomedicine. Green synthesis of nanoparticles [3] using plant extract is of significant importance as it shows an effective antimicrobial activity in both its metallic and nanoparticles forms against various multi drug resistant pathogens [2]. Phytochemical analysis has revealed the presence of alkaloids, flavonoids, polyphenols and sterols in the bell pepper extracts which could be the reason of antimicrobial capacity of the chillies [4].
Silver nanoparticles confer many advantages over other drugs. In the same concentrations which are low enough to be susceptible to human cells, it is lethal for many bacteria, viruses and other pathogenic microorganisms. Particularly being a potential bactericidal without any side effects on humans, nanosilver particles occur to be promising antimicrobial drugs. The most important application in commercial level in medical industry is as topical ointments to prevent infection in burns and open wounds [5], as it displays effective antimicrobial surface coating.

2. Materials and Methods

2.1. Sample Collection

The seeds of the two chilli varieties \textit{Capsicum annuum} and \textit{Capsicum frutescens} were purchased from Khadhi, Vellore, Tamil Nadu, India. The seeds were stored in air tight container for further processing. The clinical pathogens used in antimicrobial studies were obtained from Microbial Biotechnology Laboratory, School of BioScience and Technology, VIT University, Vellore.

2.2. Preparation of \textit{Capsicum annuum} and \textit{Capsicum frutescens} seed extracts

The seeds of both the samples were ground in a mixer grinder and the powdered samples were extracted using Soxhlet apparatus. The samples were extracted with two solvents hexane followed by acetone. Aqueous extract was also prepared using water as the solvent system in the Soxhlet apparatus, for both the test samples. Following extraction filtration was performed using Whatman filter paper No. 1 and the obtained extracts were subjected to concentration vacuum evaporater. The dried extracts were stored at 4°C for further analysis.

2.3. Antibacterial assay of the seed extracts

The well-diffusion method was performed to test the sensitivity of the clinical pathogens to the hexane, acetone and aqueous extracts by measuring the zone of inhibition of given concentration of the seed extracts. Clinical bacterial isolates i.e, \textit{Escherichia coli}, \textit{Serratiamarcescens}, \textit{Salmonella} sp., \textit{Shigellasp.}, \textit{Enterobacter} sp., \textit{Staphylococcus aureus}, \textit{Klebsiellapneumoniae}, \textit{Pseudomonas aeruginosa} and \textit{Proteus mirabilis} were swabbed on Mueller Hinton agar plates and wells were cut and loaded with 100µl of seed extracts prepared. Plates were incubated at 37°C for 24-48 h and were observed for zone of inhibition. After incubation period results were recorded in the form of a clear zone of inhibition.

2.4. Thin Layer Chromatography (TLC)

TLC plates were prepared using silica gel slurry on the glass slides. The samples were loaded on the TLC plates and after it was air dried, the plates were placed in the solvent system (hexane:formicacid:acetic acid, 2:1:1) prepared within the chamber. When the solvent front reached three-fourth, the plates were air dried and observed under UV. The distance of the sample run was measured and the Rf value was calculated.

2.5. High Performance Liquid Chromatography (HPLC)

HPLC was done for both the extracts. The mobile phase used was acetone:water (60:40). 20µl of the sample was injected into HPLC unit (Waters 1525 binary HPLC pump, Milford, USA) on a Symmetry C18 column (Waters 5 µm, 4.6 mm × 150 mm). The mobile phase was isocratic consisting of acetonitrile:water in the ratio 60:40 with a flow rate of 1 mL/min. The run time was 20 min and the absorbance was recorded at a wavelength of 220 nm [6].
Cytotoxicity of silver nanoparticles from *Capsicum* sp.

### 2.6. Biosynthesis of silver nanoparticles (AgNPs)

20g of dry seed powder from *Capsicum annuum* and *Capsicum frutescens* were boiled individually in 100 mL of de-ionized water for 5 min., cooled and filtered. The extracts were stored at 4°C for further experiments. The filtrates were used as stabilizing agent for 1 mM of silver nitrate (AgNO₃). Further, 10 mL of aqueous extracts were added to 90 mL each of aqueous solution of AgNO₃ (1 mM) The Erlenmeyer flasks were incubated at 37°C for reduction of Ag⁺ to Ag⁰ nanoparticles for 24-72 h [7]. For AgNPs, the solution turned from yellowish to bright yellow and dark brown. Periodic sampling was carried out at 15min, 30min, 45min, 60min, 90min, 24h, 48h and 72h for further characterization of AgNPs. All the experiments were carried out in dark conditions.

### 2.7. Characterization of silver nanoparticles

UV-vis spectrophotometer (HITACHI, Model U-2800 spectrophotometer) was used at a wavelength of 300-540nm to determine the time point of maximum production of AgNPs and the de-ionized water served as blank.

Atomic Force Microscopy (Model- Nanosurfeasyscan 2 AFM, made in Switzerland) was used to characterize the samples from the maximum time point of production of AgNPs for its detailed size, morphology and agglomeration of silver. AFM image was taken with silicon cantilevers with force constants 0.02–0.07 N/m, tip height 10–15 nm contact mode.

For XRD measurements the AgNPs were dried in the oven at 60°C [8]. The phase formation and purity were recorded using X-ray Diffractometer (Model-D8 Advance, made in BRUKER Germany). The binding properties of AgNPs synthesized by the seed extracts were investigated using FTIR analysis. Freeze dried and powdered AgNPs were diluted with potassium bromide in the ratio 1:100 and recorded the spectrum in FTIR.

For analysis of nanostructure the samples were coated with gold on sputter coater (JEOL, Model 1600 Ion sputter) and observed in SEM (JEOL, Model 6390) individually. Further, chemical composition of the nanoparticles was characterized by Energy Dispersive X-Ray Spectroscopy (EDX) spectrum of sample at ACC voltage 20.0 kV.

### 2.8. Antioxidant assay

DPPH (1,1-Diphenyl-2-picryl-hydrazyl) assay was done to calculate the total antioxidant content of the extract. DPPH was purchased from Sigma-Aldrich. Ascorbic acid was taken as the standard for the assay. Stock solution of DPPH was made in methanol to a concentration of 200 mM and 0.1M, pH 5.5 acetate buffer was also prepared freshly. 100µl of the nanoparticle extracts (10mg/mL) were added with 100µl of DPPH reagent and 2.8mL of acetate buffer. The reaction tubes were wrapped in aluminium foil and kept for incubation in dark for 30 min. Later absorbance was read at 517 nm using UV-Vis spectrophotometer [9].

\[
\text{Percentage scavenging activity} = \frac{A_0 - A}{A_0} [10]
\]

A₀ – Absorbance of standard
A – Absorbance of sample

FRAP (Ferric reducing antioxidant power) assay was done in accordance with modified Benzie and Strain [11]. The stock solutions were prepared fresh, acetate buffer, pH 3.6, 10mM TPTZ (2,4,6-tripyridyl-s-triazine) obtained from Sigma-Aldrich in 40mM HCl and 20mM FeCl₃.6H₂O. The FRAP reagent was made by mixing these solutions in 10:1:1 ratio respectively. The nanoparticle extracts 10mg/mL (100µL) were allowed to react with 2.9 mL FRAP reagent for 30 min in dark conditions. Ascorbic acid was used as the standard. After incubation absorbance was recorded at 593 nm due to the colored product formation [12]. Further FRAP value of the nanoparticles was calculated as:
FRAP value = $\frac{A}{A_0} \times$ FRAP value of standard*

*FRAP value of ascorbic acid = 2
$A_0$ – Absorbance of standard
A – Absorbance of sample

2.9. Cytotoxicity assay of the extracts

MTT assay was performed to determine the cytotoxic property of the synthesized nanoparticles against MG 63 (osteosarcoma cell line). It was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). The cells were maintained and the cell suspension was diluted with medium containing 5% FBS to give final density of $1 \times 10^5$ cells/mL. Further cell line was seeded into 96-well titre plates and incubated to allow for cell attachment at 37°C, 5% CO$_2$, 95% air and 100% relative humidity. Different concentrations of the extracts were made and added to the wells already containing 100µl of the culture medium and incubated for 48 h at 37°C. Non-treated cells were taken as control and all the trials were done in triplicates.

After 48 h, 15 µL of MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide) (5 mg/mL) in PBS was added to each well and incubated at 37°C for 4 h [13]. The tetrazolium salt is used to determine cell viability in assays of cell proliferation and cytotoxicity [14]. The medium with MTT was then removed and the formed formazan crystals which were solubilised in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The percentage of cell inhibition was determined using the following formula.

$$\text{Percentage Cell Inhibition} = 100 - \left(\frac{A}{A_0}\right) \times 100$$

where, A – Absorbance of sample
$A_0$ – absorbance of control

Nonlinear regression graph was plotted between Percentage of Cell inhibition and Log concentration; IC50 was determined using GraphPad Prism software.

3. Results and Discussion

3.1. Isolation of the extract

The extracts were obtained from two species of chilli Capsicum annuum (C1) and Capsicum frutescens (C2) using the Soxhlet extractor. Two solvents were used in the extraction process, which were hexane and acetone. After extraction and filtration, the samples were dried and the dried sample was used for further analysis.

3.2. Synthesis of silver nanoparticles

The filtered aqueous extract of both the Capsicum sp. inoculated with seed extracts and AgNO$_3$ were checked for silver nanoparticle synthesis based on the change in color of the solution. Aqueous silver ions were reduced to AgNPs when added to biomass of the crude chilli seed extracts of both the Capsicum sp. It was observed that the color of the solution turned from cream to brown upon synthesis of AgNPs. Higher the concentration of AgNO$_3$, faster is the nanoparticle synthesis
3.3. Thin layer chromatography (TLC)

The samples were separated by using thin layer chromatography. Hexane: formic acid: acetic acid in a ratio of 2:1:1 was used as the mobile phase as formic acid has the strongest elution strength followed by acetic acid and hexane. The Rf value of C1 sample extract in acetone was 0.70 and 0.81 and 0.94 in hexane. The Rf value of C2 sample was 0.82 with acetone and 0.81 with hexane. The Rf values obtained with the combination of solvents used refer to the presence of triterpenes in the sample extract [15].

3.4. High performance liquid chromatography (HPLC)

The C1 sample when dissolved in acetone demonstrated a broad retention peak at 1.806 min. A low intensity peak was observed at 2.622 min and 3.082 min (Figure 1a) while the Cl in hexane demonstrated a retention peak at 3.142 min and 2.703 min. A low intensity peak was observed at 1.839 min (Figure 1b). In HPLC analysis the retention peak was at 1.828 min for C2 sample with acetone (Figure 2a) and 2.758 min and 3.192 min (Figure 2b) for C2 sample with hexane.

![Figure 1. HPLC of Capsicum annuum (a) acetone extract (b) hexane extract](image1)

![Figure 2. HPLC of Capsicum frutescens (a) acetone extract (b) hexane extract](image2)

3.5. Biochemical characterization of silver nanoparticles

3.5.1. UV-Vis Spectrophotometer

The characterization was done using UV-Vis spectrophotometer and the formation and stability of the reduced AgNPs in the colloidal solution at various time intervals 24h, 48h and 72h was recorded. The absorbance was read between 200-600 nm and the values were corrected to 10mm path length. The result showed an increase in absorption in the consecutive time intervals. UV-Vis spectra for C1 AgNPs which showed strong peaks at 400-440 nm confirming the presence of the nanoparticles.
showing the silver surface plasmon resonance band at 400-440nm as shown in figure 3a. Furthermore, C2 AgNPs also projected peaks between 420-440 nm in the UV-Vis spectra revealing the existence of nanoparticles (Figure 3b).

The UV-Vis absorption spectrum can be considered as a promising method for AgNPs processing. Due to excitation of surface plasmon vibrations in the metal nanoparticles, AgNPs exhibit a yellowish brown colour in water. The results indicate that secondary structures of proteins is not affected by the consequence of reaction involving the Silver (Ag⁺) ions or while binding with silver nanoparticles. The biological molecules are capable of performing function to form and stabilize AgNPs in aqueous medium. The protein can bind to AgNPs through free amino groups in the proteins. So there is a possibility of stabilization of AgNPs by surface-bound proteins.

**Figure 3.** UV-Vis spectrum of (a) *Capsicum annuum* AgNPs (b) *Capsicum frutescens* AgNPs.

### 3.5.2. Antimicrobial activity of Silver nanoparticles

The zone of inhibition using well diffusion method was performed to evaluate the antimicrobial activity of the crude extract and the AgNPs synthesized against clinical pathogens. Pathogenic strains such as *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter* sp., *Serratia marcescens*, *Shigella* sp., *Salmonella* sp., *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Klebsiella pneumoniae*. With the crude extract, the well diffusion method showed a zone of inhibition against *Salmonella* sp. with the C1 (acetone) sample (Table 1). The antimicrobial activity was also monitored for the samples C1 (hexane), C2 (acetone) and C1 (hexane) against *Shigella* sp. (Table 1).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Pathogen</th>
<th><em>Capsicum annuum</em>(Zone of inhibition in mm)</th>
<th><em>Capsicum frutescens</em>(Zone of inhibition in mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Staphylococcus aureus</em></td>
<td>10 (acetone extract)</td>
<td>- (hexane extract)</td>
</tr>
<tr>
<td>2</td>
<td><em>Escherichia coli</em></td>
<td>- (acetone extract)</td>
<td>- (hexane extract)</td>
</tr>
<tr>
<td>3</td>
<td><em>Enterobacter</em> sp.</td>
<td>- (acetone extract)</td>
<td>- (hexane extract)</td>
</tr>
<tr>
<td>4</td>
<td><em>Serratia marcescens</em></td>
<td>- (acetone extract)</td>
<td>10 (acetone extract)</td>
</tr>
<tr>
<td>5</td>
<td><em>Shigella</em> sp.</td>
<td>20 (acetone extract)</td>
<td>15 (hexane extract)</td>
</tr>
<tr>
<td>6</td>
<td><em>Salmonella</em> sp.</td>
<td>14 (acetone extract)</td>
<td>- (hexane extract)</td>
</tr>
<tr>
<td>7</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>- (acetone extract)</td>
<td>- (hexane extract)</td>
</tr>
<tr>
<td>8</td>
<td><em>Proteus mirabilis</em></td>
<td>- (acetone extract)</td>
<td>- (hexane extract)</td>
</tr>
<tr>
<td>9</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>- (acetone extract)</td>
<td>- (hexane extract)</td>
</tr>
</tbody>
</table>

The samples C1 and C2 containing 100 µl AgNPs showed zone of inhibition in case of *Klebsiella pneumoniae* and *Serratia marcescens* at both 48h and 72h (Table 2). The 72 h samples showed more promising results, thus further AFM, XRD, FTIR, SEM, EDX and cytotoxicity assays were performed for these samples.
Table 2. Antimicrobial activity of 100 µl of AgNPs synthesized with Capsicum seed extracts

<table>
<thead>
<tr>
<th>S No.</th>
<th>Pathogen</th>
<th>Capsicum annuum AgNPs</th>
<th>Capsicum frutescens AgNPs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Zone of inhibition (mm)</td>
<td>Zone of inhibition (mm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24h</td>
<td>48h</td>
</tr>
<tr>
<td>1</td>
<td>Staphylococcus aureus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Escherichia coli</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Enterobacte r sp.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Serratiamarcescens</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>Shigella sp.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Salmonella sp.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Pseudomonas aeruginosa</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Proteus mirabilis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Klebsiella pneumoniae</td>
<td>-</td>
<td>11</td>
</tr>
</tbody>
</table>

3.5.3. Atomic Force Microscopy (AFM)

The nanoparticles synthesized were observed by AFM and the size of the C1 AgNPs was measured to be 64.8 nm (Figure 4a) and C2 AgNPs was 102 nm (Figure 4b). This confirms the presence of nanoscale sized particles in the samples processed. AFM results confirmed the size to be in nanosclae of 100 nm.

![Figure 4](image-url)

3.5.4. X-Ray Diffraction (XRD)

XRD graphs of the samples revealed three distinct peaks for Capsicum annuum (C1) as well as Capsicum frutescens (C2). The observed peaks of C1 sample at 20 of 32.31°, 46.28° and 77.75° corresponds to (121), (231) and (311) planes respectively (Figure 5a). Similarly for C2 samples the 20 values of 32.35°, 46.38° and 76.80° falls in (121), (231) and (311) planes respectively (Figure 5b). X-ray diffraction at 20 values showed similar results at (121), (131) and (311) planes corresponding to the results reported by Mohseniazar et al. [16] and Vivek et al. [17], indicating the peaks corresponding to these planes.
3.5.5. Fourier Transform Infrared Spectroscopy (FTIR)

Presence of bands by FTIR was showed at 1629 cm\(^{-1}\) and 1633 cm\(^{-1}\) corresponding to C1 and C2 samples (72 h) respectively (Fig. 6a and 6b). The analysis revealed the presence of amide groups corresponding to 3332.99 cm\(^{-1}\) in C2 sample, which are found to be relevant with the presence of AgNO\(_3\) [14] (C=C) stretches are found in both the samples whereas (O-H) is observed in C1 sample indicating the presence of hydroxyl group. The results indicate that secondary structures of proteins is not affected by the consequence of reaction involving the Silver (Ag\(^+\)) ions or while binding with silver nanoparticles.

![Figure 6. FTIR spectra of (a) Capsicum annuum AgNPs (b) Capsicum frutescens AgNPs.](image)

3.5.6. Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Spectroscopy (EDX)

Figure 7a gives the SEM picture of C1 Ag NPs. It is clearly evident that the size of the particles is in nano range and it is also observed that the nanoparticles produced are aggregated in nature. The EDX result confirms the presence of silver nanoparticles as shown in figure 7b. SEM picture of C2 AgNPs (Figure 8a) clearly indicates the shape to be irregular and of nano range and EDX (Figure 8b) confirms the presence of silver nanoparticles. Through SEM images it can be said that the C1 AgNPs form aggregates in nature and C2 AgNPs are irregularly shaped but both are in nano range.
Cytotoxicity of silver nanoparticles from *Capsicum* sp.

Figure 7. *Capsicum annuum* (a) AgNPs SEM image (b) AgNPs EDX.

Figure 8. *Capsicum frutescens* (a) AgNPs SEM image (b) AgNPs EDX.

3.6. Antioxidant assay of nanoparticles

DPPH assay has advantages of being stable, sensitive, simple and more feasible. The DPPH assay deals with the ability of the extract to donate hydrogen to the DPPH radical resulting in bleaching of the DPPH solution. The greater the bleaching action by the nanoparticles indicates higher the antioxidant property [18]. The changes in the free radical scavenging activity of the crude nanoparticle extracts vary according to the percent inhibition. The ferric reducing antioxidant potential (FRAP) assay is another simple and inexpensive procedure that tally with the total antioxidant levels. According to the FRAP assay, it is evident that C2 shows greater FRAP values than C1. The higher the FRAP value, the greater is the antioxidant property.

In both the DPPH radical scavenging assay as well as FRAP assay, the activity is observed as change in the color of the solution after the incubation time is over. It is clearly observed that C2 has better antioxidant properties than C1. As concentration of extract increases, the antioxidant potential also shows a positive response. Similar kind of study has been reported in *Cyphomandra betacea* fruit by Hasan and Bakar [19] and *Nicotiana tabacum* L. by Nacoulma et al. [20], belonging to same family of Solanacea.

3.7. Cytotoxicity assay of the extracts

The *in vitro* screening of the silver nanoparticles of both the chilli varieties showed potential cytotoxic activity against the osteosarcoma cell lines. The result revealed that MG 63 cells proliferation were inhibited significantly at IC$_{50}$ value of 64.99 µg/mL for C1 sample extract and 60.42 µg/mL for C2 sample extract. The assay was performed from 12.5µg/mL to 200µg/mL extracts in triplicates and it showed promising anticancer effects of both the extracts, C2 being more effective, revealing the fact that it can be used as an anticancer drug (Fig. 14). There are reports of silver nanoparticles possessing antitumor potential against THP-1 cancer cell lines [21], Hep2 cells, MCF7 cells and HT29 cells [16], etc., synthesized from different sources. To the best of our knowledge this is the first report on the cytotoxic effects of silver nanoparticles from two different *Capsicum* sp. against osteosarcoma cell
lines (MG63). Moreover, Faedmaleki et al. [22] illustrated that nano-silver had 44 times more inhibition effect on the growth of cancerous cells (HepG2 cell line) as compared to the normal cells (primary liver cells of mice), which might further be justified that AgNPs form a potential candidate for cancer treatment.

4. Conclusion

Silver nanoparticles were synthesized using two Capsicum sp. with AgNO₃ as a source of Ag⁺ ions. The method presented was very simple and resulted in enhanced antibacterial activity of the extracts. The UV result was obtained between 400-1000 nm and all the reactions were carried out under dark conditions. Present investigation has an additional advantage as antioxidant activities of the extracts was also confirmed. Further, good response was seen against osteosarcoma MG63 celllines, which might be due to AgNPs as well as other metabolites of the extract. Thus, it is suitable for clinical applications. There are no conflicts of interests to declare.

References

Cytotoxicity of silver nanoparticles from *Capsicum* sp.


