

Biosynthesis and Characterization of Silver Nanoparticles using *Catharanthus roseus* Leaf Extract and its Proliferative Effects on Cancer Cell Lines

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Abstract

Green synthesis is one of the most rapid, reliable, and best routes for the synthesis of silver nanoparticles (AgNPs). Previous studies demonstrated that active compounds in *Catharanthus roseus* were responsible for bioreduction during the synthesis of spherical AgNPs. In the present study, synthesis of AgNPs using *C. roseus* aqueous extract was optimized. To prepare the extract, dried *C. roseus* leaves were boiled at 100°C for 5 min or soaked in a water bath at 40°C for 24 h. The extracts at concentrations of 10% and 20% were mixed with AgNO₃ solutions of 1 and 5 mM. The resulting AgNPs were characterized by ultraviolet-visible (UV-Vis), X-ray diffraction (XRD), and transmission electron microscope (TEM). Cell proliferation of Jurkat (human acute T-cell leukemia) and HT-29 (human colorectal adenocarcinoma) cell lines in solutions containing synthesized AgNPs or *C. roseus* aqueous extract was measured by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine metho sulfate] assay using the double dilution approach (concentrations ranging from 3.91 to 1000 µg/ml). UV-Vis spectra analysis indicated that the AgNPs at 5 mM AgNO₃ + 10% extract produced the highest surface Plasmon resonance peak at approximately 500 nm. The XRD analysis confirmed the presence of AgNPs with a face-centered cubic structure. TEM analysis showed that the AgNPs ranged in size from 20 to 50 nm with spherical shapes. Based on the MTS assay, the IC₅₀ values of AgNPs ranged from 13.68 to 46.88 µg/ml for both cancer cell lines, whereas the IC₅₀ values of *C. roseus* aqueous extract ranged from 62.50 to 312.50 µg/ml for the Jurkat cell line and no IC₅₀ values for HT-29 cell lines. Further studies should be conducted to validate the potential application of AgNPs as anti-cancer agents.

Keywords: *C. roseus*; Silver nanoparticles; Characterization; Anti-cancer

Introduction

Nanotechnology is an emerging field of nanoscience that is expected to be the starting point of many technological developments in the twenty-first century. The application of nanoscale materials and structures is gaining more and more attention due to their wide applicability, especially in biomedical fields (e.g., in the diagnosis and treatment of human cancers) [1]. The special characteristics of nanomaterials and their biologic effects suggest that nanoparticles may become potential alternative treatments of disease [2]. Gold, platinum, and silver nanoparticles are among the common types of nanoparticles that have been used widely in products that directly come in contact with the human body, including soaps, shampoos, shoes, detergent, tooth paste, cosmetic products, and medical and pharmaceutical devices [3]. Silver gained popularity when researchers found they could produce it at the nanoscale level. Ultrafine particles of metallic silver at the nanometer (nm) scale were found to exhibit distinctive morphologies and characteristics [2]. Traditional methods of synthesizing nanoparticles include radiation, chemical or photochemical methods, electrochemical techniques, and Languir-Blodgett approaches [4]. However, these methods are extremely expensive and time consuming and can be dangerous to human health and the environment because of the application of hazardous substances [5]. Therefore, there is a growing need to develop cost-effective and environmentally friendly approaches for rapid synthesis of nanoparticles.

Biological approaches to the synthesis of metal nanoparticles using microorganisms and plant extracts have been suggested as valuable alternatives to chemical synthesis and physical methods [6,7]. Kotakadi [4] have described the use of natural materials such as plants, bacteria,

fungi, yeast, and honey for synthesizing gold and silver nanoparticles. For example, Saifuddin [8] studied the applicability of bacteria *Bacillus cereus* and Mukherjee [9] on fungal species *Trichoderma asperellum*, for synthesizing nanoparticles.

The rate of synthesis of nanoparticles by plant extracts is higher than that of chemical methods and green synthesis by microorganisms [10]. In addition, the use of plant materials for the synthesis of nanoparticles does not require elaborate processes such as intracellular synthesis and multiple purification steps or the maintenance of microbial cell cultures [3]. Moreover, the use of plants for synthesis of nanoparticles is rapid, low cost, eco-friendly, and a single-step process [11]. Many previous studies reported the biosynthesis of silver nanoparticles (AgNPs) using extracts of leaves of various plants, including *Pterocarpus santalinus* [1], *Moringa oleifera* [5], *Duranta repens* [10], *Oleo europaea* [12], Loquat leaf extract [13], *Annona squamosa* [14], *Rhinacanthus nasutus* [15] and *Catharanthus roseus* [16]. *Catharanthus roseus* is an erect procumbent herb or undershrub containing latex that grows up to 1m tall in subtropical areas [3]. This perennial herb is grown commercially for medicinal uses in India, Australia, Africa, and Southern Europe. It

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contains alkaloids, mainly of the indole type. The root extract (alkaloid alstonine) is used to reduce hypertension, and reserpine, ajmalicine, and serpentine are alkaloids with antiplasmodic and hypotensive properties [17]. *C. roseus* also has antibacterial, anti-inflammatory, antidiuretic, cytotoxic, antifertility, hyperglycemic, antifungal, anti-malarial, and antiviral effects [16]. The alkaloids vinblastine and vincristine in *C. roseus* has been used as anti-cancer drugs in the treatment of different types of cancers, such as lymphomas, Hodgkin's lymphoma, breast cancer, acute lymphocytic leukemia, soft tissue sarcomas, multiple myeloma, and neuroblastoma [4]. Kotakadi [4] and Ponarulselvam et al. [17] successfully synthesized AgNPs from *C. roseus* leaf extract and its antimicrobial activity was tested. However, in the present study, the ability of extracts made from the dried leaves of *C. roseus* to synthesize AgNPs was evaluated, followed by a preliminary study of anticancer activity of these nanoparticles on selected cancer cell lines.

Materials and Methods

Plant materials

C. roseus plants were collected from Teluk Air Tawar, Butterworth, Penang. The plants were sent to the Herbarium Unit, School of Biological Sciences, and Universiti Sains Malaysia (USM) for identification. The voucher material was deposited at the same herbarium with reference number 10933.

Preparation of *C. roseus* aqueous extract

Two methods were used to prepare *C. roseus* aqueous extracts. Fresh leaves were washed and then dried in 40°C oven before being ground into powder form. Fifty grams of dried leaf powder were dissolved in 1000 mL distilled water for use in each method. Method 1 was performed according to Ahmad [18]. The mixture was incubated in a water bath shaker at 40°C for 24 h. Method 2 was performed according to Gopinath [1], with slight modification. The mixture was boiled at 100°C on a hot plate for 5 min. Following incubation for either 24 h or 5 min, the extracts were centrifuged at 2000 rpm for 15 min and filtered using Whatman filter paper No. 1 (Sartourie, Germany) to remove debris before being freeze dried.

Synthesis of silver nanoparticles

Four concentrations of *C. roseus* -AgNP solutions were prepared (Table 1). Different concentrations of *C. roseus* aqueous extracts and AgNO₃ solutions were prepared by dissolution in distilled water. Five ml of *C. roseus* aqueous extract (w/v) was dissolved in 45 mL of AgNO₃ solution (w/v) in a Scott Duran bottle and left at room temperature for 24 h. A brown-yellow solution was formed, which indicated the presence of AgNPs (Figure 1). The solution was then centrifuged at 6000 rpm for 15 min. The supernatant was discarded and washed twice with distilled water. The pellet was dried in an oven at 40°C for 48 h.

Ultraviolet-Visible (UV-Vis) spectra analysis of silver nanoparticles

Four concentrations of *C. roseus*-AgNP solutions were

Concentration of <i>C. roseus</i> aqueous extract (w/v)	Concentration of AgNO ₃ (w/v)
10%	1 mM
10%	5 mM
20%	1 mM
20%	5 mM

Table 1: Concentrations of *C. roseus* aqueous extracts (w/v) and AgNO₃ solutions (w/v).



Figure 1: Color changes at 24 h of incubation after the addition of 10% (w/v) and 20% (w/v) of *C. roseus* aqueous extract to 1 mM and 5 mM AgNO₃ solution.

characterized using UV-Vis spectroscopy (UV-1650, Shimadzu, Japan) after incubation for 0, 2, 4, 22, 24, and 48 h. The bioreduction of the silver ions was monitored by measuring the absorbance of 1 ml aliquots of the reaction mixture in a wavelength range between 400 and 600 nm with 1 nm resolution.

X-ray diffraction (XRD) analysis

One gram of synthesized *C. roseus*-AgNPs was sent to the School of Physics at USM for phase identification of crystalline material and unit cell dimension measurement using the XRD D8 (Bruker, USA) operated at a voltage of 30kV and a current of 30 mA with CuK α radiation in a θ - 2θ configuration. Further analyses were performed to determine the average size of the AgNPs using Dp Calculator software from <http://mahendrakoppolu.blogspot.com/2013/07/online-crystallite-size-calculator.html>

Transmission electron microscope (TEM) analysis

Size and shape of synthesized *C. roseus*-AgNPs were observed using a TEM CM12 (Phillips, USA) at the School of Biological Sciences, USM. The *C. roseus*-AgNPs were transferred into a new vial using a spatula. A

suspension was made by adding 95% alcohol, followed by 15 min ultrasonication in an ultrasonic water bath (Elmasonic S 80H, Elma, Singen (Hohentwiel), Germany). A drop of suspension was loaded onto a carbon-coated grid and allowed to evaporate before viewing.

Preparation of cell lines for cell proliferation assay

HT-29 and Jurkat cell lines were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured and maintained in 10% fetal calf serum (v/v) (Gibco, USA), 1% penicillin-streptomycin (v/v) (Gibco, USA), and 1% L-glutamine (v/v) (Gibco). The cells were harvested in the log phase of growth and centrifuged at 1200 rpm for 10 min. The pellet was resuspended in RPMI complete growth medium at a concentration of 4×10^5 cells/ml for Jurkat cells and 1×10^5 cells/ml for HT-29 cells.

Proliferation assay

Cell proliferation was assessed using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine metho sulfate] kit (Promega, USA) following the manufacturer's protocol. Cells were treated with either *C. roseus*-AgNPs or *C. roseus* aqueous extract at concentrations of 3.91, 7.82, 15.63, 31.25, 62.5, 125, 250, 500, and 1000 $\mu\text{g}/\text{ml}$ in 96-wells plates. The plates were incubated for 24, 48, and 72 h. Untreated cells were used as the control, and vinblastine (VB) was used as the

positive control. Each sample type was run in triplicate. Following the particular incubation time, the culture cells in each well were added with a mixture of MTS/PMS with a ratio of 20:1. The plates were read at 490 nm using an ELISA reader (Bio Tek, USA). The half maximal inhibitory concentration (IC_{50}) values were determined from the graph to obtain the sample concentration that caused 50% cell death. Cell viability (%) was calculated as follows:

$$\text{Cell viability (\%)} = \frac{\text{Mean OD of treated cells}}{\text{Mean OD of untreated (control) cells}} \times 100$$

Where OD is optical density.

Statistical analysis

Data were expressed as mean \pm SEM. Statistical analysis was performed using IBM SPSS Statistics Version 20.0. Control and treated samples were compared using one way analysis of variance. Differences at $p > 0.05$ were considered to be statistically significant.

Results and Discussion

UV-Vis spectra analysis of silver nanoparticles

Figure 1 shows the color changes at 24 h of incubation after the addition of 10% (w/v) and 20% (w/v) *C. roseus* aqueous extract to 1 mM and 5 mM of AgNO_3 solution. Neither concentration of *C. roseus*

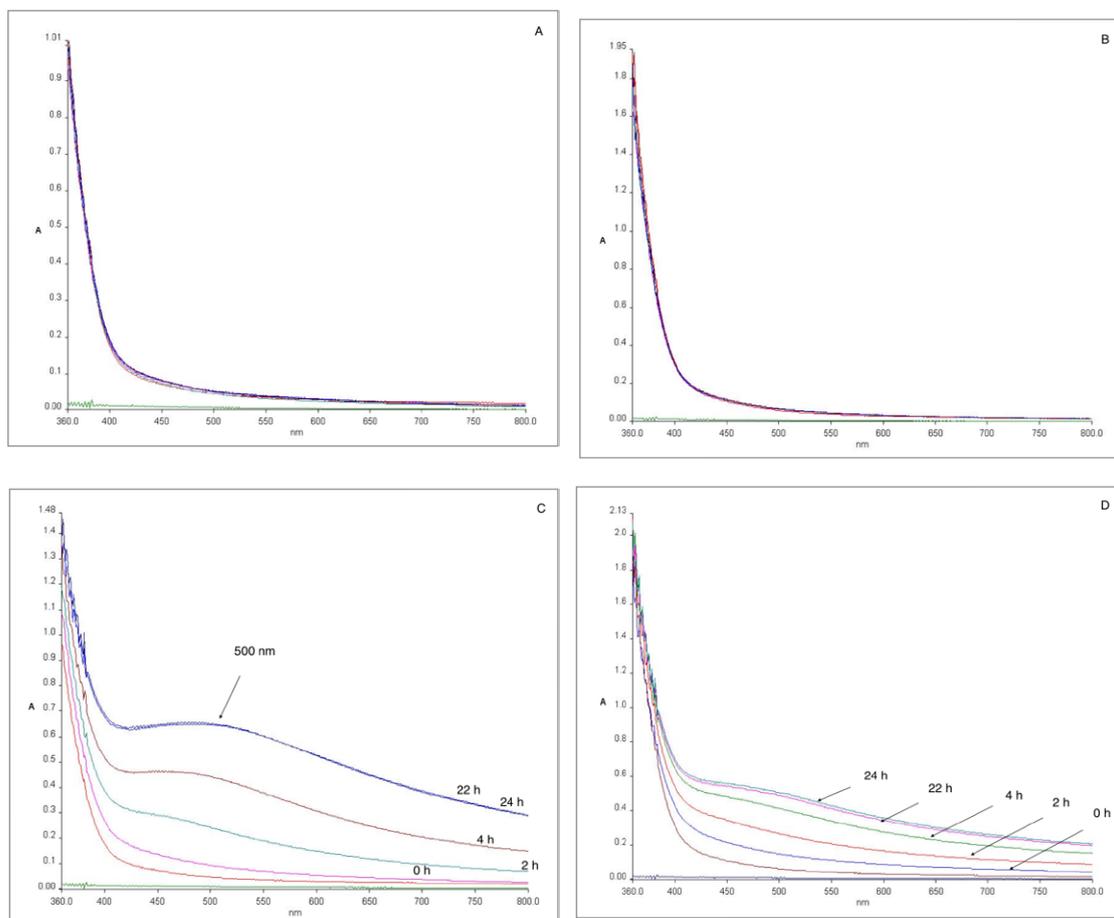


Figure 2: UV-Vis absorption spectra of (A) 1 mM AgNO_3 + 10% *C. roseus* leaf extract, (B) 1 mM + 20%, (C) 5 mM + 10%, and (D) 5 mM + 20% prepared using Method 1, at different time intervals.

aqueous extract with 1 mM AgNO₃ solution resulted in a color change. In contrast, both concentrations of *C. roseus* aqueous extract in 5 mM AgNO₃ solution showed a color change from light yellow to brown-gray after 24 h of incubation due to excitation of surface plasmon resonance (SPR). This change indicated the formation of AgNPs [17].

UV-Vis spectroscopy was used to confirm the formation and stability of AgNPs. Figure 2 shows the absorption spectra of the four different solutions prepared using method 1. No distinct peaks were observed for the solutions with concentrations of 10% (w/v) and 20% (w/v) *C. roseus* aqueous extract in 1 mM AgNO₃ solution at any time point. In contrast, broad peaks were observed for the solutions with concentrations of 10% (w/v) and 20% (w/v) *C. roseus* aqueous extract in 5 mM AgNO₃ solution. The highest absorbance peaks (at approximately 500 nm) at 24 h were observed for the 10% (w/v) *C. roseus* aqueous extract in 5 mM AgNO₃ solution, indicating the formation of AgNPs [17]. Various reports have established that the resonance peak of AgNPs appears around this region. Sileikaite et al. [19] reported that the absorption band of AgNPs is in the range of 350 to 550 nm. Figure 3 shows the absorption spectra of AgNP solutions prepared using method 2 (i.e., boiling at 100°C for 5 min). None of the AgNP solutions prepared this way showed any visible absorbance peak, which suggests that AgNPs were not produced.

Size and shape characterization of AgNPs Using TEM

According to Pasupuleti [15], the applications for AgNPs are

highly dependent on the chemical composition, shape, size, and mono dispersity of the particles. These characteristics of the AgNPs synthesized using *C. roseus* aqueous extract were evaluated by TEM. AgNPs harvested from the 10% (w/v) *C. roseus* aqueous extract in 5 mM AgNO₃ solution created using methods 1 and 2 were compared. Figure 4 shows that method 1 produced particle sizes ranging from 20 to 50 nm (average diameter, 30 nm). Gopinath [1] also successfully synthesized AgNPs with a diameter range of 20 to 50 nm. In contrast, Figure 5 shows that method 2 produced a greater range of particle sizes, which ranged from 10 to 250 nm. Basanagowda and Ashok [10] also successfully synthesized AgNPs using the boiling method to generate *Duranta repens* leaf extract, but the particle size was only 30 to 80 nm. This explains the absence of a plasmon resonance band in UV-Vis spectroscopy analysis of the AgNP solution prepared by the boiling method in recent study. However, both methods produced spherical shaped AgNPs.

XRD analysis

XRD analysis was conducted to determine the crystal structure of AgNPs. Figure 6 shows the XRD diffraction pattern of AgNPs. Four distinct diffraction peaks at 2θ with values of 38.12°, 44.31°, 64.45°, and 77.41° were indexed with the planes 111, 200, 220, and 311, respectively, which indicate that the particles were face-centered cubic silver. Further analysis was performed to determine the size of the AgNPs using Dp Calculator software. The average particle size was 29.27 nm, which matched the result obtained by TEM (30 nm).

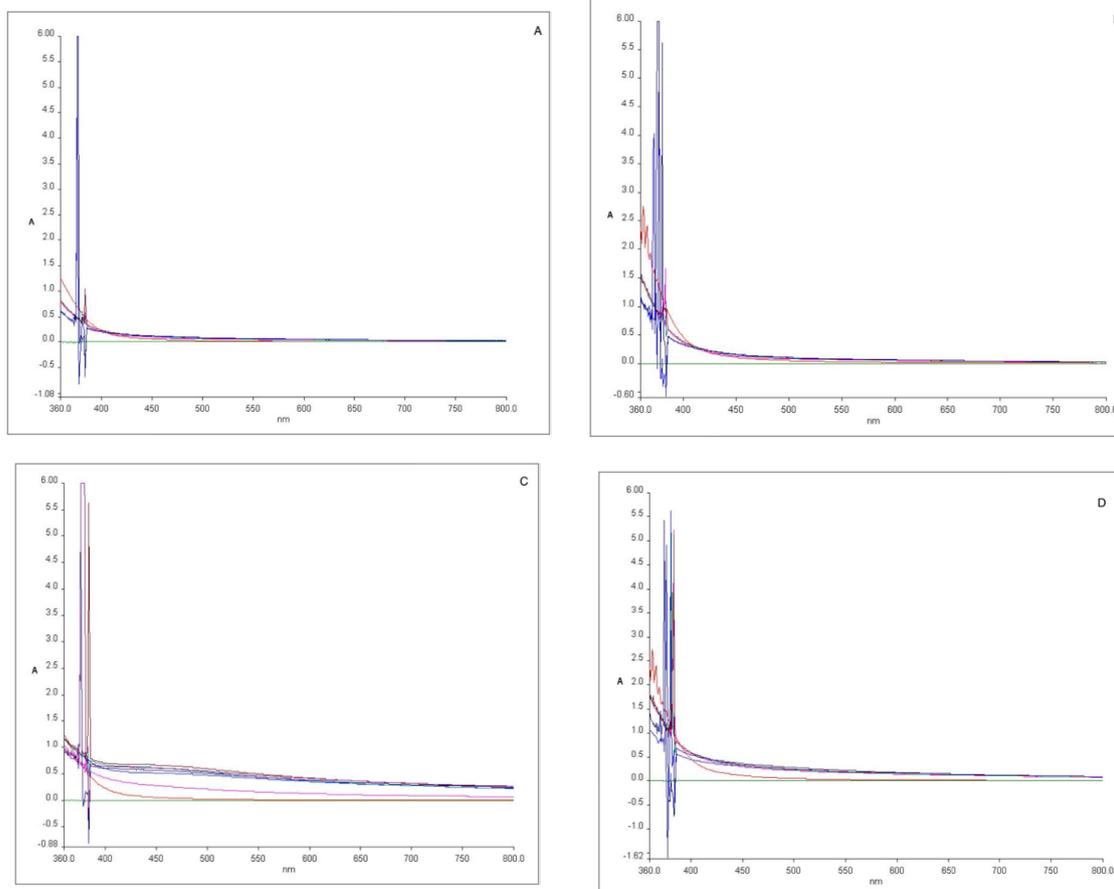


Figure 3: UV-Vis absorption spectra of (A) 1 mM AgNO₃ + 10% *C. roseus* leaf extract, (B) 1 mM + 20%, (C) 5 mM + 10%, and (D) 5 mM + 20% prepared using Method 2, at different time intervals.

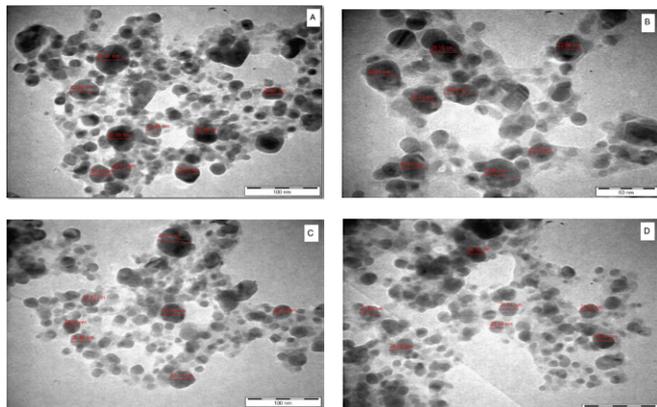


Figure 4: TEM images of AgNPs synthesized using method 1 taken from different angles.

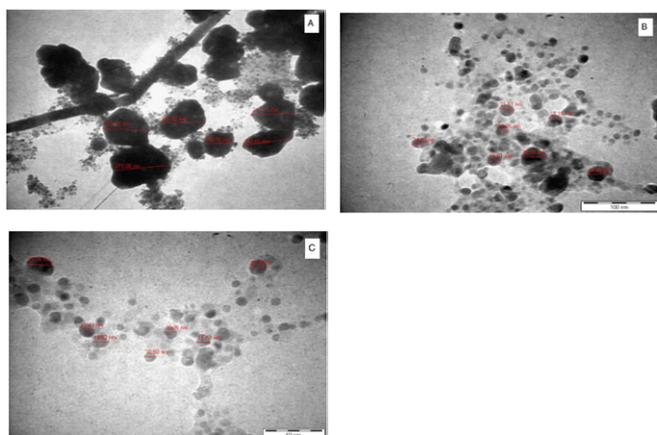


Figure 5: TEM images of AgNPs synthesized using method 2 taken from different angles.

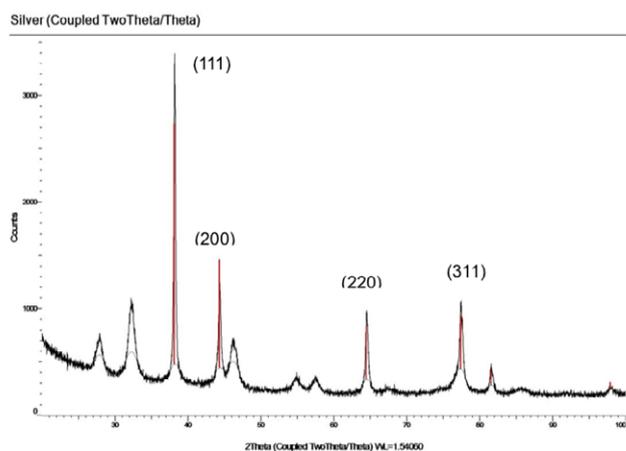


Figure 6: XRD patterns of AgNPs synthesized using *C. roseus* leaf extract.

Proliferative assay

Proliferative assay was carried out to compare the effects of AgNPs

on adherent (HT-29) and suspension (Jurkat) cancer cells. Figure 7a compares the effect of synthesized *C. roseus*-AgNP treatment on Jurkat and HT-29 cells. The IC_{50} values of treated Jurkat cells were 13.68, 27.35, and 46.88 $\mu\text{g/ml}$ of AgNPs at 24, 48, and 72 h, respectively. Significant inhibition ($p>0.05$) of cell proliferation was observed, especially at AgNP concentrations ranging from 62.5 to 1000 $\mu\text{g/ml}$ at all incubation times, with the percentage of viable cells remaining <40%. Whereas, *C. roseus*-AgNP inhibited 50% of the HT-29 cells at concentration of 23.44, 39.06 and 46.88 $\mu\text{g/ml}$ at 24, 48, and 72 h, respectively. Significant inhibition ($p>0.05$) of cell proliferation also was observed at 31.25 $\mu\text{g/ml}$ at all incubation times, but it increased in a time-dependent manner. Interestingly, cell proliferation was induced at 3.91, 7.82, and 15.63 $\mu\text{g/ml}$ at 48 and 72 h. These data show that Jurkat cells were more sensitive than HT29 cells to the toxicity of *C. roseus*-AgNPs.

Figure 7b compares the effect of *C. roseus* aqueous extract on Jurkat and HT-29 cells. The IC_{50} values for Jurkat cells were 62.5 and 312.5 $\mu\text{g/ml}$ at 48 and 72 h, respectively. Significant differences ($p>0.05$) with respect to the untreated group were observed at all concentrations. Inhibition of cell proliferation was time dependent at extract concentrations of 62.5, 250, 500, and 1000 $\mu\text{g/ml}$. The highest concentration of *C. roseus* aqueous extract tested (1000 $\mu\text{g/ml}$) produced the highest rate of cell inhibition. In contrast, no IC_{50} values were obtained for HT-29 cells, suggesting that >50% of HT-29 cells remained viable in response to the extract treatment throughout the experiment. However, significant differences ($p>0.05$) in cell inhibition with respect to the untreated group were observed at concentrations of 250 and 500 $\mu\text{g/ml}$ at all incubation times. Inhibition of cell proliferation was also observed in response to 3.91, 7.82, 15.63, 31.25, 62.5, 125, and 1000 $\mu\text{g/ml}$ of the extract, but only at 48 and 72 h. In contrast, a significant ($p>0.05$) induction of cell proliferation was observed at concentrations of 3.91, 7.82, 15.63, 31.25, and 250 $\mu\text{g/ml}$ at 24 h. Thus, these data also suggest

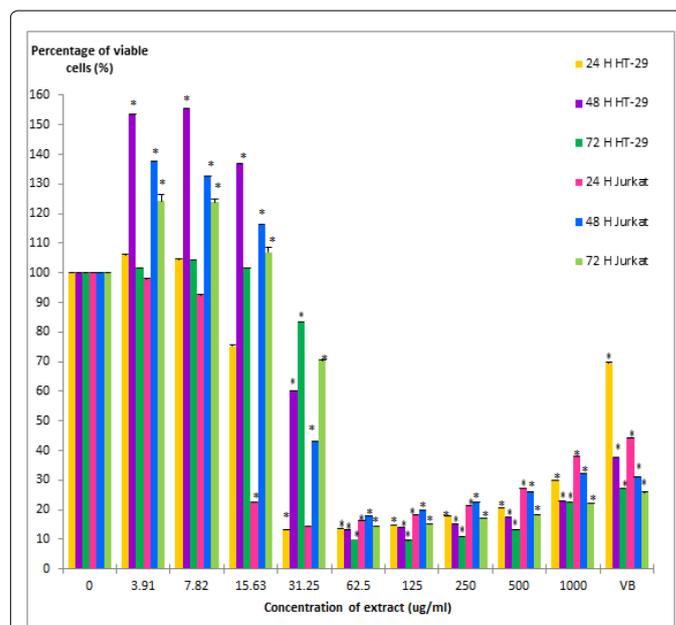
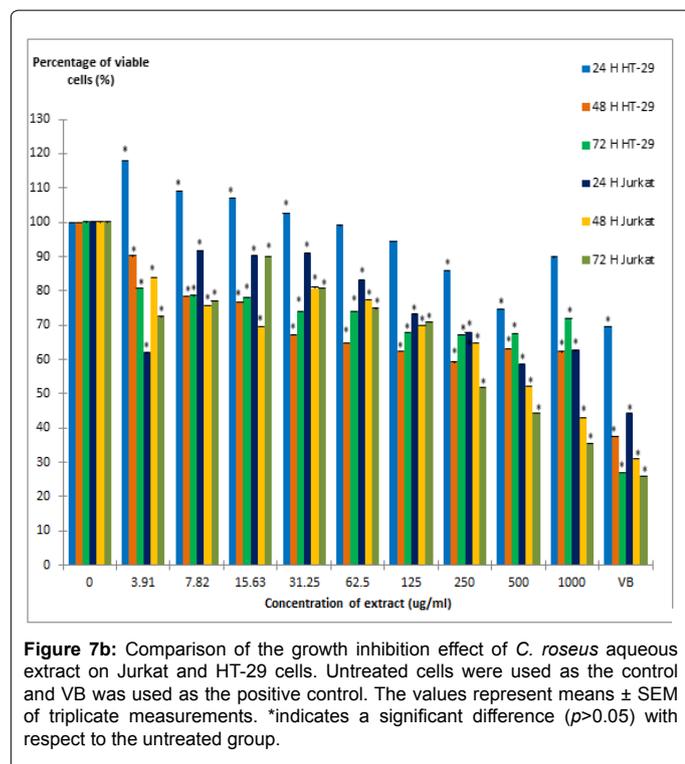


Figure 7a: Comparison of the growth inhibition effect of synthesized *C. roseus*-AgNPs on Jurkat and HT-29 cells. Untreated cells were used as the control and VB was used as the positive control. The values represent means \pm SEM of triplicate measurements. *indicates a significant difference ($p>0.05$) with respect to the untreated group.



that Jurkat cells were more sensitive than HT-29 cells to the toxicity of *C. roseus* aqueous extract.

In conclusion, the synthesized *C. roseus*-AgNPs shows higher toxicity effects compared to *C. roseus* aqueous extract on both cell types. However, both treatments are more effective in inhibiting Jurkat cells compared to HT-29 cells.

Conclusion

Herein, we demonstrated that the extract of the *C. roseus* leaf is capable of producing AgNPs extracellularly by rapid reduction of silver ions. This technique may prove useful for developing large-scale commercial production of value-added products for biomedical or nanotechnology-based industries. This green synthesis method offers a rapid and reliable way to synthesize AgNPs. Further studies should be conducted to better understand the mechanisms by which these AgNPs function as an anti-cancer therapy. These AgNPs should also be tested on normal cells, as a good anti-cancer compound should be selectively cytotoxic to cancer cells and have minimal cytotoxic activity or be non-toxic to normal cells.

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Disclosure

The authors report no conflict of interest in this work.

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