International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2014): 5.611

Biologically Synthesized Silver Nanoparticles from Latex of *Syandenium grantii* and Fresh Leaves of *Kalanchoe pinnata*: Potential Source of Cytotoxic Agents against Cervical Cancer Cells

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Abstract: ***Nanotechnlogy has become ubiquitous in every stream of science today. Biomedicine is no exception to this phenomenon. Metallic nanoparticles have been synthesized, characterized and utilized in biomedicine for imaging, wound healing and targeted drug delivery. A further improvement is the introduction of 'green nanotechnology' wherein the plant extracts are used to synthesize nanoparticles. This preparation technique has added advantages of being relatively easy, economical and faster as compared to the traditional chemical techniques. A number of studies have been reported wherein the aqueous plant extracts are used to synthesize metallic nanoparticles of varying sizes, shapes and biochemical potencies. Most of the 'green nanoparticles' have anti-oxidative, anti-microbial and anti-cancer activities. Here, we compare the anti-cancer property of different silver nanoparticle samples synthesized using two different plant extracts from Syandenium grantii and Kalanchoe pinnata using the human cervical cancer cell line HeLa. The cell viability assay was carried out using the Trypan blue dye exclusion method, while cytotoxicity was compared using the MTT assay. These nanoparticles have been earlier demonstrated to have anti-microbial, anti-oxidative properties and this study supports its anti-cancer activity.***

Keywords: Green chemistry, silver nanoparticles, anti-cancer, cytotoxicity

1. Introduction

Metallic nanoparticles have opened up a plethora of possible biological applications. These include use of nanoparticles in radio imaging, wound healing, candidates for anti-viral, anti-oxidant, anti-cancer treatment (1-8). The traditional process of nanoparticle synthesis involves the use of chemicals, reducing agents, capping agents and stabilizing agents (6). Recently, a novel method of nanoparticle synthesis using phytochemicals was reported. Since then there have been numerous reports, especially of silver nanoparticle synthesis using plant-derived phytochemicals (7) namely *Origanum vulgare*(8), *Ganoderma neo-japonicum* Imazeki (8), *Rosa damascene* (9). This so called "green nanotechnology" has numerous advantages over the traditional nanoparticle synthesis techniques such as being relatively easy, economical and faster (14, 15). Also, these phytochemicals act as capping agents for nanoparticles making them more potent biomedical agents (9, 11). Cervical cancer is one of the most common forms of cancer worldwide. According to an IARC study released in 2010, 529,828 new cases and 275,128 deaths were reported worldwide in 2008. Majority of these cases, i.e. more than 85%, were reported in developing countries, where it accounted for 13% of all female cancers (17). This study was carried out to test the anti-cancer effectiveness of different silver nanoparticles synthesized using plant extracts of *Syandenium grantii* and *Kalanchoe pinnata* (13, 14). Due to the prevalence of cervical cancer in developing countries, we decided to test the anti-cancer activity of these nanoparticles against the established human cervical cancer cell line HeLa.

2. Literature Survey

Silver nanoparticles synthesized by a modified chemical process having activity against cervical cancer cell lines HeLa and CaSki studied using flow cytometry have been reported (19). Similar studies of silver nanoparticles synthesized using the "green chemistry" demonstrated that they also have anti-cancer activity (8, 9, 10, 16). In one of the reports, *Origanum vulgare* synthesized nanoparticles were studied for their anti-microbial and anti-cancer activity (8). This report detailed their synthesis of silver nanoparticles using the *O. vulgare* plant extract and assessment of its anti-cancer activity against human larynx carcinoma cell line Hep- 2 using MTT assay. Another group reported similar synthesis of silver nanoparticles using the plant extract of *Ganoderma neo-japonicum* Imazeki (9). Apart from characterization of the nanoparticles, they also studied the anti-oxidative activity and elucidated its anticancer activity using cell viability MTT assay alongwith the lactate dehydrogenase leakage, reactive oxygen species generation, caspase 3, DNA laddering, and terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling in human breast cancer cells (MDA-MB-231). They concluded that the silver nanoparticles had inhibitory activity on cancer cell growth which was induced by increase in reactive oxidative species, cell membrane leakage and

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apoptosis. A similar study of silver nanoparticle synthesis using aqueous extract of *Rosa damascena* petals and study of its anti-cancer activity against human lung adenocarcinoma cell line A549 was reported (10). These studies indicated the „green" synthesized nanoparticles as being potent anti-oxidative, anti-microbial and anti-cancer agents (16, 18).

3. Materials and Methods

Chemicals

The cervical cancer cell line HeLa was provided by the National Centre for Cell Sciences, Pune. The cells were maintained in T-25 flasks (Nunc) containing MEM essential media (Gibco), fetal bovine serum (Gibco), Penicillin-Streptomycin (Gibco). The flasks were incubated in humidified 37°C 5% CO_2 incubator (Thermo Scientific). Other instruments and reagents used were as follows: inverted microscope (Zeiss), haemocytometer (Hausser Scientific), Trypan blue reagent(Sisco Research Laboratories), 1X Hanks" Balanced salt solution i.e. HBSS (Gibco), 6-well plate (Nest), MTT reagent (Life technologies), Trypsin (Himedia), dimethyl sulfoxide i.e. DMSO (Loba Chemicals), spectrophotometer (Shimadzu) and 0.2-µm syringe filter cartridges (Corning).

Cell Culture:

The HeLa cells were maintained in T-25 flasks containing MEM essential media, 10% heat inactivated fetal bovine serum and Penicillin-Streptomycin at 100 U/mL, 100 µg/mL respectively. The medium was renewed 2-3 times per week and the subcultivation ratio was 1:2 or 1:3.

Cell viability assay:

After the flasks were 70-80% confluent, they were treated with Trypsin to detach the cells. The cells were then re-suspended and washed once using the above mentioned complete medium and cell count was taken with Trypan blue and haemocytometer. The cells were suspended in the complete medium to a final concentration of 1 X 10^5 cells/mL. 1 mL cell suspension was added to each well in the 6-well plate which was then incubated in a humidified 37°C 5% CO_2 incubator for about 18 hours. Silver nanoparticle suspension samples derived using *S. grantii* and *K.pinnata* were filter sterilized using sterile 0.2-µm syringe filter and serial dilutions of 7 mg/mL, 1.4 mg/mL, 233.3 µg/mL, 33.3 µg/mL, 8.3 µg/mL were prepared using purified water. 500 µL of this suspension was added to the respective wells, while 500 µL purified water was added to the cell control well and the plate was further incubated for 24 hours. Then the media from the wells was discarded and washed with HBSS followed by trypsin treatment to suspend the cells. The cells were washed with HBSS and counted using Trypan blue and haemocytometer. The test was performed in triplicate for each nanoparticle suspension and data was recorded.

MTT cytotoxicity assay:

The HeLa cells were maintained and the 6-well plates were seeded similar to the method described above. Silver nanoparticle suspension samples derived using *S. grantii* and *K.pinnata* were filter sterilized using sterile 0.2-µm syringe filter and serial dilutions of 7 mg/mL, 1.4 mg/mL, 233.3 µg/mL, 33.3 µg/mL, 8.3 µg/mL were prepared using purified water. 500 µL of this suspension was added to the respective wells, while 500 µL purified water was added to the cell-control well and 500 µL medium was added to the media-control well following which the plate was further incubated for 24 hours. Then the media was discarded and wells were washed with HBSS. Fresh 500 µL HBSS and 40 µL MTT (5 mg/mL in PBS) was added to each well and incubated at 37°C 5% CO_2 for 4 hours. Formation of formazan crystals was verified under microscope and the solution was discarded. 500 µL DMSO was added to each well and placed on a plate rocker at room temperature for 15 minutes. Absorbance was measured at 540 nm using spectrophotometer and data was recorded. The test was performed in triplicate for each nanoparticle suspension.

4. Results and Discussion

Silver nanoparticles synthesized using both *S. grantii* and *K. pinnata* cause morphological changes in the cancer cells even after 24-hour exposure observed under an inverted microscope with phase contrast setting (Figure- 1). Figure 1a shows untreated healthy cells, figure 1b shows cells treated with *K. pinnata* nanoparticles having a lot of cell debris with floating cells and figure 1c shows cells treated with *S. grantii* nanoparticles which appear shrunken with discontinuous membranes.

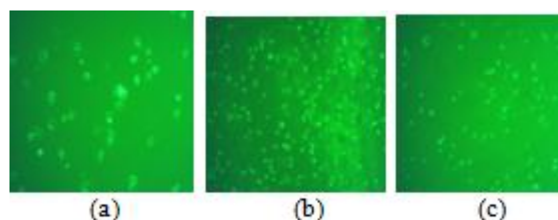

Figure 1: Effect of silver nanoparticles on morphology of HeLa cells. (a) cell control not exposed to nanoparticles, **(b)** cells exposed to *K. pinnata* nanoparticles, **(c)** cells exposed to *S. grantii* nanoparticles

The cell viability assay carried out using Trypan Blue utilizes the integrity of plasma membrane as the distinguishing factor between live and dead cells. The results showed significant cell death at higher concentrations, while the proportion of cell death did not remain consistent at lower concentrations (Figure- 2).

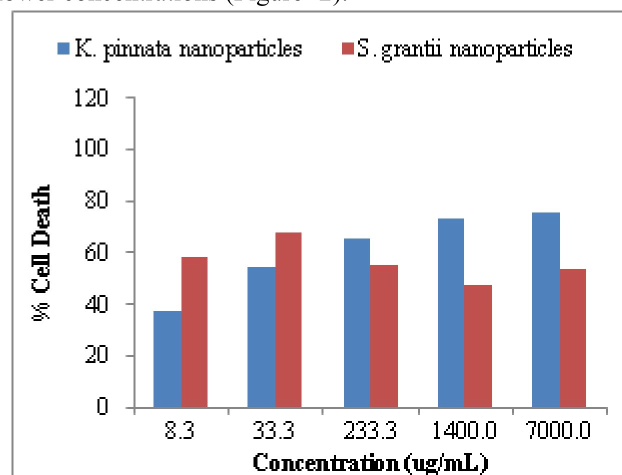

Figure 2: Graph of concentration of silver nanoparticles and percent cell death using Trypan blue. Percent cell death =

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100 – [(Cell number of control/ cell number of sample suspension)*100]

To investigate its cytotoxicity the MTT assay was performed. Higher concentrations exhibited significant cytotoxic activity which declined sharply below concentration of 1.4 mg/mL (Figure- 3).

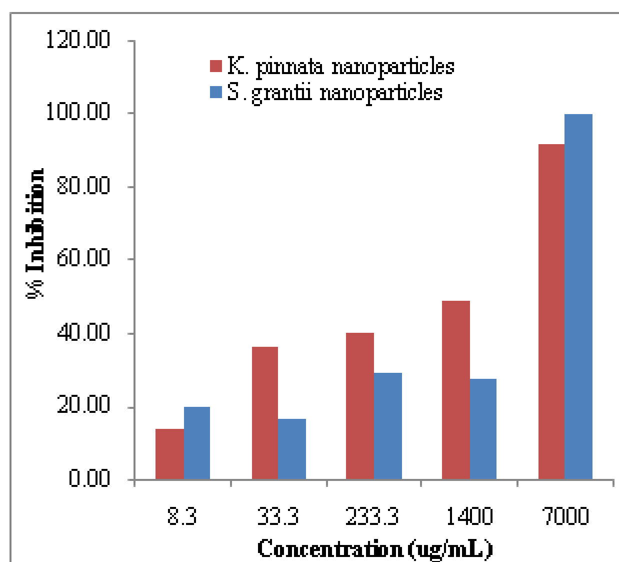

Figure 3: Graph of concentration of silver nanoparticles and percent inhibition using MTT. Percent inhibition = 100 – [(Absorbance$_{540}$ of control/ Absorbance$_{540}$ of sample suspension)*100]

Both the methods demonstrate that the nanoparticles synthesized using *K. pinnata* and *S. grantii* exhibit anti-cancer activity in HeLa cell line, though the observed effects on morphology, viable cell number and cytotoxicity are not the same.

5. Discussion

An earlier study demonstrated the synthesis of silver nanoparticles using the plant extract of *Syandenium grantii* and *Kalanchoe pinnata* separately (13, 14). In these studies, the nanoparticles were characterized using Fourier Transform Infrared spectroscopy (FTIR), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). The nanoparticles were proven to have anti-microbial and anti-oxidative properties.

The visual examination of cells after exposure to both the nanoparticles demonstrated the anti-proliferative activity of the nanoparticles (refer Fig. 1). Cells treated with *K. pinnata* nanoparticles (refer Fig. 1b) appeared to have a lot of cell debris and floating cells which indicated cell death. However, cells treated with *S. grantii* nanoparticles (refer Fig. 1c) appeared shrunken with discontinuous cell membrane with little or no cell debris. Figure 1c proved that the cells were under anti-proliferative stress but could not confirm their viability. The cell viability experiment using Trypan blue exclusion technique demonstrated that *K. pinnata* derived nanoparticles indeed had a greater cytocidal activity than *S. grantii* derived nanoparticles after 24 hours of exposure (refer figure 2). Since the trypan blue exclusion method cannot differentiate between metabolically active and inactive cells, the MTT assay was performed to test their cytotoxic effects under similar conditions. *S. grantii* derived nanoparticles showed greater cytoxicity at 7 mg/mL concentration; however, at lower concentrations the *K. pinnata* derived nanoparticles had relatively greater cytotoxicity (refer figure 3).

6. Conclusion

Even though the core material in both the nanoparticles is reduced silver, the phytochemicals bound to their surface may be different (11). Also, the anti-proliferative activity of silver nanoparticles is dependent on its size (20). These might be some of the reasons for the difference in the cytotoxicity. However, it can be concluded that the anti-proliferative activity is concentration dependent and both the nanoparticle samples have anti-cancer activity as tested on cervical cancer cell line HeLa.

7. Future Scope

Further study needs to be carried out for improving the cytotoxicity of the silver nanoparticles for example minimizing the coagulation, studying the effect of storage conditions on the level of coagulation, etc. The cytotoxicity can also be studied on different human cancer cell lines and elucidation of the molecular mechanisms involved in their cytotoxicity. This study also points to the immense potential of „green synthesis" in the field of biomedicine. Investigating the phytochemicals bound to the surface of these nanoparticles and maintaining uniformity of potency would be the further challenges in this research.

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