In vitro Studies on the Selective Cytotoxicity of Gold Nanoparticle-Prodigiosin Formulation against Cancer Cells

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Abstract

Prodigiosin is a secondary metabolite produced by different bacterial species including Serratia, actinomycetes, a few marine bacteria etc. This red-colored tripyrrole pigment possesses several pharmacological activities such as anticancer, antimicrobial, antimalarial and antialgal effects. Even though Serratia marcescens is more commonly recognized as a major producer of prodigiosin, in the present work, Serratia rubidaea isolated from leftover coconut was used to obtain Prodigiosin. Since it is already known that the hydrophobic nature of Prodigiosin limits its medical and biotechnological applications, efforts were made to formulate a Gold nanoparticle-prodigiosin Formulation (Au-Prod).

Anticancer activity of the Au-Prod against different mouse cancer cells, such as Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cells was done using the trypan blue dye exclusion method. The extent of apoptosis was determined using the apoptotic index. The results indicated significant anticancer activity while showing the least toxicity toward normal cells as shown by the results of cytotoxicity analysis in mouse splenocytes.

Keywords: Prodigiosin, Gold nanoparticle, Dalton's Lymphoma Ascites, Ehrlich Ascites Carcinoma, Apoptotic index.

Introduction

Cancer remains a leading cause of morbidity and mortality worldwide, necessitating the development of innovative therapeutic strategies to enhance treatment efficacy while minimizing side effects. Cancer treatment remains fraught with significant challenges, primarily due to issues such as drug resistance, non-selectivity and high therapeutic costs that often impede optimal clinical outcomes. Traditional therapies, while beneficial, frequently exhibit limitations that necessitate innovative solutions. Nanotechnology emerges as a promising frontier in this landscape, addressing many of these challenges by enhancing the delivery and efficacy of anticancer agents. Specifically, the incorporation of natural compounds, which have garnered attention for their therapeutic potential, faces obstacles like poor bioavailability and chemical instability. The utilization of nanocarriers offers a strategic means to improve these properties, thereby facilitating more effective cancer treatment modalities[2,12].

Recent advancements in nanotechnology have opened new avenues for targeted drug delivery, particularly through gold nanoparticles. These nanoparticles not only possess unique physicochemical properties that allow for increased cellular uptake but also can be functionalized to deliver biologically active compounds directly to tumour cells. Innovative formulations combining AuNPs with anti-cancer drugs have demonstrated selective cytotoxicity, selectively inducing apoptosis in tumor cells without adversely affecting healthy tissues. The synergistic effects of co-delivery mechanisms, particularly when AuNPs are conjugated with therapeutic compounds, can significantly inhibit cancer cell proliferation and metastasis, establishing a robust platform for future therapeutics in the fight against cancer[2,9]. Therefore, the strategic application of AuNPs represents a promising advancement in targeted cancer treatment modalities.

Prodigiosin, a potent antiproliferative agent derived from the bacterium Serratia, has garnered attention for its ability to induce cytotoxicity against cancer cells selectively[3,8]. Prodigiosin exhibits selective cytotoxicity against cancer cells while sparing normal cells, a characteristic that is vital for developing effective treatment modalities with minimal side effects. The mechanisms through which prodigiosin exerts its anticancer effects, include inducing apoptosis and inhibiting cell proliferation, as well as disrupting cellular signaling pathways that are crucial for tumor growth and survival. Its tripyrrole structure contributes not only to its vivid pigmentation but also to its medicinal properties, encompassing antimicrobial and anticancer effects[5,7,13,15]. The incorporation of Prodigiosin into innovative drug delivery systems such as magnetic nanoparticles can be used as a strategy to enhance its bioavailability and therapeutic efficacy.

This approach addresses the limitations of Prodigiosin such as its low water solubility and high hydrophobicity, which can hinder effective treatment [2, 5]. In the present work, efforts were made to develop Gold nanoparticle-Prodigiosin formulation (Au-Prod) for more effective anticancer activity. The integration of prodigiosin with gold nanoparticles is expected to enhance its therapeutic potential

by improving solubility and stability, thereby increasing bioavailability and enabling targeted delivery to cancer cells.

Material and Methods

Chemicals and Cells: All chemicals used for the study were of analytical grade and procured from reputed Indian manufacturers. Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cells and rat splenocytes were used for the cytotoxicity analysis at Amala Cancer Research Centre, Thrissur, Kerala. They were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were conducted strictly adhering to the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India.

Isolation and characterization of *Serratia* isolate: *Serratia* was isolated from coconut samples on a nutrient agar plate and made into pure culture. The culture was identified as belonging to the *Serratia* through various tests, the major identifiers being the Gram staining character of the organism, culture morphology and production of cell-associated pigment, Prodigiosin. Using 16SrRNA sequencing studies, the isolate was identified to be *Serratia rubidaea* and the UPGMA dendrograms showing phylogenetic relationships of the isolates were obtained by using MEGA.

Preparation of Gold Nanoparticles- Prodigiosin Complex: *Serratia rubidaea* was inoculated on nutrient agar plates and incubated for 24-48 hours and the cells were scrapped off from the plates. The cell pellets were inoculated into 100 ml of 1mM tetra-chloro-aurate solution ($HAuCl_4$), incubated for 10 days at room temperature and then ultra-sonicated and centrifuged to obtain the supernatant. This complex contained approximately 1 mg Prodigiosin per ml as evident from the absorption at 535 nm. The supernatant was named as Gold Nanoparticles- Prodigiosin Complex (Au-Prod) and was characterized by SEM.

Anticancer activity of Gold Nanoparticles- Prodigiosin Complex (Au-Prod), *in vitro:* The Gold nanoparticle - Prodigiosin complex was analyzed for anticancer activity. Dalton's Lymphoma Ascites (DLA) cells and Ehrlich Ascites Carcinoma (EAC) cells were used as model system for studying *in vitro* anticancer activity and rat splenocytes were used as the model system for analyzing the effect on normal cells. The cell viability was determined by Trypan blue dye exclusion method[14]. Cytotoxicity of different concentrations of Gold nanoparticles- Prodigiosin complex (Au-Prod) (10-80 µl) was analyzed using Trypan blue dye exclusion method.

Rat splenocytes were obtained as follows: the rat was sacrificed using carbon dioxide anesthesia and the spleen tissue was dissected out. It was then smashed to a single cell suspension in RPMI complete medium containing antibiotics and filtered using mesh cloth. The collected cells were washed thrice and suspended in a known volume of RPMI complete medium containing antibiotics and counted. Viable cell suspension ($1x10^6$ cells in 0.l ml) was added to tubes containing various concentrations of Au-Prod (10-80 µl) and the volume was made up to 1 ml using RPMI media. Control tube contained only cell suspension. These assay mixtures were incubated for 3 hours at 37°C. Further cell suspension was mixed with 0.1 ml of 1 trypan blue and kept for 2-3 minutes and loaded on a haemocytometer. Dead cells take up the blue colour of trypan blue while live cells do not take up the dye. The number of stained and unstained cells was counted separately.

% dead cells = (Number of dead cells/(No. of viable cells + No. of dead cells)) * 100

Study on the extent of apoptotic induction in Cancer cells *in vitro*: Gold nanoparticle - prodigiosin complex was analyzed for the extent of apoptosis induction on Dalton's Lymphoma Ascites (DLA) cells and Ehrlich Ascites Carcinoma cells (EAC). Briefly, the tumour cells (DLA and EAC) ($1X10^6$ cells in 0.l ml) were added to tubes containing 10µl of Au-Prod and the volume was adjusted up to l ml using phosphate buffered saline (PBS). The control tube contained the cell suspension only. These tubes were incubated for 4 hours at 37 °C. After the incubation period, thick smears were prepared on clean glass slide, stained with Leishman's stain, washed, dried and observed for apoptotic cells using a bright field microscope.

The cells with condensed and fragmented nuclei, blebbing of plasma membrane, reduced size and apoptotic bodies were identified as apoptotic cells[10]. Apoptotic index was calculated using the formula:

Apoptotic index = (Number of apoptotic cells / Number of total cells)*100

Results and Discussion

Isolation and characterization of *Serratia* isolate: Serratia was isolated as pure culture from coconut samples on a nutrient agar plate. The typical colour and Gram-staining nature indicated that the isolate belonged to the Serratia species. The molecular identification of the isolate was done by 16S rRNA sequencing. According to the information in the BLAST of National Center for Biotechnology Information (NCBI), the sequence obtained from the 16S rRNA sequences of the bacterial isolate corresponded to *Serratia rubidaea*. The most similar sequence corresponded to *Serratia rubidaea* NBRC103169, with GenBank accession number SUB14835937 and 99% sequence identity.

As 16S rDNA gene sequence provides accurate grouping of organisms even at subspecies level, it is considered a powerful tool for rapidly identifying bacterial species[6]. The phylogenetic analysis of 16S rRNA sequence of the isolate,

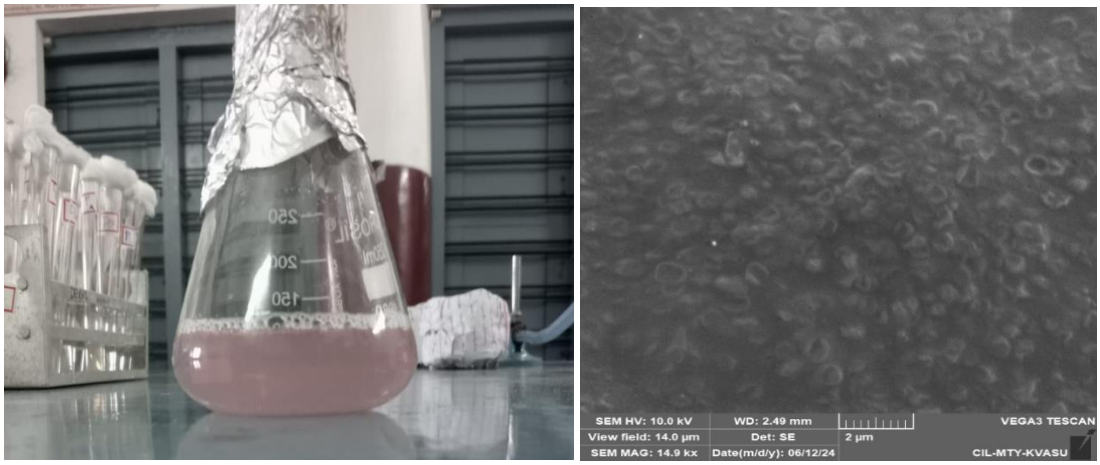
along with the sequences of the other similar strains retrieved from NCBI was carried out with MEGA 12 using the neighbor-joining method, with 1,000 bootstrap replicates. The result of phylogenetic analysis is shown in figure 2 confirming it as *Serratia rubidaea*.

Preparation of Gold Nanoparticles- Prodigiosin Complex: Since the water solubility of prodigiosin is limited, efforts were done to improve it by preparing gold nanoparticles- prodigiosin complex (Au-Prod) and it was characterized by SEM.

Effect of Gold nanoparticle-Prodigiosin complex (Au-Prod) in Dalton's Lymphoma Ascites Cells and Ehrlich Ascites Carcinoma Cells and Rat Splenocytes: Gold nanoparticle-prodigiosin complex (Au-Prod) was studied for short-term *in vitro* cytotoxicity using DLA cells and EAC cells by Trypan blue dye exclusion method. Different volumes of Au-Prod (10 - 80 µl) were used to analyse cytotoxicity. It was observed that the Au-prod caused efficient cell death. The results are shown in figures 4, 5 and 6.

Figure 1: *Serratia rubidaea* isolate

Figure 2: Phylogenetic analysis of the 16S rRNA sequence, the isolate was identified to be *Serratia rubidaea*



A **B**

Figure 3: A - Gold Nanoparticles- Prodigiosin Complex (Au-Prod) in conical flask, B - Scanning Electron Microscopic image of Gold Nanoparticles - Prodigiosin Complex (Au-Prod)

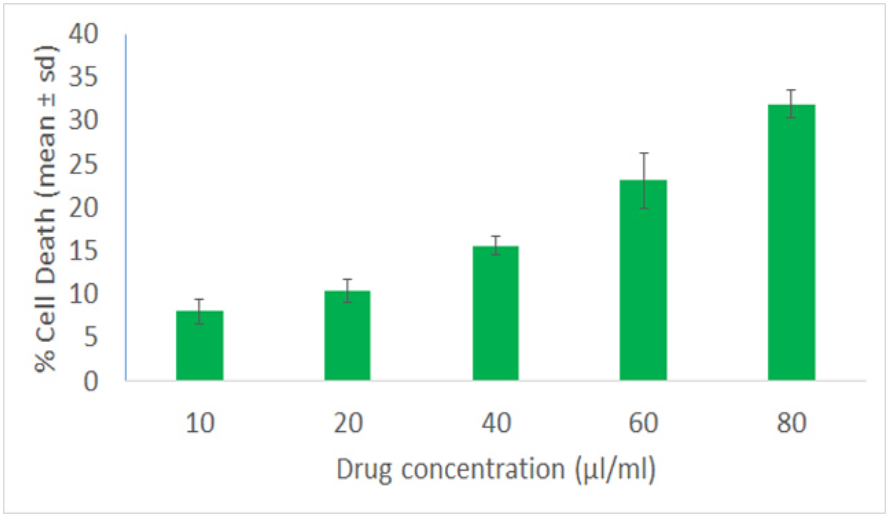


Figure 4: Effect of Different volumes (10 - 80 µl) of Au-Prod on Dalton's Lymphoma Ascites Cells as assayed by Trypan blue dye exclusion method. Results are shown as mean±sd. The experiment was done in duplicate and the count was taken 4 times.

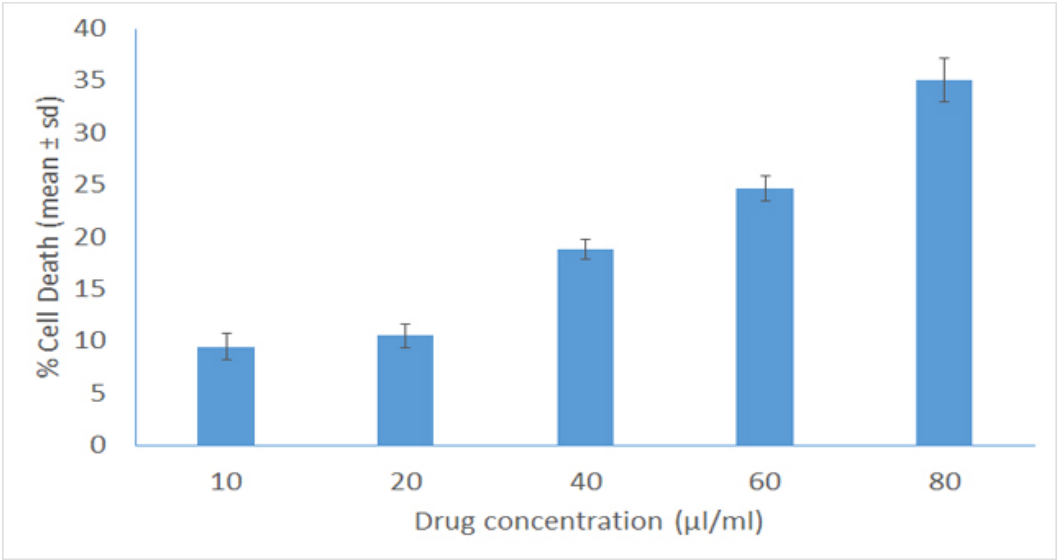


Figure 5: Effect of Different volumes (10 - 80 µl) of Au-Prod Ehrlich Ascites Carcinoma Cells as assayed by Trypan blue dye exclusion method. Results are shown as mean±sd. The experiment was done in duplicate and the count was taken 4 times.

80 µl of Au-Prod induced a percentage cell death of 31.9±1.6 in Dalton's Lymphoma Ascites Cells and 35.1±2.1 in Ehrlich Ascites Carcinoma Cells. The same concentration of Au-Prod resulted in only 8.3±1.8 percentage cell death in Rat Splenocytes showing significant and selective anticancer activity by the Gold nanoparticle-Prodigiosin complex (Au-Prod).

Induction of Apoptosis by Gold nanoparticle-Prodigiosin complex (Au-Prod) in DLA Cells and EAC Cells: Apoptosis induction by Gold nanoparticle-Prodigiosin complex (Au-Prod) was studied in in DLA cells and EAC cells. The mechanism of cancer cell killing in DLA and EAC was identified to be as the induction of apoptosis as shown in table 1. The number of apoptotic cells was calculated after 4 hours of incubation of Dalton's Lymphoma Ascites cells and Ehrlich Ascites Carcinoma cells with 10µl of Au-Prod and it was observed that more than 75 % of the cells were apoptotic as per the cell morphology.

Although prodigiosin is an excellent anti-cancer agent, its hydrophobic nature, insolubility in water, poses difficulties for medical and biotechnological applications. It has been shown that limited aqueous solubility of prodigiosin resulted in poor absorption and low bioavailability[1,11]. This study demonstrates the successful synthesis of a gold nanoparticle-prodigiosin complex (Au-Prod) using a bio-reduction approach. The Au-Prod complex significantly enhanced the anticancer activity of prodigiosin against DLA and EAC cells, while exhibiting minimal toxicity towards normal cells. The enhanced efficacy is attributed to improved solubility, cellular uptake and apoptosis induction. These findings suggest that Au-Prod holds promising potential as a novel therapeutic agent for cancer treatment.

Prodigiosin, a natural red pigment, exhibits potent anticancer properties. However, its clinical application is hindered by its inherent hydrophobicity and poor aqueous solubility, leading to low bioavailability. This study aimed to enhance the bioavailability and anticancer efficacy of prodigiosin by synthesizing a gold nanoparticle-prodigiosin complex (Au-Prod) using a bio-reduction approach with *Serratia rubidaea*. The resulting Au-Prod complex, containing approximately 1 mg/mL of prodigiosin, was characterized using Scanning electron microscopy (SEM). *In vitro* cytotoxicity assessments were conducted on Dalton's Lymphoma Ascites (DLA) cells, Ehrlich Ascites Carcinoma (EAC) cells and rat splenocytes using the trypan blue dye exclusion method. Apoptosis induction was evaluated to identify the mechanism of cell death.

In conclusion, the investigation into the selective cytotoxic effects of the gold nanoparticle-prodigiosin formulation underscores a significant advancement in cancer therapeutics. Au-Prod demonstrated a notable ability to target and induce apoptosis in cancerous cells while preserving the viability of healthy cells, suggesting their potential as a viable treatment strategy.

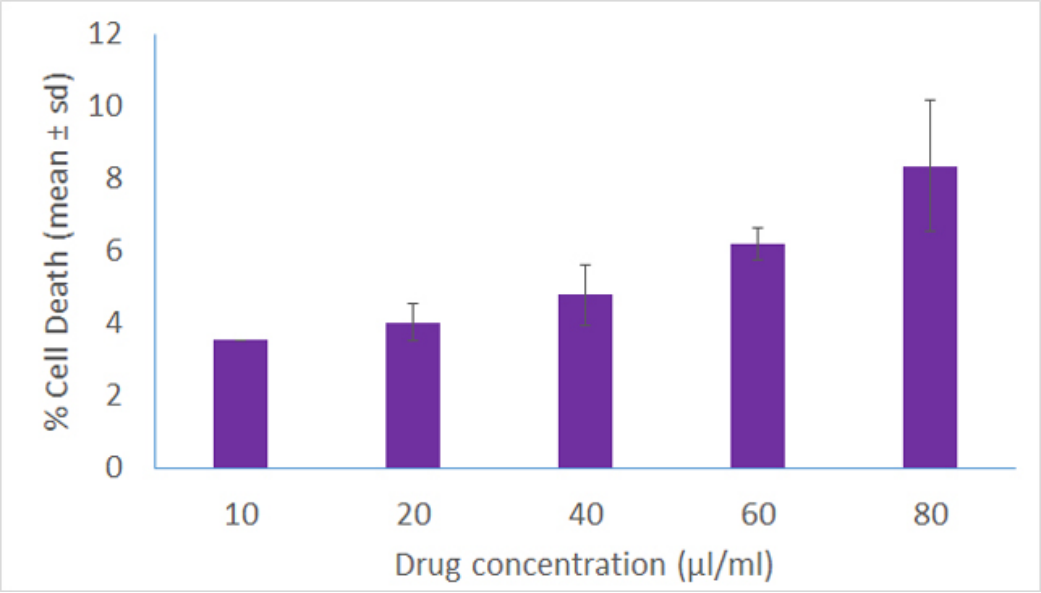


Figure 6: Effect of Different volumes (10 - 80 µl) of Au–Prod on normal Cells as assayed by Trypan blue dye exclusion method. Results are shown as mean±sd. The experiment was done in duplicate and the count was taken 4 times.

Table 1
Apoptotic index in Dalton's Lymphoma Ascites (DLA) cells and Ehrlich Ascites Carcinoma (EAC) cells incubated with 10µl of Gold nanoparticle-Prodigiosin complex (Au-Prod)

	% apoptotic index
Dalton's Lymphoma Ascites (DLA) cells	**76**
Ehrlich Ascites Carcinoma (EAC) cells	**90**

This approach not only enhances the therapeutic efficacy of prodigiosin but also capitalizes on the unique properties of gold nanoparticles, such as their surface plasmon resonance and ease of functionalization, to improve drug delivery and bioavailability. By facilitating targeted cytotoxicity in tumor cells, the gold nanoparticle-prodigiosin complex can significantly minimize the collateral damage to healthy tissues, a common side effect in traditional chemotherapy.

Furthermore, the synergistic interaction between prodigiosin and gold nanoparticles may enhance apoptotic mechanisms within malignant cells, thereby increasing sensitivity to treatment. This dual mechanism underscores the potential for improved patient outcomes and paves the way for future research into nanoformulated therapeutics, positioning gold nanoparticle-prodigiosin formulations as promising candidates in the ongoing battle against cancer.

The findings align with emerging trends in nanomedicine where nanomaterials are gaining attraction for their unique physicochemical properties, which enhance their efficacy in therapeutic applications. This promising approach paves the way for future research that could further refine these formulations, potentially leading to innovative and effective cancer therapies aligned with biocompatibility and targeted delivery principles [4].

Conclusion

The present study underscores the significant potential of the gold- nanoparticle- prodigiosin formulation in enhancing anticancer efficacy and bioavailability of prodigiosin. The complex exhibited selective cytotoxicity towards Dalton's Lymphoma Ascites (DLA) cell sand Ehrlich Ascites Carcinoma (EAC) cells, while preserving the viability of healthy splenocytes. The mechanism of action was associated with selective apoptosis induction, indicating its potential as a targeted therapeutic agent.

By leveraging the unique properties of gold nanoparticles, such as surface plasmon resonance and enhanced cellular uptake, the Au-Prod complex minimizes off-target effects, making it a promising candidate for future cancer treatments. These findings contribute to the growing field of nanomedicine, emphasizing the importance of nanoparticle-based drug delivery systems in improving treatment outcomes and reducing the side effects of conventional chemotherapy.

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