RESEARCH ARTICLE

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Physiological Investigation of Sap-AgNPs' Cytotoxic and Gene-Modulatory Effects in Oral Squamous Cell Carcinoma

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Abstract

Background: One of the most prevalent oral cancers, oral squamous cell carcinoma (OSCC), is distinguished by its rapid growth, invasiveness, and high metastatic potential. Green AgNPs are important because they can reduce systemic toxicity by inducing oxidative stress, cytotoxicity, and apoptosis in cancer cells. The goal of this study was to use saponins as natural stabilizers to create AgNPs, and the detrimental apoptotic effects on cancer cells were examined using high-content screening (HCS) assays such as TNI, CMP, and VCC. **Methods:** The size and distribution of AgNPs were determined using saponins as natural reducing and stabilizing agents, respectively. The cytotoxic effects on OSCC-25 cells were assessed using the MTT assay, alongside real-time quantitative PCR (RT-qPCR) to identify changes in gene expression associated with apoptosis. High-content screening (HCS) was used to confirm the induction of apoptosis and to measure concentration-dependent changes in several cellular parameters. All statistical analyses were performed for each experiment. **Results:** The results showed that the average diameter of the generated nanoparticles was 75.87 ± 15.69 nm, facilitating cellular uptake due to their narrow size distribution. Saponin-induced AgNPs significantly increased cytotoxicity and cancer cell death in OSCC-25 cells in a dose-dependent manner. Compared with the control group, treatment with 125 and 500 μg/mL resulted in a significant decrease in fluorescence intensity ($p < 0.05$). However, doses of 250 μg/mL and 1000 μg/mL had no significant effects. RT-qPCR analysis revealed a significant increase in the expression of IL1R, highlighting its role in apoptotic signaling. **Conclusion:** The findings suggest that the combination of the bioactive properties of saponins with the inherent cytotoxicity of AgNPs has therapeutic potential against oral squamous cell carcinoma. These results support the need for future preclinical and clinical studies and highlight the promise of integrating natural compounds with nanotechnology to develop safer and more effective anticancer therapies.

Keywords: Saponins- OSCC-25 cells- Silver nanoparticles- qPCR- AFM

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Introduction

Oral squamous cell carcinomas (OSCC) account for about 90% of lip cancers and this type of cancer is more likely to metastasize to other tissues and lymph nodes if it is not detected early. It remains a major concern in oral oncology due to its mortality and high incidence [1]. Surface-level lesions, difficulty swallowing, and ulceration were the most prevalent symptoms. Brief Response: Numerous tools have been developed to either treat the disease rapidly or postpone diagnosis. A lack of insurance might cause cancer cells to spread to other tissues and organs in the nose, lips, eyelids, or ear. In addition to performing a biopsy of the questionable tissue for microscopic inspection, I went over the text on the tongue and mouth. Among the most crucial techniques for identifying illness are blood tests, computed tomography (CT), magnetic resonance imaging (MRI), and medical imaging [2, 3].

In the field of antimicrobials, nanotechnology has been developed as an innovative method for fighting resistant microorganisms. By combining chemistry, biology, and materials science to create nanoparticles with distinctive chemical and physical characteristics, nanotechnology has shown promise as a cancer treatment [4]. Any method used at the nanoscale, where a material is reorganized at the atomic and molecular levels with sizes ranging from 1 nm to 100 nm, is referred to as nanotechnology. The physical and chemical properties of a material can undergo significant modifications when its dimensions decrease from a large size to below 100 nm [5].

Platinum, copper, silver, and gold are examples of metallic nanoparticles that have a variety of antibacterial properties against bacteria, fungi, and other illnesses.

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These properties enable changes in molecular pathways, greater cellular absorption, and improved therapeutic efficacy [6]. Silver nanoparticles (AgNPs) are the most significant nanomaterials that have demonstrated encouraging outcomes because of their antioxidant, antibacterial, and anticancer properties. However, there are also concerns that the severe temperatures and hazardous reagents used in classical synthesis procedures may have an impact on clinical applications and biocompatibility [7].

Green synthesis, an eco-friendly substitute that enhances therapeutic potential and biocompatibility, uses molecules produced by plants as reducing and stabilizing agents. This has led to the development of alternative techniques to provide a safe environment. Additionally, Siberian State Medical University researchers discovered that when nanoparticles were injected into the blood of mice and used microscopy to track their penetration into tumors in real time, the nanoparticles quickly accumulated along the blood vessel walls of the tumor before entering the tumor directly through cancerous vascular cells. The findings of this experiment might serve as the foundation for creating more successful cancer treatment strategies [8].

Saponins are among the most important plant compounds that contribute significantly to the synthesis of green nanoparticles. Its structure, which consists of a lipophilic triterpene or steroid aglycone connected to one or more lipophilic sugar molecules, has a variety of biological effects. Owing to their limited solubility, which restricts their direct clinical application, they are encouraged to be included in nanocarriers to enhance their transport and efficacy [4]. Blood lipid levels are lowered and blood glucose reactions are slowed by saponins. Additionally, they have been used in diets high in saponins, which inhibit platelet aggregation and tooth decay. Owing to their cytotoxic, anticancer, and immunomodulatory properties, they also lower the risk of cancer. By employing and synthesizing AgNPs and saponins as natural reducing and stabilizing agents, this study aimed to concentrate on the physical and chemical characteristics of nanoparticles and evaluate their cytotoxic and apoptotic effects in OSCC-25 cells. Additionally, it explores the cellular and molecular mechanisms that underlie their anticancer activity, making them naturally occurring bioactive and one of the contemporary technologies for safer and more successful treatment of oral cancer.

Materials and Methods

Materials

Silver nitrate ($AgNO_3$)

Analytical grade, obtained from Sigma-Aldrich.

Saponin extract

Isolated from Quillaja bark or commercially obtained with ≥90% purity.

Cell line Human oral squamous carcinoma cells

(SCC-25) were obtained from an accredited cell repository.

Cell culture reagents

These reagents include Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin solution, phosphate-buffered saline (PBS), fetal bovine serum (FBS), and the MTT assay.

Silver Nanoparticle Synthesis

AgNPs were prepared via eco-friendly green synthesis using saponin as a reducing and stabilizing agent. A1mM solution of silver nitrate was prepared in deionized water, followed by slow addition of 1% (w/v) aqueous saponin under continuous magnetic stirring at room temperature. Stirring was continued until the solution turned yellowish brown for approximately 3–4 h, indicating the formation of nanoparticles. The colloidal suspension was centrifuged for 20 min at 10,000 rpm. To form pure nanoparticles, the precipitate was rinsed thrice with distilled water and dried [9].

Nanoparticle Characterization

Dynamic Light Scattering (DLS)

DLS was used to assess homogeneity and polydispersity to calculate the particle size distribution, and normal distribution curves, mean diameter, and standard deviation were produced.

Atomic Force Microscopy (AFM)

AFM was used to analyze the topography and surface morphology. Particle shape, surface properties, and dispersion homogeneity were evaluated by scanning across an area of 3000 × 3000 nm at a resolution of 512 × 512 pixels.

Cell Culture

In a humidified incubator at 37°C with 5% CO2, SCC-25 cells were cultured in DMEM supplemented with 10% bovine serum and 1% penicillin-streptomycin. The standard trypsinization technique was used to subculture cells to approximately 80% confluence [10].

Cytotoxicity Assessment (MTT Assay)

Cells were seeded in 96-well plates and exposed for one day to different amounts of saponin-treated AgNPs. The MTT assay was used to measure the cell viability. Absorbance was measured at 570 nm after dissolving the formazan crystals in dimethyl sulfoxide. The percentage viability was calculated compared with the untreated control group [11].

High-Content Screening (HCS) Assay

Using the Thermo Scientific Cellomics kit was used to evaluate HCS, apoptosis, and cellular changes. Sap-NPs (125, 250, 500, and 1000 μM) were applied to the SCC-25 cells. Cytochrome c (primary antagonist), DyLightTM 649 (secondary antagonist), Hoechst dye, PBS, and permeabilization solution (PBS + 1% Triton X-100) are important reagents. Fluorescence signals for nuclear integrity, membrane permeability, mitochondrial function, and other apoptotic indicators were measured using an ArrayScan XTI Cell Imaging System (Zeiss, 40×, 0.75 NA) [12].

Analysis of Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from SCC-25 cells, treated with Trizol reagent (Sigma-Aldrich), and quantified using a NanoDrop spectrophotometer (A260/A280 = 1.8-2.0). The RNA was reverse transcribed to cDNA using a commercial kit. qPCR amplification of the IL-1 Receptor (IL-1RA) was performed using SYBR Green Master Mix and gene-specific primers on a StepOnePlus Real-Time PCR System (Applied Biosystems). Thermal cycling included an initial activation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. GAPDH was used as the housekeeping gene. The relative gene expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method [13, 14].

Statistical Analysis

Data are expressed as the mean ± standard deviation (SD) from at least three independent experiments. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used for multiple comparisons using GraphPad Prism 9.0. Statistical significance was set at $p < 0.05$.

Results

Atomic Force Microscopy (AFM) Analysis

Atomic Force microscopy imaging confirmed the effectiveness of the synthesis at the nanoscale and revealed a relatively homogeneous distribution of saponin-containing AgNPs on the scanned surface (Figure 1), with particle diameters ranging from 2 to 13.45 nm. The substrates of individual nanoparticles or small clusters are marked by bright spots marked as bright spots by black areas. No agglomeration was observed, demonstrating the effectiveness of saponin as a stabilizing or reducing agent. Controlled growth was evidenced by the smoothness and moderate roughness of the surface. These features enhance the utility of the nanoparticles for antibacterial and medical applications.

Particle Size Distribution

Sap-AgNPs had an average diameter of 75.87 ± 15.69 nm and a size range of 50–110 nm. The bulk of the particles had sizes between 60 and 80 nm according to a dynamic light scattering (DLS) study, which suggests that the synthesis was quite homogeneous (Figure 2). This selection of sizes is suitable for cancer treatment and biomedical applications, as particles smaller than 100 nm are effectively absorbed by cells and increase treatment [15].

Anticancer Activity: MTT Assay

The MTT assay was used to evaluate the cytotoxic effects of Sap-AgNPs on OSCC-25 cells. The result show during over 24 hours that cell viability gradually decreased after treatment with doses of 100, 200, 300, 400, and 500 µg/ml (Figure 3), while the IC^{2}_{0} was determined to be 400 µg/ml. These findings led to the selection of doses of 200 and 300 µg/ml for further mechanistic studies.

Multiparametric Cytotoxic Assessment
High-Content Screening (HCS)

Sap-AgNPs were further evaluated using HCS at concentrations of 125, 250, 500, and 1000 µg/ml, as well as the viable cell count (VCC), total nuclear fluorescence intensity (TNI), and cell membrane permeability (CMP).

Viable Cell Count (VCC)

Untreated cells exhibited the highest VCC (3660.5 ± 14.85). Exposure to 500 and 1000 µg/ml significantly reduced cell viability ($p < 0.01$), whereas 125 and 250 µg/ml showed no significant change (Table 1, Figure 4 (A)).

VCC, Viable Cell Count; TNI

Total Nuclear Intensity; MMP: Mitochondrial Membrane Potential; CMP: Cell Membrane Permeability; CC: Cytochrome C. Letters (a, b, and c) are significant at $p \leq 0.05$, (n = 3).

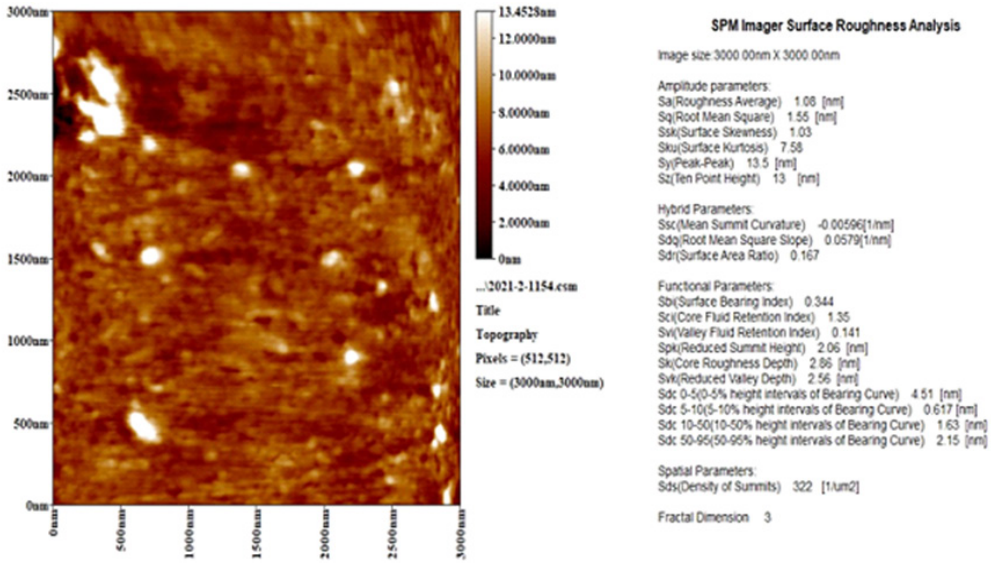

Figure 1. Analysis of Atomic Force Microscopy (AFM).

Table 1.Cytotoxic Effects of Sap-Mediat Nanoparticals on OSSC Cells Using HCS

Conc (µg/ml)	VCC		TNI		CMP	
	Mean	SD	Mean	SD	Mean	SD
Untreated	3660.5	14.84924	457	2.828427	122	4.242641
1000	2062	22.62742	673.5	23.33452	118.5	2.12132
500	2371	272.9432	549.5	16.26346	108	1.414214
250	3375	298.3991	438.5	4.949747	114.5	2.12132
125	3491	275.7716	435.5	20.5061	107.5	4.949747

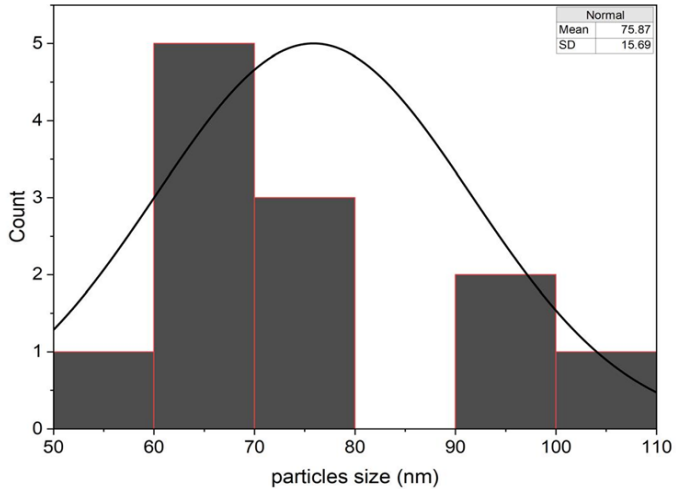

Figure 2. Particle Size Distribution.

Total Nuclear Fluorescence Intensity (TNI)

Significant increases were observed at 500 µg/ml ($p < 0.01$) and 1000 µg/ml ($p < 0.001$), reflecting nuclear alterations associated with apoptosis or cellular damage (Figure 4 (B)).

• This case offers a valuable learning point for clinicians to consider malignant etiologies, including SCLC, in patients presenting with a chylothorax.

• This effectively reinforces the importance of correlating clinical, radiological, and pathological findings in rare presentations.

• Cell Membrane Permeability (CMP): CMP analysis indicated significant changes at 125 and 500 µg/ml ($p < 0.05$), while 250 and 1000 µg/ml showed no significant difference (Figure 4(C)).

These findings support a dose-dependent cytotoxic effect of Sap-AgNPs, including decreases in cell viability and changes in nuclear and membrane integrity suggestive of apoptosis or cell damage, and they are in agreement with the MTT assay.

Gene Expression Analysis (IL-1RA)

The effect of Sap-AgNPs on IL-1 Receptor Antagonist (IL-1RA) expression in OSCC-25 cells was evaluated at 0, 125, 250, 500, and 1000 µg/ml (Figure 5). *IL-1RA* expression increased in a concentration-dependent manner, with significant upregulation at 125 µg/ml compared to the controls, and further increased at 250 µg/

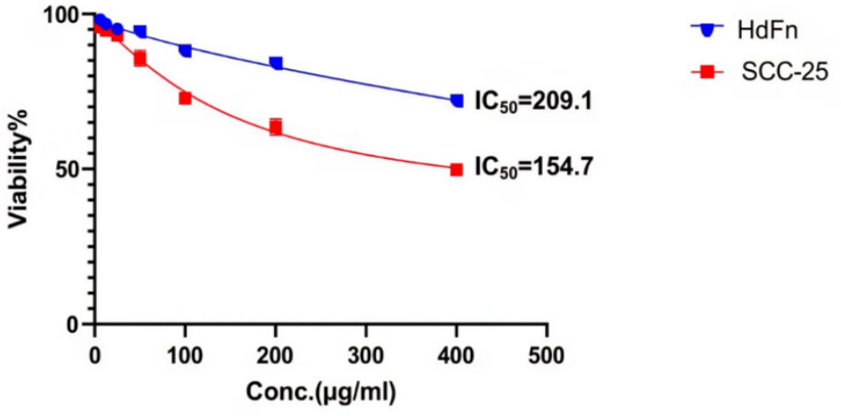

Figure 3. The Effect of Ethanol Oedogonium Extract Solution on the Cell Lines A549 and HdFn was Studied Using the MTT Method.

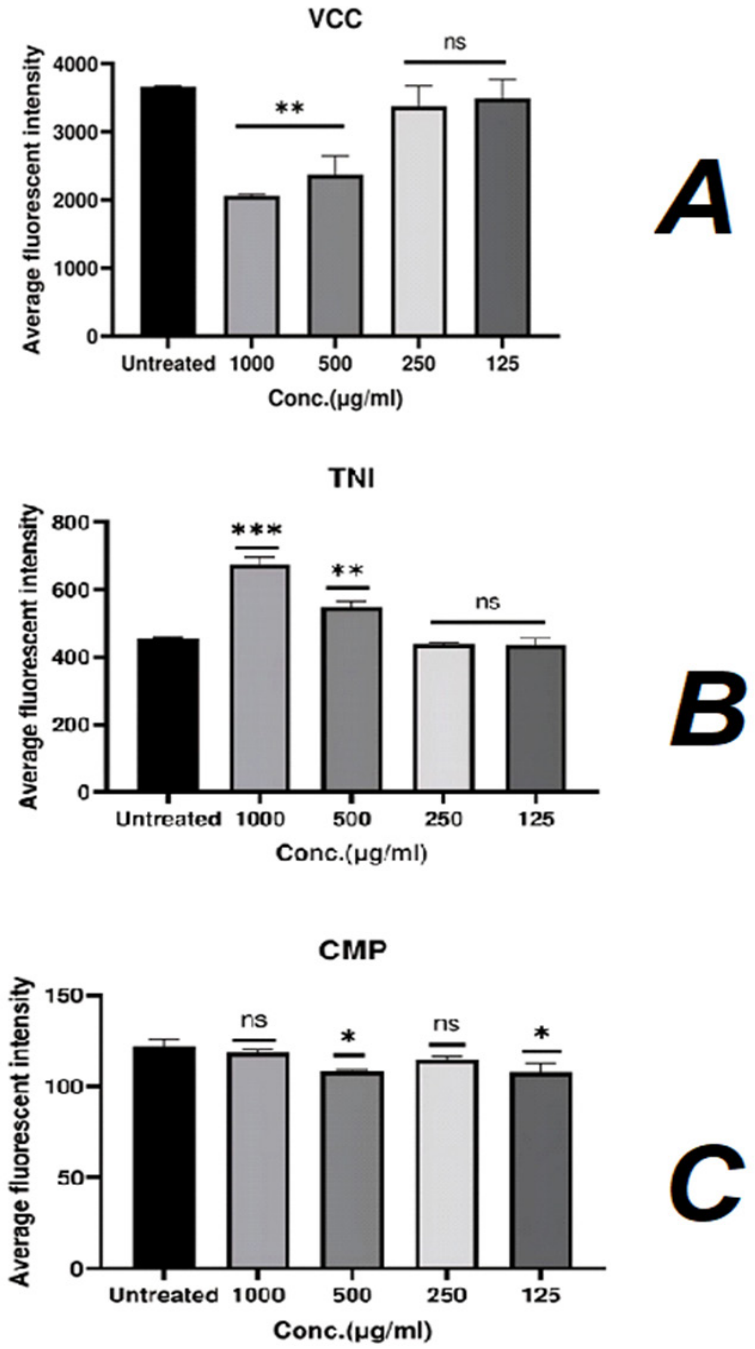

Figure 4. A: Cytotoxicity effect of Sap-AgNPs on viable cell count of OSSC-25 cell line on multipara system High Content Screening at 37 °C; B: Cytotoxicity effect of Sap-AgNPs on Total Nuclear Intensity of SSC-25 cell line on multipara system High Content Screening at 37 °C; C: Cytotoxicity effect of Sap-AgNPs on cell membrane permeability of SSC-25 cell line on multipara system High Content Screening at 37 °C. Data expressed as (mean ± SD)*,,* $p \leq 0.05$, SD: Standard Deviation, (n =3) NS: non-significant.

ml. The maximum expression was observed at 500 µg/ml, followed by a slight decrease at 1000 µg/ml, which remained higher than the control levels. These findings indicated that Sap-AgNPs modulated apoptosis-related pathways in a dose-dependent manner.

Discussion

The results of the laboratory tests on SCC-25 cells revealed that Sap-AgNPs had cytotoxic effects and decreased cell viability. The most significant factor affecting the anticancer effect is likely the nanoscale size of AgNPs, which promotes cellular internalization, the increase of reactive oxygen species (ROS), the activation of apoptosis, and mitochondrial dysfunction. Saponins have demonstrated synergistic cytotoxic properties with minimal effects on normal cells owing to their unique anti-inflammatory and anticancer properties. In addition, DLS and AFM studies confirmed successful internalization due to the spherical shape and narrow size distribution of the nanoparticles, which disrupt membranes and break DNA. This is consistent with previous studies [15].

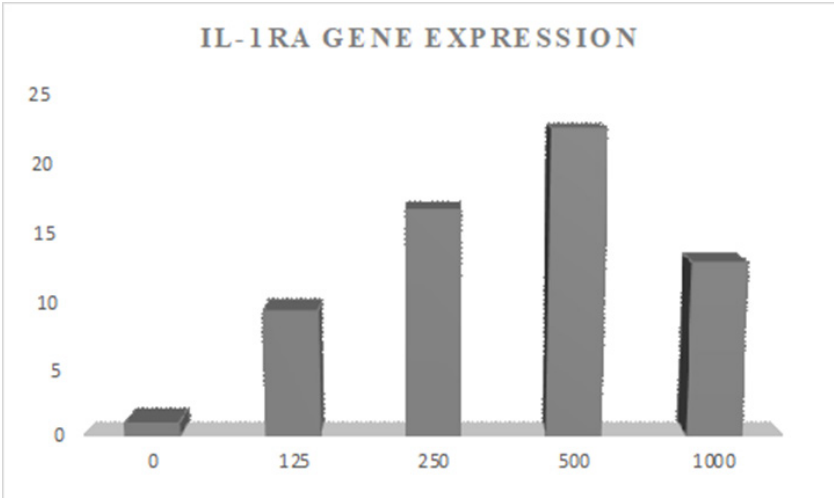

Figure 5. Expression of IL1RA in Oral Squamous Carcinoma Cell Line in Response to Exposure with Sap-Nanoparticles Comparison with Control.

The concentration-dependent detrimental effect of Sap-AgNPs was demonstrated by high-content screening (HCS), altered cell membrane permeability (CMP), increased total nuclear fluorescence intensity (TNI), and decreased viable cell count (VCC). Two of the most crucial components of apoptosis, nuclear condensation and DNA fragmentation, were significantly enhanced by 500 and 1000 µg/ml TNI. CMP analysis revealed concentration-dependent differences. While lower concentrations (125 µg/ml) caused minor membrane rupture without significantly decreasing cell viability, higher concentrations (500 µg/ml) caused significant damage, leading to apoptosis [16]. These results are consistent with the effect of AgNPs on the integrity of membranes and cytotoxicity induced by ROS.

Analysis of cell membrane permeability (CMP) revealed notable variations at 125 and 500 µg/ml; however, there were no discernible variations at 250 and 1000 µg/ml, indicating concentration-dependent variations in the manner in which the particles impacted the membrane. At high doses, damage may result in rapid cell death via apoptosis or necrosis, making permeability measures less sensitive. At low concentrations, subtle alterations in the cell membrane may occur without an immediate impact on cell viability.

This finding is consistent with previous research demonstrating that AgNPs can directly or indirectly affect cell membranes by generating reactive oxygen species (ROS), and the multi-parameter evaluation results verify that Sap-AgNPs cause apoptosis and cellular damage in SSC-25 cells by combining the nuclear and membrane pathways. The significance of selecting a suitable concentration to maximize therapeutic efficacy while reducing toxicity is further highlighted by the dose-response relationship [17].

Gene expression modification was performed to further explore the immunomodulatory effects of Sap-AgNPs. Treatment increased IL-1RA levels in SCC-25 cells in a dose-dependent manner, peaking at 500 µg/ml. IL-1RA, which opposes IL-1 receptors, is a significant anti-inflammatory mediator that reduces inflammation associated with tumor formation. The elevated IL-1RA levels at low to moderate doses suggest that Sap-AgNPs may improve the tumor microenvironment by reducing inflammatory signals and stopping the development of cancer cells. IL-1RA levels decreased slightly at the highest concentration (1000 µg/ml), which could be due to cytotoxic effects or negative feedback regulation. These findings are in line with earlier findings that saponins can affect pathways that regulate cell survival and death as well as modify inflammation [18].

The results showed that silver nanoparticles (Sap-AgNPs) have two distinct effects: direct cytotoxicity through apoptosis, oxidative stress, and immunomodulatory effects through upregulation. These results demonstrate the potential of saponin-assisted green dye-synthesized silver nanoparticles as multifunctional anticancer agents with improved selectivity and minimal systemic toxicity, which can modulate the inflammatory environment surrounding the tumor. Cancer cell survival (500 µg/ml) is the ideal concentration for balancing immunomodulation and cytotoxicity, making it a good target for preclinical research or potential therapeutic uses [19, 20].

In conclusions, this study showed that saponin-stabilized AgNPs had cytotoxic effects on SCC-25 oral cancer cells, causing a concentration-dependent decrease in the number of viable cells, changes in the permeability of the cell membrane, and an increase in the intensity of total nuclear fluorescence, which indicated the induction of apoptosis and cell damage. Concurrently, the expression of the apoptosis-associated IL-1RA gene was markedly elevated by saponin-stabilized AgNPs, indicating both cytotoxic and immunomodulatory effects. Additionally, the findings showed that targeted treatment of oral cancer using AgNPs is a potential approach. According to this study, nanoparticles offer promising qualities and may be utilized as a substitute for cancer medications that have unfavorable side effects. Nevertheless, further research is required to demonstrate its clinical effectiveness.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

Conflicts of interest

The authors declare no conflict of interest regarding this article.

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