



Solanum trilobatum leaf extract-derived silver nanoparticles downregulate the PI3K/AKT/mTOR signaling pathway and attenuate oral squamous cell carcinoma cell proliferation

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ABSTRACT

Introduction: Oral cancer's aggressive nature poses a significant health risk, demanding timely diagnosis and effective treatment. Despite progress in conventional therapies like chemotherapy and surgery, their limitations drive the exploration of alternative strategies. This study assessed *Solanum trilobatum*-derived silver nanoparticles (AgNPs) in impacting cancer cell cycles, inducing apoptosis, and modulating key pathways, including the phosphoinositide 3-kinase (PI3K)/Protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway, leveraging phytotherapy and nanotechnology—a promising frontier in cancer treatment.

Methods: AgNPs were synthesized through the reduction of ions and stabilized using aqueous leaf extracts of *S. trilobatum*. After the characterization of AgNPs, the mRNA Gene Expression and mitochondrial membrane potentials (MMPs) were assessed. DNA fragmentation was done and DNA pattern by gel documentation system was observed. The study also assessed the modulation of the PI3K/Akt/mTOR cascade, impacting tumor growth and proliferation.

Results: Biosynthesized AgNPs were characterized using UV-visible spectrophotometry (UV-vis), energy-dispersive X-ray spectroscopy (EDX), scanning electron microscopy (SEM), and Fourier-transform infrared (FTIR) spectroscopy. DNA fragmentation exhibited a typical ladder pattern. Dose-dependent changes in MMP were observed in the treated oral cancer cells. The effect of *S. trilobatum*-derived AgNPs in targeting the cell signaling pathway correlated significantly with their anticancer potency ($P < 0.001$).

Conclusion: This study reveals *S. trilobatum* leaf extract-based AgNPs as a natural cytostatic agent against oral squamous carcinoma. Utilizing nature's resources and nanoscale science, they hold promise for enhancing oral cancer treatment outcomes and survival rates.

Implication for health policy/practice/research/medical education:

The utilization of *Solanum trilobatum* leaf extract-derived AgNPs to downregulate the PI3K/AKT/mTOR signaling pathway showcases a promising avenue in combating oral squamous cell carcinoma (OSCC) cell proliferation. This innovative strategy holds potential for targeted OSCC therapies, hindering tumor progression and introducing new avenues for clinical interventions against oral cancer.

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Introduction

Metal nanoparticles have a wide varied range of applications in many fields mainly due to their promising and unique properties owing to their high surface

area to volume ratio and limited effort to surface functionalization (1). In recent years silver nanoparticles (AgNPs) have drawn considerable interest because of their unique properties. They are synthesized by various

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methods like chemical, physical, electrochemical, photochemical, and biological methods. Since most of these methods involve hazardous chemicals, they can lead to chemical toxicity and environmental contamination (2). Due to these detrimental effects, the focus is now on green chemistry and biological methods for the synthesis of AgNPs (AgNPs). Green chemistry concentrates on creating products and processes in chemistry and chemical engineering that reduce or remove the use of harmful substances. AgNPs combined with various plant extracts have undergone extensive experimentation due to their adaptability, organic composition, and multifaceted application in biomedicine (3).

Oral cancer is one of the most common forms of head and neck cancers and most of the cases of oral cancer globally are reported from south Asia. The most common variant of oral cancer is oral squamous cell carcinoma (OSCC), which comprises ~91% of all cases. The overall five-year survival rate has been below 50% in the past few decades and various treatment modalities are being tried and identified, albeit with numerous shortcomings. Numerous alternative treatment protocols have also been studied in recent years and natural biologically active compounds with potential health benefits like phytochemicals have shown promising results with reduced side effects and similar anticancer effects. Various mechanisms have been explored by phytochemicals in regulating epigenomics, targeting cancer stem cells, improving human immunity, promoting cancer cell apoptosis, cell cycle arrest, down regulation of cell cycle regulators, as well as enhancing tumor suppression (4,5).

The phosphoinositide 3-kinase (PI3K)/Protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway is an important pathway in carcinogenesis since it has central impacts in regulating metabolic pathway, cell proliferation, survival, invasion, angiogenesis, autophagy, gene expression, and protein synthesis. This pathway is reported to be dysregulated in various types of cancers, including oral cancer. The corroboration of preclinical and clinical studies has shown that inhibitors of PI3K/AKT/mTOR are future therapeutics in the treatment of various cancers and the encouraging outcomes have shown remarkable prospects in the future management of oral cancer (6).

Solanum trilobatum, belonging to the family Solanaceae, is a thorny creeper with a bluish violet flower, which is commonly available in Southern India and has been used to treat various diseases. It is a well-known medicinal herb, especially in India, where people often collect it from the wild for various home remedies. Additionally, it is sold as a fresh vegetable in local markets and is used as a food source. The plant is extensively studied for its various pharmacological activities like hepatoprotective, anti-inflammatory, anti-microbial, haemolytic, immunomodulation, and antibacterial activities. Various

chemical compounds have been identified in *Solanum* species, including flavonoids, sterols, saponins, alkaloids, phenolics, and glycosides. The secondary compounds of alkaloids such as soladunalinidine and tomatidine were isolated from the leaf and stem of *Solanum* species (7). Plants of the same family have been vastly elucidated for various therapeutic applications and phyto-insecticidal and cytotoxic properties. *Solanum trilobatum* contains saponins, which are types of steroids that show significant effects in inhibiting laryngeal cancer cells (HEP2 cell lines). In experiments conducted with rats, the introduction of Di Ethyl Nitrosamine increased the serum levels of liver marker enzymes like Aspartate Amino Transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH). However, the extracts from *S. trilobatum* were found to reduce these elevated enzyme levels, indicating its potential to counteract the effects of carcinogens (8). In the current literature, there are no available studies exploring the anticancer activity of *S. trilobatum* leaf extract on oral cancer.

The challenge is to identify effective, cost-effective, and sensitive lead molecules that have cell-targeted specificity and increase the sensitivity. Recently, AgNPs have been shown much interest because of their therapeutic applications in cancer as anticancer agents. The present study aims to investigate the AgNPs that were green synthesized from *S. trilobatum* against oral cancer cells and to identify their impacts on signaling AKT, mTOR, and PI3K pathways using real-time reverse transcription-polymerase chain reaction (RT-PCR) and the analysis of mitochondrial membrane potential (MMP) by using fluorescent rhodamine derivatives.

Materials and Methods

Biosynthesis of silver nanoparticles

Solanum trilobatum leaves were purchased from a utilized centre in Tenkasi, Tamil Nadu, India and were identified by a Professor of Botany at a government University in Tenkasi, Tamil Nadu. A voucher specimen (No. VSN: 4548) was deposited there. The procedure involved taking the leaves, washing them with tap water, and subsequently rinsing them with double distilled water (DDH₂O) before finely chopping them into small pieces. The leaves were dried in the shade and powdered. Ten grams of the powdered plant sample was added to 100 mL DDH₂O and bubbled at 80°C for 10 minutes. The gathered extract underwent filtration using Whatman no.1 filter paper, with the resulting filtrate collected in a 250 mL Erlenmeyer flask. The extractions were stored at room temperature to be used for further studies. Then, 50 mL of *S. trilobatum* leaf extract was added to 50 mL of 1 mM Silver nitrate (AgNO₃) solution at room temperature and the reduction of AgNPs was observed. Confirmation of AgNPs was obtained with the

color change to brown. These AgNPs after characterization were further used for RNA expression and MMP.

Cell culture maintenance

Oral cancer (KB) cell lines were procured from the cell repository of the National Centre for Cell Sciences (NCCS), Pune, India. Dulbecco's Modified Eagle Media (DMEM) was used for maintaining the cell line, which was supplemented with 10% fetal bovine serum (FBS). Penicillin (100 U/mL), and streptomycin (100 µg/mL) were added to the medium to prevent bacterial contamination. The medium with cell lines was maintained in a humidified environment with 5% CO₂ at 37 °C.

Mitochondrial membrane potential

Bhosle et al (9) described a method, which was used to evaluate the MMP using Rhodamine-123. Rhodamine-123 is a lipotropic cation dye that is explicit to mitochondria. The oral cancer cells treated with AgNPs were transferred into appropriate well plates and mixed with 30 µg/mL of phycoerythrin (PE), 20 µg/mL PE-AgNPs and 30 µg/mL of 5-fluorouracil (5-FU). The plates were maintained for 24 hours and then, Rh-123, a fluorescent dye (5 mmol /L), was supplemented to the cells and incubated for 30 minutes. The cells were then washed with Phosphate Buffered saline and visualized under a fluorescent microscope with a blue filter.

DNA fragmentation

Oral squamous carcinoma cells were incubated with AgNPs consisting of concentrations appropriate to their CC50 value. DNA extraction was done after 48 hours with a DNA isolation kit (Genei, Bangalore, India) and analyzed on 0.8% agarose gel using ethidium bromide to determine the DNA pattern by gel documentation system.

mRNA gene expression

RNA extraction was done from the oral cancer cell line by Trizol Reagent (Life Technologies, USA) per the procedure laid out by the manufacturer. RNA quantification was done with a NanoDrop ND1000 spectrophotometer

(NanoDrop Technologies, USA). Reverse transcription of RNA was done using primers at 42°C up to 50 min with 200 U/µL of M-MLV reverse transcriptase (Promega, USA). Amplification of the cDNA was done in PCR with Brilliant II Ultra-Fast SYBR Green qPCR Master Mix as per the instructions set in manufacturer-provided protocol with a Stratagene Mx-3000P Real-Time PCR System (Agilent Tech, USA). The PI3K/AKT/mTOR genes primer sequences used for the study are described in Table 1. The optimal concentration of the primers was determined initially for polymerase chain reaction assay and the resultant products were run on agarose gel (2%) to check the sizes.

Results

AgNP synthesis was successfully achieved using aqueous leaf extracts of *S. trilobatum*. After storage at room temperature, when 50 mL of 1 mM AgNO₃ solution was mixed with the leaf extract, a reduction in AgNP₃ was noted in less than 20 minutes.

Characterization of biosynthesized silver nanoparticles

UV-visible spectrophotometry (UV-vis) of the AgNP₃ solution presented a strong optical density peak at around 500 nm, which is the typical surface plasmon resonance peak of AgNPs and thus it confirms the formation of AgNPs synthesis. Fourier transform infrared (FTIR) analysis clearly showed that the participation of polyphenols available in the plant extract was mostly responsible for the biological reduction of silver ions (Ag⁺) into AgNPs (Ag⁰) along with other bio-compounds such as flavonoids, phenols, alcohols, aromatics, alkaloids, etc., which play a major role in stabilizing AgNPs. Scanning electron microscopy (SEM) analysis described that green synthesized Ag NPs were spherical shaped along with an average size of 20 nm. The energy dispersive X-ray (EDX) analysis of elemental composition exhibited a prominent signal solely from the silver (Ag) region, affirming the pure crystalline nature of the particles composed entirely of silver

Mitochondrial membrane potential

The control cells showed bright green fluorescence, which appealed to the well-being of the cell. In contrast, the MMP of cells depleted on treatment and was dose-dependent. A greater MMP depletion was noticed at 4 µg value of the complexes. Our result showed apoptotic and necrotic cells in the treated cell population when compared to the untreated controls indicating the activation of apoptotic cell receptors in oral carcinoma cells and arresting the life cycle because of the increased gene activation. Figure 1 shows loosely associated (rough) apoptotic cells being necrotic in the treated groups while the untreated groups clearly show tightly packed, smooth cells with clumped morphology.

Table 1. Real-time reverse transcription-polymerase chain reaction (RT-PCR) primer sequences for selected targets

Primer name	Primer sequence
ACT F	GATGATGATATCGCCGCGCT
ACT R	CCTCGTCGCCACATAGGAA
AKT F	GTGCTGGAGGACAATGACTACG
AKT R	AGCAGCCCTGAAAGCAAGGA
mTOR F	GTGAAACAGGACCCATGAA
mTOR R	CCATTCAGCCAGTCATCTT
PI3K F	GTATCCCGAGAAGCAGGATTTAG
PI3K R	CAGAGAGAGGATCTCGTGAGAA

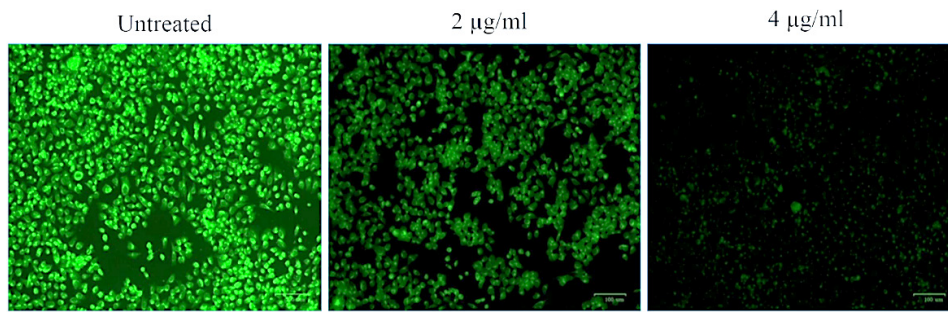


Figure 1. Effect of synthesized silver nanoparticles (AgNPs) on the mitochondrial membrane potential (MMP) by Rhodamine-123 staining. The control cells showed bright green fluorescence, indicating to the well-being of the cell. In contrast, the MMP of treated cells demonstrated a very minimal green fluorescence compared to the control cells showing apoptotic and necrotic cells in the treated cell population.

DNA fragmentation

DNA fragmentation clearly shows the DNA ladder pattern in oral cancer cells treated with AgNPs, which is believed to be due to cell death. Oral cancer cells treated for 24 h depicted a prominent DNA ladder on agarose gels (Figure 2). DNA laddering is a precise test used to assess cytogenetic damage. It is a visual indication seen during gel electrophoresis, showing the distinct pattern of DNA fragments formed due to apoptotic DNA fragmentation.

mRNA gene expression

RT-PCR analysis revealed the downregulation of PI3K/ Akt/mTOR when compared to the untreated control samples. When compared to the untreated (control) group, the protein expression of PI3K, Akt, and mTOR was decreased in the AgNPs groups (Figure 3). The changes in the gene expression are noticeable with the AgNPs level

of 3.7 µg/mL. The results indicate the activation of PI3K/ AKT/mTOR pathway.

Discussion

In recent years, the synthesis of AgNPs has evolved through various methods such as chemical reduction, radiation, hybrid processes, photochemical reduction, sono-electrochemical, micro-emulsions, microwave-based systems, and notably, green synthesis routes. While these physiochemical methods exhibit durability and technical feasibility, their large-scale application is constrained due to the use of hazardous chemicals, elevated costs, high energy consumption, and time-intensive procedures. The emergence of green synthesis and utilizing non-toxic chemicals have significant progress in economical and environmentally safe AgNP production (10). This approach represents an eco-friendly

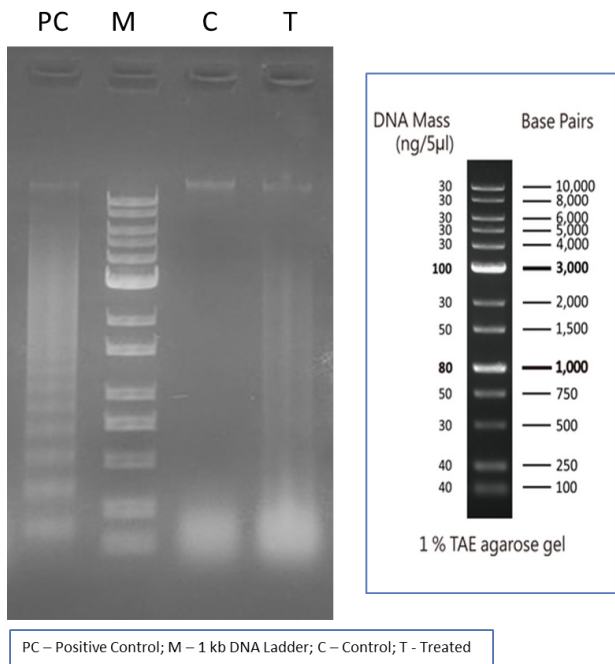


Figure 2. Synthesized-AgNPs-induced DNA fragmentation. DNA fragmentation by agarose gel electrophoresis clearly shows the DNA ladder pattern in oral cancer cells treated with AgNPs (silver nanoparticles).

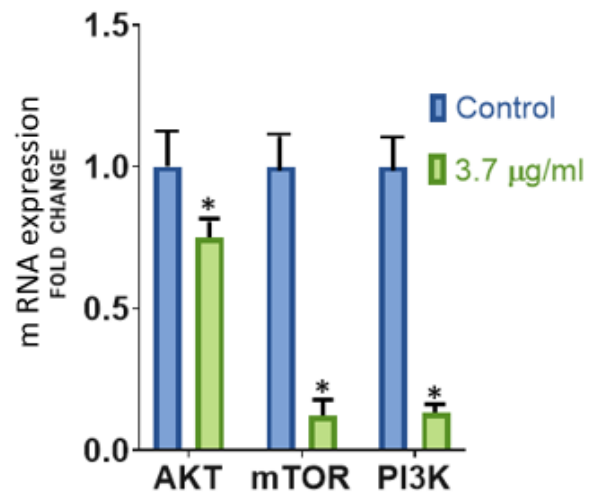


Figure 3. The expression pattern of cell signaling proteins (phosphoinositide 3-kinase (PI3K)/ Protein kinase B (AKT)/mammalian target of rapamycin (mTOR)) analyzed by RT-PCR. RT-PCR analysis showed that the synthesized AgNPs induced a decrease in the protein expression of PI3K, Akt, and mTOR compared to the control group. The figure shows a noticeable decrease with an AgNPs level of 3.7 µg/mL. The bars represent mean ± standard deviation. *P < 0.001 versus control group.

paradigm shift in chemistry, aiming to eliminate toxic waste, reduce energy consumption, and employ ecological solvents. The synthesis of AgNPs involves the reduction of silver ions (Ag⁺) from AgNO₃, facilitated by the extracts containing chemicals capable of this reduction. Typically conducted at high temperatures, this process triggers a visual change from yellow to dark brown due to surface Plasmon vibration, a unique trait of noble metals. Characterization of these nanoparticles post-synthesis is crucial, as their physicochemical properties significantly influence their biological behaviors. Parameters such as size, shape, distribution, surface area, solubility, and aggregation must be evaluated before assessing their toxicity or compatibility in biological systems (11,12).

Green synthesis minimizes hazardous byproducts by utilizing natural capping agents for stabilization. Furthermore, leveraging microorganisms, plants, and algae for green synthesis proves a natural, biocompatible, and environmentally safe paradigm. The mechanisms involving plant extracts containing functional molecules like phenols, terpenoids, ketones, enzymes, and flavones contribute to the reduction of metal ions, showcasing the potential of biological means for AgNPs production (13,14). Controlled structures with uniform size, morphology, and functionality in AgNPs are pivotal for diverse biomedical applications. Our study focuses on *S. trilobatum*, abundant in Tamil Nadu, investigating its anti-cancer properties via the inhibition of the PI3K/AKT/mTOR pathway against OSCC growth.

Cancer originates from genetic alterations disrupting normal cell function. Mitochondria play a pivotal role in cell viability, and changes in their membrane potential serve as indicators of cellular health. Induction of mitochondrial permeability transition leads to cell death through the loss of MMP, which is irreversible and crucial in cancer research. The fluorescent dyes that are cationic are generally used to assess MMP. In our study, we used Rhodamine-123 to determine the changes in the mitochondrial membrane in AgNPs-treated oral squamous carcinoma cells. Various other cell-permeable fluorescent dyes are available such as 3',3'-dihexyloxycarbocyanine iodide, tetraethyl rhodamine methyl, rhodamine-123, and ethyl esters (15). MMP was analyzed after the absorption of the fluorescence dye Rhodamine 123 (16,17). Mitochondrial oxygen consumption was measured to evaluate the effect of AgNP on mitochondrial function. Rhodamine 123 dye interferes with the inherent pathway of the cancer cell cycle. The dye binds to the damaged mitochondrial membrane and gets attached to the damaged cells. Suppressive genes are activated in the cancer cells and decrease their production. Apoptosis is indicated when the Rhodamine 123 dye enters the mitochondrial membrane after which an intracellular leakage occurs. Our result has shown apoptotic and necrotic cells when compared to the untreated controls indicating the activation of apoptotic cell receptors in oral

carcinoma cells and arresting the life cycle because of the increased gene activation. The results closely match the previously reported findings by Gandhi et al (18).

DNA fragmentation is the separation or breaking of DNA strands into pieces. High ROS levels lead to chromatin dysfunction like DNA fragmentation leading to cell death through necrosis or apoptosis. ROS-mediated DNA fragmentation is accelerated by hydroperoxides through lipid peroxidation. The most common method to analyse DNA fragmentation is gel electrophoresis, which helps to detect apoptosis and differentiate it from necrosis, which never causes DNA laddering. DNA damage in cells is also assumed to be due to alteration in replication and various reports are available to show alteration in biochemical and molecular changes due to green synthesis of AgNPs (19). Laddering DNA is a well-known method to determine the endonuclease cleavage products of apoptosis. Reactive oxygen species are considered to act as a signal to promote molecules' cell-cycle progression and to induce DNA damage due to oxidative stress (20). Apoptosis can be determined by (1) irregularity in cell size, and (2) DNA fragmentation (21). To confirm the apoptosis induction by AgNPs-treated oral cancer cells, a DNA fragmentation analysis was performed, which depicted the DNA ladder pattern in oral cancer cells treated with AgNPs, which could be the reason for cell death. Amirghofran et al showed a DNA ladder pattern in treated cells, which was detected after its exposure to Jurkat and K562 cells (22). Studies have confirmed the dose-dependent DNA damage. Studies have also shown that caspase-3, a key player in apoptosis, is known to cleave both cytoplasmic and nuclear substrates, ultimately leading to cell death by inducing DNA damage. The upregulation of the gene is appreciated in the cancer cells treated with AgNPs that activate apoptosis (23,24).

It is well known that apoptosis promotes fragmentation of DNA in a characteristic oligonucleosomal fragment in multiples of 250 base pairs. Oral cancer cells treated for 24 hours depicted a prominent DNA ladder on agarose gels in our study. However, the DNA ladder intensity was based on the dose and incubation time (25). The DNA ladder fragmentations and morphologic changes indicate the cytotoxic effect of the extract that mediates to induce apoptosis of cancer cells (26). Therefore, the DNA ladder formation indicates the cytotoxic effect of AgNPs causing inhibited growth in the oral cancer cells through apoptosis. A study was previously reported, which had similar results of using AgNPs in gastric cancer cells and OSCC cell lines that demonstrated apoptosis in response to the nanoparticles (27). It is believed that inhibited cell proliferation and apoptosis induction are processes that regulate cell survival or death based on physiological stress. The pattern of banding indicates the presence of DNA shearing in the cells treated with AgNPs that relates to apoptosis induction. Putative transcripts from *S.*

trilobatum leaf extracts have previously been reported to study the molecular basis (28).

PI3K/Akt/mTOR is a major signaling pathway that plays important role in intracellular functions. It is the contributors of physiological and pathological processes. PI3Ks belong to the lipid kinases family that phosphorylate the 3'hydroxyl group in phosphoinositide and phosphatidylinositol. Akt is a protein kinase that is serine/threonine-specific and is called protein kinase B. mTOR is also a serine-threonine kinase that belongs to phosphatidylinositol kinase-related kinase. In most cancer types the activation of PI3K/Akt/mTOR signaling pathway with elevated expression of mTOR has been shown. The pathway is responsible for regulating cell growth, proliferation, cell size, motility, and metabolism. The majority of the studies previously reported have shown an increase in Akt expression from about 50% to 70% because of the PI3K/Akt/mTOR signaling (29). Drug targets for cancer are majorly developed to target this signaling pathway though there is limited progress in achieving the target drug due to the redundant ways of generating the monotherapies. Comments of developing novel molecular mechanisms to target PI3K, Akt, and mTOR are of the current needs to achieve tumor growth inhibition. The Akt protein serves as a substrate, playing a crucial role in mediating cell multiplication. It is actively involved in the progression of the cell cycle, contributing to the corresponding advancement of cell growth. The modified forms of PI3K/Akt signaling cascade are altered in various human cancers. Thus, Akt protein is central to allowing the proliferation and survival of cancer cells, along with the downstream protein mTOR, which plays a crucial role in cellular processes. Involvement of the Akt/mTOR pathway is widely found in OSCC (30). However, PI3K3 has three lip kinases, with class IA PI3Ks, which most frequently gets altered in cancer subjects. Tyrosine kinase receptor activates PI3K and the activated PI3K subsequently activates the Akt further. The activated Akt phosphorylates downstream PDK1 and mTOR that activates the transcription factors, supporting the cell survival, proliferation, and growth. Factors that contribute to the activation of PI3K/AKT/mTOR signaling pathways are epidermal growth factor (EGF), insulin, calmodulin (CaM), and insulin-like growth factor 1 (IGF-1). In cancers, it is believed that the PI3K/AKT/mTOR pathway is highly active, which thus reduces apoptosis but promotes cell proliferation. While various inhibitors of the signaling pathway are under evaluation for both animal and human studies, the current demand is to achieve a cost-effective, and lesser side-effect compound that can be used safely. A recent study showed that AgNPs induce HiF-1 α activation, and ultimately activate autophagy via the AMPK-mTOR pathway in prostate cancer cells (31). Similarly, in another study, ROS-independent autophagy was induced through

the AMPK-mTOR signaling pathway by using sublethal doses of AgNPs. Therefore, it is strongly believed that the downregulation of PI3K/Akt/mTOR leads to the activation of autophagy that subsequently inhibits cell proliferation. Our experiment aimed to confirm whether the PI3K/Akt/mTOR signaling pathway had a major role in apoptosis influenced by AgNPs. RT-PCR analysis revealed the downregulation of PI3K/Akt/mTOR when compared to the untreated control samples. When compared to the untreated (control) group, the protein expression of PI3K, Akt, and mTOR was decreased in the AgNPs groups indicating the activation of the PI3K/AKT/mTOR pathway.

The effects of *S. trilobatum*-derived AgNPs in arresting the cell signaling pathway directly corresponds to its anticancer property and the present study has reported the downregulation of PI3K/Akt/mTOR signaling pathway by the green synthesized AgNPs from *S. trilobatum*, which is predominantly available in southern part of India.

Conclusion

In this study, *S. trilobatum* leaf extract was used for green synthesis of AgNPs. Different characterization techniques were used for the confirmation and analysis of synthesized AgNPs. The results clearly showed AgNPs had significant cytotoxicity on oral cancer cells, which blocks the cell cycle and eventually induces apoptosis through the mitochondrial pathway. AgNPs were also capable of inhibiting cell proliferation, arresting cell cycle, and generating apoptosis by downregulation of the PI3K/Akt/mTOR pathway. In the future, AgNPs should be subjected to extensive research, including in animal models to gain more insights into the toxicity/biocompatibility profile of the nanoparticle. It may be concluded that the advancement in nanotechnology would be safe, reliable, and accurate by giving better results in cancer therapeutics than conventional treatments. In the long run, investigating the biocompatibility of AgNPs and understanding how they interact with cells and tissues is crucial to guarantee their safe use in humans.

Collectively, the results of this study provide novel insights to discover the underlying mechanisms and overlay a strong foundation to develop safe therapeutic applications of AgNPs to encounter cancer. Our data confirms the potential of *S. trilobatum* as a natural therapeutic agent with cytostatic activity against human oral squamous carcinoma. The data suggests that the extract from the leaves of the plant may hold anticancer properties, and therefore is of valuable source for application in drug products.

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Supervision: Gautham Kumar N.

Validation: Anuradha G.

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Writing-original draft: Gautham Kumar N.

Writing-Review and editing: Anuradha Ganesan, Gautham Kumar N and Prabhu Manickam Natarajan.

Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

This research did not entail the utilization of animal or human subjects. The authors have diligently adhered to ethical standards, ensuring the avoidance of plagiarism, misconduct, data fabrication, falsification, double publication, or redundancy.

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