RESEARCH ARTICLE

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Anti- Cancer Potential of Diosgenin, a Steroidal Saponin, against Human Oral Cancer Cells

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Abstract

Objective: Oral squamous cell carcinoma (OSCC), affecting the lip and oral cavity, is one of the most prevalent cancers worldwide with the highest morbidity rate in the North-Eastern region of India. The current treatment options including surgery and chemotherapy are plagued by adverse side-effects and emergence of chemo-resistance, particularly against drugs like cisplatin and 5-fluorouracil. The present study focuses on demonstrating the effects of diosgenin, a naturally occurring steroidal saponin, on the survival, proliferation, and migration of OSCC cells in vitro. **Methods:** Preliminary *in vitro* screening of diosgenin in OSCC cells was performed using MTT, colony formation, cell cycle arrest, PI-FACS, live/dead, and wound-healing assays. In addition, the potential of diosgenin in regulating the expression of critical proteins involved in OSCC was evaluated using western blot analysis. **Result:** The present study shows that diosgenin exhibited selective anti-proliferative activity on OSCC cell lines SAS and HSC3 compared to normal kidney cell line HEK-293T. In addition, it enhances the chemosensitivity of SAS cells to cisplatin and 5-FU. This compound also displayed anti-clonogenic, cell cycle arrest, cytotoxic, and anti-migratory effects on SAS cells in a dose-dependent manner. Further, diosgenin regulated the expressions of *COX-2, CXCR-4, VEGF, TWIST-1, p-AKT, and AKT*, which are critical proteins for the development and progression of OSCC. **Conclusion:** These findings support the therapeutic potential of diosgenin, thereby opening a new avenue for oral cancer therapy. Nonetheless, the data needs to be further validated in in vivo and clinical settings.

Keywords: OSCC- diosgenin- chemosensitivity- cisplatin- 5-fluorouracil

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Introduction

Oral cancer refers to malignant neoplasms that develop on the lips or within the oral cavity [1]. Since 90% of malignant tumors in dentistry originate from squamous cells, it is most commonly referred to as oral squamous cell carcinoma (OSCC) [2]. Globally, oral cancer is one of the most common types of cancer, with nearly one-third of all cases occurring in India. Additionally, India holds the second-highest number of OSCC cases among countries globally [3]. The buccal mucosa accounts for about 32% of oral cancer cases, followed by the tongue (22%), lower lip (11%), palate (11%), vestibule (8%), alveolus (5%), floor of the mouth (5%), and gingiva (3%) [4].

Oral carcinogenesis is an intricate and multifactorial process involving both genetic and environmental risk factors [5]. It occurs when various genetic mutations in epithelial cells lead to the accumulation of genetic and epigenetic alterations in oncogenes and/or tumor suppressor genes [6]. Two widely established risk factors that are independent of one another are alcohol consumption and tobacco products for oral cancer and oral potentially malignant disorders [7]. Tobacco, alcohol usage or human papillomavirus infections can result in genetic alterations which trigger the stromal cell transformation, immunological suppression and persistent inflammation [8]. Currently, available treatments for OSCC include surgery, radiation therapy, and chemotherapy or a combination of these, based on pathological findings and disease grade. However, these treatment modalities often come with adverse side-effects, development of chemo-resistance, and sometimes even recurrence [9]. Thus, the need arises to identify natural compounds that are safe, efficacious and cost-effective for the treatment of OSCC.

The utilization of natural products, particularly steroid compounds, has been gaining increasing attention, both as therapeutic agents and as lead compounds in drug development approaches [10]. Amongst these compounds, diosgenin (3-hydroxy-5-spirostene) is a well-known

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steroidal sapogenin, which is obtained by hydrolysis of the saponin dioscin. Diosgenin, a phytosterol saponin, is a substantial bioactive component present in the roots of wild yam (*Dioscorea villosa*) and the seeds of fenugreek (*T. foenum-graecum*) [11]. Various ethnic communities living in North-East India have incorporated yams into their diet and medicinal practices [12]. Several studies have showed that the tubers of Dioscorea are utilized for addressing a range of health issues including fever, headache, dysentery [13], gastrointestinal disorders, abdominal discomfort, wounds, cough, among others [14, 15]. Various plant species, such as *Costus speciosus, Smilax menispermoidea,* species of *Paris, Aletris, Trillium,* and *Dioscorea*, contain high concentrations of diosgenin [16]. According to Nguyen et al. [17], sapogenins are a class of compounds commonly found in natural products in their glycoside form and contribute to overall health. Steroidal sapogenins, also known as spirostans, are among the most potent bioactive substances derived from natural sources. Most steroidal sapogenins exhibit anti-cancer activity *in vitro* and in preclinical animal models, along with a broad range of pharmacological properties. Numerous studies have demonstrated diosgenin's diverse biological activities, including cholesterol-lowering, anti-inflammatory, antiproliferative, blood sugar-lowering, and potent antioxidant effects [18]. In 2010, Jung et al. observed that murine macrophages produced reduced levels of inflammatory chemicals, including nitric oxide and interleukins 1 and 6 when pre-exposed to diosgenin and then stimulated with lipopolysaccharide/interferon-γ [19]. Diosgenin has also demonstrated strong inhibition of continuously active inflammatory and survival-promoting signaling pathways in various cancer cells, leading to apoptosis [20]. Additionally, it has been noted that diosgenin prevents Tumor Necrosis Factor-alpha (TNF-α)-induced Nuclear Factor Kappa B (NF-κB) activation and hinders osteoclast formation in RAW 264.7 macrophage cells [21]. Since, diosgenin has already displayed a wide range of biological activities, this study aimed to evaluate the anti-cancer activity of this compound against OSCC cells.

Materials and Methods

Reagents

Diosgenin was obtained from TCI, Japan. 5-Fluorouracil (5-FU) and Cisplatin were obtained from Sigma Aldrich, USA. A 50 mM stock solution of diosgenin was prepared in dimethyl sulfoxide (DMSO) (Merck Life Sciences Pvt. Ltd.). 10 mM stock of 5-FU was prepared in sterile MilliQ water and 100 mM cisplatin stock was prepared in saline water. Penicillin-streptomycin (Penstrep), Dulbecco's minimum essential medium (DMEM), and fetal bovine serum (FBS) were sourced from Gibco, USA. MTT and propidium iodide (PI) were purchased from Sigma Aldrich, USA. Antibodies against *AKT, pAKT, COX-2, CXCR-4, VEGF,* and *TWIST-1* were acquired from Cell Signaling Technologies, USA. The live and dead assay kit was obtained from Invitrogen, USA. Bovine serum albumin (BSA) and crystal violet was obtained from HiMedia, India and SRL Pvt. Ltd., India. Prestained marker, Bradford reagent, and Clarity Western ECL Substrate was purchased from BioRad Laboratories.

Cell lines

Human OSCC cells, SAS was obtained from Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, India. HSC3 cell line was generously provided by Dr. Renu Wadhwa from the National Institute of Advanced Industrial Science and Technology (AIST), Japan and human normal embryonic kidney cells, HEK-293T was gifted by Dr. Suresh Kumar Rayala, Indian Institute of Technology Madras, India. The cells were cultured in DMEM, supplemented with 10% FBS and 1% Penstrep and incubated at 37°C in a humidified chamber with 5% CO2 until reaching 60% to 70% confluence.

Cell proliferation assay

The effect of diosgenin on OSCC cells was evaluated using an MTT assay. Briefly, $2x10^3$ cells per well were plated in 96-well plates and incubated for 18–24 hours at 37°C. They were then treated with diosgenin at concentrations of 0, 10, 25, 50, 75 and 100 µM and incubated for 0 and 72 hours. Each well received 10 µl of a 5 mg/ml MTT solution and was incubated for 2 hours. After removing the growth media, 100 µl of DMSO was added to each well to dissolve the MTT-formazan product, which was then incubated for 1 hour at room temperature. Absorbance of the colored solution was measured at 570 nm using a microplate reader (TECAN Infinite 200 PRO multimode reader). The percentage of cell proliferation, indicating the growth inhibition caused by diosgenin, was assessed for SAS, HSC3, and HEK-293T cells.

Similarly, *in vitro* drug sensitivity of Cisplatin and 5-FU was also assessed by MTT assay. To investigate the effect of diosgenin in combination with Cisplatin and 5-FU, SAS cells were treated with different concentrations of diosgenin (15 and 30 µM), Cisplatin (150 and 200 µM), 5-FU (1 and 2 µM) and in combination – Diosgenin:Cisplatin (15µM:150µM, 15µM:200µM, 30µM:150µM, 30µM:200µM), Diosgenin:5FU (15µM:1µM, 15µM:2µM, 30µM:1µM, 30µM:2µM), for 72 hours.

The percentage of cell proliferation was obtained by using the following equation [22]:

$$\% \text{ proliferation} = \frac{\text{A570 of treated cells}}{\text{A570 of untreated cells}} \times 100$$

Colony formation assay

The clonogenic capacity of OSCC cells treated with diosgenin was assessed using a colony formation assay. In brief, SAS cells were plated at a low density (approximately $5x10^2$ cells per well) in 6-well plates and treated with diosgenin for 24 hours at specified concentrations (0, 5, 50 µM). After treatment, the medium was replaced with fresh medium, and the cells were incubated for 10 days. Following this, the plates were fixed with 70% ethanol and stained with 0.5% crystal violet. The survival fraction was determined by quantifying images of each well using ImageJ software.

Cell cycle analysis by flow cytometry

For measurement of cellular DNA content, flow cytometric analysis was performed. Briefly, the cells were plated in 6-well plates in the concentration of 1×10^5 cells per well and allowed to incubate overnight. Cells were exposed to different concentrations of diosgenin (0, 20, and 40 µM). After drug treatment, both adhered and suspended cells were harvested, washed with PBS and fixed in 70% ethanol at -20˚C overnight. Cells were washed twice by ice cold PBS and incubated in PI-RNase solution at room temperature for 30 min in dark. The cell cycle stages in drug-treated groups in comparison with control were measured by flow cytometric analysis (Becton- Dickinson, San Jose, CA). The percentage of cells residing in different phases of cell cycle was determined by fitting the model using FCS Express software.

PI- flow cytometric assay

PI, a fluorescent dye that binds to nucleic acids and produces red fluorescence, was employed in flow cytometry to assess cell viability [23]. SAS cells were plated in 6-well dishes with a density of $1x10^5$ cells per well, allowed to incubate for 24 hours, and subsequently treated with specified concentrations of diosgenin (0, 25, 50, and 100 µM) for 72 hours. Following treatment, all cells were collected, rinsed with PBS, and exposed to 5 µl of 1 mg/ml PI. After a 10-minute incubation, the impact of diosgenin on the cells was examined using flow cytometric analysis (Becton- Dickinson, San Jose, CA). Viable cells, characterized by intact cell membranes, exhibit low PI permeability and emit minimal fluorescence, while dead cells display heightened red fluorescence due to damaged plasma membranes.

Live/Dead assay

To assess diosgenin induced cytotoxicity, a live/dead assay was conducted to evaluate intracellular esterase activity and plasma membrane integrity. Calcein-AM, a non-fluorescent polyanionic dye, is retained by viable cells, leading to intense green fluorescence upon enzymatic (esterase) conversion. Additionally, ethidium homodimer enters the cells with damaged membranes, binds to nucleic acids and generates bright red fluorescence in dead cells [24]. Briefly, $2x10^3$ cells per well were incubated to specified concentrations of diosgenin (0, 50 and 75 µM) for 48 hours at 37°C. Subsequently, cells were stained with 10 µl live/dead reagent and incubated at 37°C for 20 minutes. Cells were then analysed using an inverted fluorescence microscope (Olympus, Japan).

Scratch wound healing assay

This assay is conducted to assess cell migration by creating an artificial gap in a confluent cell monolayer, and then monitoring the migration of cells from the edges over time [25]. The extent and rate of scratch wound healing reflect the migratory capabilities of the cells. A total of $2x10^5$ cells per well were seeded in 6-well plates and cultured until a confluent monolayer (95%) was established. After monolayer formation, the medium was replaced with serum-free medium, and the cells were incubated for 8 hours. Subsequently, the medium was aspirated, and a 200 µl pipette tip was used to create a wound in the midline of the culture well. Following this, cells were washed twice with PBS to eliminate any debris. The cells were then exposed to specified concentrations of diosgenin (0, 30, and 50 µM). Migration was assessed by observing changes in the scratch wound area using an inverted microscope (Nikon T1-SM, Japan). Images were captured at 0 and 24 hours and analyzed using ImageJ software.

Western blot analysis

Western blotting was performed to investigate the effect of diosgenin in regulating the expression level of different proteins involved in the development and progression of OSCC. SAS cells were seeded in 6- well plates ($2x10^5$ cells per well) and following overnight incubation, the cells were treated with indicated concentrations of diosgenin (0, 30, 50, and 75 µM) for 24 hours. The cells were lysed in ice cold lysis buffer. The cell lysates were centrifuged at 13000 rpm for 10 minutes to recover the supernatant. The supernatant containing proteins was taken as the cell extract and the protein concentration was quantified using Bradford assay. The extract containing equal amount of protein were subjected to 10% Sodium Dodecyl Sulphate –Polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were electro-transferred to a nitrocellulose membrane and protein bands were visualized by staining with Ponceau S Stain. The membrane was incubated in blocking buffer containing 5% milk for 2 hours, washed with 1xTBST three times and then probed with primary antibody overnight at 4˚C. Afterwards, membranes were washed in TBST three times, and then with an appropriate peroxidase- conjugated secondary antibody at room temperature for 1 hour. The expression of proteins was detected by enhanced chemiluminescent (ECL) reagent [26].

Statistical analysis

Statistical analyses were performed using Student's t-test. Results are presented as mean ± standard deviation (SD) with a p-value of < 0.05 vs. control, considered statistically significant. All analyses were conducted and graphs were plotted using Microsoft Office Excel Version 2015.

Results

Anti-proliferative effect of diosgenin on SAS and HSC3 cells

We first examined whether diosgenin affected the cell proliferation of OSCC cells, SAS and HSC3, and human embryonic kidney epithelial cells, HEK-293T, using the MTT assay. The results demonstrated that treatment with diosgenin at concentrations below 25 µM for 72 hours did not significantly affect the proliferation of SAS and HSC3 cells. However, at 100 µM, diosgenin effectively reduced SAS cell proliferation, indicating a strong cytotoxic effect (Figure 1). The IC_{50} value for SAS cells was determined to be 31.7 µM. Similarly, diosgenin inhibited the proliferation of HSC3 cells with an IC_{50} value of 61 µM.

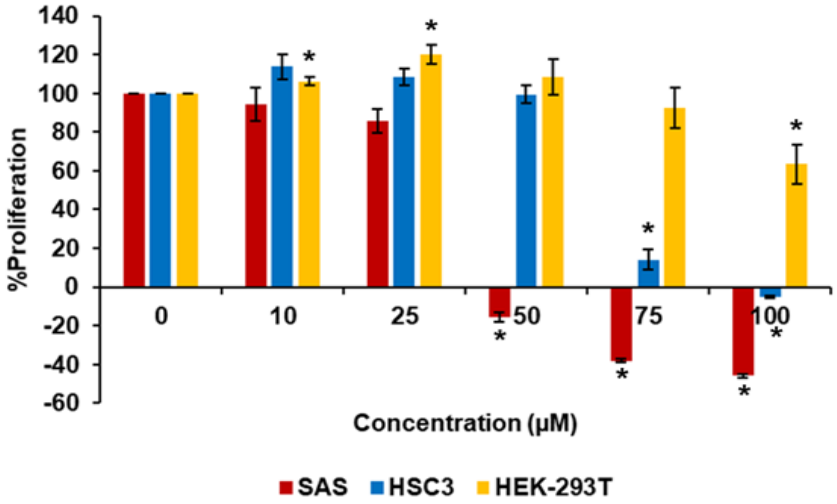

Figure 1. Effect of Diosgenin on the Proliferation of SAS, HSC3, and HEK-293T Cells Using MTT Assay. Cells were treated with 0, 10, 25, 50, 75 and 100 μM diosgenin for 72 hours. %Proliferation for control was taken as 100%. Data is presented as mean ± SD, *p < 0.05 vs. control.

However, diosgenin did not cause significant cytotoxicity in normal human embryonic kidney epithelial cells (HEK-293T), suggesting a selective effect on cancer cells. As diosgenin demonstrated stronger cytotoxic effect and more effective inhibition of cell proliferation in SAS cells than in HSC3 cells, all further experiments were conducted using the SAS cell line.

Chemosensitizing effect of diosgenin in combination with 5-FU and cisplatin on SAS cells

The proliferation of the SAS cell was significantly decreased by diosgenin when treated in combined doses of cisplatin and 5-FU (Figure 2 a and b). The data indicated that treatment with diosgenin and 5-FU at a dose of 30 μM and 2 μM respectively and diosgenin and cisplatin at a dose of 30 μM and 200 μM respectively, caused higher cytotoxicity to SAS cells. Treatment of SAS cells with diosgenin shows enhanced cytotoxicity when combined with chemotherapeutic drugs 5-FU and cisplatin as compared to when it is used alone.

Effect of diosgenin on the clonogenic property of SAS cells

To investigate the effect of diosgenin on the clonogenic potential of SAS cells, a colony formation assay was performed. After exposure to different concentrations of diosgenin, the colony-forming ability of both SAS cells was suppressed in a dose-dependent manner (Figure 3). These results revealed that diosgenin reduced the clonogenic potential of SAS cells in dose dependent manner.

Effect of diosgenin in inducing cell cycle arrest in SAS cells

To evaluate the effect of diosgenin on the phases of the cell cycle of SAS cells, flow cytometry-based cell cycle

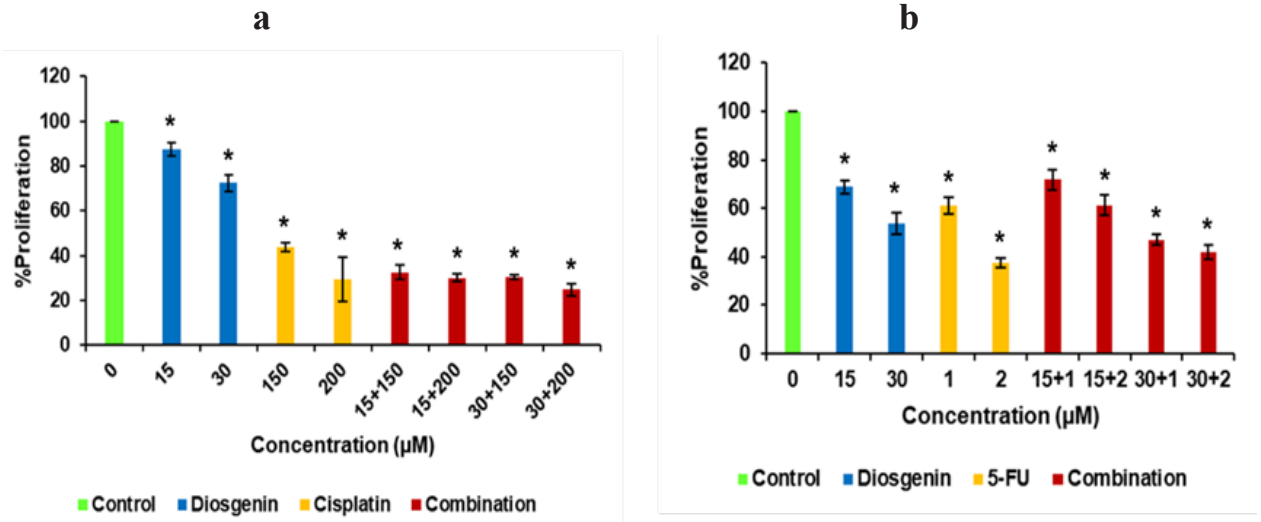

Figure 2. Effect of Diosgenin on the Proliferation of SAS Cells in Combination with (a) Cisplatin, (b) 5-FU. Cells were treated with diosgenin (15 and 30 μM), Cisplatin (150 and 200 μM), 5-FU (1 and 2 μM) and in combination – diosgenin:Cisplatin (15μM:150μM, 15μM:200μM, 30μM:150μM, 30μM:200μM), diosgenin:5FU (15μM:1μM, 15μM:2μM, 30μM:1μM, 30μM:2μM), for 72 hours. %Proliferation for control was taken as 100%. Data is presented as mean ± SD, *p < 0.05 vs control.

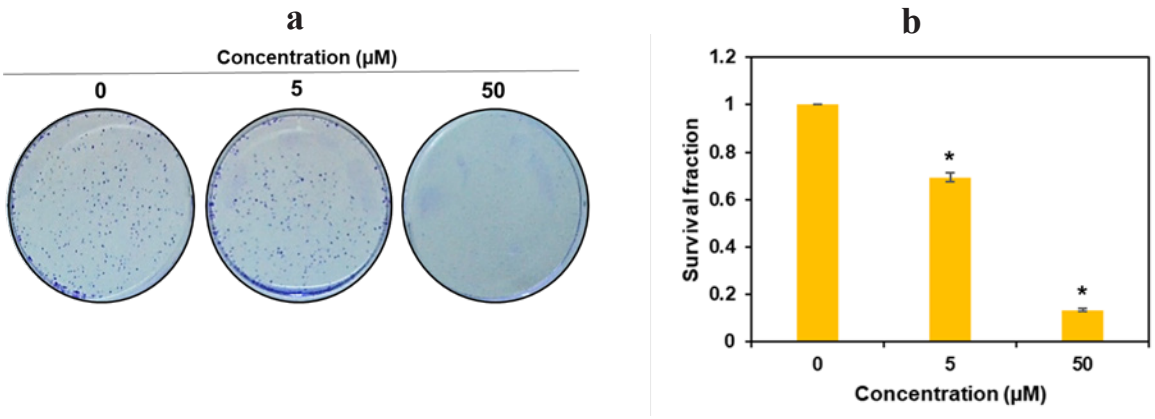

Figure 3. Effect of Diosgenin on the Clonogenic Property of SAS Cells. (a) SAS cells were treated with diosgenin: 0, 5, 50 µM for 24 hours and monitored for up to 10 days for colony formation; (b) Survival fraction vs control for SAS cells were plotted. Data is presented as mean ± SD, *p < 0.05 vs control.

analysis was performed at two different doses – 20 and 40 µM. The results showed that diosgenin induced cell cycle arrest at the S-phase of the cell cycle which led to an increase in the S-phase subpopulation and a decrease in the G1 and G2/M phase subpopulation (Figure 4). The effect was more pronounced at 40 µM concentration.

Effect of diosgenin in inducing cell death in SAS cells

In order to evaluate the potential of diosgenin in inducing cell death in SAS cells, PI-FACS analysis was performed. It was observed that disogenin exhibited a dose dependent cytotoxicity, with more than 50 % cell death at 100 µM concentration (Figure 5 a and b). The same has been visually evident from the live/dead staining assay, where diosgenin treatment for 48 hours caused a dose-dependent toxic effect on SAS cells (Figure 5 c).

Effect of diosgenin in inhibiting the migration of SAS cells

Diosgenin was observed to inhibit the migration of SAS cells into the denuded area at a concentration of 30 and 50 µM after 24 hours of incubation. These results indicated that diosgenin significantly reduced the motility of SAS cells at 50 µM (Figure 6).

Effect of diosgenin in regulating the expression of different proteins involved in SAS cells

In the cells treated with diosgenin, downregulation of the following proteins was observed: *COX-2, CXCR-4, VEGF, TWIST-1, p-AKT,* and *AKT* (As shown in Figure 7). These proteins are involved in the major pathways regulating cell growth and proliferation.

Discussion

Diosgenin is a well-known steroidal sapogenin that has been used in the treatment of cancers, inflammation, and infections due to its wide range of biological activities and medicinal properties [27, 28, 11]. It expresses by acting on different target proteins such as *CxCr4, COX-2, EF-kB, ERK, JNK, AKT,* etc. which regulate important processes in cancer cells like survival, invasion, proliferation, metastasis, chemoresistance, and cell death [19].

Notably, this is the first report to describe the anti-cancer potential of diosgenin against OSCC. Our findings demonstrated the efficacy of this steroidal sapogenin in influencing the viability, clonogenic potential, and migration of OSCC cells, potentially through the modulation of *AKT, COX-2,* and *CXCR4*-regulated gene products, which are pivotal in regulating various cell

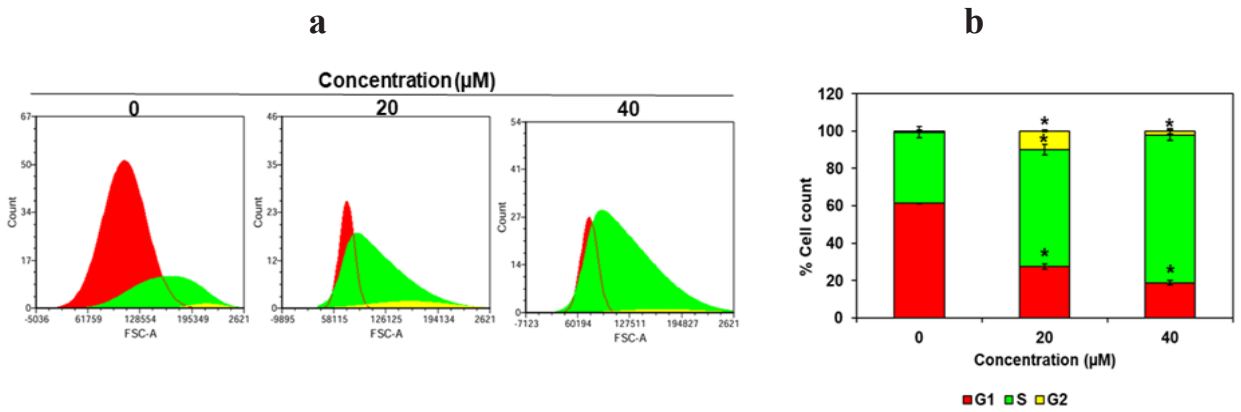

Figure 4. Diosgenin Induced Cell Cycle Arrest in SAS Cells. (a) SAS cells were treated with 0, 20, and 40 µM diosgenin for 48 hours followed by flow cytometric analysis. (b) % cell count vs. Concentration was plotted. Data is presented as mean ± SD, *p < 0.05 vs control.

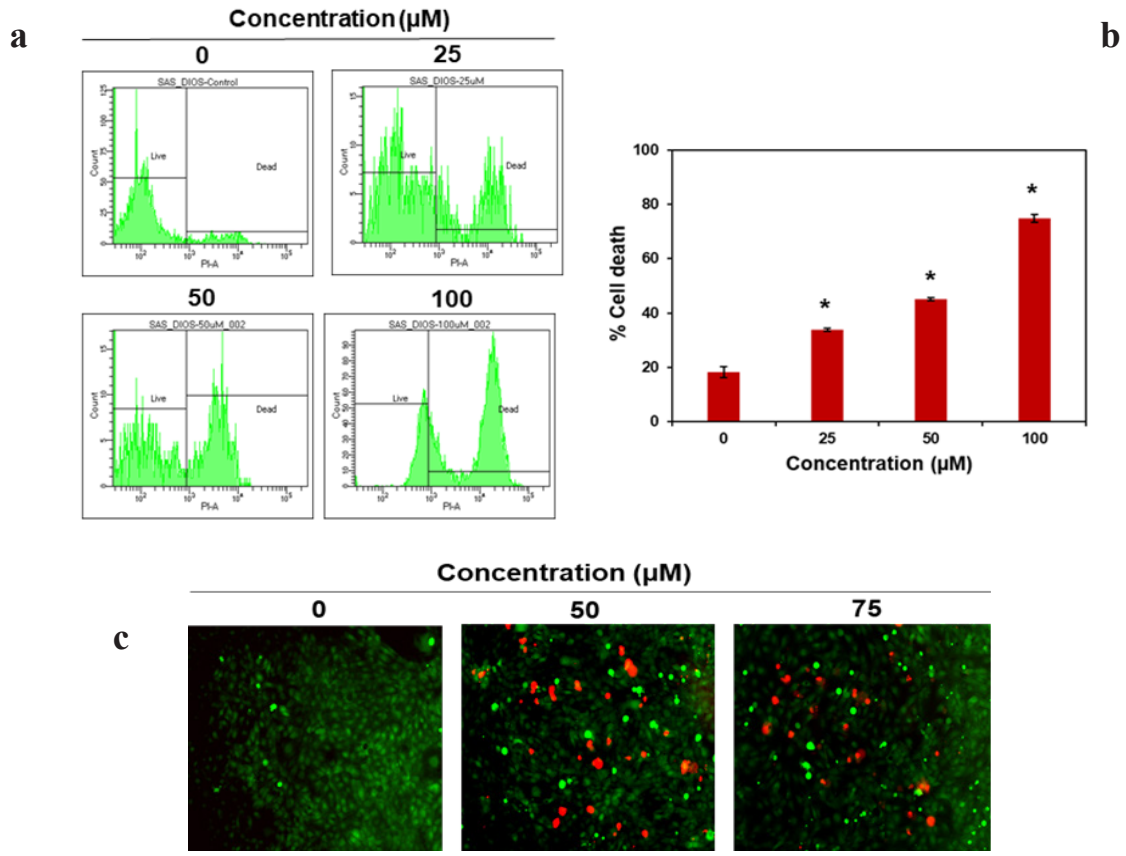

Figure 5. Diosgenin Induces a Cytotoxic Effect on the SAS Oral Cancer Cells. (a) SAS cells were treated with 0, 5, 10, 25, 50, and 100 µM diosgenin for 72 hours, followed by PI staining and FACS analysis for the cell death profile. (b) % cell death vs. Concentration was plotted. (c) Cytotoxic effect of diosgenin analysed by Live/Dead staining assay. SAS cells were treated with 0, 50, and 75 µM diosgenin for 48 hours The live cells are stained with calcein AM (green) and dead cells are stained with ethidium homodimer-1 (red). Data is presented as mean ± SD, *p < 0.05 vs control.

signalling pathways in cancer.

The current study suggests that diosgenin treatment exerts antiproliferative and cytotoxic effects on OSCC cells. Similarly, previous findings indicate that diosgenin inhibits cell growth, induces cell cycle arrest, and activates apoptosis through p53 activation in various human cancer

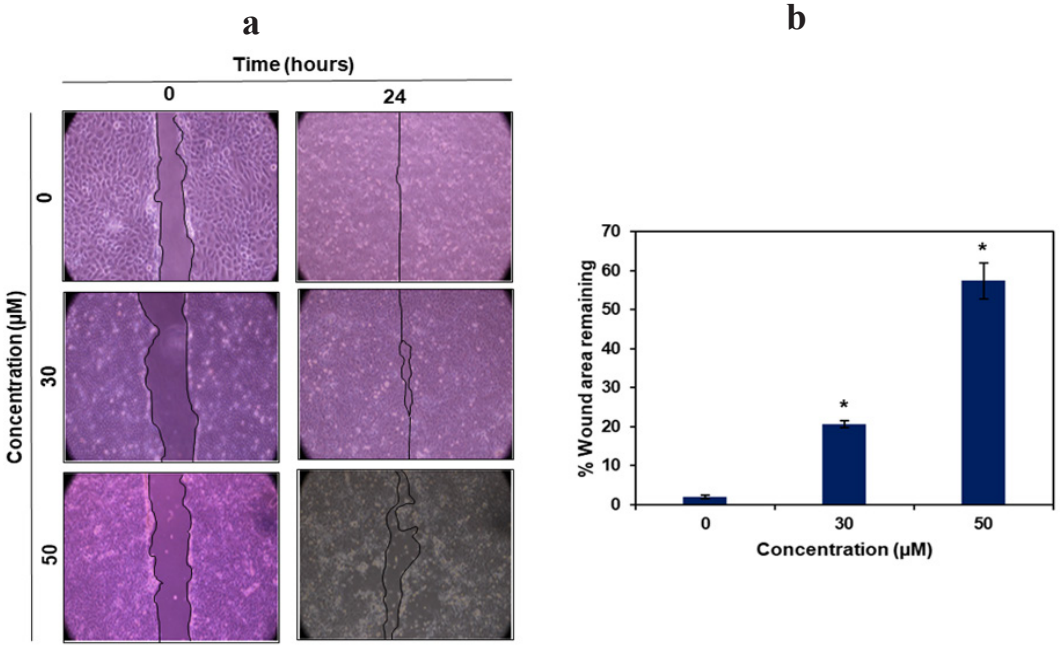

Figure 6. Effect of Diosgenin on the Migration of SAS Cells. (a) SAS cells were treated with 0, 30, and 50 µM diosgenin for 24 hours. (b) % wound area healed was calculated using ImageJ software. Data is presented as mean ± SD, *p < 0.05 vs control.

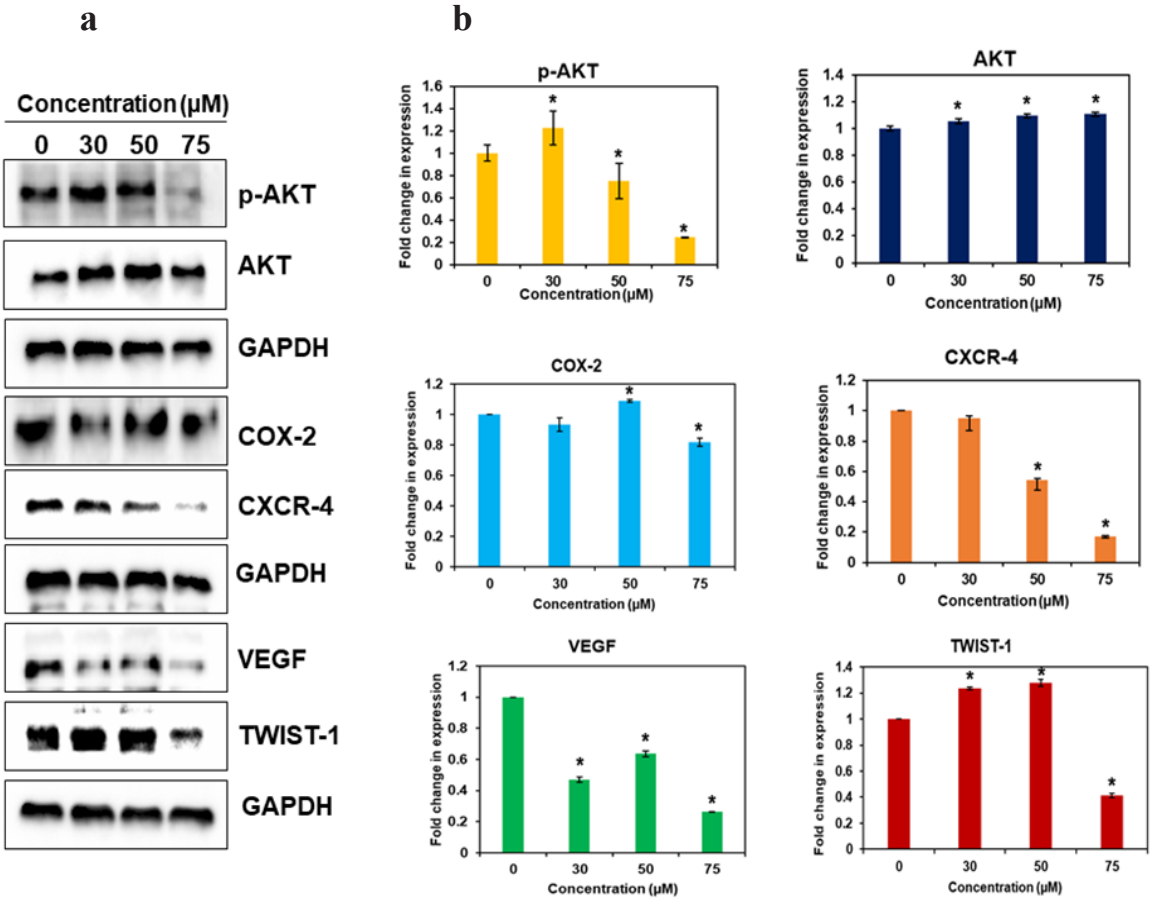

Figure 7. Effect of Diosgenin on Various Proteins Involved in OSCC. (a) SAS cells were treated with 0, 30, 50 and 75 μM concentrations of diosgenin for 24 hours followed by Western blot analysis. (b) Densitometry plots of Fold change in expression vs. concentration were plotted. Blots were visualized and analyzed using Image Lab software. GAPDH was used as the housekeeping control. Data are presented as mean ± SD, *p < 0.05 vs control.

cell lines, including laryngocarcinoma and melanoma cells [29]. Moreover, diosgenin has demonstrated antiproliferative activity in cervical cancer cells [30]. For instance, Selim and Al Jaouni, [31] observed that treatment of macrophages or lymphocytes with diosgenin (250μg/ml) led to a 3.2-fold and 2.1-fold induction in cell proliferation, respectively, compared to the control. Further, it decreased the ability of invasion and survival in cobalt chloride-treated BGC-823 cells effectively [32]. Diosgenin inhibited cell proliferation, induced G0/G1 phase arrest, and blocked IGF-1-induced cell proliferation in primary human thyroid cells [33].

Cytotoxicity refers to a sequence of molecular events disrupting macromolecular synthesis, causing substantial damage to cellular function and structure [34]. In this study, the anti-proliferative potential of diosgenin was examined in both normal (HEK-293T) and cancer cell lines (SAS and HSC3) using the MTT test. The MTT method is a sensitive indicator for assessing cytotoxicity, reflecting both cell number and metabolic activity, and is favoured for its simplicity, speed, repeatability, and lack of dependence on radioisotopes [22, 35, 36]. The results of cytotoxic studies revealed that the application of diosgenin led to a decrease in the percentage of cell proliferation in both cancer cell lines, while minimal changes were observed in the normal cell line. The IC_{50}, indicating the concentration at which 50% of cells are inhibited, was determined for the SAS cell line as 31.7 μM and for the HSC3 cell line as 61 μM. The minimum inhibitory concentration against SAS cells significantly differed from that of normal epithelial lung (L132) cells, suggesting that this drug may pose a lower risk of damage to normal cells at appropriate doses. Additionally, the findings indicate that diosgenin demonstrates effectiveness against oral cancer cell lines over a broad concentration range.

Chemotherapy frequently results in undesirable side effects as the drugs lack the ability to differentiate between healthy and tumour cells, leading to harm to normal tissues. In this study, diosgenin was combined with well-established chemotherapeutic drugs, Cisplatin and 5-FU, to explore diosgenin's chemosensitizing effect. The combinations exhibited a synergistic effect on the anti-proliferative activity of diosgenin.

Diosgenin downregulated the expression of cancer-related protein markers such as *AKT, pAKT, VEGF, TWIST-1, COX-2,* and *CXCR-4*, indicating cancer suppressive role. These findings suggest that diosgenin can effectively regulate crucial cell signalling pathways to reduce tumour cell survival. *COX-2* is associated with carcinogen formation, tumour promotion, inhibition of apoptosis, angiogenesis, and the metastatic process [37]. It influences apoptosis, angiogenesis, and invasion, playing

a crucial role in carcinogen production. Typically, cancer cells exhibit high *COX-2* expression levels [38]. In vitro studies have shown the potential role of COX inhibitors as single agents in preventing tumour occurrence [39]. Liu et al. (2001) [40] were the first to describe tumorigenesis induced by *COX-2* overexpression. AKT/protein kinase B (PKB) is a central node in various signalling cascades crucial for normal cellular physiology and various disease states [41, 42]. AKT signalling regulates cell proliferation and survival, cell growth (size), glucose metabolism, cell motility, and angiogenesis [43]. In recent years, an expanding body of literature has documented the common occurrence of hyperactivation of AKT kinases across a wide spectrum of human solid tumors and hematological malignancies [44, 45]. Furthermore, a set of refined investigations employing animal models has illustrated that abnormal signaling through the AKT pathway can lead to malignancy either independently or in conjunction with specific genetic alterations, thereby promoting a more aggressive cancer phenotype [46, 47, 48]. Several oncoproteins and tumor suppressors interact with the AKT signal transduction pathway and become activated or deactivated, respectively, in cancer [49].

The collective findings from various studies emphasize the pivotal role of CXCR4 in OSCC and its potential impact on the diverse aspects of OSCC progression. Almofti et al. (2004) [50] proposed that the SDF-1/CXCR4 signalling pathway in oral squamous cell carcinoma cells may contribute to various actions of oral SCC, including invasion or micrometastasis at the primary site and lymph node metastasis. This aligns with the observations of Uchida et al. (2003) [51], who suggested a potential role of stromal-cell-derived factor-1/CXCR4 signalling in the lymph node metastasis of oral squamous cell carcinoma. The study conducted by Meng et al. (2010) [52] proposes CXCR4 as a novel biomarker for evaluating the biological behaviour of oral SCC. Furthermore, they suggest that CXCR4 inhibitors or antagonists could serve as potential anticancer agents to suppress tumour proliferation. Ishikawa et al. (2006) [53] found a highly significant correlation between CXCR4 expression and lymph node metastasis in OSCC, reinforcing the notion that CXCR4 may indeed play a crucial role in the lymph-node metastasis of oral squamous cell carcinoma. In line with these findings, Uchida et al. [54] suggested that the blockade of CXCR4 in oral squamous cell carcinoma inhibits lymph node metastases, indicating the potential therapeutic utility of CXCR4 inhibition against metastatic spread. A study by Yu et al. (2011) [55] showed that the knockdown of CXCR4 significantly reduces Tca8113 cell migration and invasion, accompanied by a decrease in MMP-9 and MMP-13 expression. Additionally, the inhibition of ligand binding to CXCR4 by a specific antagonist (TN14003) led to reduced cancer cell migration and invasion, highlighting the potential of CXCR4 blockade as an anti-metastatic therapy against lymph node metastases in CXCR4-related OSCC [56]. The observed downregulation of CXCR4 aligns with the previously mentioned findings that highlight the crucial role of CXCR4 in different aspects of OSCC progression.

A study reported VEGF expression in OSCC tissues and found a significant association between VEGF-A expression and tumour stage, as well as patient age. VEGF-A and VEGF-C were found to be overexpressed in OSCC, which were linked to tumour progression, invasion, and metastasis via the mTOR–HIF-1α–VEGF pathway [57]. Another study has shown that VEGF can indirectly induce osteoclast formation by upregulating RANKL expression in OSCC cells via VEGF-Flt-1 signalling. This pathway may contribute to bone invasion commonly observed in OSCC cases [58]. A study demonstrated that TWIST1 overexpression in OSCC tissues correlates with higher rates of lymph node and lung metastases, as well as poorer patient survival suggesting its potential to serve as a prognostic marker for OSCC progression [59].

Our study also demonstrates that diosgenin effectively inhibits the proliferation of SAS oral cancer cells by inducing cell cycle arrest at the S-phase. This arrest leads to a concentration-dependent reduction in cell survival, colony formation, and migratory behaviour, as evidenced by MTT, colony formation and wound healing assays. Additionally, PI-FACS analysis revealed an increase in cell death percentages correlating with higher diosgenin concentrations. The induction of S-phase arrest by diosgenin aligns with findings in other cancer cell types. A study reported that diosgenin-treated HEp-2 cells exhibits S-phase arrest at 24 hours, while M4Beu cells showed G2/M phase arrest after 12 hours of treatment [60]. Beyond cell cycle arrest, diosgenin has been shown to inhibit migration and invasion in various cancer cells. In ovarian cancer cells (OVCAR-3), diosgenin treatment led to significant inhibition of cellular proliferation in a dose-dependent manner and decreased cell migration and invasion by modulating gene expression of invasion-related markers [61]. Similarly, Diosgenin induced G1 phase cell cycle arrest in both ER+ and ER− breast cancer cells by suppressing the expression of cyclin D1, CDK-2, and CDK-4, leading to inhibited cell proliferation and induction of apoptosis [62].

In conclusion, the plant saponin diosgenin has demonstrated significant anti-proliferative, cytotoxic, and anti-migratory effects by influencing various gene products such as *pAKT, AKT, VEGF, TWIST-1, COX-2*, and *CXCR-4*. These proteins are essential for the growth, survival, invasion, and metastasis of cancer cells, including OSCC, as was previously indicated. This study is the first to demonstrate diosgenin's anti-cancer properties in OSCC and the potential underlying mechanisms. However, further in vivo studies and clinical validation are required to validate the potential of this compound and the respective combination of drugs in order to design a novel drug that can effectively prevent and manage oral cancer progression and recurrence as well as successfully overcome chemoresistance.

Author Contribution Statement

Uma Dutta contributed to the study design, conceptualisation, supervision, draft writing, review editing, material preparation, data collection, and analysis. Ajaikumar B. Kunnumakkara contributed to the study

design, fund acquisition, conceptualisation, review editing and laboratory facilities. Sonali Dey contributed to draft preparation, data analysis, figure and table preparation and bibliographic study. Monikongkona Baruah and Bethsebie Lalduhsaki Sailo contributed to data collection, table and figure preparation and some experimental part of work. Bhaigyaroti Muchahary contributed to bibliographic search and preparation. The first draft of the manuscript was written by Uma Dutta, Monikongkona Boruah, Sonali Dey and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material. Raw data that support the findings of this study are available from the corresponding author, upon reasonable request.

Ethical Declaration

This article does not contain any studies involving animals and human participants performed by any of the authors.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

OSCC: Oral Squamous Cell Carcinoma
TNF-α: Tumor Necrosis Factor-alpha
NF-κB: Nuclear Factor Kappa B
5-FU: 5-Fluorouracil
CIS: Cisplatin
DMSO: Dimethyl Sulfoxide
DMEM: Dulbecco's Minimum Essential Medium
FBS: Fetal Bovine Serum
PI: Propidium Iodide
BSA: Bovine Serum Albumin
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
PBS: Phosphate-Buffered Saline
FACS: Fluorescence-Activated Cell Sorting
SAS: Human Oral Squamous Cell Carcinoma Cell Line
HSC3: Human Oral Squamous Cell Carcinoma Cell Line
HEK-293T: Human Embryonic Kidney 293T Cells
ECL: Enhanced Chemiluminescence
SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TBST: Tris-Buffered Saline with Tween 20
SD: Standard Deviation
IC_{50}: Half-Maximal Inhibitory Concentration
PKB: Protein Kinase B
RANKL: Receptor Activator of Nuclear Factor Kappa-B Ligand
Flt-1: Fms-like Tyrosine Kinase-1

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