Toxicology Reports 13 (2024) 101833

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Pro-apoptotic and anti-proliferative activities of cassane diterpenoids on squamous carcinoma cells: An in vitro and in silico study

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ARTICLE INFO

Keywords:
Cassane Diterpenoids
Squamous Carcinoma
Apoptosis
Caesalpinia pulcherrima
Cancer
Cell cycle

ABSTRACT

Squamous carcinoma of the head and neck is characterized by aberrant apoptosis that prolongs the proliferative capacity of the cells and by uncontrolled cell growth. This study aimed to examine the pro-apoptotic and anti-proliferative effects of *Caesalpinia pulcherrima* cassane diterpenoids on squamous carcinoma cells *in vitro*. The cytotoxicity of four (4) cassane diterpenoids {Six-cinnamoyl- 7-hydroxyvouacapen-5-ol(1), pulcherrimin A(2), C (3), and E(4)} isolated from C. pulcherrima was determined in squamous carcinoma cell lines (CAL33, FaDu, and Detroit 562) and in non tumorigenic fibroblast cells. The results showed that compounds 1 and 4 had the highest cytotoxic potential, significantly reducing cell viability in all squamous cell lines in a concentration dependent manner. Compounds 1 and 4 may inhibit the proliferation of CAL33 cells by reducing their ability to divide, decreasing PCNA expression, and suppressing migration. Additionally, treatment with compounds 1 and 4 led to an activation of Caspase 3 expression in FaDu cells. Molecular docking analysis revealed strong binding affinities of compounds 1 and 4 to the Caspase 3 receptor, with values of −8.5 and −8.8 kcal/mol, respectively. These results suggest that Pulcherrimin E and 6-cinnamoyl-7-hydroxylvouacapen-5-ol have potential antitumor effects based on their selective cytotoxic effect on squamous carcinoma cells in vitro.

1. Introduction

Head and neck squamous carcinoma (HNSC) is characterized by unregulated cell growth with abnormalities of apoptosis that prolong their proliferative capacity. Apoptosis is a highly regulated process of cell death characterized by "nuclear condensation, cell shrinkage, membrane blebbing, and DNA fragmentation"[1]. Caspases, a group of cysteine proteases, are key regulators of apoptosis. They are classified into two categories: initiator caspases [2, 8, 9, 10] and executioner caspases [3, 6, 7]. The initiator caspases respond to pro-apoptotic signals and activate the executioner caspases by cleaving them. Once activated, executioner caspases target and modify various proteins, including poly (ADP-ribose) polymerase (PARP), lamin α/β, and actin, which are involved in the process of programmed cell death and serve as markers of apoptosis [2]. Treatment options for HNSC are surgical resection, radiation, chemotherapy, but patients suffer recurrence or metastasis

Abbreviations: Akt, Protein Kinase B; BCA, Bicinchoninic Acid; Bcl-2, B-cell lymphoma; *C. pulcherrima,*, *Caesalpinia pulcherrima*; CDK, cyclin-dependent kinases; COX-2, cyclooxygenase-2; CO_2, carbonIV oxide; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DNA, deoxyribonucleic acid; EDTA, Ethylenediaminetetraacetic acid; EGFR, Epidermal Growth Factor Receptor; ERK, extracellular signal-regulated kinases; FACS, Fluorescence-activated cell sorting; HNSC, Head and neck squamous carcinoma; HRP, horseradish peroxidase; IgG, Immunoglobulin G; INOS, inducible nitric oxide synthase; MMPs, metalloproteinases; MTOR, mammalian target of rapamycin; 3–4, 5-dimethylthiazol-2-yl-5–3-carboxymethoxyphenyl-2-4-sulfophenyl-2H-tetrazolium; NF-kB, nuclear factor kappa B; PAGE, Polyacrilamide gel electrophoresis; PARP, Poly ADP-ribose polymerase; PBS, Phosphate buffered; PCNA, Proliferating cell nuclear antigen; PDB, protein data bank; PI3K, phosphoinositide 3-kinase; PMSF, phenylmethylsulfonyl fluoride; PVDF, Polyvinylidene fluoride; RIPA, Radioimmunoprecipitation assay; Rb, retinoblastoma; SDS, sodium dodecyl sulphate, saline; TBS, Tris-Buffered Saline; VEGF, vascular endothelial growth factor.

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https://doi.org/10.1016/j.toxrep.2024.101833
Received 9 January 2024; Received in revised form 19 November 2024; Accepted 25 November 2024
Available online 29 November 2024

and side effects from orthodox therapy. To circumvent this challenge, our present research is designed to use the application of natural plant-based compounds to treat squamous carcinoma.

It is reported that anti-cancer compounds exert their anti-proliferative effect through diverse mechanisms to inhibit cell growth [3]. Some compounds inactivate enzymes associated with the pro-survival signaling pathway or target major apoptosis mediators and B-cell lymphoma (Bcl-2) family members to inhibit carcinoma cell growth [4]. The commonly influenced pro-survival pathways include *nuclear factor kappa B* (NF-kB) pathway, phosphoinositide 3-kinase (P13K/Protein Kinase B (Akt)/ mammalian target of rapamycin (mTOR) signaling pathway. Targeting these pathways with therapeutic agents has demonstrated promising results[5–8].

Caesalpinia pulcherrima L, commonly called Pride of Barbados is an ornamental shrub belonging to the family Caesalpiniaceae. In folk medicine, the plant is used mainly as a stimulant, emmenagogue, and as an abortifacient [9,10]. Other uses in ethnomedicine include treatment of fever, malaria, diarrhea, asthma, and skin diseases [11,12]. Pharmacological evaluations of various parts of the plant have shown antimicrobial [13], cytotoxic, antioxidant [14], anti-inflammatory and analgesic [15], antiulcer [16], antiplasmodial [17], and anthelminthic activities [18]. The plant has been found to contain phytochemicals such as flavonoids, carotenoids, glycosides, peltogynoids, terpenoids, and steroids. Notable among the phytochemicals present in the plant are the diterpenoids, mostly the cassane diterpenoids. The cassane diterpenoids are the most common class of diterpenoids; they consist of a tricarbocyclic skeleton which occasionally has a furanoid group attached to ring C (furanocassane diterpenoids). Erharuyi et al. [19] isolated cassane diterpenoids from the root bark of *Caesalpinia pulcherrima*, and investigated that the root of *C. pulcherrima* exhibited cytotoxic, anti-inflammatory, and leishmanicidal activities. Additionally, the cassane diterpenoids were derivatized, leading to the discovery of three new derivatives with enhanced cytotoxic and leishmanicidal activities. Building on this, we investigated the effects of 6β-cinnamoyl-7α-hydroxyvouacapen-5α-ol (1), Pulcherrimin A (2), Pulcherrimin C (3), and Pulcherrimin E (4), all isolated from the root bark of *Caesalpinia pulcherrima*, on squamous cell carcinoma.

2. Materials and methods

2.1. Plant collection, extraction, and isolation

The collection of *C. pulcherrima* roots, extraction, and isolation of compounds **1 – 4** from the root bark have been described previously [19,20]. Compounds were dissolved in Dimethylsulfoxide (DMSO), to maintain constituency, DMSO was used as control.

2.2. Cell culture

Squamous carcinoma cell line CAL33 (CVCL_1108), Detroit 562 (ATCC- CCL-138), FaDu (ATCC- HTB-43) and non-tumorigenic associated cell L929 (Fibroblast) (ATCC- HTB-22) were obtained from the America Type Culture Collection (Manassas VA, USA). These cell lines originated from different tumor sites within squamous cell carcinomas, representing a variety of anatomical locations. CAL33 was derived from a tongue tumor, Detroit 562 and FaDu derived from a pharyngeal and hypopharyngeal carcinomsa respectively within the head and neck region. Fibroblasts are non-cancerous associated cells and are valuable for comparison with cancer cells, providing a baseline to assess the specific effects of compounds or treatments. Cells were maintained at 37°C and in a 5 % CO_2 atmosphere in a monolayer in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Germany) with 10 % fetal bovine serum (PAA Laboratories GmbH, Germany) and 1 % gentamycin (Ratio pharm, Germany). Confluent cells were passaged by treating them with 0.05 % trypsin/ 0.02 % EDTA. The medium was changed every two days. Cultivation conditions were described previously [21].

2.3. Cell viability assays and IC_{50} value calculation

The cell viability of the squamous cell lines (CAL33, Detroit 562, FaDu as well as non-tumorigenic associated Fibroblast cells (L929) was quantified with the Cell Titer 96®Aqueous One Solution Cell Proliferation Assay (MTS-assay) according to the manufacturer's instructions (Promega Corp, Madison, USA) as described previously [22]. At least, eight technical replicates with corrected background absorbance were performed. For calculation of the IC_{50} value (Compounds concentration: 1, 10, 25 and 50 μM) were plotted on concentration-response curves. Afterward concentration-responsiveness was determined via non-linear regression using excel statistical package.

2.4. Cell cycle analysis

Proliferation changes and apoptosis induction following exposure to the compounds 6-β-cinnamoyl-7-α-hydroxylvouacapen-5-α-ol (1) and Pulcherrimin E (4) were assessed using cell cycle analysis via flow cytometry (FACS Calibur, BD Biosciences). Cells were stained with propidium iodide (50 μg/mL, Roche Diagnostics, IN, USA) as previously described [23]. Data were acquired and histograms were generated using FlowJo software (version 7.6.5, Tree Star). A minimum of 15,000 ungated events were recorded, and doublets and clumps were excluded by gating DNA pulse width versus pulse area displays. For statistical analysis, the sum of cells in the S and G2/M phases was considered as proliferative events, while the sub-G1 peak in the histogram was used to identify apoptotic cells.

2.5. Immunofluorescence and microscopy

Fluorescence microscopic imaging was performed to analyze the cytoskeletal structure and nuclear staining of squamous cells, focusing on the effects of the compounds on cell morphology and organization. Following the method described by Anuradha et al. [24], squamous cells were seeded on glass coverslips and cultured for 24 hours. After 24 hours of treatment with the compounds, the cells were fixed with 4 % paraformaldehyde for 15 minutes, washed three times with phosphate-buffered saline (PBS), and then permeabilized with 0.1 % Triton X-100 for 15 minutes. The cells were then incubated with 100 μL of 6.6 μM Alexa Fluor488 phalloidin (Invitrogen, Germany) for 60 minutes in the dark at room temperature to stain the actin cytoskeleton. After another wash, the cells were counterstained with DAPI (Roche Diagnostics GmbH, Germany) to visualize the nuclei for 15 minutes. The cells were washed four times with PBS and embedded in mounting medium.

The fluorescence signals, including those for the actin cytoskeleton and nuclei, were examined using an inverted confocal laser scanning microscope (LSM780, Carl Zeiss, Germany) with a helium/neon-ion laser and a ZEISS 63 × oil immersion objective. Hoechst dye (Invitrogen, Germany) was used for additional nuclear staining. These analyses provided insights into how the compounds affect cell structure and nuclear morphology.

2.6. Migration assay

Migration assays are essential for studying metastasis, the process by which cancer cells spread from the primary tumor to distant parts of the body. In this study, migration assays were performed using squamous cell lines (CAL33, Detroit 562, and FaDu)[25]. The cells were pre-incubated in assay medium for 48 hours in 6-well plates (Greiner, Germany) to allow for adaptation. A scratch wound was created using Ibidi culture inserts (a 35 mm micro-dish with an Ibidi polymer coverslip bottom for high-end microscopy; Ibidi GmbH, Martinsried, Germany), following the recommended procedure. Once the cell layers reached confluence, the culture insert was removed, and the cells were treated with compounds 1 and 4 or a control vehicle. Photographs of the wound

gap were taken at various time points (0, 12, 24, 48 hours) using a bright-field microscope (Axiovert 40, Carl Zeiss, Jena, Germany) equipped with an ICC1 camera (Carl Zeiss, Jena, Germany).

2.7. Western blot analysis

Western blot analysis was conducted to investigate the expression of PCNA, a marker of cell proliferation, and Caspase 3, a marker of apoptosis, in squamous cells (CAL33, Detroit 562, and FaDu) treated for 24 hours with 6-β-cinnamoyl-7-α-hydroxylvouacapen-5-α-ol (1) and Pulcherrimin E (4). The cells were lysed using RIPA buffer containing sodium chloride (150 mM), NP-40 (1.2 %), sodium deoxycholate (0.6 %), SDS (0.2 %), tris hydrochloric acid (50 mM, pH 8.0), EDTA (10 mM), and PMSF (1 mM) for 40 minutes at 4°C. The lysate was then centrifuged at 12,000 x g for 25 minutes, and the protein concentration was measured with a BCA assay kit.

Proteins were separated on a 10 % SDS-PAGE gel and transferred to PVDF membranes. The membranes were blocked for 2 hours with a mixture of TBS, Tween 20 (0.1 %), and milk (5 % w/v) to prevent nonspecific binding. After blocking, the membranes were incubated overnight at 4°C with primary antibodies against PCNA and Caspase 3 (catalog numbers CPP324–1–18 and sc-53407, respectively, from Santa Cruz Biotechnology). Following primary antibody incubation, the membranes were washed with PBS and then incubated for 1 hour at room temperature with HRP-conjugated anti-rabbit IgG secondary antibodies. Band intensities were quantified using ImageJ software.

2.8. Molecular docking

Molecular docking studies will be conducted on caspase 3 using AutoDock Vina software, following the method described by Trott and Olson [26]. The crystal structure of caspase 3, obtained from the Protein Data Bank (PDB), was selected with PDB ID 1GFW [23] and a resolution of 2.8 Å. The active binding site of caspase 3 will be defined using AutoDock Tools [27] with a grid size of 28 × 28 x 28 and a grid spacing of 1.00 Å. AutoDock Tools' graphical user interface, provided by MGL Tools [28], will be used to add Gasteiger charges. Prior to docking, the ligand structures will be optimized using Gaussian 09 to achieve their minimal energy conformations [29].

2.9. Statistical analysis

Data were expressed as the mean ± SD using Microsoft Excel 2010. Statistical significance was calculated by one-way analysis of variance by multiple comparisons between untreated tumour cells and tumor cells treated with compounds. Differences between means were estimated by Duncan's multiple range tests and a value of $p < 0.05$ was taken as statistically significant.

3. Results

3.1. Characterization of isolated compounds

The physical properties and spectra data of compounds 1 – 4 were as previously reported [17]. The compounds were characterized as 6β-Cinnamoyl-7β-hydroxyvouacapen-5α-ol (1), Pulcherrimin A (2), Pulcherrimin C (3), and Pulcherrimin E (4) (Fig. 1).

3.2. Initial screening on cell viability

The cytotoxic properties of four cassane diterpenoids isolated from *C. pulcherrima* root bark on squamous (Detroit 562, CAL33, and FaDu) cancer cell lines in comparison with Fibroblast cells were evaluated. The cells were treated with 50 μM of compounds **1, 2, 3, 4** and incubated for 24 h to determine the cell viability via MTS assay (Fig. 2). Compounds **1** to **4** caused a significant reduction (40 – 75 %) of cell viability in all cell lines. On Fibroblasts (L929), compounds **1** and **4** induced a slight decrease in cell viability while **2** and **3** showed a significant decrease of 40.25 and 25.15 %, respectively. These results suggest that the compounds **1** and **4** have inhibitory potential on head and neck cancer since it is cytotoxic to Detroit 562, CAL33, and FaDu cell lines and exert a minimal effect on fibroblast cell line

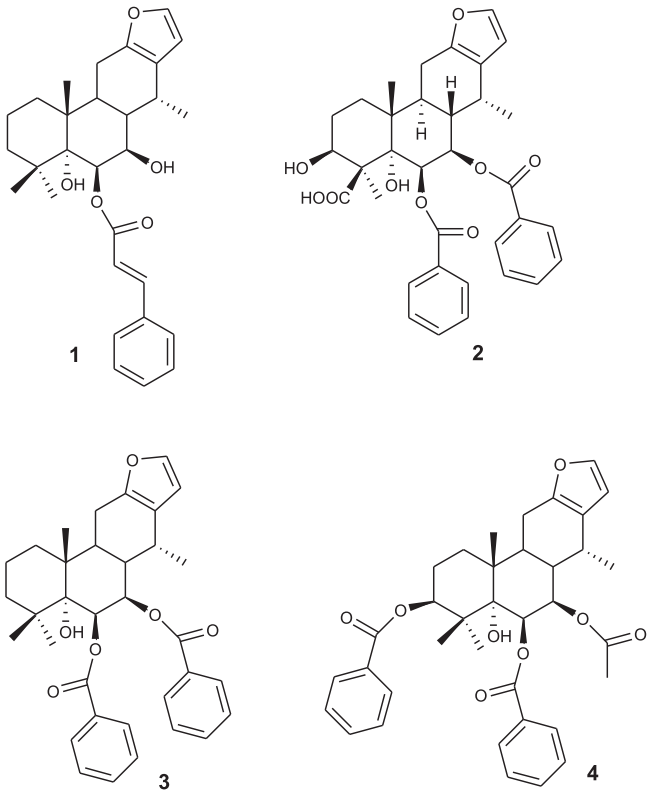

Fig. 1. : Chemical structures of compounds 1 – 4.

3.3. Inhibitory effects of 6-β-cinnamoyl-7-α-hydroxylvouacapen-5-α-ol (1) and Pulcherrimin E (4) on squamous carcinoma cell viability

The effects of 6-β-cinnamoyl-7-α-hydroxylvouacapen-5-α-ol (1) and Pulcherrimin E (4) on fibroblasts were not significant at all concentrations (Fig. 3). However, treatment of CAL33, Detroit 562, and FaDu cells with gradient concentrations (1, 10, 25, and 50 μM) of compounds **1** and **4,** after 24 h of incubation significantly reduced the proliferative growth of the squamous cells in a concentration-dependent manner (Figs. 4–6). The reduction of proliferative potential by compound 1 was more prominent in CAL33 followed by FaDu cells compared to Detroit 562 cells. At 24 h, Detroit 562, CAL33, and FaDu proliferative potential was suppressed to 19.10 ± 3.23 %, 46.6 ± 5.67 % and 64.12 ± 0.29 %, respectively relative to the control cells on treatment with 50 μM of Compound **1**. The highest concentration of 50 μM reduced the cell viability by 60–80 % in all tumorigenic cells when compared to control cells.

Similarly, at 24 h, Detroit 562, CAL33, and FaDu proliferative potential was suppressed by compound **4** (50 μM) and their percentage cell viability were 72.09 ± 4.49 %, 33.37 ± 1.13 % and 38.44 ± 5.45 %, respectively relative to the control cells. The 50 % growth inhibition concentrations of 6-β-cinnamoyl-7-α hydroxylvouacapen-5-α-ol (1) for CAL33, Detroit 562, FaDu and Fibroblast cell lines were 16.45, 40.87, 47.00 and >50 μM, respectively, while that of Pulcherrimin E (4) were 26.91, 13.94, 42.00, >50 μM, respectively (Table 1).

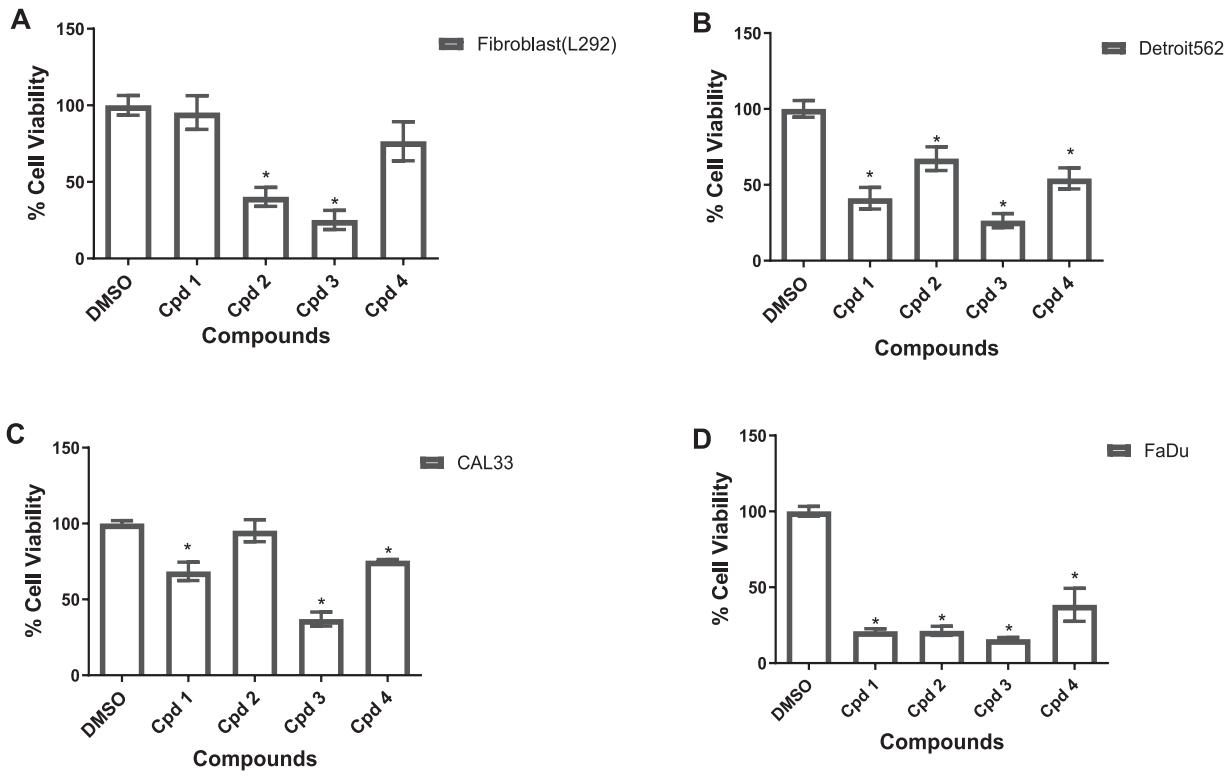

Fig. 2. Determination of cell viability. Measurement of cell viability via MTS assay after exposure to 50 μM compounds for 24 h on respective normal cells **A**) (Fibroblast, L929) and squamous cancer cell lines, **B**)Detroit 562, **C**) CAL33 and **D**) FaDu). As control treatment, the vehicle DMSO was used at a final concentration of 0.1 % (w/v). Samples were compared using one-way ANOVA. Error bars indicated mean ± SD, n = 8, *p < 0.05, significantly different compared to control.

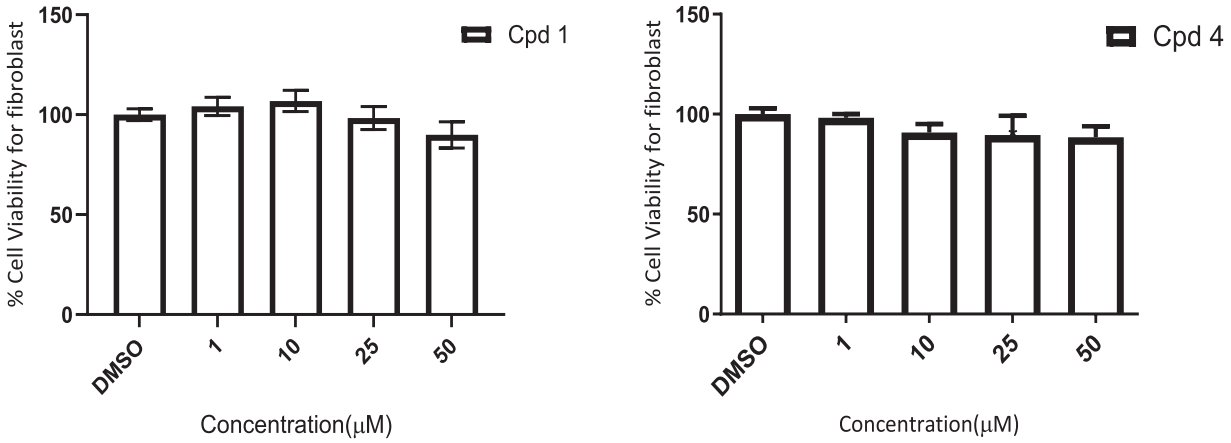

Fig. 3. Effect of 6-*β*-cinnamoyl-7-*α*-hydroxylvouacapen-5-*α*-ol (1) and Pulcherrimin E (4) on fibroblasts. The cells after 24 h of exposure to gradient concentrations (1, 10, 25, and 50 μM) of Cpds 1 and 4 were assessed for proliferation by MTS assay. As control treatment, the vehicle DMSO was used at a final concentration of 0.1 % (w/v). Samples were compared using one-way ANOVA. Error bars indicated mean ± SD, n = 8, p > 0.05, not statistically different when compared to the control.

3.4. Influence of 6-β-cinnamoyl-7-α-hydroxylvouacapen-5-α-ol (1) and Pulcherrimin E (4) on the cell cycle phase of squamous cells

In addition to the evaluation of the suppressive abilities of the compounds from *C. pulcherrima* root bark, influence on cell growth and induction of apoptosis are imperative in the understanding of their anticancer properties. Therefore, cell cycle analyses were performed to determine the influence on the proliferation behavior (G2/M + S phase) and apoptosis initiation by DNA strand breaks (sub G1 phase) using propidium iodide staining and flow cytometry assay as shown in (Fig. 7). Treatment with 6-*β*-cinnamoyl-7-*α*-hydroxylvouacapen-5-*α*-ol (1) on Detroit 562 caused a significant decrease in proliferation rate of 26.8 % compared to control (untreated cells), while treatment with Pulcherrimin E (4) resulted in no significant reduction in proliferation rate compared to control. The inhibition of proliferation rate triggers cell cycle arrest at the G1 phase and reduces the proportion of cells in S phase. Similarly, treatment with compounds **1** and **4** caused a significant increase in the sub-G1 phase of 22 % and 12 %, respectively compared to the untreated cells (2.24 %) (Table 2). The proliferative and apoptotic changes in CAL33 and FaDu were also evaluated (Fig. 7). Treatment with 6-β-cinnamoyl-7-α-hydroxylvouacapen-5-α-ol (1) and Pulcherrimin E (4) at 25 μM for 24 h reduced the proliferation rate of CAL33 cells

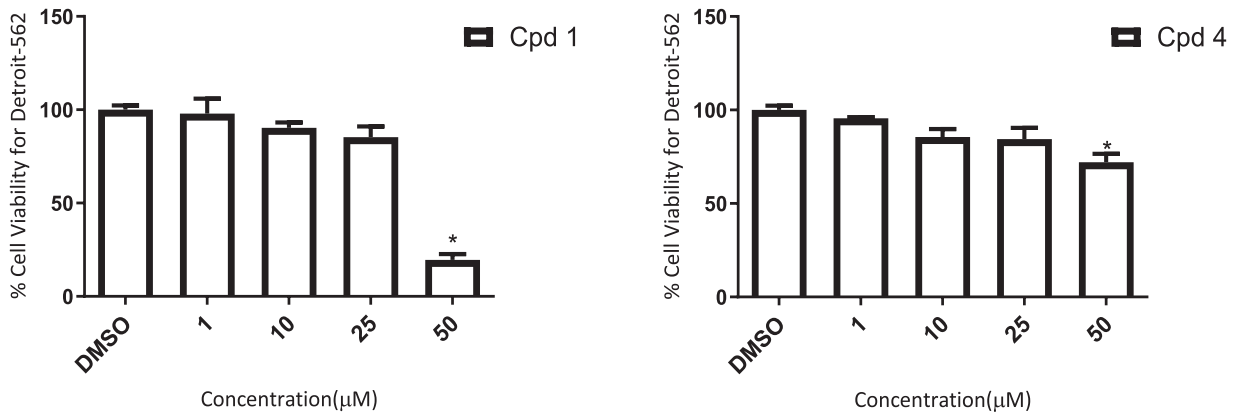

Fig. 4. Effect of 6-*β*-cinnamoyl-7-*α*-hydroxylvouacapen-5-*α*-ol (1) and Pulcherrimin E (4) on Detroit 562. The cells after 24 h of exposure to gradient concentrations (1, 10, 25, and 50 μM) of Cpds 1 and 4 were assessed for proliferation by MTS assay. As control treatment, the vehicle DMSO was used at a final concentration of 0.1 % (w/v). Samples were compared using one-way ANOVA. Error bars indicated mean ± SD, n = 8, *p < 0.05, significantly different compared to control.

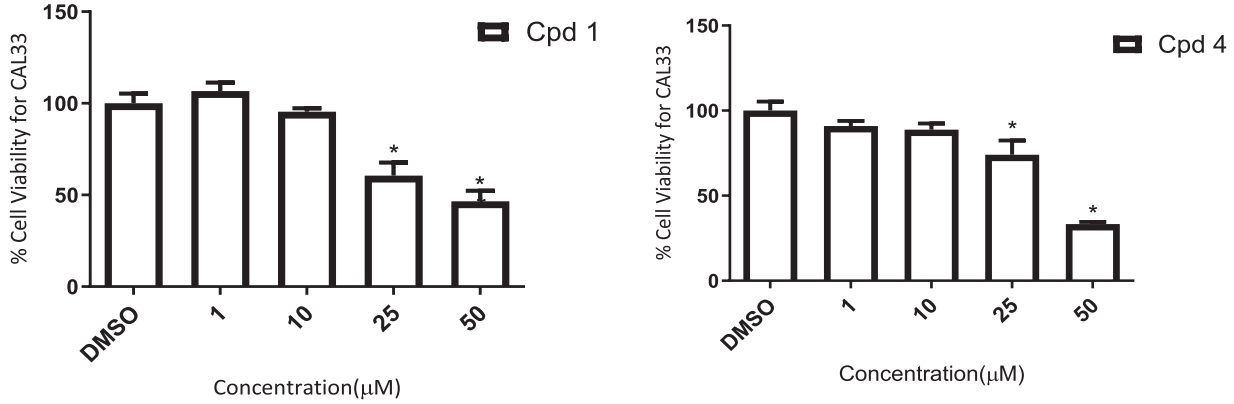

Fig. 5. Effect of 6-*β*-cinnamoyl-7-*α*-hydroxylvouacapen-5-*α*-ol (1) and Pulcherrimin E (4) on CAL33. The cells after 24 h of exposure to gradient concentrations (1, 10, 25, and 50 μM) of Cpd 1 and 4 were assessed for proliferation by MTS assay. As control treatment, the vehicle DMSO was used at a final concentration of 0.1 % (w/v). Samples were compared using one-way ANOVA. Error bars indicated mean ± SD, n = 8, *p < 0.05, significantly different compared to control.

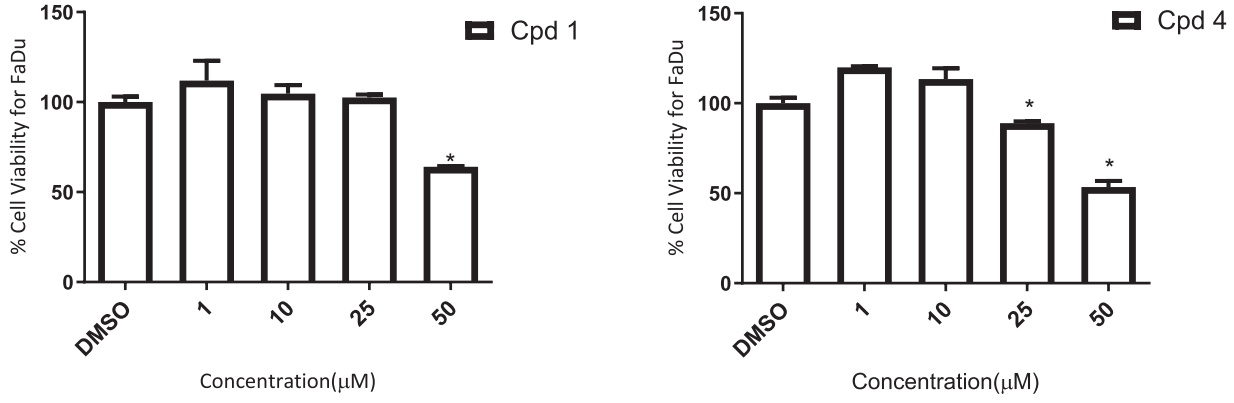

Fig. 6. Effect of 6-*β*-cinnamoyl-7-*α*-hydroxylvouacapen-5-*α*-ol (1) and Pulcherrimin E (4) on FaDu. The cells after 24 h of exposure to gradient concentrations (1, 10, 25, and 50 μM) of Cpds 1 and 4 were assessed for proliferation by MTS assay. As control treatment, the vehicle DMSO was used at a final concentration of 0.1 % (w/v). Samples were compared using one-way ANOVA. Error bars indicated mean ± SD, n = 8, *p < 0.05, significantly different compared to control.

to 20.82 % and 23.84 %, respectively.relative to 30.38 % in untreated cells. There was also a reduction in proliferation rate in FaDu cells for Cpd **1** (22 %) and Cpd **4** (26 %) when compared to the untreated cells (37 %). Consequently, treatment with 6-*β*-cinnamoyl-7-*α* hydroxylvouacapen-5-*α*-ol (1) and Pulcherrimin E (4) at 25 μM for 24 h increased apoptosis in CAL33 cells to 25.2 % and 20.8 %, respectively relative to 1 % in untreated cells. The apoptosis in FaDu cells was 17.8 % on treatment with 6-*β*-cinnamoyl-7-*α*-hydroxylvouacapen-5-*α*-ol (1) for 24 h relative to 2.8 % in control cells. In fibroblasts, the compounds could not increase the percentage of apoptotic cell.

3.5. Actin stress fiber formation

Actin, a major component of the cytoskeleton, primarily exists as a

Table 1
IC_{50} values of 6-β-cinnamoyl-7-α-hydroxylvouacapen-5-α-ol (1) and Pulcherrimin E (4) on squamous cancer cell lines in comparison with the non-tumorigenic associated cells.

Compound	IC_{50} (μM)
Fibroblast	
Compound 1	>50
Compound 4	>50
CAL33	
Compound 1	16.45
Compound 4	26.91
Detroit 562	
Compound 1	40.87
Compound 4	13.94
FaDu	
Compound 1	>50
Compound 4	42.00

fibrous polymer and plays a crucial role in determining the structure and stiffness of cells, as well as serving as a scaffold for signaling proteins. In squamous cancer cells and non-tumorigenic associated fibroblast cells, actin fibers typically exhibit a cortical arrangement of F-actin, as shown in Fig. 8. This cortical arrangement means that actin is concentrated around the periphery of the cell, and there is an absence of stress fiber formation within the cell body. However, after treatment with compounds 1 and 4, significant changes were observed in the actin cytoskeleton. In most cells, the F-actin reorganized into long stress fibers that spanned throughout the cell. Stress fibers are bundles of actin filaments that are associated with increased cellular tension and rigidity, indicating a major reorganization of the actin cytoskeleton in response to the compounds. An exception to this observation was noted in Detroit 562 cells, where the F-actin did not form long stress fibers upon treatment with compounds 1 and 4. Instead, the actin structure in these cells remained similar to that of untreated cells, with no significant stress fiber formation.

3.6. Effect of 6-β-cinnamoyl-7-α-hydroxylvouacapen-5-α-ol (1) and Pulcherrimin E (4) on migration ability

The effect of 6-β-cinnamoyl-7-α-hydroxylvouacapen-5-α-ol (1) and Pulcherrimin E (4) on migration assay is shown in Figs. 9 to 11. At day 0, the migration rate of the untreated FaDu cells was 100 %, but showed a significant decrease in migration rates after 24 h (66.69 %) and 48 h (39.51 %) of incubation time, respectively. Treatment of FaDu and Detroit 562 cells with 6-β-cinnamoyl-7-α-hydroxylvouacapen-5-α-ol (1) at 25 μM significantly prevented the gap for closing up after 48 h, which suggest that Cpd **1** inhibited FaDu cells substantially, but hindered the Detroit 562 cells motility while Pulcherrimin E (4) could not inhibit migration of both FaDu and Detroit 562 cells which was not different from the untreated cells after 48 h of incubation. Similarly, treatment of

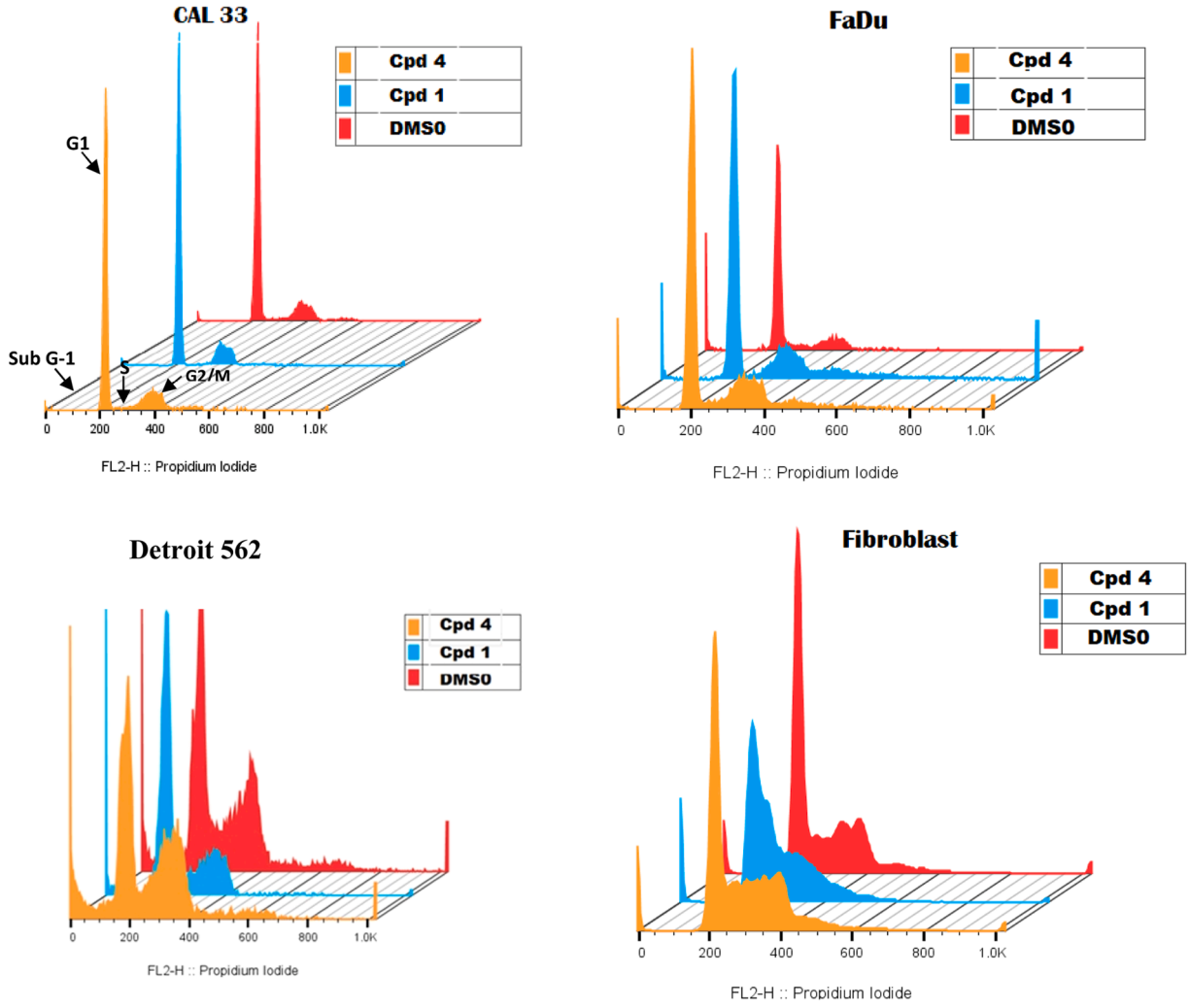

Fig 7. Cell cycle measurement via propidium iodide staining of different cells after treatment with 6-β-cinnamoyl-7-α-hydroxylvouacapen-5-α-ol (1) and Pulcherrimin E (4) at a concentration of 25 μM for 24 h.

Table 2
Cell cycle distribution of squamous cells after treatment with compounds **1** and **4** from *C. pulcherrima*.

Samples	G1(%)	G2(%)	S(%)	Sub-G1 (%)
Detroit562 (Control)	50.1 ± 0.12	20.2 ± 0.14	14.5 ± 0.1	2.24 ± 0.01
Detroit562 + Cpd **1**	29.5 ± 0.02**	18.9 ± 0.04 ns	7.9 ± 0.01*	21.5 ± 0.10**
Detroit562 + Cpd **4**	31.2 ± 0.01**	26.1 ± 0.13*	9.3 ± 0.01*	11.5 ± 0.11**
CAL33(control)	61.7 ± 0.15	18.9 ± 0.01	11.5 ± 0.02	1.0 ± 0.001
CAL33 + Cpd **1**	36.9 ± 0.11**	19.9 ± 0.01 ns	0.92 ± 0.015**	25.2 ± 0.12**
CAL33 + Cpd **4**	38.2 ± 0.14**	21.8 ± 0.11 ns	2.04 ± 0.001**	20.8 ± 0.03**
FaDu (Control)	61.0 ± 0.18	30.3 ± 0.15	6.7 ± 0.005	2.8 ± 0.001
FaDu + Cpd **1**	41.2 ± 0.03*	21.1 ± 0.05*	1.3 ± 0.001*	17.8 ± 0.04**
FaDu + Cpd **4**	55.6 ± 0.25*	23.9 ± 0.12*	2.1 ± 0.002*	6.6 ± 0.002*
Fibroblast(Control)	49.0 ± 0.25	22.3 ± 0.15	18.2 ± 1.25	0.76 ± 0.001
Fibroblast + Cpd 1	55.6 ± 0.01 ns	21.4 ± 0.03 ns	14.2 ± 0.01*	1.43 ± 0.010 ns
Fibroblast + Cpd 4	41.0 ± 0.01*	29.2 ± 0.11*	13.0 ± 0.02*	0.87 ± 0.001 ns

Values are expressed as mean ± SD, n = 2, **P < 0.01, *P < 0.05, significantly different compared to vehicle control

CAL33 cells with 6-*β*-cinnamoyl-7-*α* hydroxylvouacapen-5-*α*-ol (1) and Pulcherrimin E (4) showed reduced cell movement, very few cells were able to migrate into the gaps. The migration rate for Cpd **1** and **4** after 48 h were 93.45 % and 86.57 %, respectively.

3.7. 6-β-Cinnamoyl-7-α-hydroxylvouacapen-5-α-ol (1) and Pulcherrimin (4) suppresses proliferating cell nuclear antigen

Treatment with 6-β-cinnamoyl-7-α-hydroxylvouacapen-5-α-ol (1) and Pulcherrimin (4) suppressed levels of PCNA in squamous cells after 24 h relative to the control (Fig. 12). The PCNA in Cpd 1 treated FaDu and Detroit 562 cells was lowered relative to the control cells, while the cells treated with Cpd 4 showed significant increase in PCNA levels in both FaDu and Detroit 562 cells when compared to the untreated cells. However, the PCNA level in CAL33 was markedly lowered relative to the control cells when treated with compounds **1** and **4.**

3.8. 6-β-cinnamoyl-7-α-hydroxylvouacapen-5-α-ol (1) and Pulcherrimin (4) stimulate Caspase 3 activation

The changes in Caspase 3 activation by treatment with compounds 1 and 4 in FaDu, Detroit 562, and CAL33 cells were assessed using western blot (Fig. 14). Caspase 3 activation typically occurs when the procaspase is cleaved into its active forms. However, the antibody used in this study recognizes full-length Caspase 3, not the cleaved (active) form. In CAL33 and Detroit562 cells, there was no observable reduction in full-length Caspase 3 following treatment with compounds 1 and 4, indicating that Caspase 3 was not activated through cleavage. This suggests that these compounds do not induce apoptosis via the Caspase 3 pathway in these cell lines. In contrast, in FaDu cells, a significant decrease in full-length Caspase 3 was observed after 24 hours of treatment with compounds 1 and 4, indicating the activation of Caspase 3 and the induction of apoptosis through this pathway.

3.9. Molecular docking analysis: binding of 6-β-cinnamoyl-7-α-hydroxylvouacapen-5-α-ol (1) and Pulcherrimin (4) in the Caspase 3 binding site

Binding of 6-*β*-cinnamoyl-7-*α* hydroxylvouacapen-5-*α*-ol (1) and Pulcherrimin (4) in the Caspase 3 binding site is shown in Fig. 14. The docking model of compound **1** and **4** in caspase 3 showed that it fits snuggly in the binding site. Docking studies showed that compound 4 binds to key amino acids residues with higher binding affinity, though a slight difference in binding energy compared to compound 1 (Table 3). Fig. 14 revealed that compounds 1 and 4 binds in the active pocket (Fig. 15A) and interacted with active site amino acids of caspase 3 through hydrogen bonding.(Fig. 15B) Compounds 1 and 4 activate caspase 3 *via* hydrogen bonding with ARG207 and Pi-Pi sigma interactions.

4. Discussion

In recent years, plant derivatives have received much attention for their possible utility in the treatment of tumors [30]. The demand for new drugs to treat squamous carcinoma (Head and Neck) is irrefutable as current therapy is exploited especially in advanced tumor stages. Drugs that have low side effects, with the ability to specifically inhibit proliferation and metastatic invasion in squamous cells are the candidate drugs [31,32]. We were able to evaluate the ability of cassane diterpenoids: 6β-cinnamoyl-7α-hydroxyvouacapen-5α-ol (1), Pulcherrimin A [2], Pulcherrimin C [3], and Pulcherrimin E (4) (Fig. 1) to affect the survival, mobility, and cell cycle characteristics of squamous cell lines compared to response seen in non tumour- associated as well as on non-tumorigenic associated fibroblasts.

Initially, the compounds were screened against squamous cancer cell lines of different origins such as Detroit 562, CAL33, and FaDu carcinoma and non-tumorigenic associated fibroblasts using MTS assay. It was observed that pulcherrimin A and C were highly cytotoxic against fibroblasts as compared to 6*β*-cinnamoyl-7*α*-hydroxyvouacapen-5*α*-ol and pulcherrimin E which showed moderate toxicity (Fig. 2). To further investigate and evaluate this potentially specific cytotoxic effect observed in this initial viability assay, concentration-response were performed with compounds **1** and **4**, for their effects on cell viability and the concentration at which 50 % cell growth inhibition (IC_{50}) was evaluated. The lower the IC_{50} value the higher the cytotoxic activity. The 50 % growth inhibition concentration of Cpd **1** for CAL33, Detroit 562, and FaDu cell lines were 16.45, 40.87 and >50 μM respectively, while that of Cpd **4** were 26.91, 13.94 and 42.00 μM, respectively. However, in comparison with cancer cells, fibroblasts were not strongly affected by compound **1** and **4** in concentrations less than 50 μM. It was observed that Cpd **4** was able to significantly suppress cell proliferation in Detroit 562 cells with the lowest IC_{50}, while Cpd **1** and **4** showed reduced cell viability in CAL33 compared to other cells. The anti-proliferative potential of compounds 1 and 4 on HNSC cells may be attributed to the decreased expression of Epidermal Growth Factor Receptor (EGFR) and its downstream target, mTOR and extracellular signal-regulated kinases (ERK). The activation of mTOR and ERK has been proven to promote tumor progression [33]. The compounds may act by inhibiting the proliferation of malignant cells after targeting EGFR and its downstream molecular proteins.

The regulation of eukaryotic cell division and growth involves molecular circuits known as “checkpoints” that guarantee the correct timing of cellular events [34]. Movement through a checkpoint from one cell cycle phase to the next involves an organized set of proteins that check cell growth and DNA integrity. However when cell growth is not controlled, mutated DNA can contribute to tumorigenesis. During the G1 phase, retinoblastoma (Rb) which is a tumor suppressor gene binds to the transcription factor E2F, thereby halting the progression of the cell from G1 to S phase. This is possible because “phosphorylation of Rb by cyclin D-bound cyclin-dependent kinases (CDK 4 and 6) in late G1

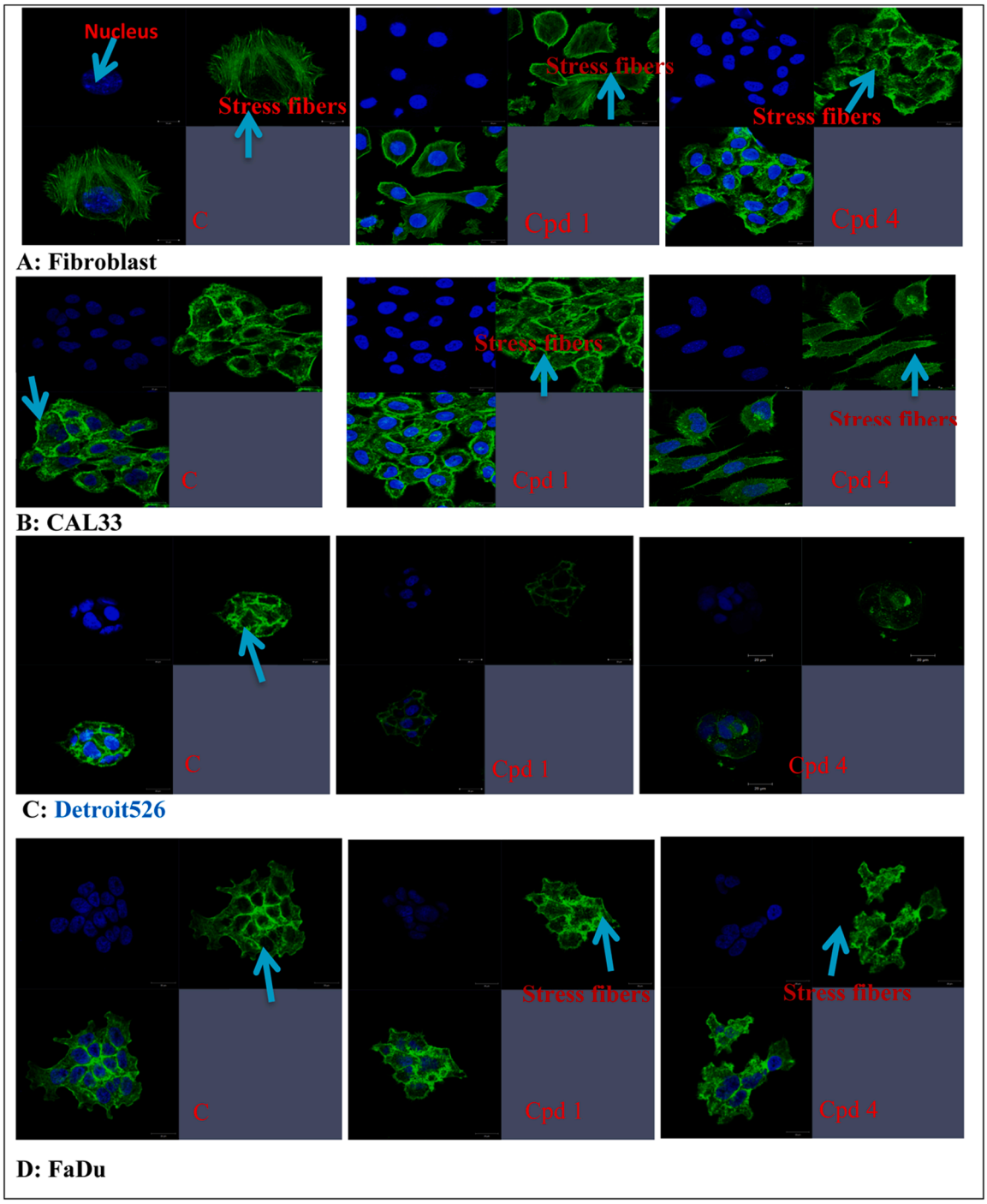

Fig. 8. Visualization of actin filament in fibroblasts (**A**), CAL33 (**B**), Detroit 526 (**C**), and FaDu (**D) cells** under control conditions (vehicle 0.1 % DMSO) and after treatment with 25 μM of **Cpd 1** and **Cpd 4** for 24 h. Confocal images of actin stained cells in a monolayer (LSC 780, Carl Zeiss, bar 20 μm). Actin filaments stained with green while the nucleus is stained blue.

stimulate the dissociation of Rb and allows E2F mediated transcription of S phase promoting genes" [35]. DNA damage activates pathway through checkpoint kinases to impede CDK activity, and in the process causing cell cycle arrest. Alteration of the gene is mainly responsible for the development of cancer. Changes associated with the mutated genes are uncontrolled expression of oncogenes, inactivation of tumor suppressor genes, and mutations of other genes involved in the apoptotic pathway, which may cause instability of the genome and abnormal growth of the cells [36]. Many of these mutations are located in genes involved in the regulation of cell cycle G1 phase progression [37].

6β-cinnamoyl-7α-hydroxyvouacapen-5α-ol (1) and Pulcherrimin E (4) reduced the proliferation rate of squamous cells, triggering cell cycle arrest at the G1 phase and reducing the proportion of cells in S phase (Fig. 7, Table 2), Pulcherrimin E was not able to influence Detroit 562 cells. This was consistent with findings from Chan *et al.* [38] that apigenin inhibited the progression of squamous cells (SCC-25 OSCC) from the G1 phase to the S phase.

Furthermore, the compounds (**1** and **4**) were able to stimulate apoptosis in squamous cells. There was increased apoptosis in CAL33 cells to 25.2 % and 20.8 %, respectively relative to 0.97 % in untreated

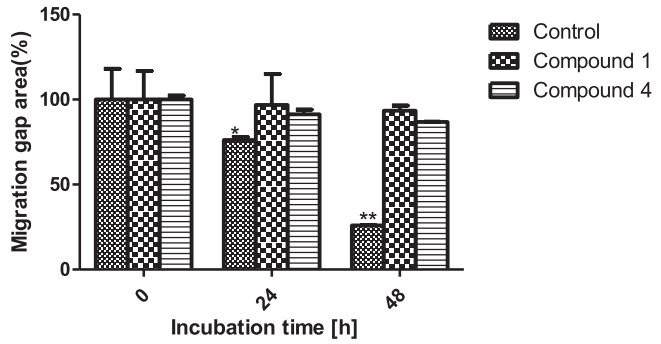

Fig. 9. : Evaluation of cell migration capacity of CAL33 cells during compounds 1 and 4 (25 μM) treatment for 2 days compared to the control (0.1 % DMSO). Mean ± SD, n = 3. *p< 0.05, significantly different compared to vehicle control.

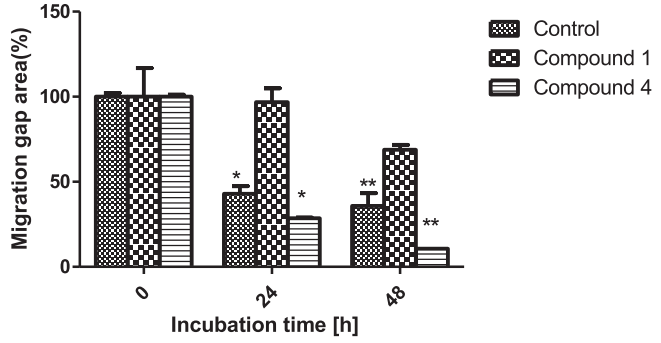

Fig. 10. : Evaluation of cell migration capacity of Detroit562 cells during compounds **1** and 4 (25 μM) treatment for 2 days compared to the control (0.1 % DMSO). Mean ± SD, n = 3. **p< 0.01, *p< 0.05significantly different compared to vehicle control.

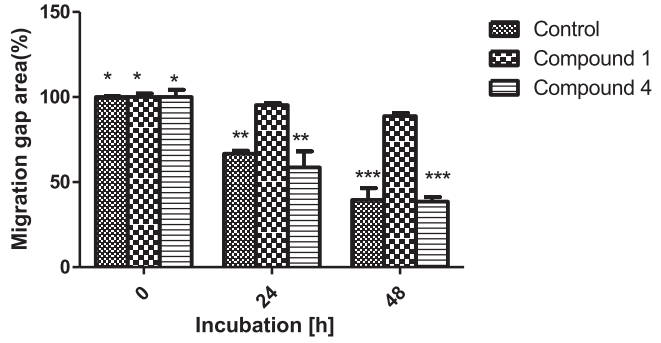

Fig. 11. : Evaluation of cell migration capacity of FaDu cells during compounds **1** and **4** (25 μM) treatment for 2 days compared to the control (0.1 % DMSO). Mean ± SD, n = 3. *p< 0.05, significantly different compared to vehicle control.

cells. The apoptosis in FaDu cells increased to 17.8 % on treatment with 6-*β*-cinnamoyl-7-*α*-hydroxylvouacapen-5-*α*-ol (1) for 24 h relative to 2.8 % in control cells. In fibroblasts, the compounds could not increase the apoptotic cell percentage. This was in agreement with Hou *et al.* [39] that a natural diterpenoid ovatodiolide induces cell cycle arrest and apoptosis in human oral squamous carcinoma cells.

Cytoskeletal signaling regulates several important cellular processes such as cell division, adhesion, cell shape, proliferation, migration, and survival. Microfilaments are major structural components of the cytoskeleton and consist of fibrous polymers of actin called F-actin. In the present study, after exposure of the cells to compounds **1** and **4**, the F actin showed long stressed fibers throughout the cell except for Detroit 562 cells (Fig. 8). Also, confocal imaging revealed that untreated fibroblast cells possess cortical actin, some well-defined stress fibers, and cell polarity as shown by the presence of lamellipodia (Fig. 8A), but treatment with compounds **1** and **4** caused a remarkable change in cell shape. The filament number and total filament length of the actin were significantly increased in the treated fibroblast cells compared to the untreated cells. Normally, cancer cells possess a cortical cytoskeleton with short or no stress fibers which enable cells to grow, migrate and adhere much faster than normal epithelial squamous cells [37]. However, long F-actin filaments create stabilization of the cells and become stiff and inflexible, thereby disrupting cell-cell contact which includes adherent junction, tight junctions, and focal adhesions (cell-matrix) [40].

It was shown that Cpd **1** and Cpd **4** strongly inhibited cell migration ability. Fig. 9 demonstrates a decrease in the migration rate of CAL33 cells after treatment with 25 μM of compounds **1** and **4** in standard scratch assays, when compared to control cells after 48 h of incubation. It was observed that the untreated cells were able to close the gap as a sign of high migratory capacity, but exposure to Cpd **1** inhibited substantially cell motility in Detroit 562 and FaDu cells (Figs. 10 and 11). The inhibition of cell migration capacity of squamous cells may be attributed to the inhibition of EGFR by **c**ompound**s 1** and **4.** Nuclear factor-kappaB (NF-κB) has been identified as an important regulator of cancer cell invasion, metastasis, and angiogenesis and is a downstream target of EGFR [41,42]. Again the compounds may have caused a down-regulation and inactivation of the NF-κB pathway, which inhibited the invasion of HNSC cells. NF-κB-targeted proteins, such as matrix metalloproteinases (MMPs), cyclooxygenase-2 (COX-2), inducible nitric oxide s ynthase (iNOS) and vascular endothelial growth factor (VEGF), also, have been implicated in cancer cell migration.

The expression of PCNA (Proliferating Cell Nuclear Antigen) in squamous cells was observed when not treated, but treatment with compounds **1** and **4** was able to down-regulate the expression of PCNA in CAL33 cells. **Cpd 1** was only able to suppress the expression of PCNA in FaDu and Detroit 562 cells, while **Cpd 4** up-regulated PCNA expression in FaDu and Detroit 562 cells (Fig. 13). However, treatment with compounds 1 and 4 did not lead to a reduction in full-length Caspase 3 in CAL33 and Detroit562 cells, indicating a lack of Caspase 3 activation via cleavage in these cell lines. This observation suggests that apoptosis was not induced through the Caspase 3 pathway in CAL33 and Detroit562 cells. In contrast, FaDu cells exhibited a notable decrease in full-length Caspase 3 with the same compounds, suggesting Caspase 3 activation and apoptosis induction through this pathway. These differential responses highlight the potential for cell line-specific mechanisms in the activation of apoptosis by compounds 1 and 4. The reason could be that the compounds don't act through the downstream pathway (Caspase 3, 6, and 9), but the cascade of the event may go through the upstream pathway (Caspase 2, 8, and 10) which is at the beginning of the cascade, often called the initiators. The result from these finding correlate with Engel *et al.* [43], that extracts from *Pyrenacantha staudtil* and *Picralima nitida* were not able to cleave Caspase 3. However, it was revealed under the microscope (slides not presented) that the treated squamous cells with Cpd 1 and 4 showed condensation of chromatin, fragmentation of DNA, changes of nuclear shape, and membrane blebbing. The activation of Caspase 3 an indication of apoptosis was supported by the molecular docking analysis carried out on compound **1** and **4** with strong binding affinities of −8.5 and −8.8 kcal/mol respectively. This suggests that the compounds may have induced apoptosis at the sub G1 phase. This was in agreement with Iida et al. [44] that docetaxel activate caspase 3 in human oral squamous cell carcinoma cell lines.

Using the CAL33, Detroit 562, and FaDu cell lines to evaluate the antiproliferative effects of cassane diterpenoids offers both significant advantages and some limitations. These cell lines, derived from squamous cell carcinomas of the head and neck (HNSCC), are highly relevant models for studying compounds targeting this specific cancer type. Their

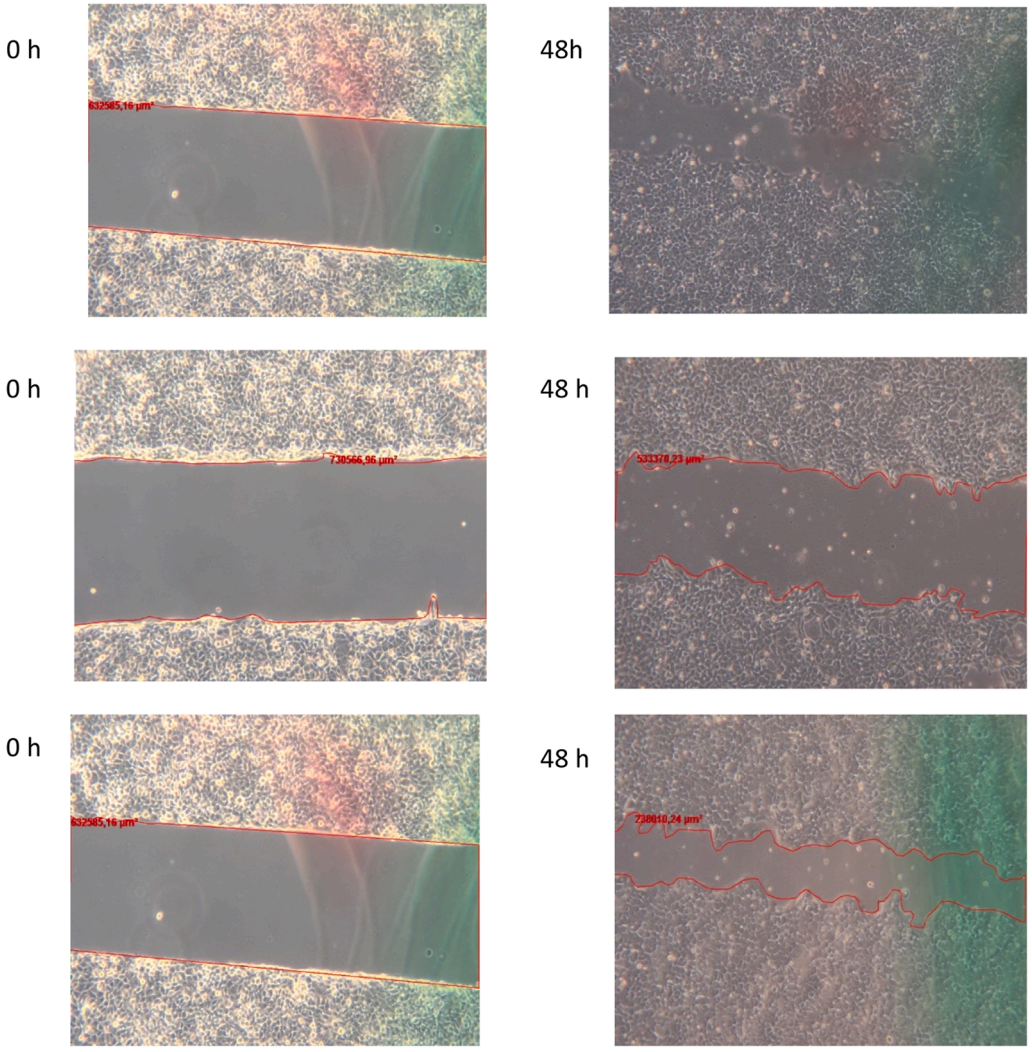

Fig. 12. Evaluation of cell migration capacity of FaDu cells during compounds 1 and 4 (25 μM) treatment for 2 days compared to DMSO (0.1 %).

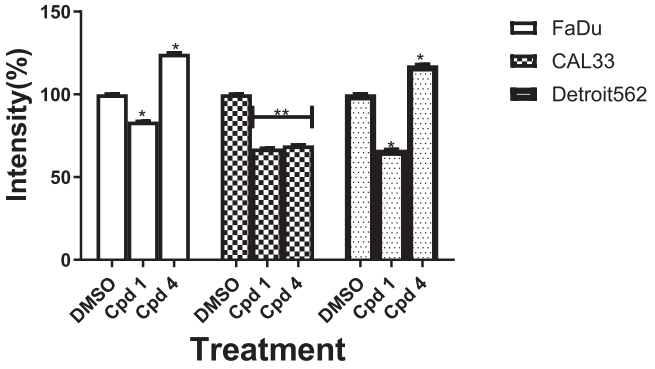

Fig. 13. : Changes in PCNA by Cpd 1 and 4 in squamous cells, FaDu, CAL33, and Detroit 562 were treated with 6-*β*-cinnamoyl-7-*α*-hydroxylvouacapen-5-*α*-ol (1) and Pulcherrimin (4) at 25 μM for 24 h and then subjected to western blotting for detection of PCNA.

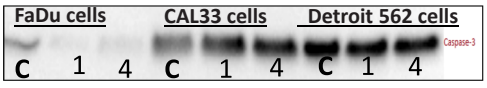

Fig. 14. : Changes in Caspase 3 activation by compound 1 and 4 in FaDu, CAL33 and Detroit562 cells.

Table 3
Molecular docking analysis of 6-*β*-cinnamoyl-7-*α*-hydroxylvouacapen-5-*α*-ol (1) and Pulcherrimin (4) with caspase 3.

Compounds	Binding affinity (kcal mol^{-1})
1	−8.5
4	−8.8

diverse origins allow for a broader exploration of cassane diterpenoid efficacy across different HNSCC subtypes. Furthermore, the unique genomic mutations in each line, such as TP53 and PIK3CA mutations in CAL33 and KRAS mutations in Detroit 562, provide an opportunity to assess potential genotype-specific responses to the compounds. These cell lines are well-established models with known proliferation and apoptosis markers, facilitating the assessment of the antiproliferative and pro-apoptotic effects of cassane diterpenoids [45]

However, the use of these cell lines also has inherent limitations. As in vitro models, they do not fully replicate the complex tumour microenvironment, immune interactions, or heterogeneity seen in actual tumours [46]. This may limit the generalizability of the findings to in vivo settings. Additionally, while these cell lines represent HNSCC, their utility in predicting the effects of cassane diterpenoids on other cancer types remains limited. Moreover, the homogeneous nature of cancer cell lines oversimplifies the genetic and cellular diversity found in real tumours, potentially masking resistance mechanisms or variant responses

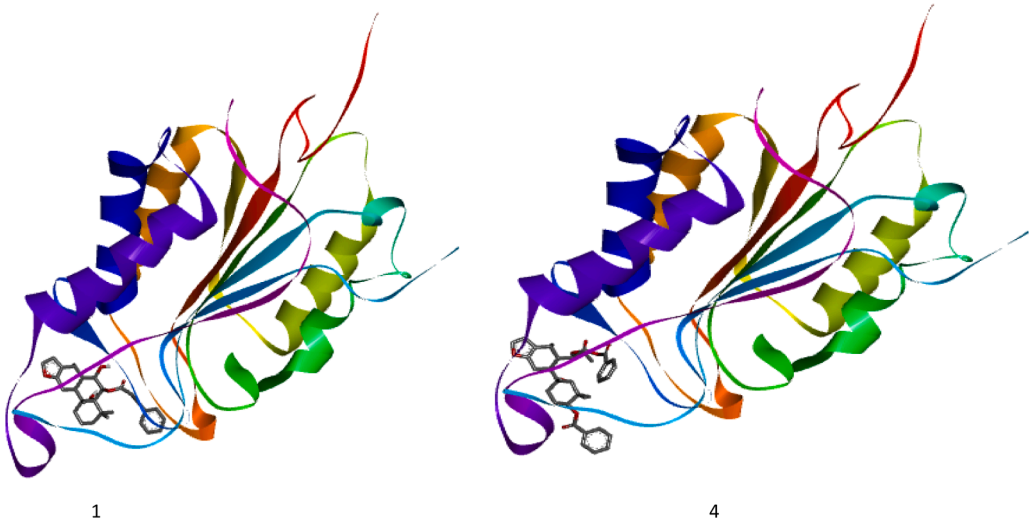

Fig. 15A. : 3D structure of compounds 1 and 4 in complex with caspase3 around the active pocket of the enzyme.

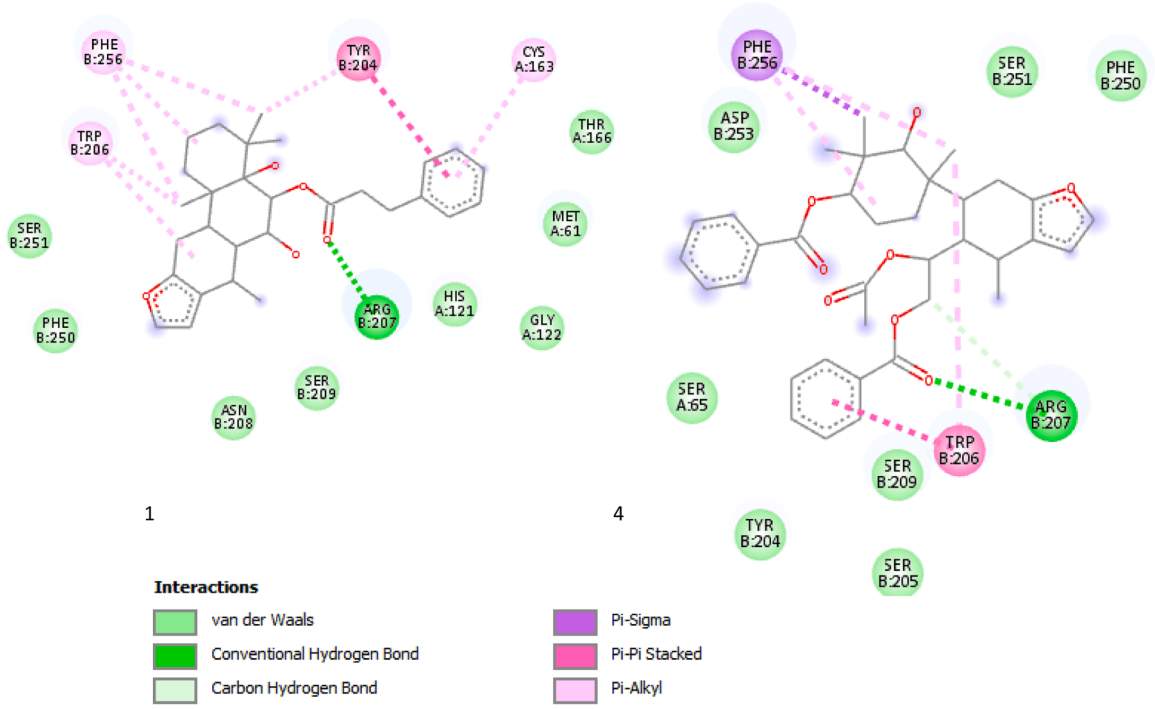

Fig. 15B. : A 2-D representation of compounds 1 and 4 displaying the key interactions with amino acid residues.

that could emerge in a more heterogeneous tumour setting [47]. Despite these drawbacks, these cell lines provide a valuable initial platform for screening and understanding the molecular effects of cassane diterpenoids. However, further studies in animal models and clinical trials would be necessary to confirm their broader therapeutic potential.

5. Conclusion

In the present study, we have shown that 6β-cinnamoyl-7α-hydroxyvouacapen-5α-ol (1) and Pulcherrimin E (4), naturally occurring cassane diterpenoid compounds, potently decreased the cell viability of the squamous cell lines, and the effect is likely to be the result of induction of apoptosis and cell cycle arrest. 6β-cinnamoyl-7α-hydroxyvouacapen-5α-ol (1) executed the anti-proliferative effect via down-regulation of PCNA activation and stimulation of caspase 3 activation in FaDu cells.

Authors Agreement

On behalf of other authors, I wish to confirm that all authors agree to the publication of this article with no conflicts of interest. Authors have approved the manuscript to be submitted to Toxicological Reports

Credit Author Statement

Oriakhi K performed the experiment. Engel N designed the project and supervised the experiments, Eharuyi O isolated the compounds. Interpretation of data: Oriakhi K, Orunmwensodia O.O and Engel N. Drafting of manuscript: Oriakhi K, Erharuyi O, Essien E.E and Engel N. Critical revision of manuscript: Oriakhi K, Erharuyi O, Essien E.E, Frerich B, Falodun A, Uadia P. O, and Engel Nadja. All authors reviewed the manuscript.

Funding

This research was funded by Deutsche Forschungs Gemeinschaft, Third World Academy of Science (DFG-TWAS 2018/19)

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We are grateful to Deutsche Forschungs Gemeinschaft, Third World Academy of Science (DFG-TWAS 2018/19) awarded to Kelly Oriakhi, Department of Medical Biochemistry, University of Benin. We also appreciate the department of Oral and Maxillofacial Surgery, Facial Plastic Surgery, Rostock University, Medical Center, Schillingallee, Germany for providing the facilities

Data availability

The authors do not have permission to share data.

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