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SF1: A Standardised Fraction of *Clinacanthus nutans* that Inhibits the Stemness Properties of Cancer Stem-Like Cells Derived from Cervical Cancer

(SF1: Fraksi Piawaian *Clinacanthus nutans* yang Merencat Sifat Stem Sel Menyerupai Sel Stem Kanser Diperolehi daripada Kanser Serviks)

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ABSTRACT

Cancer stem cells (CSCs) are a small population of tumour cells that are responsible for tumour initiation, metastases, recurrence, and resistance to conventional therapy. Hence, targeting CSCs is crucial in the fight against cancer. SF1, a standardised fraction from *Clinacanthus nutans* leaf extract, has been reported to exhibit potent and selective antineoplastic effects against cervical cancer cells. In this study, the potential of SF1 to inhibit the stemness of cervical cancer stem-like cells has been evaluated. SF1 extraction was carried out using the dry column vacuum chromatography technique. SiHa cell lines were cultured as spheres in CSC-conditioned medium (cervospheres), and the IC_{50} of SF1 against cervospheres was determined using the OZBlue Cell Viability Kit. The effects of SF1 on the cervosphere's stemness markers, including CD49f, CK17, SOX2, OCT4, and NANOG, were assessed using a flow cytometry assay. Self-renewal inhibition and anti-tumorigenesis effects of SF1 in cervospheres were evaluated using a sphere formation assay and a xenograft mouse model. The present study shows that SF1 treatment at an IC_{50} of 17.07 µg/mL inhibited the proliferation, self-renewal, and tumorigenic capacity of SiHa cervospheres *in vitro* and *in vivo*. A decrease in the expressions of CK17, SOX2, CD49f, and OCT4 in cervical CSCs indicated that SF1's inhibitory effects were also associated with the suppression of stemness markers. These results suggest that SF1 possesses an antitumor effect against cervical CSCs and may be regarded as a promising approach to the development of targeted anticancer agents for cervical cancer.

Keywords: Anticancer; cancer stem cells; cervical cancer; *Clinacanthus nutans*; stemness

ABSTRAK

Sel stem kanser adalah populasi kecil daripada keseluruhan sel tumor yang bertanggungjawab mencetus pertumbuhan awal tumor, perebakan, pengulangan dan kerintangan terhadap rawatan konvensional. Oleh itu, sasaran rawatan terhadap sel stem kanser adalah penting bagi memerangi kanser. SF1, fraksi yang diseragamkan daripada ekstrak daun *Clinacanthus nutans*, dilaporkan menunjukkan kesan anti-kanser yang kuat dan selektif terhadap sel kanser serviks. Dalam kajian ini, potensi SF1 untuk merencatkan ciri stem sel yang menyerupai sel stem kanser serviks telah dikaji. SF1 diekstrak menggunakan teknik kromatografi vakum lajur kering. Sel SiHa telah dikultur sebagai sel sfera di dalam kultur media khusus untuk pembentukan sel stem kanser (sel sfera kanser serviks) dan nilai IC_{50} SF1 terhadap sel sfera kanser serviks ditentukan menggunakan kit kelangsungan hidup OZBlue. Kesan SF1 pada penanda stem di dalam sel sfera kanser serviks, termasuk CD49f, CK17, SOX2, OCT4 dan NANOG, dianalisis menggunakan

ujian flositometri. Perencatan pembaharuan diri dan kesan anti-tumorigenesis SF1 dalam sel sfera kanser serviks dinilai menggunakan ujian pembentukan sel sfera dan model tetikus xenocantum. Kajian ini menunjukkan bahawa rawatan SF1 pada IC_{50} 17.07 µg/mL merencat proliferasi, pembaharuan diri dan keupayaan tumorigenik sel sfera kanser serviks SiHa *in vitro* dan *in vivo*. Kesan perencatan SF1 turut disertai oleh penurunan penanda stem untuk sel stem kanser serviks, seperti yang ditunjukkan oleh penurunan dalam pengekspresan CK17, SOX2, CD49f dan OCT4. Hasil kajian ini menunjukkan bahawa SF1 mempunyai kesan anti-tumor terhadap sel stem kanser serviks dan berpotensi sebagai pendekatan baharu untuk membangunkan agen anti-kanser yang bersasar terhadap kanser serviks.

Kata kunci: Anti-kanser; *Clinacanthus nutans*; kanser serviks; sel stem kanser; sifat stem

Introduction

Cervical cancer is one of the leading causes of cancer-related death in women worldwide and represents a global public health burden, particularly in low- and middle-income countries (Singh et al. 2023). Despite the advances in treatment modalities, rates of cervical cancer recurrence, therapy resistance, and metastases have remained high (George et al. 2022). It is increasingly evident that these predicaments are caused by the presence of cancer stem cells (CSCs) in the niche areas of the tumours (Aramini et al. 2022; Chang 2016). The 'clonal evolution' theory of carcinogenesis posits that cervical cancer is initiated when cells from a single clone indefinitely proliferate in an uncontrollable manner (Messerschmidt, Bhattacharya & Messerschmidt 2017). Nonetheless, there is emerging evidence that cervical cancer possesses intratumoral heterogeneity (Sun et al. 2022). The existence of CSCs is one factor that contributes to cervical cancer heterogeneity. Cervical CSCs (CCSCs) are slow-cycling cells that reside in specific niches in the tumour microenvironment. They are endowed with self-renewal and differentiation capabilities and are accountable for initiating malignant transformation and maintaining tumour progression in cervical cancer, leading to distant metastasis. Cancer cells of varying types exhibit distinct molecular and phenotypic traits that have been associated with unfavourable responses to chemotherapy and/or radiotherapy; these traits are correlated with an increased likelihood of lymph node metastases and pelvic recurrence in cases of cervical cancer (Di Fiore et al. 2022; Huang & Rofstad 2017).

The human papillomavirus (HPV) infection has been identified as the single most significant etiological agent of cervical cancer. HPV 16 and 18 are high-risk HPV subtypes that can cause up to 99.7% of cervical cancer cases (Burmeister et al. 2022). Cells located in the transformation zone between the endocervix and ectocervix, also known as the squamo-columnar (SC) junction, are found to be more susceptible to high-risk HPV infection and more likely to undergo cervical intraepithelial neoplasia (CIN) and carcinogenesis. Multiple subtypes of cervical cancer, including squamous cell carcinoma and adenocarcinoma, which account for 70% and 25% of cases, respectively, have been demonstrated to arise within or near the SC junction (Balasubramaniam et al. 2019; Rositch et al. 2022). The unique morphology and stemness traits of these particular SC junction cells distinguish them from the adjacent endocervical and ectocervical epithelium and have led to their designation as the CCSC niche. Furthermore, they also share a unique expression profile with CINs and cervical cancer, including both squamous cell carcinomas and adenocarcinomas, indicating that multiple cervical cancer subtypes are derived from the SC junction cells, or CCSCs (Di Fiore et al. 2022; Huang & Rofstad 2017). CCSCs are known to express high levels of CD49f and CK17 on their surface, which draw the high-risk HPV to bind to the cells, leading to virus internalisation, viral genome integration into the host genome, and subsequently viral oncoprotein (E6 and E7) synthesis. These oncoproteins, in turn, stimulate the overexpression of stemness-related genes such as SOX2, OCT4, and NANOG while decreasing endogenous tumour suppressor proteins, ultimately leading to apoptosis resistance, aberrant cell proliferation, and cancer progression (Organista-Nava et al. 2019). Furthermore, overexpression of CD49f, CK17, SOX2, OCT4, and NANOG has been demonstrated in primary cervical tumours and is implicated with the disease progression and prognosis (Bigoni-Ordóñez et al. 2018; Ortiz-Sánchez et al. 2016). Hence, developing efficient therapeutic agents to specifically target CSCs can potentially impede the propagation of HPV-related CINs and cervical carcinomas.

Clinacanthus nutans, a tropical herb belonging to the Acanthaceae family, has been widely used as herbal medicine in Southeast Asia for treating various diseases, including skin rashes, animal bites, diabetes, and gout. A number of studies that reported high anti-inflammatory, antiviral, antioxidant, and anti-diabetic activities in this plant species (Alam et al. 2016; Yahaya et al. 2015) also support its medicinal value. At the moment, this plant has gained attention among researchers for its potential in cancer therapy. *C. nutans* exerted a significant impact on cancer cells originating from the liver, lungs, breast, brain, and cervical regions. The induction of DNA damage, cell cycle arrest, apoptosis, immunomodulation, and stimulation of antioxidant and free radical scavenging activities served to demonstrate these effects (Huang et al. 2015; Ng et al. 2017; Yong et al. 2013). In previous studies, a fraction from *C. nutans* was successfully isolated, which exhibited potent cytotoxic and antiproliferative effects against cancer cells, specifically SF1. This standardised fraction was found to induce cervical cancer cell death via apoptosis and cell growth inhibition at the G1 phase checkpoint. In particular, SF1 has shown a selective inhibitory effect on cancer cells while sparing normal cells (Zainuddin et al. 2020, 2019). Therefore, the potential of SF1 as a safe and effective anticancer agent is promising. Hence, the present study aimed to determine the effectiveness of this fraction in inhibiting the CCSC subpopulation by way of evaluating the cell viability, sphere formation efficiency, stemness characteristics, and tumorigenicity in xenograft mouse models. The SiHa cell line, a well-established cell line for cervical squamous cell carcinoma, was used to generate cervical cancer stem-like cells in this study.

Material and Methods

PLANTS MATERIALS

The aerial parts of *C. nutans* (Burm. F.) were collected from Pengkalan Chepa, Kelantan, and a voucher specimen number PIIUM 0238-2 was deposited at the Herbarium of the Kulliyyah of Pharmacy, International Islamic University Malaysia.

EXTRACT PREPARATION AND FRACTIONATION OF SF1

The extraction was performed in accordance with the published protocol (Zainuddin et al. 2019). Briefly, the collected leaves were dried and ground to a coarse powder. The dried powdered leaves were soaked in hexane (HMBG, Germany) overnight at 60 °C. The mixture was then filtered, and the leaf remnants were then extracted with chloroform (HMBG, Germany) using the Soxhlet extractor for 48 h. The chloroform filtrates were concentrated using a rotary evaporator to give the chloroform extract. For the isolation of SF1, the chloroform extract was further subjected to fractionation using two steps of the dry column vacuum chromatography technique. Firstly, the chloroform extract was chromatographed on a silica gel 60 (250 g) (Merck, Germany), and the column was eluted with hexane-ethyl acetate (1:1) (Merck, Germany) in order to collect and isolate the active fraction, specifically F11. The F11 compound was concentrated and further purified by loading it onto the second column chromatography using acetonitrile-methanol (2:8) (Merck, Germany) as the elution solvent to isolate the SF1 compound. The collection of fractions for the first and second column chromatography was monitored by spotting them on a thin-layered chromatography (TLC) plate (Merck, Germany), using chloroform-methanol (1:1) and chloroform-methanol (2:8) as the mobile phases, respectively. The retention factor (Rf) was calculated using the following formula: Rf = distance moved by solute/distance moved by solvent; this was compared with the standard (previous SF1) to ensure the obtainment of a similar fraction.

CELL LINES AND CULTURE

Human cervical cancer cell lines, SiHa cells (ATCC@-HTB-35, squamous cell carcinoma, HPV-16), were acquired from the American Type Culture Collection (ATCC, USA) and cultured in T75 tissue culture flasks. The monolayer cells were maintained in DMEM media (Nacalai tesque, Japan) supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich, USA) and 100 U/mL penicillin/streptomycin (Nacalai tesque, Japan) in a humidified atmosphere of 5% CO_2 at 37 °C.

ENRICHMENT OF CERVOSPHERES

To enrich undifferentiated tumorspheres derived from cervical cancer cell lines (cervospheres), the monolayer cells were cultured in serum-free media using a non-adhesive culture system described by Chen et al. (2012) with minor modifications. Briefly, the monolayer cells were grown to 70-80% confluence and then harvested, counted, and washed with phosphate buffer solution (PBS). Then, the cells were cultured with DMEM/F-12

media (Nacalai tesque, Japan) supplemented with 20 ng/mL epidermal growth factor (Sigma-Aldrich, USA), 20 ng/mL basic fibroblast growth factor (Roche, USA), 10 µL/mL B27 (50x, Thermo Fisher Scientific, USA), and 100 U/mL penicillin/streptomycin (Nacalai tesque, Japan) in 1.5% agarose-coated dishes at a density of 1×10^4 cells/mL. Cervosphere formation was monitored daily, and one-third of the media was replaced every three days. For maintenance, the cervospheres were subjected to gentle centrifugation at 300 × g for 5 min, washed with PBS, disaggregated with 0.05% trypsin (Thermo Fisher Scientific, USA) to generate single cell suspensions, and passage into new plates every 7 days. Only cervospheres in the third passage and above were used for further tests.

CYTOTOXICITY ASSAY

Prior to cell treatment, the SF1 was diluted in DMSO (Nacalai Tesque, Japan) and followed by a serial dilution ranging from 0.2 to 100 µg/mL. The selection of these concentrations was based on the cytotoxicity studies previously conducted for SF1 (Zainuddin et al. 2019). Cisplatin (Sigma-Aldrich, USA) was used as a positive control, whereas DMSO was used as a negative (vehicle) control with a concentration less than 0.1%. Cell cytotoxicity was determined by the OZBlue Cell Viability Kit (OZ Biosciences, USA) following the supplier's instructions. Briefly, cervosphere cultures were collected, centrifuged, and dissociated with 0.05% trypsin to form single-cell suspensions. Then, the cells were seeded in agarose-coated 96-well plates at an initial number of 5×10^3 cells/well (100 µL/well) and allowed to grow for 7 days. After time, the cells were exposed to various concentrations of SF1 and cisplatin for 72 h. Then, the OZBlue Cell Viability Kit solution was added to each well at a volume equal to 10% of the culture media volume, and the plates were incubated for another 8 h at 37 °C. The absorbance was measured using a microplate reader at 570 nm. The IC_{50} was determined from curves constructed by plotting the percentage cell viability versus the drug concentrations. The IC_{50} obtained was used for the downstream study.

SPHERE-FORMATION ASSAY

After 7 days of culture to allow cervosphere generation, the cells were treated with the IC_{50} of SF1 and cisplatin separately for 72 h. Then, the cells were gently collected, dissociated into single cells, and replated at a density of 1×10^4 cells/mL in agarose-coated 6-well plates (Hydragene, USA), and new medium was added every 3 days. Following 7 days of culture, the sphere number of each well with a diameter greater than 100 µm was manually counted. The ratio of spheres to seeded cells, expressed as a percentage, is the sphere-forming efficiency (SFE).

ANALYSIS OF PROTEIN MARKERS BY FLOW CYTOMETRY

For CD49f and CK17 protein markers, cervospheres treated with SF1 at 17.07 µg/mL and cisplatin at 4.29 µg/mL for 72 h, as well as untreated cervospheres, were collected separately, washed, and dissociated into single cells as previously mentioned. For each primary antibody, 1×10^6 cells were incubated with the appropriate dilution of anti-CD49f-PE (Miltenyi Biotec, Germany) and anti-CK17-FITC (Santa Cruz Biotechnology, USA) in 100 µL flow buffer (PBS 1 × 0.5% BSA) (Vivantis, Malaysia) on ice in the dark. After 30 min, cells were washed twice with flow buffer, spun down at 300 × g for 5 min at room temperature, and fixed with 4% p-formaldehyde (Nacalai Tesque, Japan) in PBS.

For SOX2, NANOG, and OCT4 protein markers, cervosphere cells treated or untreated with SF1 and cisplatin for 72 h were collected, washed, and counted. Before incubation with anti-SOX2, anti-NANOG, and anti-OCT4, cells were fixed with 4% p-formaldehyde for 10 min and permeabilized with 0.1% Triton-X (Sigma-Aldrich, USA) for 15 min at room temperature in the dark. Then, cells were washed with flow buffer and incubated with the primary antibody. For each primary antibody, 1×10^6 cells were incubated with anti-SOX2-AlexaFluor488 (BioLegend, USA), anti-OCT4-PE (Miltenyi Biotec, Germany), and anti-NANOG-PE (Miltenyi Biotec, Germany) in 100 µL flow buffer for 30 min on ice in the dark. Subsequently, cells were washed with flow buffer, spun down at 300 × g for 5 min at room temperature, and resuspended in PBS. All staining cells were read in the BD FAC Scan™. At least 10,000 events were recorded for each flow cytometry measurement. FlowJo™ v10 software was utilised for analysing data.

IN VIVO TUMORIGENIC ASSAY

Female athymic nude mice (BALB/c nu/nu) were used in this work to test the tumorigenic capacity of CCSC-like cells, treated and untreated. The animals were 4-6 weeks old, weighed 20-25 g, and were obtained from NOMURA, Thailand. The mice were housed in laminar flow cabinets under pathogen-free conditions. They were kept at room

temperature. The USM Institutional Animal Care and Use Committee approved the animal protocols (Approval Number: USM/IACUC/2019/(120)(1021)).

After 1 week of acclimatisation, the mice were randomly assigned to five experimental groups (5 mice/group) as follows: (1) mice received untreated monolayer cells at a concentration of 1×10^6 cells; (2) mice received untreated cervosphere cells at a concentration of 1×10^6 cells; (3) mice received SF1-treated cervosphere cells at a concentration of 1×10^6 cells; (4) mice received cisplatin-treated cervosphere cells at a concentration of 1×10^6 cells; and (5) mice received PBS as the vehicle control group. Prior to cell inoculation, SiHa monolayer cells and their respective cervosphere cells, either treated or untreated with SF1 and cisplatin, were collected, enzymatically dissociated, washed in PBS, and kept at 4 °C until subcutaneously injected into the mice. 1×10^6 cells in 200 µL PBS were inoculated subcutaneously into the mice on the dorsal side between the scapulae using the 25-gauge needle. Tumour growth as well as secondary tumour incidences were inspected by observation and palpation every 3 days for 12 weeks following cell injection. For subcutaneous tumours in mice, the maximal allowable size is 2 cm in diameter ('Tumour Policy for Mice and Rats', Boston University Research Compliance). At the end of 12 weeks, the mice were sacrificed by cervical dislocation, and the tumour tissues were dissected and measured using a digital Vernier calliper. Tumour volume was calculated using the formula $V = ½ (L \times W^2)$, where L is the largest diameter and W is the smallest.

STATISTICAL ANALYSIS

Statistical analysis was conducted using GraphPad Prism software (version 9.0, GraphPad Software, USA). All results are representative of experiments performed in triplicate and are expressed as the mean ± SD. Evaluation of the statistical significance between the groups was performed using one-way analysis of variance (ANOVA) with a post-hoc Tukey test for normally distributed data and Kruskal-Wallis with a post-hoc Dunn's pairwise test for abnormally distributed data. Categorical variables were analysed using Fisher's exact test. Statistical significance was assumed at $p < 0.05$.

RESULTS AND DISCUSSION

CYTOTOXIC EFFECT OF SF1 ON CERVOSPHERE

SF1 is a semi-purified fraction isolated from the leaves of *C. nutans* using bioassay-guided fractionation. The fraction has demonstrated potent anticancer effects on cervical cancer cells by causing DNA damage, cell cycle arrest, and triggering apoptosis (Zainuddin et al. 2019). Alkaloids with functional group amines emerged as the dominant phytochemical class in the analysis of SF1 using Fourier transform infrared spectroscopy (FTIR) and liquid chromatography-mass spectrometry (LC-MS), followed by alkanes, alkenes, esters, and aromatics (Zainuddin et al. 2020). Alkaloids are well-established as significant sources of novel drugs and leads for anticancer drug discovery due to their potent antineoplastic effects. Several alkaloids, including vinblastine, vincristine, vinorelbine, and vindesine have already been used for the clinical treatment of cancer, and many others are currently under clinical trials (Mondal et al. 2019). Therefore, SF1's potential to be developed as an anticancer agent is promising, and its biological activities in cancer should be further explored.

The CSC population forms a tiny fraction of the whole tumour and shares the common features of normal stem cells, including self-renewal, proliferation, and differentiation into multiple lineages. Several emerging theories have proposed that CSCs arise from mutated normal stem cells or progenitor cells, leading to aberrant and dysregulated stemness properties and ultimately the expansion of malignant cell populations (Li & Neaves 2006). Hence, cancer studies have been focusing on the development of new approaches to eliminate these highly tumorigenic and multipotent CSCs by targeting their stemness characteristics and pathways. To screen for potential drug sensitivity in CSCs, isolation and enrichment of CSCs are crucial (Lee et al. 2016). One of the methods used for these purposes is the tumour-derived spheroids culture system, which has been established as a reliable model of CSCs for basic and preclinical studies (Ishiguro et al. 2017). This method has been applied in this work, where a human cervical cancer cell line was cultured as floating cells in serum-free media (without FBS) and in the presence of mitogens including EGF, bFGF, and B27 to enrich the cervical cancer stem-like cells and prevent cell differentiation. Under this culture condition, only a small minority of the observed parental cells were able to survive, developing into tumorspheres, regenerating, and retaining their proliferation for the long term. This reflects the presence of a subset of cancer cells with CSC characteristics, including the ability to grow under anchorage-independent conditions, escape anoikis, and proliferate by synthesising higher levels of growth factors and extracellular matrix receptors (Liu et al. 2013).

Previous studies have demonstrated that SF1 has potent inhibitory effects against cervical cancer cell lines and the proposed mechanisms (Zainuddin et al. 2020, 2019). In an attempt to bring this research forward, the effect of the SF1 fraction in CCSCs was further evaluated. To evaluate the cytotoxicity effect of SF1 on SiHa cervospheres (CCSC-like cells), the cells were exposed to various SF1 concentrations. Cisplatin was used as a positive control, as it is one of the first-line chemotherapeutic agents for cervical cancer treatment. As shown in Figure 1(A), treatment with SF1 reduced the viability of cervospheres in a dose-dependent manner. The IC_{50} of SF1 against cervospheres was 17.07 ± 1.63 µg/mL, which is higher compared to cisplatin (IC_{50} = 4.29 ± 1.95 µg/mL) (Table 1). Additionally, it was observed that cervospheres treated with SF1 and cisplatin were smaller, loosely compact, and irregularly conformed compared to the untreated cervospheres (Figure 1(B)-1(D)). Furthermore, the changes in cervosphere morphology following SF1 treatment were also consistent with apoptosis features, including cell shrinkage, disaggregation, chromatin condensation, and apoptotic body formation (Pandrangi et al. 2022). Thus, the results showed that SF1 has a potent cytotoxicity effect on SiHa cervospheres by decreasing viability and preventing further proliferation of the cells. The IC_{50} obtained in this test was used for the downstream study.

INHIBITION OF CERVOSPHERES SELF-RENEWAL BY SF1

The sphere formation assay serves as one of the important tools to assess the effect of drugs or novel agents on the self-renewal of CSC in 3D culture. Consistent with the cytotoxicity assay findings, SF1 was also found to inhibit the self-renewal capacity of cervospheres by interfering with the formation and proliferation of tumourspheres. As shown in Figure 2, SF1 and cisplatin significantly reduced the SFE of cervospheres ($p < 0.0001$ and $p = 0.0001$, respectively) compared to the untreated cervospheres. In addition, the SFE of cervospheres treated with SF1 and cisplatin did not differ significantly, indicating that the inhibitory effect of SF1 at 17.07 µg/mL on sphere formation was comparable to that of cisplatin treatment at 4.29 µg/mL. This suggests that SF1 may exhibit a potent inhibitory effect against CCSC self-renewal. The self-renewal feature is considered a hallmark of CSC stemness, and it is directly correlated with their capacity to regenerate tumours after cancer therapy (Bahmad et al. 2018). Inhibition of CSC self-renewal can be attributed to downregulation of Hedgehog (Hh), Wnt, Notch, and B-cell-specific Moloney murine leukaemia virus integration site 1 (BMI1) pathways, which have been proposed as the key players in maintaining the self-renewal and proliferation of CSCs (Borah et al. 2015).

SUPPRESSION OF CCSC PHENOTYPIC MARKERS BY SF1

CD49f and CK17 have been used to characterise CCSC populations and have been proposed as potential targets for the development of novel anticancer agents (Bigoni-Ordóñez et al. 2018; Ortiz-Sánchez et al. 2019). CD49f is a transmembrane protein expressed in a number of normal stem cells and CSCs. Upregulation of CD49f in cancer cells was associated with enrichment of stem-like properties, including self-renewal, tumour-forming capacity, and resistance to chemotherapeutic drugs and radiation (Lopez et al. 2012). In cervical cancer, CD49f was described as a target during HPV entry into host cells and was associated with the cancer's progression (Ammothumkandy et al. 2016). From the findings in Figure 3, the expression of CD49f in cervospheres was slightly reduced with SF1 treatment in comparison with the untreated cervospheres. On the contrary, a marked decrease in CK17 expression was noted in cells treated with SF1 compared to the untreated ones ($p < 0.05$). Apart from that, cisplatin treatment more noticeably suppressed the levels of both CD49f and CK17 expressions in cervospheres compared to SF1.

This flow cytometry analysis demonstrated that SF1 was capable of reducing the expression of CCSC-specific markers, primarily the CK17 protein, in SiHa cervospheres. CK17 is a marker for sub-columnar reserve cells of the uterine cervix, which also become the target for HPV binding (Martens et al. 2004). This marker was reported to induce metaplasia in cervical tissues and was positively correlated with the degree of cervical intraepithelial neoplasia (CIN) lesions (Mitra et al. 2017). Additionally, CK17 was implicated in the chemoresistance and metastases of cervical cancer and served as an important diagnostic and prognostic biomarker for this disease (Wu et al. 2017). Since CK17 plays a crucial role in the progression of cervical cancer, it is hypothesised that inhibiting this marker may delay or halt the progression of the disease.

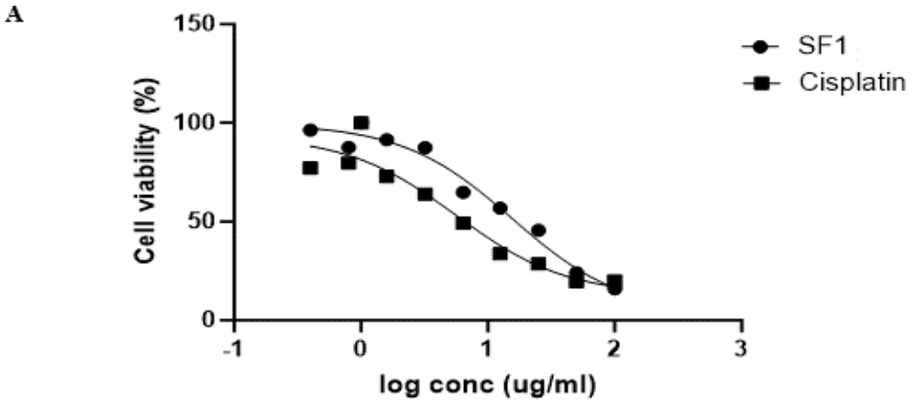
SUPPRESSION OF CCSC PLURIPOTENT MARKERS BY SF1

SOX2, NANOG, and OCT4 transcription factors are important regulators of embryonic stem cell pluripotency, differentiation, and self-renewal (Swain et al. 2020). In

cervical cancer, the tumour spheres derived from both primary tumours and cell lines strongly expressed SOX2, NANOG, and OCT4 (Feng et al. 2009). The upregulation of these markers was closely linked to augmentation of self-renewal, tumorigenicity, apoptosis inhibition, cell migration, and chemoresistance in CCSCs (Ding et al. 2016; Kim et al. 2015). The present study's results show that SF1 may inhibit the CCSCs by selectively suppressing their stemness marker proteins. As shown in Figure 4, SF1 treatment significantly reduced the protein level of SOX2 when compared to its untreated counterparts ($p < 0.05$). Suppression of OCT4 protein expression was also observed in SF1-treated cervospheres, although not to a significant level. Additionally, similar trends were observed with cisplatin treatment for both stemness markers. However, no change was found in NANOG expression in either SF1 or cisplatin-treated cervospheres compared to the untreated ones. Overall, the findings show that SF1 downregulated SOX2 and OCT4 expressions in cervospheres, with a limited effect on NANOG expression. Recent studies have shown that co-expression of SOX2 and OCT4 plays important roles in promoting tumour radioresistance and recurrence relative to SOX2 and OCT4 alone. Additionally, both markers have been proposed as candidate markers for predicting the prognosis of cervical cancer (Shen et al. 2014). Taken together, suppression of the CCSC phenotypic and pluripotent markers by SF1 may enhance the patients' response to cancer treatment and prevent further relapse.

INHIBITION OF CERVOSPHERES *IN VIVO* TUMORGENICITY BY SF1

Xenotransplation is recognised as the gold standard for assessing the tumorigenic capacity of cancer cells. *In vivo*



Compound	IC_{50} values (µg/ml)
SF1	17.07 ± 1.63*
Cisplatin (CP)	4.29 ± 1.95

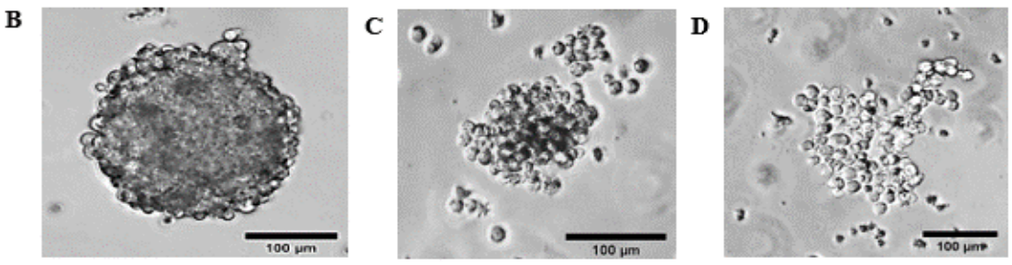


FIGURE 1. SF1 exhibited cytotoxic effects on SiHa cervospheres. (A) Treatment with SF1 at various concentrations inhibited the viability of cervospheres in a dose-dependent manner. Cisplatin was used as a positive control. Data are expressed as mean ± SD (n = 3 independent assays). The asterisk indicates a significant difference with respect to the control (*$P < 0.05$). (B) Microphotographs (X20 magnification) of untreated cervospheres. (C) Microphotographs (X20 magnification) of SF1-treated cervospheres. (D) Microphotographs (X20 magnification) of cisplatin-treated cervospheres

tumorigenicity has been found to positively correspond with the sphere formation capacity of cancer cells *in vitro* (Wang et al. 2018). Hence, to validate the present study's *in vitro* findings, xenotransplantation of treated and untreated cervosphere cells and monolayer cells was performed in immunodeficient mice. As shown in Table 1, inoculation of athymic nude mice with 1×10^6 untreated cervosphere cells promoted tumour growth in 3/5 mice ($p < 0.01$). Tumours begin to develop within 14-26 days after inoculation, and the tumour volumes ranged from 1.67 to 151.08 mm^3 (Figure 5). Interestingly, after 12 weeks of monitoring, no tumour growth was found in other groups, including SiHa monolayers, SF1-treated cervospheres, cisplatin treated cervospheres, and the group receiving PBS only (vehicle control). No decrease in body weight or any adverse events were seen throughout the experiment.

The results indicated that the sphere-forming cells exhibited more potent self-renewal and tumour-initiating properties *in vivo* compared to the monolayer cells. Consistent with the present study's observation, it was reported that lower cell numbers of cervospheres, as few as 1×10^4, were able to propagate tumours in immunodeficient mice, in contrast to monolayer cells (Zhang et al. 2012). Besides, SF1 was found to have been able to impede the self-renewal and tumorigenicity of CCSCs and prevent tumour development *in vivo.* This finding was also in line with the outcomes of the *in vitro* sphere formation assay. Besides, few studies have outlined the positive association between CCSC stemness markers such as SOX2, Olig2, and nesting and their tumorigenic potential *in vivo* (Bhattacharjee et al. 2022). Hence, it was proposed that the suppression of tumour growth in xenograft mice be linked to the downregulation of stemness markers, mainly SOX2, in SF1-treated CCSCs.

Apart from that, SF1 had a comparable effect to cisplatin under these study conditions in terms of suppressing the viability, self-renewal, stemness, and tumorigenic capability of CCSCs. Despite cisplatin's reputation as a potent chemotherapeutic agent, its efficacy is often limited by its deleterious side effects and the emergence of drug resistance (Bhattacharjee et al. 2022). Moreover, numerous studies have demonstrated that CSCs are resistant to cisplatin, necessitating a higher concentration to inhibit the cells, which is clinically impractical and may exacerbate the deleterious side effects of the drug (Cortes-Derickse et al. 2010). Meanwhile, several studies have found that *C. nutans* extracts, including SF1 possess potent and selective anticancer effects on cancer cells while preserving normal cells (Fong et al. 2016; Zainuddin et al. 2019). Hence, SF1 may serve as a promising alternative to cisplatin in cervical cancer treatment.

TABLE 1. The number of tumours developed *in vivo* (n = 5/group)

Group	No. cells injected	Tumour incidence	Latency (Days)
Monolayer	1×10^6	0/5	0
Cervosphere	1×10^6	3/5**	14-26
Cervosphere + SF1	1×10^6	0/5	0
Cervosphere + cisplatin	1×10^6	0/5	0
PBS (Vehicle)	0	0/5	0

Data are expressed as counts and the asterisk indicates a significant difference with respect to the control and treatment groups (**$P < 0.01$)

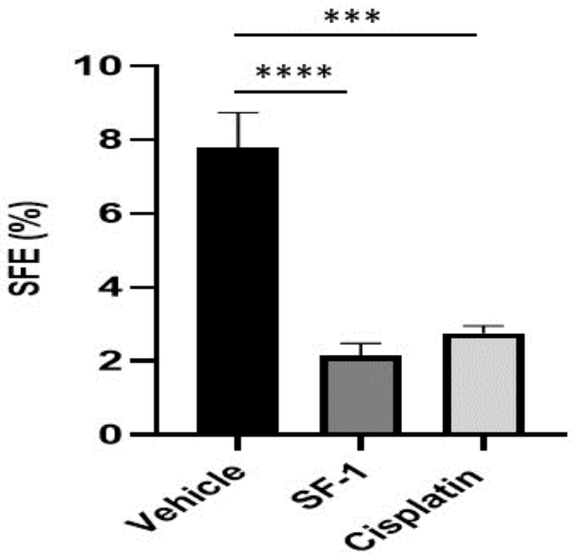


FIGURE 2. SF1 inhibited the self-renewal and sphere-forming efficiency of cervospheres *in vitro* at an IC_{50} of 17.07 µg/mL. There is no significant difference in SFE between SF1 and cisplatin-treated cervospheres. Data are expressed as mean ± SD (n = 3 independent assays), and the asterisk indicates a significant difference with respect to the control (***P = 0.0001, ****P < 0.0001)

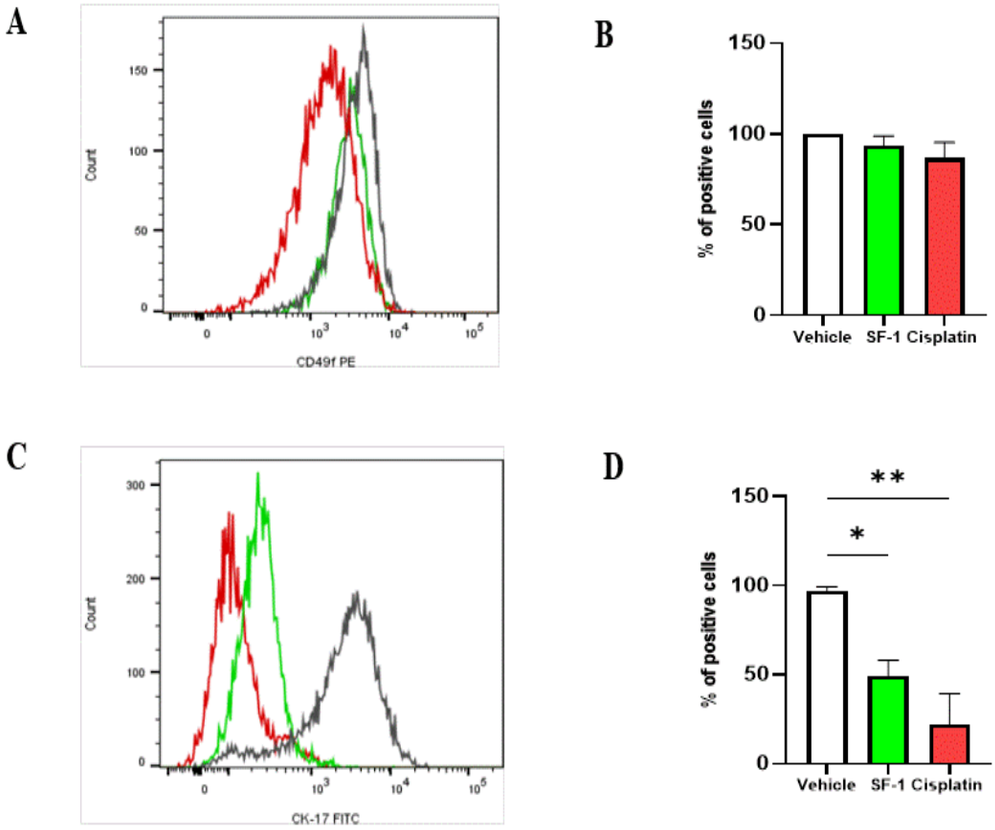


FIGURE 3. SF1 reduced the expressions of putative CCSC phenotypic markers, CD49f and CK17, in SiHa cervospheres. Cervospheres were treated with SF1 (green line), cisplatin (red line), and without treatment or vehicle control (black line). CD49f (A and B) and CK17 (C and D) expressions were analysed using flow cytometry. Data are expressed as mean ± SD (n = 3 independent assays), and the asterisk indicates a significant difference with respect to the control (*P <0.05, **P < 0.01) (B and D)

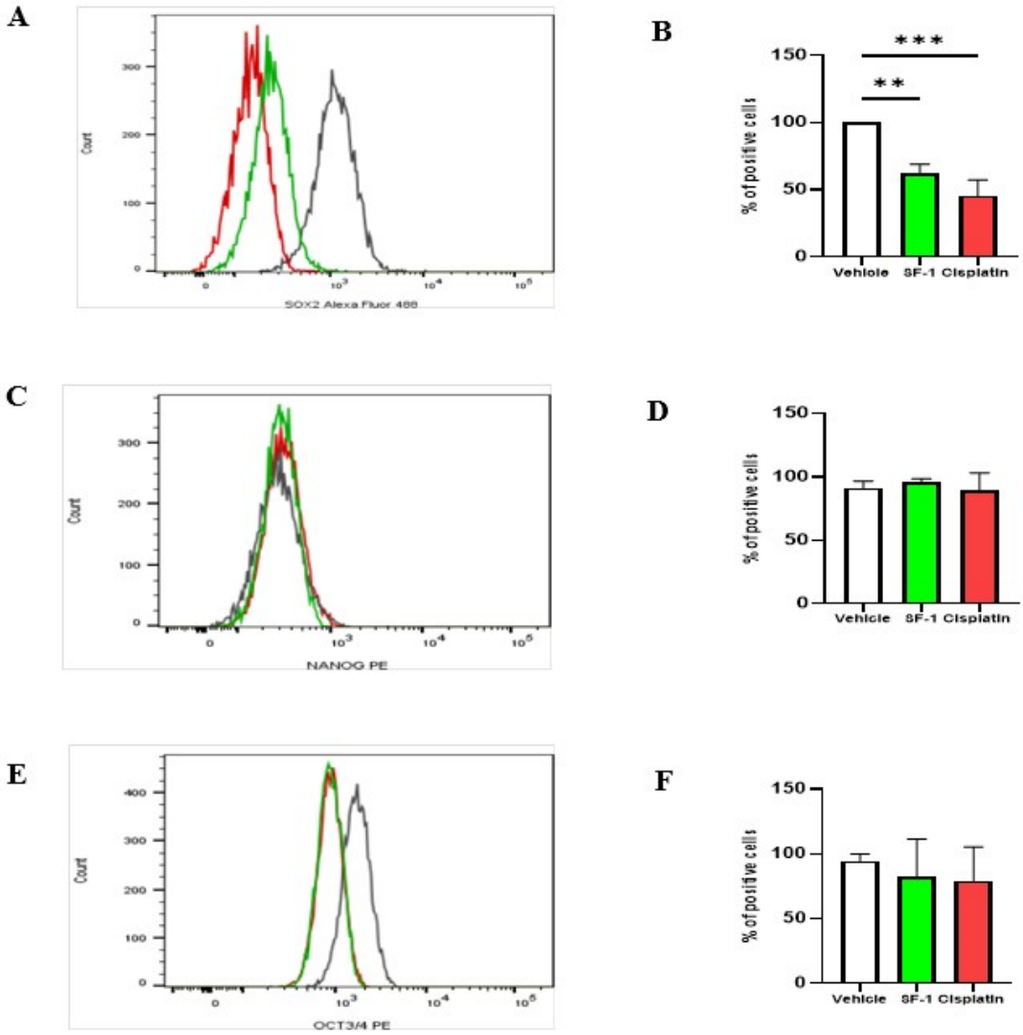


FIGURE 4. SF1 reduced the expression of stemness markers, SOX2, and OCT4 in cervospheres, except for NANOG. SiHa cervospheres were treated with SF1 (green line), cisplatin (red line), and without treatment or vehicle control (black line). SOX2 (A and B), NANOG (C and D), and OCT4 (E and F) expressions were analysed using flow cytometry. Data are expressed as mean ± SD (n = 3 independent assays), and the asterisk indicates a significant difference with respect to the control (**P <0.01, ***P < 0.001) (B, D, and F)

FIGURE 5. Tumour growth in Balb/c nude mice inoculated with untreated SiHa cervospheres. SiHa monolayer cells and cervosphere cells, treated or untreated with SF1 or cisplatin for 72 h, were subcutaneously inoculated into the mice on the dorsal side between the scapulae (n = 5)

Conclusions

To the best of our knowledge, this is the first kind of research that demonstrated the role of *C. nutans* extract on cancer stem cells. In summary, treatment with SF1, a standard fraction isolated from *C. nutans,* induced cytotoxicity in CSC derived from cervical cancer cell lines and inhibited their self-renewal properties *in vitro* and *in vivo*. A selective suppression of cervical cancer stemness characteristics, primarily the CK17 and SOX2 protein markers, was present along with these effects. Hence, this study provides an experimental basis for the potential of SF1 as a natural anticancer agent to eliminate both cancer cells and cancer stem cells. Nevertheless, further preclinical studies are required to assess the anticancer effect of SF1 and the underlying mechanisms.

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References

Alam, A., Ferdosh, S., Ghafoor, K., Hakim, A., Juraimi, A.S. & Sarker, Z.I. 2016. *Clinacanthus nutans*: A review of the medicinal uses, pharmacology and phytochemistry. *Asian Pacific Journal of Tropical Medicine* 9(4): 402-409.

Ammothumkandy, A., Maliekal, T.T., Bose, M.V., Rajkumar, T., Shirley, S., Thejaswini, B., Giri, V.G. & Krishna, S. 2016. CD66 and CD49f expressing cells are associated with distinct neoplastic phenotypes and progression in human cervical cancer. *European Journal of Cancer* 60: 166-178.

Aramini, B., Masciale, V., Grisendi, G., Bertolini, F., Maur, M., Guaitoli, G., Chrystel, I., Morandi, U., Stella, F., Dominici, M. & Haider, K.H. 2022. Dissecting tumor growth: The role of cancer stem cells in drug resistance and recurrence. *Cancers (Basel)* 14(4): 976.

Bahmad, H.F., Cheaito, K., Chalhoub, R.M., Hadadeh, O., Monzer, A., Ballout, F., El-Hajj, A., Mukherji, D., Liu, Y.N., Daoud, G. & Abou-Kheir, W. 2018. Sphere-formation assay: Three-dimensional *in vitro* culturing of prostate cancer stem/Progenitor sphere-forming cells. *Frontiers in Oncology* 8: 347.

Balasubramaniam, S.D., Balakrishnan, V., Oon, C.E. & Kaur, G. 2019. Key molecular events in cervical cancer development. *Medicina (Lithuania)* 55(7): 384.

Bhattacharjee, R., Dey, T., Kumar, L., Kar, S., Sarkar, R., Ghorai, M., Malik, S., Jha, N.K., Vellingiri, B., Kesari, K.K., Pérez de la Lastra, J.M. & Dey, A. 2022. Cellular landscaping of cisplatin resistance in cervical cancer. *Biomedicine and Pharmacotherapy* 153: 113345.

Bigoni-ordóñez, G.D., Ortiz-sánchez, E., Rosendo-chalma, P., Valencia-González, H.A., Aceves, C. & García-Carrancá, A. 2018. Molecular iodine inhibits the expression of stemness markers on cancer stem-like cells of established cell lines derived from cervical cancer. *BMC Cancer* 18(1): 928.

Borah, A., Raveendran, S., Rochani, A., Maekawa, T. & Kumar, D.S. 2015. Targeting self-renewal pathways in cancer stem cells: Clinical implications for cancer therapy. *Oncogenesis* 4(11): e177.

Burmeister, C.A., Khan, S.F., Schäfer, G., Mbatani, N., Adams, T., Moodley, J. & Prince, S. 2022. Cervical cancer therapies: Current challenges and future perspectives. *Tumour Virus Research* 13: 200238.

Chang, J.C. 2016. Role in tumor growth, recurrence, metastasis, and treatment resistance. *Medicine* 95(S1): S20-S25.

Chen, S.F., Chang, Y.C., Nieh, S., Liu, C.L., Yang, C.Y. & Lin, Y.S. 2012. Nonadhesive culture system as a model of rapid sphere formation with cancer stem cell properties. *PLoS ONE* 7(2): e31864.

Cortes-Dericks, L., Carboni, G.L., Schmid, R.A. & Karoubi, G. 2010. Putative cancer stem cells in malignant pleural mesothelioma show resistance to cisplatin and pemetrexed. *International Journal of Oncology* 37(2): 437-444.

Di Fiore, R., Suleiman, S., Drago-Ferrante, R., Subbannayya, Y., Pentimalli, F., Giordano, A., & Calleja-Agius, J. 2022. Cancer stem cells and their possible implications in cervical cancer: A short review. *International Journal of Molecular Sciences* 23(9): 5167.

Ding, Y., Yu, A.Q., Wang, X.L., Guo, X.R., Yuan, Y.H. & Li, D.S. 2016. Forced expression of Nanog with mRNA synthesized *in vitro* to evaluate the malignancy of HeLa cells through acquiring cancer stem cell phenotypes. *Oncology Reports* 35(5): 2643-2650.

Feng, D., Peng, C., Li, C., Zhou, Y., Li, M., Ling, B., Wei, H. & Tian, Z. 2009. Identification and characterization of cancer stem-like cells from primary carcinoma of the cervix uteri. *Oncology Reports* 22(5): 1129-1134.

Fong, S.Y., Piva, T., Dekiwadia, C., Urban, S. & Huynh, T. 2016. Comparison of cytotoxicity between extracts of *Clinacanthus nutans* (Burm. f.) Lindau leaves from different locations and the induction of apoptosis by the crude methanol leaf extract in D24 human melanoma cells. *BMC Complementary and Alternative Medicine* 16: 368.

George, I.A., Chauhan, R., Dhawale, R.E., Iyer, R., Limaye, S., Sankaranarayanan, R., Venkataramanan, R. & Kumar, P. 2022. Insights into therapy resistance in cervical cancer. *Advances in Cancer Biology – Metastasis* 6: 100074.

Huang, D., Guo, W., Gao, J., Chen, J. & Olatunji, J.O. 2015. *Clinacanthus nutans* (Burm. f.) Lindau ethanol extract inhibits hepatoma in mice through upregulation of the immune response. *Molecules* 20(9): 17405-17428.

Huang, R. & Rofstad, E.K. 2017. Cancer stem cells (CSCs), cervical CSCs and targeted therapies. *Oncotarget* 8(21): 35351-35367.

Ishiguro, T., Ohata, H., Sato, A., Yamawaki, K., Enomoto, T. & Okamoto, K. 2017. Tumor-derived spheroids: Relevance to cancer stem cells and clinical applications. *Cancer Science* 108(3): 283-289.

Kim, B.W., Cho, H., Choi, C.H., Ylaya, K., Chung, J.Y., Kim, J.H. & Hewitt, S.M. 2015. Clinical significance of OCT4 and SOX2 protein expression in cervical cancer. *BMC Cancer* 35(5): 2643-2650.

Lee, C.H., Yu, C.C., Wang, B.Y. & Chang, W.W. 2016. Tumorsphere as an effective *in vitro* platform for screening anti-cancer stem cell drugs. *Oncotarget* 7(2): 1215-1226.

Li, L. & Neaves, W.B. 2006. Normal stem cells and cancer stem cells: The niche matters. *Cancer Research* 66(9): 4553-4557.

Liu, H., Zhang, W., Jia, Y., Yu, Q., Grau, G.E., Peng, L., Ran, Y., Yang, Z., Deng, H. & Lou, J. 2013. Single-cell clones of liver cancer stem cells have the potential of differentiating into different types of tumor cells. *Cell Death & Disease* 4(10): e857.

López, J., Poitevin, A., Mendoza-Martínez, V., Pérez-Plasencia, C. & García-Carrancá, A. 2012. Cancer-initiating cells derived from established cervical cell lines exhibit stem-cell markers and increased radioresistance. *BMC Cancer* 12: 48.

Martens, J.E., Arends, J., Van der Linden, P.J, De Boer, B.A. & Helmerhorst, T.J. 2004. Cytokeratin 17 and p63 are markers of the HPV target cell, the cervical stem cell. *Anticancer Research* 24(2B): 771-775.

Messerschmidt, J.L., Bhattacharya, P., Messerschmidt, G.L. 2017. Cancer clonal theory, immune escape, and their evolving roles in cancer multi-agent therapeutics. *Curr. Oncol. Rep.* 19(10): 66.

Mitra, S.K., Misra, R.K., Varshney, B., Kumari, A. & Rai, D. 2017. Role of p16, Ki67 and CK17 in differentiating benign lesions, cervical intraepithelial neoplasia (CIN) and atypical immature squamous metaplasia (AIM) of uterine cervix. *Tropical Journal of Pathology and Microbiology* 3(1): 13-19.

Mondal, A., Gandhi, A., Fimognari, C., Atanasov, A.G. & Bishayee, A. 2019. Alkaloids for cancer prevention and therapy: Current progress and future perspectives. *European Journal of Pharmacology* 858: 172472.

Ng, P.Y., Chye, S.M., Ng, C.H., Koh, R.Y., Tiong, Y.L., Pui, L.P., Tan, Y.H., Siew, C., Lim, Y. & Ng, K.Y. 2017. *Clinacanthus nutans* hexane extracts induce apoptosis through a caspase-dependent pathway in human cancer cell lines. *Asian Pacific Journal of Cancer Prevention* 18(4): 917-926.

Organista-Nava, J., Gómez-Gómez, Y., Garibay-Cerdenares, O.L., Leyva-Vázquez, M.A. & Illades-Aguiar, B. 2019. Cervical cancer stem cell-associated genes: Prognostic implications in cervical cancer. *Oncology Letters* 18(1): 7-14.

Ortiz-sánchez, E., Santiago-López, L., Cruz-Domínguez, V.B., Toledo-Guzmán, M.E., Hernández-Cueto, D. & Muñiz-Hernández, S. 2016. Characterization of cervical cancer stem cell-like cells: Phenotyping, stemness, and human papilloma virus co-receptor expression. *Oncotarget* 7(22): 31943-31954.

Pandrangi, S.L., Chittineedi, P., Chalumuri, S.S., Meena, A.S., Neira Mosquera, J.A., Sánchez Llaguno, S.N., Pamuru, R.R., Mohiddin, G.J. & Mohammad, A. 2022. Role of intracellular iron in switching apoptosis to ferroptosis to target therapy-resistant cancer stem cells. *Molecules* 27(9): 3011.

Rositch, A.F., Levinson, K., Suneja, G., Monterosso, A., Schymura, M.J., Mcneel, T.S., Horner, M.J., Engels, E. & Shiels, M.S. 2022. Epidemiology of cervical adenocarcinoma and squamous cell carcinoma among women living with human immunodeficiency virus compared with the general population in the United States. *Clinical Infectious Diseases* 74(5): 814-820.

Shen, L., Huang, X., Xie, X., Su, J., Yuan, J. & Chen, X. 2014. High expression of SOX2 and OCT4 indicates radiation resistance and an independent negative prognosis in cervical squamous cell carcinoma. *Journal of Histochemistry and Cytochemistry* 62(7): 499-509.

Singh, D., Vignat, J., Lorenzoni, V., Eslahi, M., Ginsburg, O., Lauby-Secretan, B., Arbyn, M., Basu, P., Bray, F. & Vaccarella, S. 2023. Global estimates of incidence and mortality of cervical cancer in 2020: A baseline analysis of the WHO global cervical cancer elimination initiative. *The Lancet Global Health* 11(2): e197-e206.

Sun, Q., Wang, L., Zhang, C., Hong, Z. & Han, Z. 2022. Cervical cancer heterogeneity: A constant battle against viruses and drugs. *Biomarker Research* 10(1): 85.

Swain, N., Thakur, M., Pathak, J. & Swain, B. 2020. SOX2, OCT4 and NANOG: The core embryonic stem cell pluripotency regulators in oral carcinogenesis. *Journal of Oral and Maxillofacial Pathology* 24(2): 368-373.

Wang, H., Paczulla, A.M., Konantz, M. & Lengerke, C. 2018. *In vitro* tumorigenic assay: The tumor spheres assay. *Methods in Molecular Biology* 1692: 77-87.

Wu, L., Han, L., Zhou, C., Wei, W., Chen, X., Yi, H., Wu, X., Bai, X., Guo, S., Yu, Y., Liang, L. & Wang, W. 2017. TGF-β1-induced CK17 enhances cancer stem cell-like properties rather than EMT in promoting cervical cancer metastasis via the ERK1/2-MZF1 signaling pathway. *FEBS Journal* 284(18): 3000-3017.

Yahaya, R., Dash, G.K., Abdullah, M.S. & Mathews, A. 2015. *Clinacanthus nutans* (burm. F.) Lindau: An useful medicinal plant of South-East Asia. *International Journal of Pharmacognosy and Phytochemical Research* 7(6): 1244-1250.

Yong, Y.K., Tan, J.J., Teh, S.S., Mah, S.H., Ee, G.C.L., Chiong, H.S. & Ahmad, Z. 2013. *Clinacanthus nutans* extracts are antioxidant with antiproliferative effect on cultured human cancer cell lines. *Evidence-Based Complementary and Alternative Medicine* 2013: 462751.

Zainuddin, N.A.S.N., Muhammad, H., Nik Hassan, N.F., Othman, N.H. & Zakaria, Y. 2020. *Clinacanthus nutans* standardized fraction arrested SiHa cells at G1/S and induced apoptosis via upregulation of p53. *Journal of Pharmacy and Bioallied Sciences* 12(2): 768-776.

Zainuddin, N.A.S.N., Hassan, N.F.N., Zakaria, Y., Muhammad, H. & Othman, N.H. 2019. Semi-purified fraction of *Clinacanthus nutans* induced apoptosis in human cervical cancer, SiHa cells via up-regulation of Bax and down-regulation of Bcl-2. *Sains Malaysiana* 48(9): 1997-2006.

Zhang, S.L., Wang, Y.S., Zhou, T., Yu, X.W., Wei, Z.T. & Li, Y.L. 2012. Isolation and characterization of cancer stem cells from cervical cancer HeLa cells. *Cytotechnology* 64(4): 477-484.

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