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Cytotoxic Effect of *Clinacanthus nutans* Semi-purified Fraction (SF1) in Combination with Cisplatin against Human Cervical Cancer

(Kesan Sitotoksik Fraksi Separa Tulen (SF1) *Clinacanthus nutans* dalam Gabungan dengan Cisplatin terhadap Kanser Pangkal Rahim Manusia)

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ABSTRACT

Cervical cancer is one of the most diagnosed malignancies in the world, and it is associated with HPV virus infection and invasion. A standard fraction from the medicinal plant *Clinacanthus nutans* was successfully separated, isolated, and identified as SF1 (semi-purified fraction). According to the previous study, SF1 has shown cytotoxic action against the cervical cancer cell line, SiHa. This study aims to further annotate the effect of SF1 in combination with cisplatin against SiHa to increase the selectivity of the anti-cancer treatment. The MTT assay was used to analyse the combination treatment's cytotoxicity, and its index value was calculated using the Chou-Talalay method. A cell survival test was conducted to evaluate the reproducibility of cancer cells after being treated. The combination treatment has enhanced cytotoxic action and inhibited SiHa's cell proliferation the most (IC_{50} value = 5.10 ± 0.86 µg/mL) compared to the individual cytotoxic activity of SF1 (IC_{50} value = 9.60 ± 0.20 µg/mL) and cisplatin (IC_{50} value = 3.60 ± 0.60 µg/mL). Simultaneously, the combination study has shown lesser cytotoxic activity towards normal cells, Vero (IC_{50} value = 10.30 ± 3.10 µg/mL) compared to SiHa cells. The combination also showed an antagonism effect with CI values of 2.60 to 1.50 and fractional inhibition (Fa) of 0.50 to 0.90. The findings have demonstrated that in contrast with single-agent therapy, the treatment of SF1 and cisplatin in combination has increased the efficacy because it selectively targets cancer cells by antagonism action.

Keywords: Antagonism; *Clinacanthus nutans;* cisplatin; cytotoxicity; SiHa

ABSTRAK

Kanser pangkal rahim adalah salah satu kanser yang paling banyak didiagnosis di dunia dan ia dikaitkan dengan jangkitan dan pencerobohan virus HPV. Fraksi daripada tumbuhan ubatan *Clinacanthus nutans* berjaya dipisahkan, diasingkan dan dikenal pasti sebagai SF1 (fraksi separa – penulenan). SF1 telah menunjukkan tindakan sitotoksik terhadap titisan sel kanser pangkal rahim, SiHa menurut kajian terdahulu. Kajian kali ini bertujuan untuk menjelaskan lagi kesan SF1 dalam penggabungan dengan cisplatin terhadap SiHa untuk meningkatkan selektiviti untuk rawatan anti kanser. Asai MTT digunakan untuk menganalisis sitotoksisiti rawatan gabungan dan nilai indeks gabungannya dihitung menggunakan kaedah Chou-Talalay. Ujian kemandirian sel telah dijalankan untuk menilai kebolehulangan sel kanser selepas dirawat. Rawatan gabungan telah meningkatkan tindakan sitotoksik dan menghalang pembiakan sel SiHa paling banyak (nilai IC_{50} = 5.10 ± 0.86 µg/mL) berbanding tindakan sitotoksik secara individu SF1 (nilai IC_{50} = 9.60 ± 0.20 µg/mL) dan cisplatin (nilai IC_{50} = 3.60 ± 0.60 µg/mL). Pada masa yang sama, kajian gabungan telah menunjukkan tindakan sitotoksik yang lebih rendah terhadap sel normal, Vero (nilai IC_{50} = 10.30 ± 3.10 µg/mL) berbanding sel SiHa. Gabungan tersebut juga menunjukkan kesan antagonis dengan nilai CI 2.60 hingga 1.50 dengan perencatan pecahan (Fa) 0.50 hingga 0.90. Penemuan telah menunjukkan bahawa berbeza dengan terapi agen tunggal, rawatan gabungan SF1 dan cisplatin telah meningkatkan keberkesanan kerana ia secara selektif menyasarkan sel-sel kanser melalui tindakan antagonisme.

Kata kunci: Antagonisme; cisplatin; *Clinacanthus nutans*; SiHa; sitotoksiksiti

INTRODUCTION

Cervical cancer is an invasive form of cancer that is the fourth - most prevalent cancer among women worldwide, with an estimated 570,000 new cases in 2018 and an age - standardized rate (ASR) of 13.1 per 100,000 women, and 340,000 women died from this cancer, with 90% of deaths occurring in low - and middle - income countries (Arbyn et al. 2020). Cervical cancer is the third most predominant and the fourth most fatal type of cancer in women in Malaysia (Ferlay et al. 2020). Geographical differences in the incidence of cervical cancer result in the accessibility, coverage, and effectiveness of preventive measures, including the human papillomavirus (HPV) vaccine, which targets adolescent girls aged 9 to 14 as a primary prevention strategy, and precancerous lesion screening and treatment as a secondary measure. The World Health Organisation (WHO) declares invasive cervical cancer clinical therapy and diagnosis as a tertiary preventative (World Health Organization 2020). The clinical treatments also underscore how frequently risk factors occur. Approximately 5.4% of all human carcinomas have been reported to be linked to HPV infection (de Martel et al. 2020). Women who engage in sexual activity frequently could develop high-risk HPV infections, and cervical cancer is the most severe malignancy linked to HPV (Castanheira et al. 2020). HPV is also one of the most prominent sexually transmitted disease (STD) agents worldwide. It can result in a wide range of clinical problems, including asymptomatic infections and benign and malignant genital diseases (Kaliterna et al. 2023). Although it is sexually transmitted, transmission and infection of HPV does not require penetrative sexual contact. Skin-to-skin genital contact is a robust mode of transmission (Okunade 2020). Higher parity, smoking, multiplication number of sex partners, and HIV infection are some other significant risk factors (Stelzle et al. 2021). It was found that most women in Malaysia still have low awareness of cervical cancer prevention methods, including HPV vaccination, healthy diet, and smoking cessation practices (Seng et al. 2018).

In the 1950s, cervical cancer screening programs were established in the United States (Miller et al. 2020). Malaysia initiated a national vaccination program in 2010 with a 3 - dose schedule for all 13 year - old females but revised to a 2 - dose schedule in 2015 (Muhamad et al. 2018) and having more than 80% coverage across the country (Ministry of Health Malaysia 2020). The WHO's global strategy to eradicate cervical cancer has set a target to immunise at least 90% of girls before they turn 15 by 2030 as a preventive measure (World Health Organization. 2021). This is because immunisation against HPV could be envisioned to avert more deaths per person that have been immunised (Li et al. 2021). The rate of parental authorisation for the teenage girls' HPV vaccination programme is extremely satisfactory (96 - 98%), and the country's school - based HPV immunisation programme achieves a coverage rate of over 95% (Muhamad et al. 2018). The careHPV™ test, a straightforward and relatively affordable DNA test based on HPV DNA signal amplification, allows the identification of 14 high - risk genital HPV (HR - HPV) genotypes, and offers significant benefits over other conventional molecular methods. Results might be obtained in 2 - 5 h using this method, which also works without electricity or water supply and does not require costly molecular laboratory equipment (Paboriboune et al. 2022). The prediction based on DNA test results could potentially be feasible to circumvent cervical cancer (Roden & Stern 2018). Although the current effect of HPV vaccination on the prevention of the development of cervical cancer is quite limited because most of the target population for vaccination has not reached the targeted high - risk age group and vaccine uptake is very low in most countries, therefore, it will be the most important way to reduce the burden of cervical cancer in the future long term with reasonable intake of vaccine (Lin et al. 2021).

Currently, chemotherapy, radiation, locally targeted therapy, and surgery are the main conventional approaches to treatment for cervical cancer. Cervical cancer's high mortality and morbidity rates continue to be a significant obstacle to scientific study. However, the available treatment options are limited, and patients with advanced and recurrent/metastatic cervical cancer have a terrible prognosis with 5 - year survival rates of only 16 to 58% in the most advanced stages (Han, Chang & Xia 2022). Cisplatin has become a commercial chemotherapy drug that is often used to treat cervical cancer. However, various adverse effects are obtained from this drug, and studies have found that it can reduce the quality of life of patients (PDQ® Adult Treatment Editorial Board 2023). The most important side effects associated with cisplatin exposure may include anaemia, gonad toxicity, hepatotoxicity, neurotoxicity, ototoxicity and nephrotoxicity (Ghosh 2019). Therefore, it is crucial and necessary to develop medicines for the treatment of cervical cancer that possess better safety profiles as well as greater efficacy. The development of complementary and alternative medicine treatments generated from natural extracts from plant is currently receiving more attention and effort (Keene et al. 2019). Studies demonstrate that this natural extract has pharmaceutical potential and is a precursor to bioactive compounds that exhibit cytotoxic or antiproliferative effects against different types of cancer by targeting particular proteins involved in cell growth and metabolic signalling pathways (Ma et al. 2021). Additionally, the natural extract can be regarded as a multi - target drug to combat medical conditions like cancer due to the existence of diverse chemical compounds (Kumar et al. 2019).

Extracts from the leaves and stems of *Clinacanthus nutans* have recently received extensive recognition and utilisation especially among Asians as alternative treatments for cancer and other medical conditions. As a result, lots of anti - cancer research involving *C. nutans*

extract has been conducted recently. Its extract has been scientifically confirmed to be a promising alternative cancer treatment and prevention for some specific forms of cancer, and the study examined its effect on a various of cancer cell lines (Bong et al. 2021). Apart from that, according to a non - scientific and unpublished exploration or study on the ethnobotanical employment of medicinal plants, *C. nutans* is one of the top five herbs used in tropical countries like Malaysia, Thailand, Brunei, and Singapore for treating diabetes, hypertension, inflammation, and antioxidant deficiencies. There were also reports of further pharmacological effects, including anti - venom, anti - cancer, anti - bacterial, anti - fungal, and anti - analgesic actions (Tan et al. 2020). In Malaysia, this plant is popular in most states including the Borneo archipelago and is known as the snake plant or the local name *'belalai gajah'*. Based on preceding study, a semi - purified fraction of *C. nutans* has been successfully isolated and named as SF1 (standardised fraction) which produced a cytotoxic effect against human cervical squamous cell line, SiHa (Zainuddin et al. 2019).

Malignant tumours are frequently difficult to treat with chemotherapy drugs alone. However, it has been discovered that compounds primarily derived from medicinal plants might lessen the toxicity of chemotherapeutic drugs when use them in combination. When natural compounds are combined with chemotherapeutic drugs, the combination could potentially render cancer cells more susceptible to chemotherapeutic drug like cisplatin (Wu et al. 2023). In the past study, Huang et al. reported in 2019 that the combination of *Scutellaria baicalensis* (SB) and cisplatin had a synergistic impact on Lewis lung cancer (LLC) cells, and that SB reversed the effect of cisplatin on cachexia and acute kidney injury. Cachexia and severe renal damage were the two major side effects of acquiring cisplatin alone (Chen et al. 2015). SB amplified and potentiated the efficacy of cisplatin to suppress tumour growth *in vitro* and *in vivo*. In animal studies, SB has been demonstrated to restore murine body weight loss and gastrocnemius muscle, increasing blood urea nitrogen levels, and injury to renal tubules in mice following cisplatin treatments (Huang et al. 2019). This potential study merits evaluation and in - depth research. Therefore, the primary objective of this study was to further enhance the sensitivity to the treatment of SiHa by combining SF1 and cisplatin, which could potentially reduce the severe side effects of cisplatin on normally functioning and healthy cells.

MATERIALS AND METHODS

CELL LINES AND CULTURE CONDITIONS

Human cervical squamous cell carcinoma epithelial (SiHa) and normal African green monkey kidney epithelial (Vero) cell lines from ATCC were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (ThermoFisher Scientific's Gibco, USA) with 10% fetal bovine serum (FBS) (ThermoFisher Scientific's Gibco, USA) and 1% penicillin – streptomycin (Pen – strep) (ThermoFisher Scientific's Gibco, USA) and incubated in incubator at 37 °C and 5% CO_2.

COLLECTION AND AUTHENTICATION OF *C. NUTANS*

The garden - fresh leaves of *C. nutans* were pristinely harvested at Kelantan, Malaysia. The specimen's authenticity was verified by the Botanist Dr. Shamsul Khamis from Herbarium Kulliyyah of Pharmacy, Universiti Islam Antarabangsa Malaysia (UIAM). The *C. nutans* was prepared as a voucher specimen (PIIUM 0238 - 2) and placed in the herbarium. The fresh leaves weighing 1 kg were cleaned before being dried in a drying oven for one night at 50 °C. Using a laboratory grinder, the dried leaves of *C. nutans* were ground to a coarse powder weighing 150 g. The methodology was slightly revised in accordance with earlier studies (Zainuddin et al. 2019).

EXTRACTION, ISOLATION AND PURIFICATION OF *C. NUTANS* STANDARDISED FRACTION (SF1)

Hexane (Merck, Germany) and chloroform (Merck, Germany) have been utilised in turn to extract the dried, powdered leaves of *C. nutans*. The powdered leaves of *C. nutans* weighing 150 g was macerated in 150 mL of Hexane solution and incubated overnight in water bath at 60 °C. The leaves powder of 20 g was put into a Soxhlet extractor thimble and run with 400 mL of chloroform. The extract was concentrated in a vacuum chamber at 40 °C to produce dried chloroform extract. The chloroform extract weighing 4 g was first chromatographed on a silica gel 60 (250 g) column using a dry vacuum liquid chromatographic method to isolate *C. nutans*. Hexane - ethyl acetate (Merck, Germany) (1:1) prepared in 1 L was used to elute the column, and only the active fraction, designated as F11, was collected and isolated (Zakaria & Abdullah 2018). Thin - layer chromatography (TLC) was used to collect the fraction while with the ratio of chloroform - methanol (HMBG, Germany) (1:1). Purification of the F11 was concluded by utilizing a dry vacuum liquid chromatographic method on silica gel 60 (200 g) with the goal to characterize the F11 further. The column was loaded with around 2 g of F11. The acetonitrile – methanol (Merck, Germany) (2:8) solution of 1 L was used to elute the column. A portion of each fraction weighing 1 g was separated using preparative TLC using chloroform - methanol (2:8), and the retention factor (Rf) for each TLC plate was computed as (distance moved by solute)/(distance moved by solvent). The technique was effectively applied using existing techniques invented by earlier research colleagues, and SF1 fractions were acquired (Zainuddin et al. 2019).

CYTOTOXICITY ASSAY

By employing the yellow tetrazolium salt or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay to establish significant cell inhibitory properties, the SF1 fraction was examined for cytotoxicity activity against SiHa and Vero cells. The RPMI 1640 medium with addition of 10 % FBS and 1% pen – strep used to maintain both SiHa and Vero cells. The cell lines were kept at 37 °C in an incubator with humidified 5% CO_2. In 96 - well plates, around 90% of confluent cells were seeded the night before treatment at a density of 5×10^4 cells per well for SiHa cells and 3×10^4 cells per well for Vero cells. In separate instances, cisplatin and SF1 were dissolved in DMSO (Dimethylsulfoxide) 98 % (Nacalai Tesque, Japan), and then successive two – fold dilutions between 0.4 and 10 mg/mL were applied. Cisplatin was utilised as a positive control and DMSO was performed as a negative control. The plates were incubated for 72 h at 37 °C with humidified 5% CO_2. After the incubation period was over, 50 µL of MTT reagent (Nacalai Tesque, Japan) at 2 mg/mL was meticulously added to each well. A further 4 h were spent incubating the plate. Shortly after removing the MTT solution, including the old media, 200 µL of DMSO was subsequently added to dissolve the purple formazan that had developed at the well's bottom. The plates were thereupon shaken for 5 min and the absorbance at 570 nm was measured on a spectrophotometric microplate reader. The percentage of cell viability was computed as (treated optical density) / (optical density negative control) ×100%. IC_{50} values were calculated using dose - response curves engineered by PRISM Graph Pad Version 9.0 software.

$$\% \text{ Cell Viability} = \frac{(\text{Treated optical density}) \times 100\ \%}{(\text{Optical density negative control})}$$

COMBINATION INDEX ANALYSIS

Using the Chou and Talalay method (Chou 2006), the drug - drug interaction between SF1 and cisplatin was investigated, and the results of combination index (CI) values were calculated using CompuSyn software based on IC_{50} values. The interaction between the two drugs was considered synergism when the CI value existed between 0 and 1 and the CI value was negatively related to the extent of synergism: < 0.1, very strong synergism; 0.1 - 0.3, strong synergism; 0.3 - 0.7, synergism; 0.7 - 0.85, moderate synergism; 0.85 - 0.90, slight synergism; 0.9 - 1.1, nearly additive; > 1.1, antagonism.

CLONOGENIC ASSAY

The culture was established by plating 2×10^5 cells of SiHa into a 25 cm^2 flask and incubating overnight (around 90% confluent) before the treatment process. There were three parts of the experiments. In the first part (P1), cells were treated with SF1 for 6 h before proceeding with the cisplatin. In the second part (P2), the SF1 was introduced after 6 h of treatment with cisplatin. Meanwhile, in the third part (P3), cells were treated with SF1 and cisplatin at the same time. The untreated SiHa cells (vehicle only) named UT also included. All the experiments were carried out for 72 h. The concentrations of SF1 and cisplatin were based on the results from IC_{50} values of the combination treatment of SF1 and cisplatin. Cells were harvested after the treatment 72 h period was over, and surviving cells were collected and calculated relative to UT from the same experiments. Later, up to 1×10^3 cells were seeded in 6 - well plates for colony growth. After that, the cells were incubated in the incubator at 37 °C for 7 days until cells in plates successfully developed colonies with substantially good size (50 cells per colony is the minimum for scoring). Then, medium cultures were removed gently from each plate by aspiration. The plates were washed with PBS 1X, and the colonies were directly fixed with a 10% neutral buffered formalin solution for 15 min. Staining was completed with 0.01 % (w/v) crystal violet (Sigma Aldrich, USA) for 30 min, the solution was diluted earlier in sterilised distilled water. The excess crystal violet solutions were discarded, and the plates were washed with sterilized distilled water and then dried at room temperature prior to observation under inverted microscope. Colonies containing more than 50 individual cells will be counted using openCFU software.

STATISTICAL ANALYSIS

Statistical analysis was performed using Graph Pad PRISM Version 9.0 Software (Inc, California, United States). Each experiment was conducted in triplicate and expressed as mean ± S.D. Statistical significances of data obtained were calculated and determined using Students' paired t - tests or one - way ANOVA with Dunnetts' multiple comparison tests. The results were considered significance, if $*p < 0.05$, $**p < 0.01$, $***p < 0.00.1$.

RESULTS AND DISCUSSION

CYTOTOXICITY STUDIES

The US National Cancer Institute Guidelines declare that plant extracts with cytotoxic activity against cancer cells should be prioritized for consideration if the 50% inhibitory concentration value (IC_{50}) procured through experiments is less than 20 µg/mL (Twilley, Rademan & Lall 2020). Based on the past study, the characterization analysis was conducted using liquid chromatography

(LC) alongside mass spectrometry (MS). The primary components discovered in SF1 were alkaloids, a class of organic nitrogenous compounds, including methysergide, calycanthine, eburnamonine, dextromethorphan, and an unknown substance, C33H37N5OS2, that does not match a record in the METLIN database. In contrast to the untreated group on SiHa cells, the SF1-treated group caused considerable DNA damage within 72 h, resulting in cell cycle arrest at G0/G1 (Zainuddin et al. 2020). While other scientists have researched *C. nutans* and its bioactive compounds as well as its combination with chemotherapeutic drugs, this study is distinct from those of other researchers because the *C. nutans* fraction underwent enhanced separation and purification techniques, progressing from F11 to SF1 (Zainuddin et al. 2019), to produce higher purity fractions for subsequent analysis.

SF1 has great potential to be an effective anti - cancer drug due to its high toxicity on SiHa. The previous study has reported that SF1 was examined and found to promote cytotoxicity against SiHa cells in a dose-and time-dependent manner (Zainuddin et al. 2019). A prominent chemotherapy drug named cisplatin is frequently used in medical practices to treat various cancers. The Food and Drug Administration (FDA) approved cisplatin as an anti - cancer drug in 1978 for a wide range of cancers, including head and neck squamous cell carcinoma, bladder cancer, cervical cancer, ovarian cancer, testicular cancer, and non-small cell lung cancer (Purena, Seth & Bhatt 2018). Because of the effects of cisplatin, DNA damage is irreversible, and cisplatin resistance is frequently inescapable. After experiencing initial response to platinum or cisplatin cycles, the majority of patients relapse, which is linked to the emergence of one or more resistance mechanisms. Resistance to cisplatin alone is a primary contributor to relapse and death (Nguyen et al. 2022). This complex process often includes intrinsic pathways (Rocha et al. 2018). The decrease in cellular drug accumulation, which lowers the number of platinated DNA adducts, is a key factor in the cisplatin resistance (Ali et al. 2022). Toxic side effects, drug resistance and cancer cell recurrence are the main challenges in the use of cisplatin in cancer patients. Other side effects such as nephrotoxicity, neurotoxicity, gastrointestinal toxicity, and ototoxicity are of serious concern to researchers (Ghosh 2019).

Combination treatment of SF1 and cisplatin might be an essential strategy in treating the medical condition like SiHa. The IC_{50} was determined in SiHa and Vero cell lines to evaluate the cytotoxicity of combination treatment between SF1 and cisplatin *in vitro*. The choice to use these two cell types was simply to facilitate the comparison between cancer and normal cells. Both cells are of the epithelial type in terms of their morphology. The cell viability was enhanced in Vero cells compared to SiHa cells. Table 1 depicts the IC_{50} values of SF1 alone, cisplatin alone and combination between SF1 and cisplatin against Vero and SiHa cells. There was no remarkable IC_{50} values were detected on Vero cells proliferation of SF1 alone treatment as the IC_{50} values for the fractions were more than the highest concentration used in this experiment. The IC_{50} value on Vero cells proliferation was more than 100 µg/mL, thus, no inhibitory effect was perceived (Zainuddin et al. 2019). The results showed that the combination treatment has exerted more vigorous cytotoxic activity on SiHa (5.10 ± 0.86 µg/mL) compared to Vero cells (10.30 ± 3.10 µg/mL) as shown in Figure 1. It has demonstrated that SF1 and cisplatin in combination have selective cytotoxic activity against SiHa cells. Combination treatment of SF1 and cisplatin could reduce ten times toxicity of cisplatin alone against Vero cells.

Furthermore, SF1 itself is non - toxic to Vero cells. The positive control, cisplatin, one of commonly used chemotherapy drugs for cervical cancers exhibited strong cytotoxic activity on all tested cell lines; 3.6 ± 0.60 µg/mL on SiHa and 1.35 ± 0.47 µg/mL on Vero cells. The results presented supplementary evidence of the beneficial potency, selectivity, and adaptability of the SF1 and cisplatin combination's ability to eradicate cervical cancer cells. However, a thorough understanding of the complex physiological mechanisms underlying the divergent responses of tumour and normal cells is still required. This SF1 and cisplatin combination formulation resulted in a higher degree of selectivity since it operated preferentially on SiHa cells, which reduces the adverse effects of chemotherapy medication, cisplatin.

DRUG COMBINATION INDEX ANALYSIS

Large - scale dose - response matrix experiments with different concentrations of single and combined drugs were used to study the effects of drugs combinations. Drug - related information contributes a significant role in understanding the behaviour and combination mechanisms of compounds, such as structural similarities and biochemical properties between the drugs. Drug - target and drug - drug interactions are further options to improve the probability that combination therapies will be feasible and efficient. Drug interactions, in particular, are significant because they might result in unpredictable pharmacological effects, such as the emergence of adverse effects associated with medications (Güvenç Paltun, Kaski & Mamitsuka 2021). Drug combinations could be categorised as additive, antagonistic, or synergistic based on the differences

in the apparent mechanisms of response (Chou 2006). Two compounds acting together can be classified as synergistic when the combined effect is greater than the potency of each of their individual agents. The additive effect also known as non - interaction happens when two types of medicine working together have the effect that is equivalent to the total of the effect of the two drugs operating individually. Contrary to synergistic effects, a combination is antagonistic if the combined effects are smaller than the responses of the standalone agents. In contradiction to antagonistic combinations, which are effective in inhibiting the emergence of resistance, synergistic combinations have an advantage at delaying the beginning of resistance (Saputra et al. 2018). Although most researchers concentrate on exploring synergistic drug combinations, antagonism may be advantageous, and attention should be invested in identifying levels of toxicity. The antagonistic action of combination therapy is preferable when it comes to toxicity, as one drug lessens the damaging side effects of the other.

The calculation of combination index (CI) value is considered as a criterion to investigate to what extent SF1, and cisplatin interact together as a great anti - cancer drug. The CI would be determined when its calculation less than 1 indicates synergism, while more than 1 shows antagonism, and equal to 1 indicates additive effect. The fraction - affect (Fa) versus log (CI) analysis showed that all the concentration points, which are above the line of additive effects, have exhibited the antagonism effect as portrayed in Figure 2. The combination treatment of SF1 and cisplatin resulted in an antagonistic as the CI values were more than one. The CI values achieved were 2.60 - 1.5 with the fractional inhibition of Fa = 0.50 - 0.90 indicated the antagonistic action of SF1 and cisplatin in combination. The antagonism action exerted by combination of SF1 and cisplatin, probably leads to the selectivity of the treatment towards cancer cells, SiHa therefore, reduce the detrimental side effects on normal cells, Vero. Natural compound like SF1 have the capacity to simultaneously supress the toxic effects of cisplatin on healthy cells and induce cytotoxic effects on cancer cells (Bijnsdorp, Giovannetti & Peters 2011). It is primarily intended to protect normal cells from lethal cytotoxic effects if one drug in combination therapy is antagonistic, which occurs when one drug effects another drug in normal cells through triggering selectivity (Blagosklonny 2005).

For certain combinations of therapies, the purpose of drug combination treatment is to accomplish an antagonism effect to reduce the adverse effects *in vivo* experiments (Bijnsdorp, Giovannetti & Peters 2011). Synergism is a pro - efficacy strategy because resistance is suppressed by eradicating cancer cells before resistance can emerge. While antagonism is a defensive (anti - resistance) strategy, it might restrict single resistant subpopulations by not giving them as much of a fitness advantage as synergism does, although antagonism may not be maximally effective to lower the number of cells in the initial stages of therapy. In some cases, treatments that do not result in a superior initial response might yet generate improved long-term results due to their ability to limit the emergence of resistance (Saputra et al. 2018).

CLONOGENIC ASSAY

Cancer cells have a propensity to grow in new adjacent regions, eliciting in the formation of additional tumour cells. Normal tissue homeostasis depends on the ability to proliferate, however in tumours, eliminating this ability is essential to averting cancer recurrence (Franken et al. 2006). Malignant cancer cells, on the other hand, have the ability to form colonies (Ge et al. 2019). At least 50 cells constitute a colony. The most practical technique to assess the efficacy of anti - cancer treatment based on cell proliferation is the clonogenic assay, also known as the cell survival (Cunha et al. 2020). This assay evaluates each cell's capacity to proliferate and develop into a metastatic colony. The most practical technique to assess the efficacy of anti - cancer treatment based on cell proliferation is through the clonogenic assay. The combined effects of SF1 and cisplatin on the capacity of cells to grow into colonies were studied to assess the potential for cell proliferation and long-term cell survival following anti - cancer treatment. Clonogenic assays were conducted as well to further investigate the growth-suppressive effects of SF1 and cisplatin in combination. Figure 3 shows the effects of combination treatment on the clonogenic potential of the negative control (UT = Untreated as negative control-vehicle only), and the treatment groups represented by P1, P2, and P3. The combination suppressed the clonogenicity of SiHa cells in a time - dependent manner in comparison to the UT, where maximal clonogenic inhibition was observed at the P2 rather than P1 and P3 treatment groups as shown in Figure 3. After treatment expulsion, the remaining of harvested viable cancer cells that had been exposed to combination treatment of cisplatin first then followed by SF1 were unable to recover most of the vitality, thus, demonstrating the existence of a resilient regulation of the combined therapy's effects. This can reduce the risk for tumour recurrence and, as a result, has significant advantages for both the development and improvement of treatment approaches for the tumours undergoing study (Núñez et al. 2018).

TABLE 1. The IC_{50} values of SF1 alone, cisplatin alone and combination of SF1 and cisplatin against Vero and SiHa cell lines. The values represented as mean ± S.D with *p<0.05

Cell Lines	IC_{50} values (µg/mL)		
	SF1 alone	Cisplatin alone	SF1 + Cisplatin
SiHa	9.60 ± 0.20* (Zainuddin et al. 2019)	3.6 ± 0.60**	5.1 ± 0.86**
Vero	> 100	1.35 ± 0.47**	10.30 ± 3.10**

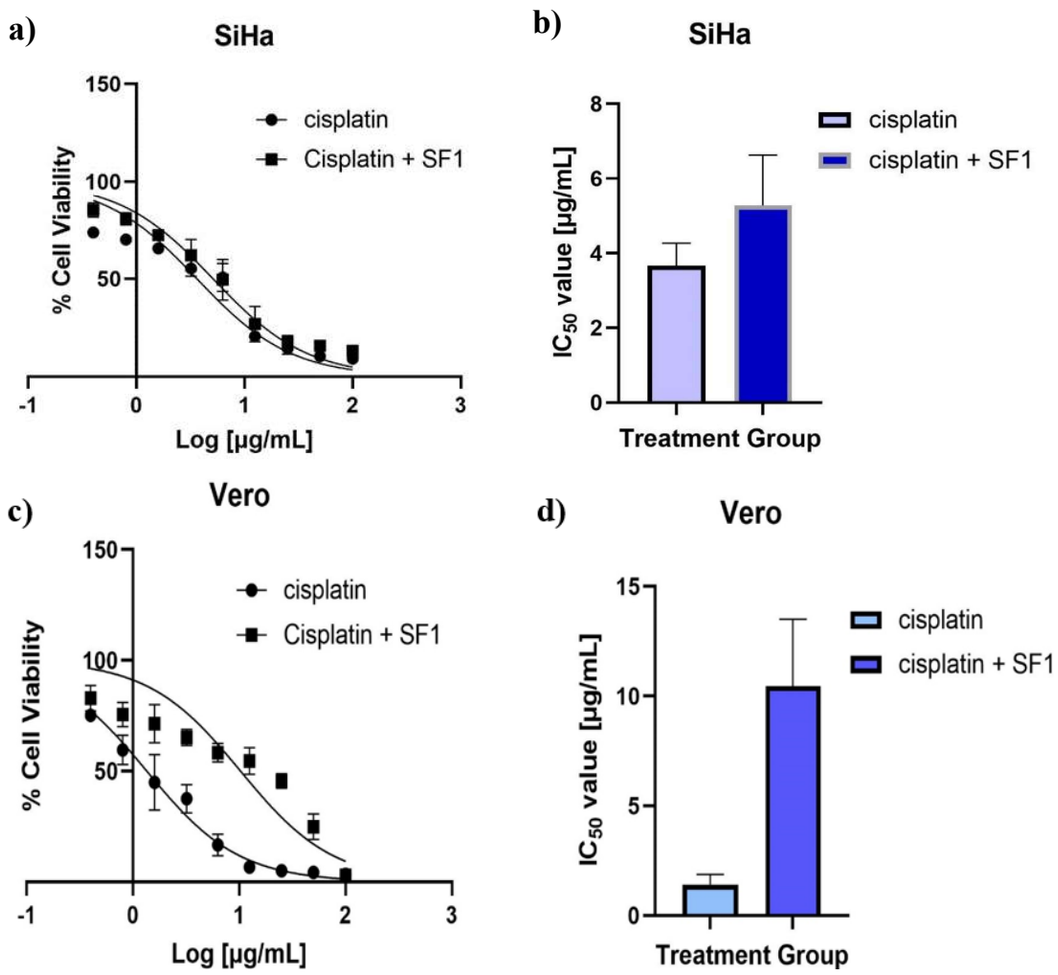

FIGURE 1. Dose response curve of combination treatment between SF1 and cisplatin towards a) b) SiHa and c) d) Vero cells. Every point represents mean ± SD for triplicate from three independent experiments (n = 3).
* p-values were obtained from a two-tailed t-test

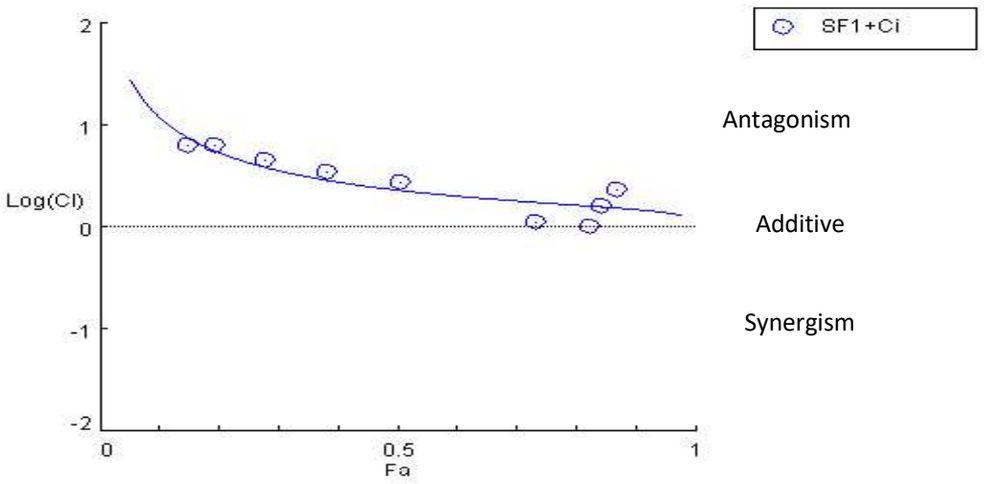

FIGURE 2. The fraction-affect (Fa) versus log (CI) analysis revealed that all the concentration points, which are above the line of additive effects exhibited antagonism effect for the combination treatment of SF1 and cisplatin

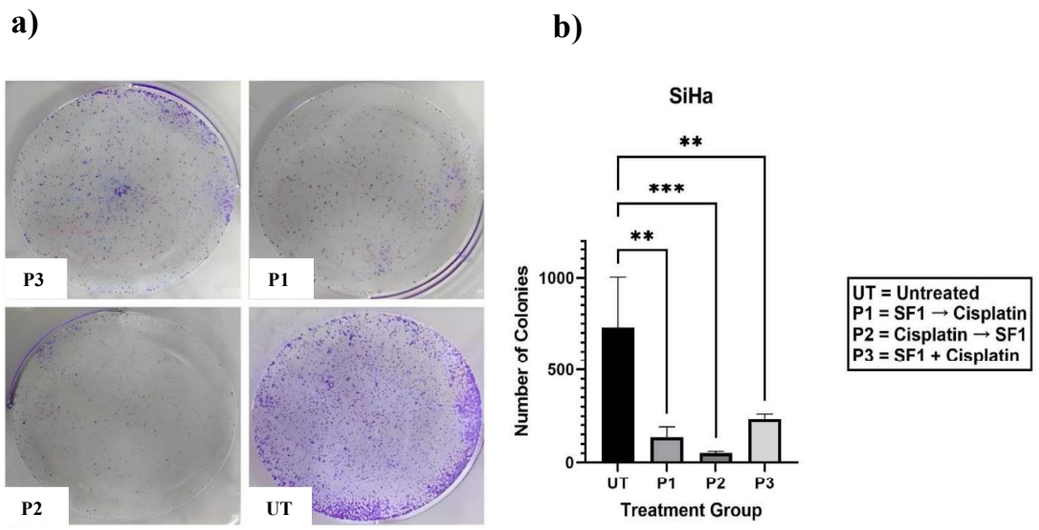

FIGURE 3. a) Representative images of SiHa colonies grew on petri dishes according to each of the treatment group. b) Number of SiHa colonies based on treatment groups performed for 7 days colony formation. The values are represented as mean ± S.D with * $p \leq 0.05$, significantly different from vehicle control

CONCLUSIONS

SF1 exerted potent anti - cervical cancer effects when administered alone or in combination with cisplatin. This study demonstrates that the combination of SF1 and cisplatin treatment has a high potency, selectivity, and tolerance cytotoxicity effect on SiHa cells and low toxicity on Vero cells. It also proves suppression of cancer cell growth and reduced colony formation. Nevertheless, more research is required to determine the specific mechanism of the response induced by the combination therapy and to affirm the treatment's effectiveness in animal model systems. The delivery of SF1 and cisplatin in combination to specific targeted cells remains a major challenge to the treatment of cervical cancer because the mechanism has yet to be further explored. One of the drug delivery systems with excellent therapeutic potential for enhancing pharmacological activity, drug solubility, and bioavailability is the employing of nanoparticles. Researchers can employ nanocarriers to load phytochemicals, which may be an achievable option for this problem given that most phytochemicals

are water - soluble, bioavailable, and stable (Gao et al. 2022). Through their method, they significantly increase the toxicity in the targeted cervical tumour cells or tissues by attaching them to specific ligands (Himiniuc et al. 2022). The coordination of the method of administration is therefore necessary for dosage control and the chronological sequence of administration of each medication combination to achieve the optimal drug combination.

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