ORIGINAL ARTICLE

Growth Inhibition of Standardized Amine Fraction From *Clinacanthus nutans* on Mice Xenograft Model for Human Cervical Cancer

Nik Aina Syazana Nik Zainuddin[1], Hussin Muhammad[1], Nik Fakhuruddin Nik Hassan[2], Yusmazura Zakaria[2]

[1] Toxicology and Pharmacology Unit, Herbal Medicine Research Centre, Institute for Medical Research, National Institutes of Health, Block C, No.1, Jalan Setia Murni U13/52 Seksyen U13 Setia Alam, 40170 Shah Alam, Selangor, Malaysia
[2] School of Health Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kota Bharu, Kelantan, Malaysia

ABSTRACT

Introduction: Cervical cancer continue to be a leading cause of cancer-related death for women. The current anticancer drugs remain inefficient due to the lack of specificity to inhibit the cancer cells growth. Plant-based drugs with potent anticancer should add to the efforts in finding drugs with lesser side effects but great potential to increase survival. Hence, this study sought to determine the tumour growth inhibition of standardized fraction (SF1) from Clinacanthus nutans as a potential alternative treatment for cervical cancer. **Methods:** SF1 was isolated from a series of bioassay-guided fractionation of C.nutans leaves. Female nude mice were xenografted with human cervical cancer cell line, SiHa, subcutaneously. When the tumour volume reached 100mm3, SF1 was intraperitoneally injected once daily for 28 days. Body weight and tumour volume were recorded every 3 days. Tumour and liver were fixed for hematoxylin and eosin staining and immunohistochemistry using caspase-3 antibody. Blood was collected by cardiac puncture for assessment of liver enzymes level, AST and ALT. **Results:** SF1 has a great tumour inhibition with more than 50% inhibition rate compared to the negative control group (DMSO). ALT and AST levels in SF1-treated mice were maintained in normal ranges compared to the positive control group, cisplatin indicating no sign of toxicities. SF1-treated group revealed no indication of liver toxicity. The number of tumour mitosis was reduced and caspase-3, a critical mediator of apoptosis was overexpressed in SF1-treated group. **Conclusion:** SF1 demonstrated potent anticancer activity by inhibiting the tumour growth with less toxic effects, suggesting a promising candidate for preventing cancer progression.
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Keywords: *Clinacanthus nutans*, standardized fraction, cervical cancer, xenograft, apoptosis, anticancer

Corresponding Author:
Yusmazura Zakaria, PhD
Email: yusmazura@usm.my
Tel: +609-7677781

INTRODUCTION

Currently, developing countries including Malaysia are going through a rapid transformation of social life and economic changes which led to the improvement of lifestyles. As a consequence, cancer rises as one of the major health threats related to the quality of human life. The rise of prevalence in cancer has captured the attention of researchers who have developed better and effective treatments (1). Tremendous efforts have been put into developing a cure for this disease, but the number of cancer patients remains high and continuously increases as new cases arise annually. Since decades ago, cervical cancer has been the most common type of gynecological cancer malignancies worldwide (2). Throughout the world, cervical cancer is the fourth most frequent cancer in women with an estimated more than 500,000 new cases in 2020 (3). According to the National Cancer Society of Malaysia (NCSM), in Malaysia, cervical cancer is the second most frequent cancer among women between 15 and 44 years of age and ranked as the fourth leading cause of death in women. Although screening and immunisation programmes have been widely implemented, the mortality rate due to cervical cancer in Malaysia is still higher compared to United Kingdom (4). Cervical cancer is a potentially preventable and treatable disease if it is detected early and managed effectively. However, without urgent attention and further treatment, over the next ten years it is anticipated that mortality from cervical cancer will increase by 25%. The most common death due to cervical cancer exist in the developed world, where rural and poorer women are at greatest risk of

invasive cervical cancer. In addition, many women especially those who are not currently sexually active, may not experience any symptoms until the disease has progressed (5).

The current treatments of cervical cancer are surgical removal of certain reproductive organs and radiotherapy with the help of chemotherapy. However, these approaches are limited by resistance, toxicity to surrounding healthy cells in the body and expensive operation. Chemotherapy for example, is the popular choice to battle cervical cancer. However, the treatment does not distinguish between cancerous and healthy fast-growing cells in the body. The use of chemotherapy drugs is controversial mainly due to drug resistance where the immune system of patients has been compromised with chemotherapy routine treatment (6). A study by Public Health England and Cancer Research UK (2016), shows up to 50% of patients are killed by the chemotherapy drugs, not the disease, indicating the treatment, not the cancer was the cause of death. Thus, there is an urgent need to have selective therapy to prevent the increment of death by cancer especially for cervical cancer cases. The public interest in natural therapies has increases greatly, with expanding use of ethnobotanicals (7) to develop potent and various anticancer drugs (8,9). The use of natural sources in cancer treatment has been applied by many Malaysian old folks to improve survivorship (10). The medicinal plants have been an asset to Malaysian due to the richness of our herbal resources in the forests (10). In fact, medicinal plants constitute the largest and most valuable sources with the current trend. Over 50% of all modern clinical drugs are of natural origin (11). One quarter of the prescription drugs marketed in North America and Europe contain active ingredients derived from plants (12)

In this study, *Clinacanthus nutans*, has been used based on traditional claim from the old folks in combating cancer (13-14). *C. nutans* which belongs to a family of Acanthaceae, is a native herb in tropical regions of Southeast Asia. This plant is utilised in other forms such as tinctures, elixirs, poultices, powders and other formulations for chronic disease management or health-boosting purposes in the local community (13,15-16). Many researchers reported that the extract and active fractions of *C. nutans* are capable of free radical scavenging and displayed significant antiproliferative effects on various cancer cells including lung cancer, liver cancer, nasopharyngeal cancer, ovarian cancer, cervical and melanoma cancer (17-20). However, there are very limited studies on the potential of *C. nutans* as an anticancer agent in animal models. As far as the literature ascertained, one of the main components of *C. nutans* known as CN30, has a significant inhibitory effect on tumour volume and induced apoptosis in hepatoma cells and meanwhile *C. nutans* methanol extract significantly reduced tumour volume and weight in mice (21). In our previous study, the standardized amine fraction from *C. nutans* has been successfully characterized and named as SF1. SF1 has exhibited potent anticancer activity against human cervical cancer cell line, SiHa, without affecting normal cells (20). Thus, this study was carried out to determine the potential of SF1 in in vivo tumor inhibition.

In this current study, a xenograft animal model of immunodeficient mice has been utilized because this model can be easily used to monitor tumour growth for the evaluation of anticancer efficacy where it can represent solid tumour (9,22). The xenograft model of human cancer plays an important role in the screening and evaluation of candidates for new anticancer agents (23).

The cancer xenograft model in nude mice lacking thymus hence T cells has provided a convenient approach to be conducted and observed due to a shorter incubation period with the high rate of tumour occurrence (22,24). The xenograft model is developed from human tumour cell lines and is classified based on the xenotransplant site, which can be either ectopic or orthotropic xenograft. Ectopic is referred to as the transplantation at a different site from the origin of the tumour whereas orthotopic is transplantation at the same site of tumour origin (23). Tumor xenografts in animal models have been used in many relevant investigations aimed at gaining insight into the clinical activity of new anticancer drugs because the implanted cells retain the characteristics of the parental ones (25).

Hence, to govern the characterisation of SF1, a standardised amine fraction derived from *C. nutans* by the optimised methodology of bioassay-guided fractionation (26), this present study was conducted using ectopic xenograft where the cervical cancer cells were subcutaneously injected into the flank or back . The tumour inhibition effect of SF1 together with the anticancer mechanism of action were evaluated to determine the potential of SF1 as an alternative treatment for cervical cancer. Besides, to evaluate the SF1-induced hepatotoxicity, the serum biomarkers alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured.

MATERIALS AND METHODS

Sample collection and preparation of SF1

The leaves of *C. nutans* were collected from Pengkalan Chepa, Kelantan, Malaysia. The specimen was authenticated (no. PIIUM 0238-2) and deposited at Herbarium, Kulliyyah of Pharmacy, Universiti Islam Antarabangsa Malaysia (UIAM), Jalan Gombak, Selangor, Malaysia. The leaves (500 g) were dried at 50°C overnight. The dried leaves were ground and sequentially extracted with hexane and chloroform, respectively. SF1 was then derived from bioassay-guided fractionation of *C. nutans* leaves which was previously

optimized by our colleagues (20). Then, several fractions were collected and tested for cytotoxicity test by MTT Assay on various cancerous cells in which SF1 has been obtained as a potent anticancer fraction towards human cervical cancer cell line, SiHa (26) and stored at -20°C before proceeding with the next steps.

Animals

Six weeks old female athymic nude nu/nu mice (strain: nu/nu-F, body weight 20-25g) were purchased from BioLASCO, Taiwan (licensed by Charles River, USA) and kept at Animal House, Institute for Medical Research (IMR), Kuala Lumpur. The mice were housed (in a group of 5 per cage) in individually ventilated cages under specific pathogen-free conditions according to the guidelines of Animal Ethics of Animal Care and Use Committee [ACUC/KKM/02 (17/2015)], IMR. Mice have received an autoclaved standard laboratory chow pellets and water ad libitium. The mice were acclimatized to standard conditions (temperature 22 ± 2°C, relative humidity 55 ± 5%, 12 hours light and 12 hours dark cycle) for a week before the commencement of experimental procedures. Animal beddings were changed regularly, once per week.

Cell culture

Human cervical cancer cell lines, SiHa was cultured in a complete RPMI 1640 medium at 37°C in a humidified atmosphere with 5% CO_2, overnight. Cells were then harvested, and the viability of the cells was determined by CountessTM Automated Cell Counter. Cells with more than 95% viability were used for xenotransplant.

Cell xenotransplants and animal study

The calculation of concentration for administration and preparation of stock solution for SF1 were adapted from our previous studies (27,28). SF1 was dissolved in 1% DMSO in distilled water to get a stock solution of 50 mg/ml. The concentration used was based on the conversion of IC50 value from the in vitro cytotoxicity test to the in vivo animal testing which is 30 mg/kg for SF1 and 10 mg/kg for cisplatin. The prepared 5.0 x 106 cells (0.5 ml of cells suspension in PBS) were inoculated by subcutaneous injection into mice's flank using a 27-gauge needle. The mice palpated twice per week for detection of the tumour. The palpable tumours started to develop within 3 weeks after inoculation and their locations were recorded. Tumour volume was measured using automated Vernier callipers and calculated using the formula width 2 x length x 0.52 (29,30). When tumours reached volumes of 90 – 100 mm^3, mice were randomly assigned into 3 groups; (i) negative control group (vehicle control - 1% DMSO), (ii) SF1-treated group (30 mg/kg BW), and (iii) positive control group, cisplatin (10 mg/kg BW). Another one group included was the clean mice (non- bearing tumor and non-treated) labelled as untreated group. Each group consists of 10 mice (n=10). The SF1, cisplatin and DMSO were given intraperitoneally (IP) once a day for 28 days. Body weights, food and water intake, toxicity signs, physical tumour growth and secondary tumour incidences were assessed and recorded every 3-day intervals throughout the treatment period. After 28 days of treatment, all mice were euthanized with an overdose of sodium pentobarbital (60 mg/kg) intraperitoneally. The liver and developed tumour were surgically removed, excised, weighed, and recorded.

Tumour measurement

The final tumour volume and tumour weight were recorded. Relative tumour volume (RTV) for day n = 0, 3, 6, 9, 12, 15, 18, 21, 24, 27 were calculated following the formula RTV = Vt/Vo where Vt = tumour volume at day n and Vo = initial tumour volume at day 0. Tumour growth profiles for relative tumour volume against time were plotted and endpoints were calculated. The endpoints used include relative tumour growth ratio, TC (%) = TRTV/CRTV X 100 % where TRTV = test group's RTV and CRTV = common negative control group's RTV. A TC value of less than 50 % indicates antitumour activity (31). The percentage of tumour growth inhibition was calculated basd on (1 – (TRTV/CRTV)) x 100% (25).

Histopathology study by H&E staining

Tumour and liver were washed in cold phosphate-buffered saline and fixed in 10% buffered formalin. Both organs were grossly sliced and dehydrated gradually in series of ethanol solutions using an automated tissue processor. Then, the tissue specimens were embedded molten paraffin wax and sectioned (4 µm thick slices) on a rotary microtome. Later, a ribbon of sectioned tissues was immersed in a water bath at 45°C. The sectioned tissues were dried on a slide warmer (60°C) for 30 minutes and stained according to the optimized H & E staining procedure (32). The tumour samples were examined using a light microscope (Olympus Leica) under 20X magnification. Cell nuclei in the mitotic cells appear as spotted structures dark- blue in colour. The image of mitotic cells was taken using an image analyser from five different points of view. The percentage of mitotic index was calculated by dividing the total number of mitotic cells with the total number of cells (22). The severities of histopathological changes; central vein, sinusoid fatty vacuoles and hepatocytes in the liver tissues were scored as follows: none (-), mild (+), moderate (++) and severe (+++) damage (32). All the changes were reviewed by a pathologist at Pathology Department, Universiti Sains Malaysia Hospital.

Immunohistochemistry analysis

The paraffin-embedded sectioning tissues were deparaffinized and rehydrated. A few drops of hydrogen peroxide block were added to cover the tissues. Pretreatment was performed and the sectioned tissues were washed in tris buffer. Then, protein block was applied and incubated for 10 minutes at room temperature. The tissues were washed 1X in the tris buffer. Primary antibody, a caspase-3, rabbit monoclonal

EPR18297, cleaved (dilution 1:100, Abcam, USA) was applied and incubated according to the manufacturer's protocol. Then, the sectionized tissues were washed in the buffer before the secondary antibody horseradish peroxidase (HRP) conjugate, Rabbit Specific HRP/DAB Detection IHC Kit, Abcam, USA) was applied. One drop of DAB chromogen was added to 1.5 ml DAB substrate. The mixture was applied to sectionized tissues and incubated for 10 minutes before rinsing with the tris buffer. Hematoxylin was then used to counterstain the sectionized tissue. The stained tissues were then mounted using DPX mounting medium and covered with a coverslip. The samples were examined for microscopy examination using a light microscope (Olympus Leica) under 20X magnification. The caspase 3-positive cells were determined by counting (H score software) the number of positively stained cells (brown in colour) within a view and divided by the number of cells per view in each sample. The percentages of positive cells were calculated from the ratio of positive cells to total cells counted (9,36) and reviewed by the pathologist.

Analysis of liver enzymes

Blood was collected by intra-cardiac puncture and aliquoted into plain vacutainers tube for assessment of liver toxicity by liver enzymes test (ALT and AST) at the Toxicology and Pharmacology Laboratory, Institute for Medical Research (IMR), Jalan Pahang, Kuala Lumpur.

Statistical analysis

Statistical analysis was performed using Microsoft Excel data analysis tool package and Graph Pad PRISM Version 6.0 GraphPad Software Inc, California, United States. Each experiment was conducted in triplicate and the data were expressed as mean ± standard deviation (S.D). The distribution of each data was initially determined whether it was normally distributed. Statistical significances of data obtained were calculated and determined using Students' paired t-test or analysis of variance (ANOVA) with Turkey's multiple comparison tests. The data were considered significance, if * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

RESULTS

The effect of SF1 on body weight

There were no secondary tumours observed and no significant clinical toxicity signs developed in all mice. The mice remained active with no changes in behavior . However, significant changes ($p < 0.05$) were observed in the body weight of mice bearing tumour in SF1 treated groups (27.10 ± 0.64 g) as compared to the vehicle control group (DMSO) (26.88 ± 0.83 g) (Figure 1a). The body weight of vehicle control mice was similar to the untreated group (28.69 ± 0.57 g) throughout the treatment period. The body weight of SF1-treated mice was slightly higher when compared to the cisplatin-treated mice (26.87 ± 0.60 g), a positive control group. Nevertheless, the data were not statistically significant

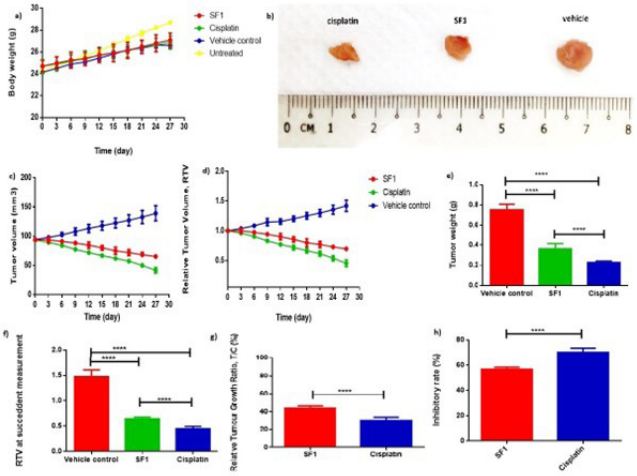

Figure 1: The effects of standardized amine fraction (SF1) on xenografted tumor model. a) Mice body weight has been recorded for 3-day intervals throughout treatment period for the vehicle control, SF1, cisplatin and untreated group. b) The isolated subcutaneous xenotransplant tumors of human cervical in nude mice on necropsy day. c) The effect of different treatment with vehicle control, SF1 and cisplatin against tumor volume of nude mice from day 1 until day 28. d) Relative Tumor Volume (RTV) at day 0, 3, 6, 9, 12, 15, 18, 21, 24, 27 upon treatment with vehicle control, SF1 and cisplatin. Each point represented as mean ± S.D, n = 10. e) The mean tumor weight after treatment with vehicle control, SF1 and cisplatin on necropsy day. f) The efficacy of tumor suppression after different treatment at necropsy day with vehicle control, SF1 and cisplatin. g) Percentage of relative tumor growth ratio (T/C) upon treatment with SF1 and cisplatin towards nude mice bearing tumor. h) The tumor growth inhibitory effects upon treatment with SF1 and cisplatin on xenotransplant tumor in nude mice. The value of bars represented mean ± SD of independent experiment with **** $p < 0.0001$ was taken as significant difference compared with the other treatment group.

in difference. The body weights were increased as observed in all treatment groups throughout 28 days of observation. Mice generally gained weight as they aged towards the end of the treatment duration thus proving the non-toxic compounds used. None of the mice have died including mice in the untreated group.

The effect of SF1 on tumour growth inhibition

Before the treatment started, the tumour volume was measured using Vernier caliper and the tumour volume for all mice were in between 90 – 100 mm^3. On day 28, the effect on the growth of tumours in vehicle control in comparison with SF1-treated mice were observed and recorded. Tumour volume was more vigorous in vehicle control as compared to mice treated with SF1 (Figure 1b). At the end of treatment day (day 28), tumour volume (Figure 1c) in SF1-treated mice (60.18 ± 2.17 mm^3) significantly reduced compared to the vehicle group (139.16 ± 12.97 mm^3). However, when compared to the cisplatin group, (41.60 ± 4.62 mm^3), the tumour volume of SF1-treated mice slightly higher ($p < 0.0001$). Similarly, decrease ($p < 0.0005$) of tumour weights (Figure 1e) were recorded in SF1 (0.36 ± 0.05 g) and cisplatin (0.23 ± 0.01 g) groups respectively,

compared to vehicle control group (0.75 ± 0.06 g). Antitumour activity was assessed based on the time taken for the relative tumour volume (RTV) to increase up to five-fold. The RTV of vehicle control, SF1 and cisplatin throughout the treatment period were shown in Figure 1d. The findings suggested that the RTV in SF1 and cisplatin group were decreased, compared to vehicle control group. This indicates that both groups showed a good tumour growth inhibition effect started from early treatment day until necropsy day. The RTV at succeeding measurement (Figure 1f) confirmed that the growth of tumour in nude mice treated with SF1 was inhibited with RTV 0.64 ± 0.03 whereas RTV 0.45 ± 0.05 was recorded for the cisplatin group. The RTV for both groups showed significantly decreased ($p < 0.0001$), compared to the vehicle control group. The efficacy of tumour growth inhibition after different treatments with vehicle control, SF1 and cisplatin at day 29 were expressed as relative tumour growth ratio, TC ratio (%) (Figure 1g). SF1 possessed a 43.74 ± 2.27% of TC ratio. Meanwhile, the cisplatin-treated group showed 30.34 ± 3.3% of TC ratio. The SF1- treated group demonstrated more than 50% of tumour inhibition rate (56.76 ± 1.65%) at the end of the treatment period (Figure 1h), which in line with the expected result due to the concentration of SF1 used in this experiment was based on IC50 value. However, the tumour inhibitory rate for cisplatin was significantly higher with 70.11 ± 3.32%.

The effect of SF1 on tumour histology by H & E staining

The histopathological examination of tumours depicts the effect of SF1 in reducing mitosis to inhibit tumour progression. The dark-blue spots represented the mitotic nuclei (Figure 2). The number of mitoses in tumour treated with SF1 and cisplatin were reduced compared to the vehicle control group which shown numerous mitotic cells. Significant decrease ($p < 0.0001$) in mitotic index was determined in SF1 (28.64 ± 6.11%) and cisplatin (19.97 ± 6.8%) treated groups as compared to the vehicle control group (72.64% ± 7.9) indicating that the mitosis was arrested due to the treatments.

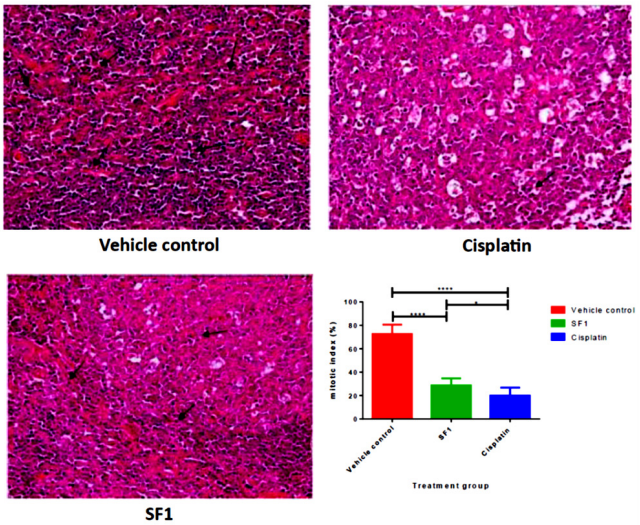

Figure 2: The histological H&E images of the tumor tissues (20x magnification) against different treatment groups; vehicle control, SF1 and Cisplatin. The blue arrow indicates mitotic cells (dark blue) in the tumor tissues. The graph showed the mitotic index in SF1 and cisplatin -treated groups compared to vehicle control on the xenotransplant tumor in nude mice. The morphological changes of tumor cells showed that mice treated with vehicle control group have numerous mitotic cells. The value of bars represented mean ± SD of independent experiment with * $p < 0.05$ and **** $p < 0.0001$ were taken as significant difference compared with the other treatment group.

The expression of apoptosis mediator, caspase-3

The positive caspase-3 expression was stained brown mainly in the cytoplasm or nuclear membrane. The results were shown in Figure 3 where the caspase-3 positive cells were significantly increased ($p < 0.0001$) in SF1 and cisplatin -treated groups, compared to a vehicle control group. The data analysis indicated that apoptotic signal, caspase-3 in the isolated tumour of mice were highly expressed in SF1 (36.33 ± 5.9%) and cisplatin (49.33 ± 5.8%) treatment groups, compared to a vehicle control group (8.34 ± 2.8%). This finding revealed that the apoptotic activity was strongly induced in both SF1 and cisplatin -treated groups through the cleavage of caspase 3.

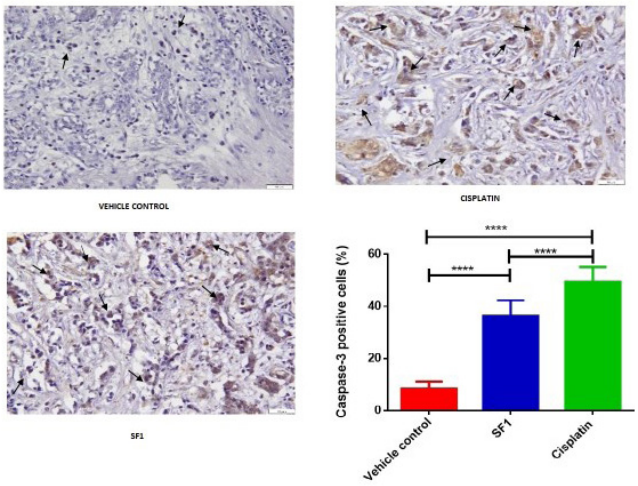

Figure 3: The immunohistochemical (IHC) analysis of caspase-3 expression in the tumor administered with vehicle control, SF1 and cisplatin, (20x magnification). The arrows indicate caspase-3 positively stained cells in the tumor tissues. The positive caspase-3 expression was stained brown mainly in the cytoplasm or nuclear membrane. The graph showed the percentage of caspase-3 positive cells in SF1 and cisplatin -treated groups and vehicle control on the xenotransplant tumor in nude mice. The value of bars represented mean ± SD of independent experiment with **** $p < 0.0001$ was taken as significant difference compared with the other treatment group.

The effect of SF1 on liver toxicity

Liver histopathology by H & E staining

The morphological changes of liver in the experimental groups were shown in Figure 4 and the score was shown in Table I. The untreated group possessed normal hepatocytes with central vein are well preserved and liver sinusoids are well structured. Mice treated with SF1 demonstrated mild remarkable changes and no signs of liver toxicity after treatment is given. Hepatocytes and

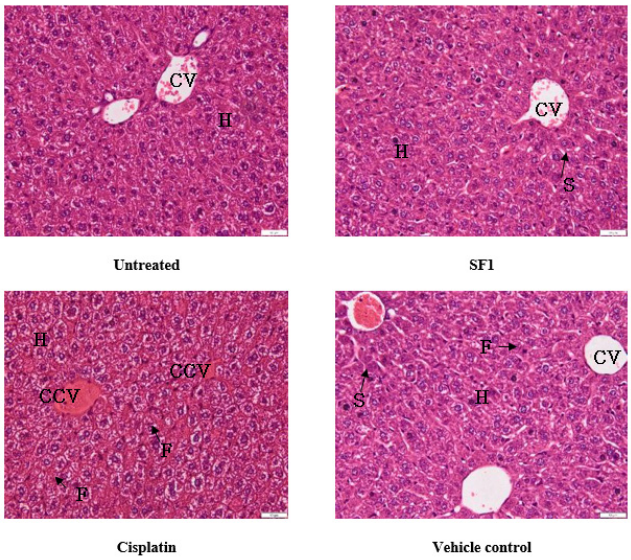

Figure 4: The histopathological H&E images of the liver (20x magnification) against different treatment groups. The untreated group possessed normal hepatocytes with central vein are well preserved and liver sinusoids are well structured. Mice treated with SF1 demonstrated mild remarkable changes but with no signs of liver toxicity. CV, central vein; CCV, congested central vein; S, sinusoid; F, fatty vacuoles, H, hepatocytes.

Table I: The scoring of the morphological changes in the liver of nude mice for different type of treatments

Groups (treatment)	Histopathological changes
Untreated	-
Vehicle control	+
SF1	+
Cisplatin	++

- = none, + = mild, ++ = moderate

sinusoidal spaces appeared near-normal characteristics with the untreated group. Similar morphology was seen in vehicle control with mild histopathological changes compared to the untreated group. However, some degenerative changes in the liver including the presence of degenerated changes in hepatocytes and the formation of fatty vacuoles indicating fatty changes occurred in the cisplatin group. The congestion of the central vein which possibly leads to liver dysfunction was demonstrated in the cisplatin group too.

Aspartate transaminase (AST) and alanine transaminase (ALT) enzymes levels

According to the veterinary encyclopedia (Wikivet), the normal range of serum liver enzymes, ALT and AST in mice are 17 – 77 U/L and 54 – 296 U/L, respectively. In this study, ALT (53.25 ± 9.58 U/L) and AST (176.0 ± 30.97 U/L) levels were in normal ranges and remained low in the untreated group (clean mice), compared to the SF1-treated group. The ALT levels were recorded slightly higher compared to untreated group after 28 days of administration with SF1 (84.88 ± 17.03 U/L) as well as in vehicle control group (91.50 ± 18.29 U/L). In contrast, the AST levels were higher in the SF1-treated group (189.0 ± 38.95 U/L) compared to the vehicle

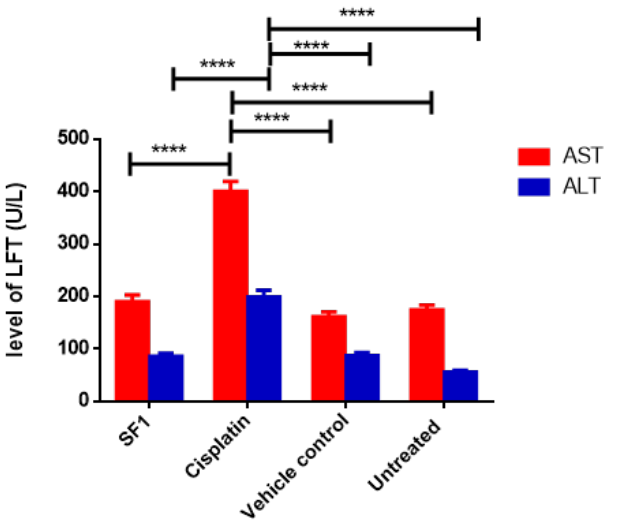

Figure 5: The level of ALT and AST in mice bearing tumor treated with vehicle control, SF1, cisplatin and untreated group on necropsy day. There was a significant increase in both serum levels of ALT and AST in the cisplatin-treated group, compared to the SF1-treated group. No significant differences for both ALT and AST in SF1 compared to vehicle control (negative control) group and untreated group (clean mice). The value of bars represented mean ± SD of independent experiment with **** $p < 0.0001$ was taken as significant difference compared with the other treatment group.

control group (164.0 ± 29.37 U/L), but the enzyme level remains in normal ranges. Nevertheless, the data were not statistically significant in differences (Figure 5). However, there was a significant increase in both serum levels of ALT (199.5 ± 40.92 U/L) and AST (396.33 ± 53.05 U/L) in the cisplatin-treated group, compared to the SF1-treated group.

DISCUSSION

The leaves of *C. nutans* which is commonly known as Sabah Snake Grass has been widely reported to possess anticancer activities (17-20,34). However, the molecular mechanisms underlying its anticancer activity in animal studies have not been fully elucidated. Previously, SF1 derived from *C. nutans* was shown to impede the growth of human cervical cancer cells, in vitro (19,26). Based on current findings, SF1 has shown its anticancer property in vivo .

In this study, the body weights of mice were closely monitored throughout the experimental period where no major changes were observed. The body weights of mice were measured in order to evaluate the systemic toxicity upon given treatment. No remarkable difference in food and water consumption were observed in all tested groups which justified that SF1 treatment did not affect the food intake of mice. All mice survived with no signs of mortality on the day of necropsy and no secondary tumour formation was recorded. No signs of behavior that indicate pain, distress or discomfort were seen from all treated groups. All mice were walking

normally, no limping was observed. All mice ate and drank as usual and no clinical diarrhea cases were recorded. In a previous study, the body weights of mice treated with ethanol extract of *C. nutans* were similar in all treated groups although different dosages were used. Other researchers also reported that there was an increase in body weight of all mice which indicates that the *C. nutans* extract did not affect the mice's appetite (15). Similarly, the body weights of mice treated with the *C. nutans* ethanol extract were significantly higher than those treated with fluorouracil, a conventional drug to treat hepatoma.

This present finding indicated SF1 inhibited the palpable tumor size, thus reducing the tumour volume as compared to the vehicle control group. This trend was also shown in a study where a 1000 mg/kg dose of *C. nutans* methanol extract significantly reduced its tumour volume and body weight in mice (21). However, SF1 was able to reduce tumour volume with a lower dosage (30 mg/kg). This finding also strengthens the view that *C. nutans* inhibits tumour progression in tumour-bearing mice models.

To further evaluate the potential of SF1 on inhibition of tumour growth, the antitumour activity was investigated. Relative tumour growth (RTV) was used as an indicator that represents the treatment efficacy in inhibiting the growth of tumour. The inhibition of tumour growth in nude mice treated with SF1, expressed as T/C ratio (%) was more than 40%. A T/C ratio that equals or less than 50% is a potent value of antitumour activity (31). Similarly, a T/C ratio of hepatocellular carcinoma in nude mice was found to be lower in total flavonoid Arachniodes exilie (TFAE) compared to a negative control group (24). At the end of the treatment period, SF1 demonstrated more than 50% inhibition rate on the tumour growth, indicating a great property of tumour inhibition compared to the negative control. Meanwhile, the tumour inhibitory rate for the commercial drug, cisplatin was significantly lower compared to SF1. Other researchers reported that treatment with ethanol extract of *C. nutans* aerial part showed a dose-dependent reduction in hepatoma tumour size with the inhibition ratios of 8.2% and 58.6% at doses of 3 and 10 mg/kg, respectively. However, the same research group also found that the inhibitory rate for the ethanol extract of *C. nutans* was lower compared to a commercial drug, fluorouracil due to the protective effects *C. nutans* on immune function.

This present study also evaluated the histological changes in the tumour by H&E staining and immunohistochemistry. H&E stains have been used for at least a century and are still essential for distinguishing various tissue types and the morphologic alterations that form the basis of modern cancer diagnosis. The number of mitotic cells is an important hallmark in the grading and diagnosis of cancer. It is used to evaluate the proliferation and aggressiveness of the tumor. Based on H&E staining, untreated mice showed normal characteristics of mitotic cells with the presence of high nuclear staining. This demonstrated that there were highly proliferating cells in the untreated group compared to SF1- and cisplatin-treated group. In both treated groups, mitosis was fewer indicating the occurrence of tumour cells damage which led to a halt of tumour growth. In comparison with other reported studies, *C. nutans* ethanol extract (10 mg/kg) and fluorouracil (20 mg/kg) have similar effects on tumour cell damages such as condensation of cytoplasm and fragmentation of nuclei upon H&E staining which indicate that the extract reduced tumour volume and growth rate of hepatoma cells compared with the drug used for positive control. Similarly, the methanolic extract of *C. nutans* leaves inhibited the common appearance of actively mitotic cells in tumours compared to the control and untreated groups, as seen from H&E staining profile (21). Thus, it can be concluded that SF1 also reduced tumour volume and growth rate of SiHa cells by arresting mitosis process. However, the H&E staining has some limitations which include the existence of substantial inter- and intra- observer variation when mitotic cells were manually counted (35). Besides, the main challenge in H&E staining is in detecting mitotically active areas or the presence of mitotic mimics such as hyperchromatic nuclei, karyorrhectic and apoptotic cells (35,36). Variations in cell density and the heterogeneity of mitotic activity in different areas might be aggravating factors as well (37-39). Therefore, the current result might be improved by incorporating both H&E and immunohistochemistry (IHC) of highly specific mitotic marker, phosphorylated histone H3 (PHH3). This dual-staining technique enables visualization of morphology and molecular profiling over the same tissue section and give optimum method for mitosis detection and counting, thus improve the overall accuracy, quality, and diagnostic precision of cancer (40). Other than that, selective histochemical stains like crystal violet, which provides a definite advantage over the H&E also can be done.

The immunohistochemistry analysis showed that caspases-3 level was highly induced and expressed in SF1-treated tissue samples, indicating apoptosis activation. Caspases are crucial mediators of apoptosis. Among other mediators, caspase-3 is known as a frequently activated death protease and acts as a downstream executioner caspase in inducing apoptotic pathway. From the current findings, caspase-3, also known as an effector caspase was responsible for the proteolytic cleavage that led to apoptosis by SF1. Although the activation of caspase-3 is not the sole factor during apoptosis, caspase-3 played an important role in this pathway. This finding was similar to the study by other researchers that showed an increased expression level of caspase-3 with the treatment of 10 mg/kg *C. nutans* ethanol extract, as evidenced by Western blot analysis. Other researchers also suggested that a flavonoid, liquiritin was able to

induce apoptosis in SiHa cells due to high levels of caspase-3 expression upon immunoblotting analysis (9). After all, the caspase-3 cleaves and activates numerous effector proteins in apoptosis including caspase -6, -7 and -9. Both extrinsic and intrinsic pathways eventually lead to caspase-3 activation and induce apoptosis (9). Besides, previous study has reported that SF1 induces apoptosis via mitochondrial pathway (26). SiHa cells treated with SF1 have shown an increased level of Bax protein activated by p53, thus stimulating the release of cytochrome C which directly activates the cascade caspases to enhance apoptosis (26). Therefore, together with this current finding, SF1 was suggested to inhibit the tumour cells progression by inducing apoptosis.

The liver is the primary drug-metabolising organ and plays an important role in the elimination of drugs (15). In this present study, the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were the focus since both are among important markers of hepatocellular damage and toxicity. These cytosolic enzymes of liver cells are used as indices of liver damage in both animals and humans (41). Although the present findings showed a slight increase in serum ALT and AST in SF1-treated mice compared to the untreated group, the changes were not statistically significant, and the AST enzyme levels remain in the normal ranges. The increase in the level of liver enzymes may temporarily occur due to hepatic biotransformation of SF1. Hepatic biotransformation mainly involves the cytochrome P-450 enzyme system. Metabolism of SF1 may inhibit the cytochrome P-450 causing the integrity changes in hepatocytes which leads to the slight increase in ALT and AST (42). Therefore, understanding mechanisms underlying the action of SF1 on cytochrome P-450 enzymes is essential especially in conjunction with other cytochrome metabolized drugs. However, the present data indicated that there is no toxicity effect on the liver of SF1-treated mice when compared to the cisplatin-treated group, which may otherwise lead to potential side effects and impairment on the liver function of the mice. Previous researchers proved that the clinical evidence of cisplatin induced liver injury has been demonstrated by elevated activities of serum enzymes and hepatotoxicity (43). Variable amounts of these enzymes are leaked into serum as a result of cellular stress, affecting cell membrane integrity (44). Oxidative stress appears to play an important role in cisplatin-induced hepatotoxicity (45) where a few mechanisms have been identified. Cisplatin causes overproduction of the generation of reactive oxygen species that leads to oxidative stress, inflammation, DNA damage and apoptosis in the liver (46). Based on these present data, SF1 showed a less toxicity effect on the liver compared to cisplatin. In comparison with other previous studies, ALT level showed no major changes when a low dosage of 75 mg/kg of *C. nutans* ethanol extract was administered compared to vehicle control, 10% DMSO (46). There was also no significant difference in AST and ALT levels between *C. nutans* aqueous extract-treated and untreated groups indicated that the extract does not affect the liver function (15).

Based on the H&E staining, the liver of SF1-treated mice showed no obvious alterations and differences compared to the untreated group. In this study, a similar trend was shown by the liver of mice in the SF1-treated group compared to the untreated group where the hepatic cells had well – preserved central vein, nucleus and cytoplasm. This indicates its non-hepatotoxicity occurred on the liver tissue of tumour-bearing nude mice. In contradiction with the cisplatin-treated group, moderate alteration of the liver was clearly shown with degenerated hepatic cord, which is related to former findings (43). In a previous study, administration of 75 mg/kg ethanol extract of *C. nutans* for 90 days induced no hepatotoxicity on treated rats (46). Other studies also showed that *C. nutans* aqueous extract at 5000 mg/kg was not toxic to the liver and no abnormalities were observed in the branches of hepatocytes cords (15). Based on these recent findings, a proposed mechanism of SF1 in inhibiting tumour growth is depicted in a schematic diagram in Figure 6. Although the activation of caspase-3 was identified, other essential proteins such as caspase-8 and caspase-9 may play a part of important role in further clarifying the overall apoptosis pathway that takes part. The combination methods of H&E and IHC should also be carried out to improve the accuracy of mitotic index scoring and provides better diagnostic quality. Nevertheless, SF1 demonstrated the ability to inhibit the growth of SiHa cells in a mice xenograft tumour model. The activation of caspase-3 without any toxicity signs was presented in accordance with biochemical data and histopathological analyses,

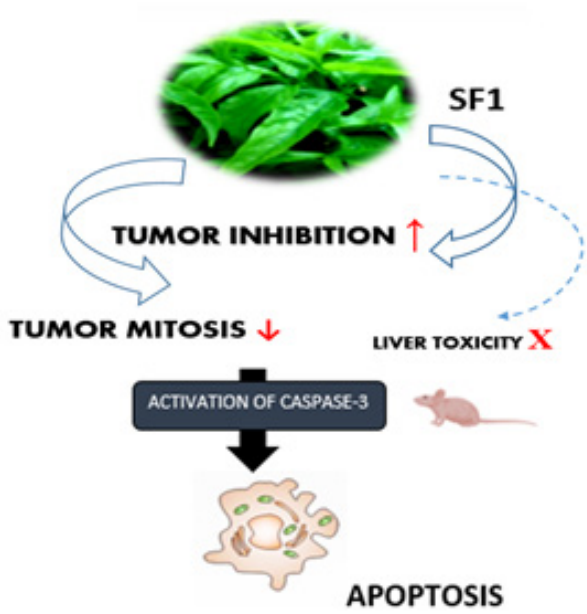

Figure 6: Schematic diagram for the suggested mode of actions of SF1. SF1 inhibits the tumor growth by activation of executioner caspase, caspase-3 which induces apoptosis program cell death.

indicating the selective targeting of SF1 on cancer cells.

However, even though the nude mice xenograft model is very useful for elucidating the biological process of tumour growth and development, the use of this model cannot mimic all the disease features. The main drawback of xenografts is that they typically lack the genetic makeup and histology of the corresponding human tumour, and that, thus far, these models have not proven as accurate predictors of treatment effectiveness as one would like (47). In addition, the use of immunodeficient mice type is suffered from the lack of immunity which provide a less realistic tumor microenvironment. Therefore, they have limited use in research that requires an intact immune system (48). This limitation can be addressed with the use of humanized mice, which are generated with a human immune system (49, 50). The use of humanized mice may further enhance the therapeutic relevance of the patient-derived tumor xenograft model particularly in cancer immunotherapy study. In addition, the development of anatomic and molecular in vivo imaging techniques such as magnetic resonance imaging and bioluminescence imaging together with genetically engineered models (GEMs) could also overcome these shortcomings (47). In the future, hopefully with the new technologies, these two additional types of animal models can be utilized in parallel to the tumor xenograft models to support the use of xenografts and help to determine the predictive analysis for human responses in order to enhance result interpretation and clinical relevance of the study.

CONCLUSION

The findings from this study demonstrated that the active amine compound from *C. nutans* known as SF1 exerted a potent anticancer activity with acts effectively in inhibition of tumour growth, in vivo. Despite the limitation, the presented data suggested that the action of SF1 in inhibition of tumour progression was by preventing the cells from undergoing mitosis process and inducing apoptosis. Hence, these current findings were able to support and justify the medicinal value of SF1 as previously reported. To further explore the potential of SF1 as an effective anticancer treatment towards cervical cancer, the study on the delivery of SF1 on targeted cells was suggested in the future.

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