Clinacanthus nutans Standardized Fraction Arrested SiHa Cells at G1/S and Induced Apoptosis via Upregulation of p53

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ABSTRACT

Introduction: Cervical cancer is a leading cause of death in women. Current cancer treatment comes with side effects. Clinacanthus nutans has been known traditionally to treat cancer. This study was aimed to characterize C. nutans standardized fraction (SF1) and to investigate its anticancer mechanism against SiHa cells. Materials and Methods: SF1 was produced by optimized methodology for bioassay-guided fractionation. Fourier transform infrared (FTIR) spectroscopy and liquid chromatography–mass spectrometry (LC-MS) were carried out to characterize the SF1. SF1 was screened for cytotoxicity activity toward HeLa, SiHa, and normal cells (NIH) cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The anticancer mechanism of SF1 was evaluated toward SiHa cells, which showed highest cytotoxicity toward SF1 treatment. The mechanism includes cell cycle progression and protein expression, which was detected using specific antibody-conjugated fluorescent dye, p53-FITC, by flow cytometry. Results: Major constituents of SF1 were alkaloids with amines as functional group. SF1 showed highest cytotoxic activity against SiHa (half-maximal inhibitory concentration \([IC_{50}] < 10 \mu g/mL\)) compared to HeLa cells. Cytoselectivity of SF1 was observed with no IC50 detected on normal NIH cells. On flow cytometry analysis, SF1 was able to induce apoptosis on SiHa cells by arresting cell cycle at G1/S and upregulation of p53 protein. Conclusion: SF1 showed anticancer activity by inducing apoptosis through arrested G1/S cell cycle checkpoint–mediated mitochondrial pathway.

KEYWORDS: Apoptosis, cervical cancer, Clinacanthus nutans, cytotoxicity

INTRODUCTION

Cancer has been known as one of the major health threats in this world. The World Health Organization reported that cervical cancer is the fourth most common cancer among women worldwide and the second most common cancer among women in Asia.¹ Current treatment of cervical cancer that is commonly used, such as surgery, radiotherapy, and chemotherapy, failed to kill cancerous cells efficiently due to resistance and toxicity to healthy cells. Chemotherapy is unable to discriminate between healthy and cancerous cells; therefore, it becomes toxic for the body. A study showed up to 50% of patients with cancer were killed by the chemotherapy drugs, not the disease itself.² Rise of prevalence in cancer has captured the attention of researchers who have been determined to find effective treatments.³ Many anticancer drugs have been developed using medicinal plants,⁴ which is believed to improve survivorship in cancer. In this study, Clinacanthus nutans has been...
chosen based on the traditional claim from old folks in combating cervical cancer. Despite numerous identified studies related to antiproliferative activity of *C. nutans* and their responsible bioactive compounds are available, less information regarding its anticancer mechanism is reported. Hence, the aim of this study was to characterize the active anticancer compounds from *C. nutans* and to study its mechanism to evaluate the potential of *C. nutans* as an alternative potential treatment for cervical cancer.

**Materials and Methods**

**Plant collection, extraction, and fractionation**
The fresh leaves of *C. nutans* were collected from Pengkalan Chepa, Kelantan, Malaysia. The specimen was authenticated (no. PIUUM 0238-2) and deposited at Herbarium, Kulliyyah of Pharmacy, Universiti Islam Antarabangsa Malaysia (UIAM), Jalan Gombak, Selangor, Malaysia. The leaves were dried in an oven at 50°C overnight. The dried leaves were ground and sequentially extracted with hexane and chloroform, respectively.[6] Standard fraction (SF1) was then produced by bioassay-guided fractionation, previously optimized by our colleagues.[7]

**Characterization of standardized fraction**

**Fourier transform infrared spectroscopy analysis**
One gram of dried SF1 was scanned at wavelength ranging from 600 to 4000 nm, and the characteristic peaks and their functional groups were detected using Tensor-II-FTIR spectrometer, Bruker, Billerica, Massachusetts. The total run time was 15 min.

**Liquid chromatography–mass spectrometry analysis**
A 10 mg/mL of SF1 was injected using Agilent-1290-Infinity liquid chromatography (LC) system and separated using a C18 column (Agilent ZORBAX Eclipse XDB-C18, Narrow Bore 2.1 mm × 150 mm, 3.5 micron). The LC was coupled to an Agilent-6520 Accurate-Mass Q-TOF mass spectrometer with a dual Electrospray Ionisation source. The total run time was 30 min. The analysis was set to mass spectrometry (MS) mode with negative and positive polarity.

**Cytotoxic activity of standardized fraction by MTT assay**
MTT assay was performed toward two types of cervical cancer cells, HeLa and SiHa, and normal fibroblast cells, NIH cells. HeLa and NIH cells were maintained in Dulbecco Modified Eagle medium, whereas SiHa cells were maintained in Roselle’s Park Memorial Institute 1640 medium, supplemented with fetal bovine serum and penicillin–streptomycin. The cells were seeded at 5 × 10^4 cells/well in 96-well plates overnight. Ten milligram of SF1 was dissolved in Dimethyl sulfoxide (DMSO) and followed by a serial dilution ranged from 0.2 to 100 μg/mL. Cisplatin was used as a positive control and DMSO acted as a negative control. The treated plates were incubated for 72 h. The old medium was discarded and MTT solution was added. The plates were incubated for 4 h. MTT solution was removed and dissolved with DMSO. The plates were shaken for 30 min, and the absorbance at 570 nm was read on microplate reader. The percentage of cell viability was calculated as follows: (optical density (OD) treated)/(OD negative control) × 100%. Dose response curves were constructed to obtain half-maximal inhibitory concentration (IC_{50}) values.

**Sample preparation for flow cytometry analysis**
The cells for each flask were seeded at 5 × 10^6 cells/mL overnight. The next day, cells were treated with IC_{50} of SF1 and IC_{50} of cisplatin for 24, 48, and 72 h. Untreated cells were used as negative control. The SF1-treated, cisplatin-treated, and untreated cells were harvested by centrifugation.

**Cell cycle analysis of cell-treated standardized fraction by flow cytometry**
After centrifugation, all the cells were fixed in cold 70% ethanol for 1 h. Pellet of cell was then resuspended with 1 mL of 1% Triton X-100 in phosphate-buffered saline (PBS) and 100 μL of 1 mg/mL RNase A. The mixture was kept at 37°C for half an hour. Cells were stained with 10 μg/mL propidium iodide solution in staining buffer. Incubation was allowed for 30 min and tubes were kept in ice. Before flow cytometric analysis, 300 μL of staining buffer was added and analyzed.

**Detection of apoptotic protein, p53, in cell-treated standardized fraction by flow cytometry**
After centrifugation, all the cells were fixed in ice cold 70% ethanol for 1 h. The cell pellet was washed twice with ×1 PBS and resuspended in a blocking buffer (2% bovine serum albumin) for 10 min. Again, washing step was carried out. Cell pellet was resuspended in PBS to a concentration of 1 × 10^6 cells/mL. Then, 100 μL of cell suspension (1 × 10^6 cells/mL) was transferred into flow cytometry tubes, and 20 μL of p-53/Fluorescein isothiocyanate-conjugated antibody was added. The tubes were mixed and left for 30 min in the dark at room temperature. The cells were washed with ×1 PBS, resuspended in ×1 PBS, and proceeded with flow cytometric analysis.

**Results**

**Fourier transform infrared spectroscopy analysis of standardized fraction**
Amines have been detected as the most abundant functional groups present in SF1, followed by alkanes, alkenes, esters, and aromatics [Figure 1]. The identified amines include aromatics amine and aliphatic amines [Table 1].
Liquid chromatography–mass spectrometry analysis of standardized fraction

Mass spectrum identification was matched with METLIN database and composition that possessed higher peaks in the chromatogram with more 95 DB, database score is referred as the closeness match of the compound with the database [Tables 2 and 3].

The major known constituents from positive polarity [Table 2] belong to alkaloids, a class of nitrogenous organic compound. They are methysergide, calycanthine, eburnanomine, and dextromethorphan. There was one unknown compound, C_{33}H_{37}N_{5}O_{5}S_{2} that does not match with METLIN database [Figure 2]. As compared to negative polarity [Figure 3], there was one known constituent identified as α-9(10)-EpODE, fatty acids, and two unknown constituents, C_{31}H_{59}N_{2}O_{4}S and C_{16}H_{26}O_{3}S [Table 3]. Molecular structure of major known compounds from positive polarity had a ring structure, whereas major known compounds from negative polarity displayed long chain structure [Figure 4].

Cytotoxicity of standardized fraction

SF1 showed potent inhibition toward SiHa cells with lowest IC_{50} values of 9.98 ± 1.24 µg/mL as compared to HeLa cells [Figure 5]. Cisplatin showed high cytotoxic activity on all tested cell lines; 2.76 ± 1.06 µg/mL on NIH cells, 2.95 ± 1.20 µg/mL on HeLa, and 2.29 ± 1.16 µg/mL on SiHa cells. No IC_{50} value was detected on normal cell, NIH, which showed no inhibition effect on the treatment with SF1. This indicated that the cytotoxicity effect of SF1 was selective toward SiHa cells. SiHa cells were also more susceptible to the SF1 treatment than HeLa cells. Hence, for subsequent experiments, SiHa cells were selected to determine the anticancer mechanism of SF1.

Table 1: Peak value of Fourier transform infrared spectroscopy and its different functional group in standardized fraction

| NO | PEAK VALUE (cm⁻¹) | FUNCTIONAL GROUPS |
|----|------------------|-------------------|
| 1  | 3399.22          | Amines            |
| 2  | 2923.87          | Alkanes           |
| 3  | 2853.27          | Alkanes           |
| 4  | 1736.37          | Aromatics, Esters |
| 5  | 1635.32          | Amines            |
| 6  | 1456.30          | Alkanes, Aromatics |
| 7  | 1376.95          | Unknown           |
| 8  | 1259.67          | Aromatic Amines   |
| 9  | 1163.91          | Aliphatic Amines  |
| 10 | 1088.95          | Aliphatic Amines  |
| 11 | 1024.69          | Aliphatic Amines  |
| 12 | 983.63           | Alkenes           |
| 13 | 896.75           | Amines, Aromatics |
| 14 | 800.59           | Amines, Aromatics |
| 15 | 719.95           | Amines, Aromatics |
| 16 | 670.01           | Amines, Aromatics |

Figure 1: Fourier transform infrared spectroscopy spectrum of standardized fraction
Cell cycle arrest by standardized fraction

Figure 6 showed DNA content profile of SiHa cells in all tested groups for 24, 48 and 72 hours. The DNA content is reflex to the presence of DNA in the cell population. The percentage of DNA content at G2/M remained high throughout all treatment durations, which indicated that the mitosis occurred as a common process in untreated SiHa cells. In SF1-treated SiHa cells [Figure 7B], the pattern of cell cycle was altered at the end of 72 h of treatment duration. The DNA content in G0/G1 phase at 24 h was 46.59% ± 1.05%, and it reduced to 31.94% ± 0.89% at the S phase. The cell population was significantly reduced ($P < 0.01$) to 21.46% ± 0.62% in the G2/M phase. This cell cycle

| Table 2: List of standardized fraction major constituents detected in positive mode |
|---|---|---|---|---|---|
| No | Formula | Compound name | Mass | RT | Base peak | Compound class | Percentage volume (%) |
| 1 | C33 H37 N5 O S2 | Unknown | 583.24 | 20.03 | 371.25 | Unknown | 22.64 |
| 2 | C18 H25 N O | Dextromethorphan | 271.19 | 14.33 | 272.20 | Alkaloids | 16.59 |
| 3 | C19 H22 N2 O | Eburnamonine | 294.17 | 11.20 | 295.18 | Alkaloids | 17.99 |
| 4 | C22 H26 N4 | Calycanthine | 346.21 | 15.89 | 347.22 | Alkaloids | 21.17 |
| 5 | C21 H27 N3 O2 | Methysergide | 353.21 | 14.31 | 354.22 | Alkaloids | 21.59 |

Figure 2: Chromatogram of standardized fraction (positive mode)

| Table 3: List of standardized fraction major constituents detected in negative mode |
|---|---|---|---|---|---|
| No | Formula | Compound name | Mass | RT | Base peak | Compound class | Percentage volume (%) |
| 1 | C16 H26 O3 S | unknown | 298.16 | 15.53 | 297.15 | Unknown | 22.43 |
| 2 | C21 H38 N4 O4 S | unknown | 442.25 | 17.80 | 441.25 | Unknown | 33.31 |
| 3 | C18 H30 O3 | $\alpha$-9(10)-EpODE | 294.22 | 16.175 | 293.21 | Fatty acids | 22.13 |
trend was similar throughout 48 and 72 h of SF1-treated SiHa cells, with 42.35% ± 1.44% and 40.56% ± 1.05% cells were arrested at G1/G0 phase, respectively. During S phase, 35.60% ± 3.31% at 48 h and 46.14% ± 2.46% at 72 h were recorded. At the end of 72 hr, there was 13.29% ± 2.38% of SF1-treated SiHa cells entered G2/M phase (P < 0.01), which indicated that less percentage of cell proliferation and could not undergo mitosis phase. Similarly in cisplatin-treated SiHa cells [Figure 7C], most of the cell population was accumulated at G1/S with 54.95% ± 3.33% and only 10.42% ± 1.29% was detected at G2/M phase.

Quantification of apoptotic protein
Figure 8 showed profile of p53 proteins expression of SiHa cells in all tested groups for 24, 48 and 72 hours. This study indicated that SF1 successfully caused highest accumulation of p53 protein up to 92.33% ± 3.56% at 72 h (P < 0.001) compared to 53.17% ± 3.19% at 48 h, and 19.50% ± 0.92% at 24 h of treatment in SiHa cells [Figure 9]. On the contrary, in untreated SiHa cells, the percentage of p53 remained at the lowest with 0.46% ± 0.45% at 24 h, 1.43% ± 0.35% at 48 h, and 3.90% ± 0.53% at 72 h. The level of p53 protein in cisplatin-treated SiHa cells was dramatically increased from 35.60% ± 4.50% at 24 h to 95.97% ± 2.30% at 48 h and 98.53% ± 0.49% at the end of 72 h.

Discussion
Bioassay-guided fractionation was carried out to produce SF1 due to the main goal to characterize the compounds present in C. nutans that are responsible as anticancer agent.[6,8] In this study, the bioassay-guided fractionation involved various extraction techniques including soaking, rotary evaporation, and column chromatography. The IR spectroscopic analysis was carried out to determine the chemical functional groups, and the MS analysis was carried out to determine the selectively accurate mass of compounds present in the SF1. A research study identified a polysaccharide–peptide complex from C. nutans leaves, which showed inhibition for gastric cells.[8] On the basis of the results of this study, major phytochemical class of SF1 was
Figure 6: DNA content of SiHa cells in untreated SiHa cells (UT), standardized fraction-treated SiHa cells (SF1), and cisplatin-treated SiHa cells (CP) for 24, 48, and 72 h. Similar plots were observed in three independent experiments ($n = 3$). Indicator: Green = G1/S, Blue = S, Red = G2/M.

Figure 7: Graph summarized the percentage of DNA content of SiHa cells in each cell cycle phase for (A) untreated SiHa cells, (B) standardized fraction (SF1)-treated SiHa cells, and (C) cisplatin-treated SiHa cells. Each point represented mean ± SD of three independent experiments, and **$P < 0.01$ and *$P < 0.05$ were taken as significantly different with treatment duration.
identified as alkaloids with functional group, amines. Alkaloids are the class of naturally occurring amines, which mostly contain organic nitrogen containing bases.[3,9] Some of the alkaloids have already been successfully developed into chemotherapeutic drugs, such as vinblastine, taxol, and vincristine. They are known for having potent anticancer activity against various cancers.[3,10] A few notable phytochemical compounds of \textit{C. nutans} that relate to anticancer property, such as 1,2-benzenedicarboxylic acid monoester, were the major chemical constituents from chloroform leaf extract, which were capable to inhibit the growth of HeLa cells.[11] High-performance LC-MS-identified flavonoids were presented in ethanol extract of \textit{C. nutans} leaves,[12] and they potentially inhibited hepatoma in mice. Hence, based on the findings, it was found that the main source of anticancer activity of SF1 was produced by secondary metabolites, alkaloids.

| Duration | 24 hours | 48 hours | 72 hours |
|----------|----------|----------|----------|
| SF1      | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| CP       | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| UT       | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |

Figure 8: Histogram profile of p53 proteins expression detected by flow cytometry analysis in untreated SiHa cells (UT), standardized fraction-treated SiHa cells (SF1), and cisplatin-treated SiHa cells (CP) for 24, 48, and 72 h. Similar profile was observed in three independent experiments ($n = 3$)
SF1 showed a better inhibition with highly toxic towards SiHa cells and nontoxic to normal cells. The ability of SF1 to selectively kill SiHa cells could lead to cell-specific drugs without the side effects. Therefore, further study on the SF1 mechanism toward the inhibition of SiHa cells was proceeded.

The regulation of cell cycle is responsible to either inhibit or induce cell growth. The relative DNA contents in all tested groups were analyzed using flow cytometer and represented as three different stages within the interphase of cell cycle known as G0/G1, S, and G2/M phases. On the basis of the findings, the distribution of SiHa cells in G0/G1 and S phase was notably decreased following treatment duration in SF1-treated group as compared to that in the untreated group. This indicated that SF1 significantly triggered the DNA damage within 72 h and caused cell cycle to stop. Thus, the current finding recommended that SF1 inhibited the proliferation of SiHa cells by promoting the cell cycle arrest at G1/S checkpoint. These data were similar with the previous findings reported on HeLa and A549 cells arrested cell cycle at G1/S phase on 72 h treatment with an active fraction of C. nutans. However, dichloromethane extract of C. nutans arrested HeLa cell at S phase after 48 h of treatment.

Many previous studies reported that cell cycle arrest induced apoptosis by the increased levels of p53. In this study, the main focus was on intrinsic pathway. The activation of this pathway is mainly regulated by the increased levels of p53 protein. It is known as the guardian of genome and is the most frequently mutated gene in almost all types of cancers. Various apoptotic signals are activated by DNA damage, cytotoxic agents, and p53 activation. On the basis of the results, the level of p53 increased gradually at 24–72 h of treatment with SF1. The percentage of p53 on treatment with SF1 was similar with cisplatin-treated SiHa cells at the end of 72 h incubation. This indicated that the SF1-activated p53 resulted from DNA damage. p53 is able to regulate the expression of proteins that plays the major roles in growth arrest and apoptosis. Therefore, SF1 induced cell death in SiHa cells via apoptosis-dependent p53-mediated pathway. Several previous studies showed that apoptosis was induced by the elevation of p53 via mitochondrial apoptotic pathway in human cervical cancer cells. The p53 plays a critical role in the G1/S checkpoint of cell cycle, and this checkpoint is recognized as being entirely p53 dependent.

CONCLUSION
These findings are pivotal as this is the first report on anticancer effect of standardized fraction extracted from C. nutans.
from *C. nutans*, SF1, against cervical cancer cell, SiHa. SF1 was successfully characterized as alkaloids with amines as functional group. SF1 has been found to have cytoselective anticancer effects toward SiHa by damaging the DNA, and it leads to p53 activation at G1/S checkpoint, which results in cell cycle arrest and apoptosis. Further investigation including elucidation of *in vivo* antitumor suppression of SF1 and the underlying mechanism are currently being carried out.

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**Conflicts of interest**

There are no conflicts of interest.

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