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Article

Anticancer and Apoptotic Activity in Cervical Adenocarcinoma HeLa using Crude Extract from Spike of *Stomopneustes variolaris*

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Abstract: Cancer is one of the world's most serious health problems and the top cause of mortality today. *S. variolaris* is a valuable sea creature that has long been used as a folk medicine to cure and prevent ailments. A subcritical water extraction was used method to obtain crude extract from spikes of *S. variolaris*. The extract had shown antiproliferative effect in HeLa cells with an IC_{50} of 723.1 ± 9.73 μg/ml, but no toxicity in HEK293. Westernblot was used to detect protein expression; Bax, caspase-8, and IκBα were increased, whereas IKKα and p-NFκB-65(Ser 536) were downregulated. RNA/mRNA expression was revealed by RT-qPCR, and fold changes of caspase-3, cytochrome-c, Bax, Apaf-1, caspase-9, and Bak genes' expression were raised. Using gel electrophoresis, the treatment groups showed more DNA fragmentation than the control group. GC-MS was used to identify the components in the crude extract; anticancer activity could be attributed to dodecanoic acid, hexadecanoic acid, and tetradecanoic acid content. In conclusion, these results showed the potential use of the crude extract of a spike from *S. variolaris* with anticancer activity against cervical cancer.

Keywords: Adenocarcinoma HeLa; Anticancer; Apoptosis; Crude extract; *Stomopneustes variolaris*.

Abbreviations

Apaf-1:	Apoptotic protease activating factor-1
cBID:	Cleaved BID
FBS:	Fetal bovine serum
GC-MS:	Gas chromatography-mass spectrometry
HEK293:	Human embryonic kidney 293
HeLa:	Adenocarcinoma of the cervix
HPV:	Human papilloma virus
IC_{50}:	50% inhibitory concentration
MEM:	Minimum Essential Medium
MOMP:	Mitochondrial outer membrane permeabilization
PARP:	poly (ADP-ribose) polymerase
PCR:	Polymerase Chain Reaction
SVCE:	Crude extract from spike of *Stomopneustes variolaris*

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WHO: World Health Organization
XIAP: X-linked inhibitor of apoptosis protein

1. Introduction

Cancer is currently one of the most serious health issues in the globe and the leading cause of death. Physical carcinogens from the environment, biological carcinogens from particular virus infections, and chemical carcinogens as such in the food are the major causes of cancer (1). Among the most common cancers, Cervical cancer is one of them in women all over the world (2). Oral contraceptive use, smoking, multiparity, HIV infection, and other variables can contribute to cervical cancer. The chronic infection of oncogenic human papillomavirus is one of the key and significant etiologies (3).

Cervical cancer is a type of invasive cancer and the most life-threatening cancer in women (4). About 80% of cervical cancers are caused by pre-existing squamous dysplasia, while 20% of invasive tumors are caused by cervical adenocarcinoma. In comparison to squamous carcinoma, adenocarcinoma is becoming increasingly common in industrialized countries (5). A history of smoking, parity, oral contraceptive use, initiation of coitus at a young age, a greater number of sexual partners, a history of sexually transmitted disease, and chronic immunosuppression are all epidemiologic risk factors for cervical cancer (6). HIV/AIDS infected females are at a significant danger of contracting HPV and, as a result, developing cervical cancer at a young age (5). Chronic HPV infection is the most common and leading cause of lethal cervical cancer (6). Vaccination against HPV is supposed to prevent cervical cancer caused by certain HPV strains by intercepting the HPV infection (6). HPV is responsible for the majority of occurrences of genital cancer, approximately 95% of malignant cervical cancers embraced HPV DNA (7). HPV serotypes 16 and 18 are said in charge of roughly 70% of incidences, with the most prevalent HPV serotypes in women with cervical cancer being 16, 18, 45, 31, 33, 52, 58, and 35, in descending order of prevalence (7). HPV genes E6 and E7 play a vital function in proteins synthesis that are important for HPV multiplication within the cervical cell (8). E6 and E7, two HPV cancer-causing proteins, inactivate the tumor suppressor proteins p53 and pRb (9), respectively. In HPV 16 or 18 infected cells, ubiquitin degradation of p53 and repression of pRb skew the key checkpoints of cell cycle (8). As a result, cervical adenocarcinomas have been found to include HPV genetic material. The primary prevention of cervical cancer is achieved by avoiding HPV infection. No matter how, the efficacy and reliability of the HPV vaccine is significantly more evolved. The initial cervical screening test is the Pap smear, while HPV-based screening is more successful in finding early cervical cancer. Each of two are useful in preventing cervical malignancies (5). The effective execution of a cervical cancer screening initiatives for identification and treatment of cervical cancer has led to a reduction in cervical cancer incidence and death (10).

Because current and standard treatments for cancer such as surgery, radiation, and chemotherapies have undesirable consequences. As a result, there is a growing desire to find natural chemicals that have fewer complications, are less toxic, and have great efficacy in cancer prevention and treatment (11). Sea urchins are members of the Echinodermata phylum and can be found in tropical and subtropical oceans all over the world (12). *S. variolaris* is an edible sea urchin species that is dark-colored, omnivorous, and lives in warm water (13). Sea urchin spines, which are attached to tests and feature primary giant spines and numerous secondary little spines surrounding their base, are mostly employed for protection and mobility (14). Sea urchin gonads, commonly known as roe, are a popular and commercially essential meal all over the world because of their high nutritional content (13). Toxins are found in the bodies of some sea urchins as a protective mechanism, but they can have medical and pharmaceutical use (14).

Numerous scientific studies on marine sources, including *S. variolaris*, have been conducted to date. However, there have been no studies on the relationship between *S. variolaris* and its anticancer activity in cervical cancer. This study has a goal to achieve and see

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if the crude extract from *S. variolaris* has anticancer action and determines the apoptotic pathway in human adenocarcinoma HeLa cells in vitro.

2. Materials and Methods

2.1. Crude extraction

P.N. Fernando & Company Pvt. LTD. in Sri Lanka supplied whole *S. variolaris* sea urchins that were delivered frozen to South Korea. Thawed the Sea urchins that had been frozen to room temperature. The spines were detached, cleaned in cold water, and then lyophilized (HyperCOOl freeze drier, HC8080, Gyrozen Co., Ltd, Daejeon, Republic of Korea). Then, making use of a mechanical grinding machine (2500Y-Dong Yi multifunctional grinder, Yongkang Boou Hardware Products Co., Ltd. China), it was crushed into powder and sieved through a stainless-steel mesh (500 µm size). The milled sample was put in an airtight bottle and kept at -20°C until it was extracted.

The extraction was performed using a small-scale subcritical water extraction (SWE) equipment. 150 mL of DW was combined with 4g of milled sample in a 200 mL capacity reactor (solid to liquid ratio of 1:37.5). After that, the reactor had been sealed and nitrogen gas was pumped into it (pressure of 50 bars). The rotator and electric heater were then turned on. 15 minutes of extraction time was allowed when the reactor reached 150°C. After the extraction was finished, the safety valve was progressively opened to allow the extract to cool in the cooler before being collected. The obtained extract was centrifuged at 7,900g at 4°C for 20 minutes and filtered through a Buchi vacuum pump V100 with F1113 grade filter paper under a mild vacuum. The extracts were then freeze-dried and kept at 4°C in the dark.

2.2. Cell culture

HeLa (ATCC, Manassas, VA, USA) and HEK293 (ATCC, Manassas, VA, USA) cell lines were employed in this investigation, and they were cultured in MEM (Mediatech, Manassas, VA USA) in the moistened incubator with 5% CO_2 at 37^0C. When they reached 80% confluence, the cells were then subcultured to passage-3 and were suitable for the experiment. 1.1% antibiotic antimycotic (Mediatech, Manassas, VA, USA) 10% and heat-inactivated FBS (Mediatech, Woodland, CA, USA) were added to MEM.

2.3. Solution preparation

The concentration of SVCE stock was prepared at 100mg/ml, first by diluting 30mg of SVCE in 150µl of DMSO, then 150µl of distilled water was added. For HeLa and HEK293 cells treatment, stock was diluted in MEM media and made at varied concentrations (600, 700, 800, and 900 µg/ml).

2.4. Cell viability assay

In testing the cytotoxicity of SVCE, cells from the control and experimental groups were sown in a 96-well plate at a density of 1×10^4 cells/well, with a blank well containing just 100µl medium and no cells, and incubated at 37^0C with 5% CO_2 for 24 h. HeLa and HEK293 were treated with various concentrations of SVCE (600, 700, 800, and 900 µg/ml) and incubated for another 24 hours. After 24 hours, the media in the blank, control, and experimental group wells were replaced by new media consisting of EZ-Cytox solution (10µl), which was also wrapped in light-protected aluminium and incubated in the dark for 2 hours at 37^0C with 5% CO_2. Cells viability was determined using an ELISA plate reader (Varioskan Lux, Finland) set at 460nm. This experiment was performed in triplicate.

2.5. Protein extraction and Westernblot

25ml of MEM constituted suspended HeLa (adjusted: $1x10^5$ cells/ml) were prepared, then 5ml were put into the 100mm cell culture dish and incubated at 37^0C with 5% CO_2

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for 24 h. The media were then changed, and both the control (no treatment) and the experiment groups were given treatments with 600, 700, 800, and 900 µg/ml of SVCE and incubated at 37^{0}C with 5% CO2 for 24 hours. After 24 h, HeLa cells were rinsed and scraped with cold PBS buffer for being transferred into conical tubes of 15ml. After that, 5 min of centrifugation at 1400 RPM was done. Cells were lysed after centrifugation by adding 35 µl cell lysis buffer (iNtRON BIOTECHNOLOGY, Cat. No. 17081, Korea). On ice, incubated for 10 min. Centrifugation at 14,000 rpm for 20 min at 4^{0}C was used to collect and clarify lysates. Albumin bovine 2 mg standard (Lot # SA242714A, Thermo Scientific, USA) and Bradford reagent for ELISA microplate reader at 595nm were used to quantify protein. SDS-PAGE was used to separate aliquots of whole-cell lysates or cytosolic fractions, which were then transferred to a nitrocellulose membrane. Thereafter, the membranes were blocked with PBST (PBS buffer and 0.5 percent Tween-20) with 5% skim milk. Primary antibodies (Cell Signaling Technology, USA) were used to probe the membranes after blocking non-specific sites, and then washed three times in PBST. Then after, the secondary antibodies were incubated with anti-rabbit IgG and anti-mouse IgG (Cell Signaling Technology, USA) for 1 h. The blots were then washed in PBST and observed using an advanced chemiluminescent detection solution (Abfrontier, Lot.QJN28, Korea) and a machine (Thermo Fisher Scientific, iBrightCL1000, USA). Eventually, Adobe Photoshop CS6 was also used to measure the band density of the blotted image.

2.6. RNA Extraction

15ml MEM medium comprised suspended HeLa (adjusted: 1x10^5 cells/ml) was prepared, then 5ml was put in every 100mm cell culture dish and incubated for 24 h at 37^{0}C with 5% CO_2. Thereafter, the media were switched. The control group (not treated), and the experiment groups were treated with 600 and 800 µg/ml of SVCE and incubated for 24 h at 37^{0}C with 5% CO2. After 24 h, the media was removed, then by adding 1ml cold PBS in every dish, the control (not treated) and SVCE (600 and 800 µg/ml) treated cells were rinsed, scraped and collected into each 1.5ml tubes; followed by centrifugation at 8000 RPM at 4^{0}C for 10 min. The RNeasy® Mini Kit (50; QIAGEN, Germany) in support of protocol was used for RNA extraction.

2.7. Reverse Transcriptase PCR

A PCR machine (SampliAmp Thermal cycler, Singapore) and a SuPrimeScript RT Premix (2X with oligo dT; GeNet Bio, Global Gene Network, Korea) with the appropriate amount of RNA and RNase-free water were used for the reverse transcription of extracted RNA to cDNA. The PCR condition was kept to incubate at (50^{0}C for 60 min, then 70^{0}C for 10 min) 1-cycle. Lastly, kept at 4^{0}C.

2.8. Real Time-qPCR

The relative fold change of gene expression in the control and treatment groups was determined using RT-qPCR. In each qPCR tube (Bioneer, Daejeon, Korea), 7 µl distilled water, primer (Table-1)(1 µl forward and 1 µl reverse) (Bioneer, Daejeon, Korea), 1 µl cDNA, and 10 µl qPCR master-mix (Bioneer, Daejeon, Korea) were mixed, then spun down. The experiment was run in triplicate using an RT-qPCR machine (Exicycler™ 96; Bioneer, Korea) with a qPCR condition: at (95^{0}C for 5 min) 1-cycle, then at (95^{0}C for 20sec; 60^{0}C for 40 sec; 72^{0}C for 30 sec) 45-cycles. The $2^{-\Delta\Delta C_T}$ approach (15) was used to determine the relative quantification result.

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Table 1. List of designed primers used for RT-qPCR.

Genes	Product size	Sequences
Caspase-3	102 bp	Forward 5'-CTGTGAACCCTGCATTTGGC-3'
		Reverse 5'-ACTTCGGAAGCTGAACCTGG-3'
Cytochrome-c	104 bp	Forward 5'-TGGCTTAATGTGTTCGCCCT-3'
		Reverse 5'-AAGCCCAAGCAAAGAGGGAA-3'
Bax	102 bp	Forward 5'-ACGAGGGTGATAGGTGGTACA-3'
		Reverse 5'-TGTTCTTCCCTTACCCACACG-3'
Apaf-1	101 bp	Forward 5'-TGGGTGACTGACCTTTGCTTT-3'
		Reverse 5'-GTCTGTGAGGATTCCCCAGTG-3'
Caspase-9	105 bp	Forward 5'-GAAGAGACCTGGCCAGAACC-3'
		Reverse 5'-ATTGCACAGCACGTTCACAC-3'
Bak	101 bp	Forward 5'-GGTTTTCCGCAGCTACGTTTT-3'
		Reverse 5'-GTTGCAGAGGTAAGGTGACCA-3'
β-Actin	104 bp	Forward 5'-TCTTCCAGCCTTGCTTCCTG -3'
		Reverse 5'-GGTGTACAGGTCTTTGCGGA-3'

2.9. DNA Fragmentation

15ml of MEM constituted suspended HeLa (adjusted: $1x10^5$ cells/ml) was prepared, then 5ml was inoculated on each 100mm cell culture dish and incubated at 37^0C with 5% CO_2 for 24 h. After 24 hours, the media were changed, and the control and experimental groups with SVCE concentrations of 700 and 800 μg/ml incubated for 24 hours. Next, the media was removed and then 1ml cold PBS was added in each dish, cells were scraped and collected into separated 1.5ml-eppendorf tubes. Genomic DNA was extracted from the control and experiment groups using a kit (AccuPrep® Genomic DNA Extraction Kit; Bioneer, Korea). Finally, DNA fragmentation is checked using 1.5% agarose gel electrophoresis with 100V for 30 min followed by an imaging system (Thermo Fisher Scientific, iBrightCL1000, USA).

2.10. GC-MS

The sample was dissolved in water, then diluted with methanol for being centrifuged and examined as a supernatant. The compounds were identified using GC-MS (Model Name: GCMS QP-2010Ultra, Shimadzu) and a column (DB-5MS Ultra (30 × 0.25 x 0.25).

2.11. Statistical analysis

All data are presented and shown as Mean ± SE. Statistical analysis was conducted with ANOVA and T-test using Microsoft Excel where the mean value of control group was individually compared with the mean value of every treated group. P-value was used as statistical significant.

3. Results

HeLa cell viability *in vitro*; the results showed that the IC_{50} of SVCE in treated HeLa was 723.1 ± 9.73 μg/ml (Figure 1) compared to the untreated group, and significant sign of toxicity of the SVCE on HEK293 cells was not observed (Figure 2). In comparison to the control group, the signal proteins affecting the change in cell viability following the treatment were identified using Westernblot (Figure 3). Bax, caspase-8, and IκBα were increased, whereas IKKα and p-NFκB65(Ser 536) were downregulated in the treated HeLa compared to the control. The expression level of apoptotic genes that are essential for cell death was quantified using RT-qPCR, as well as the ratio fold change of their expression (Figure 4) was calculated for the control and experimental groups; caspase-3 was 1.26 ± 0.63, 4.00 ± 0.63 and 3.87 ± 2.33; cytochrome-c was 1.01 ± 0.09, 2.26 ± 1.07, 3.11 ± 0.24; Bax was 1.2 ± 0.48, 9.03 ± 3.88 and 26.88 ± 9.18; Apaf-1 was 1.11 ± 0.38, 1.57 ± 0.54 and 10.56 ± 2.09; caspase-9 was 1.00 ± 0.07, 1.51 ± 0.39, 6.52 ± 1.05; Bak was 1.01 ± 0.09, 3.75 ± 0.97 and 3.29 ± 1.51, respectively. One of the features in induction of apoptosis is the disintegration

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of nuclear DNA and becoming nucleosomal fragments. This study employed gel electrophoresis method (Figure 5), which demonstrated that the SVCE treated groups had considerably higher DNA fragmentation than that of the control group. Some of the components in the crude extract were identified using GC-MS (Figure 6 and Table 2), then we looked at other studies and recommended a few compounds linked to fatty acids (Figure 7), which might be responsible for the anticancer and apoptotic effects, and we encourage more research.

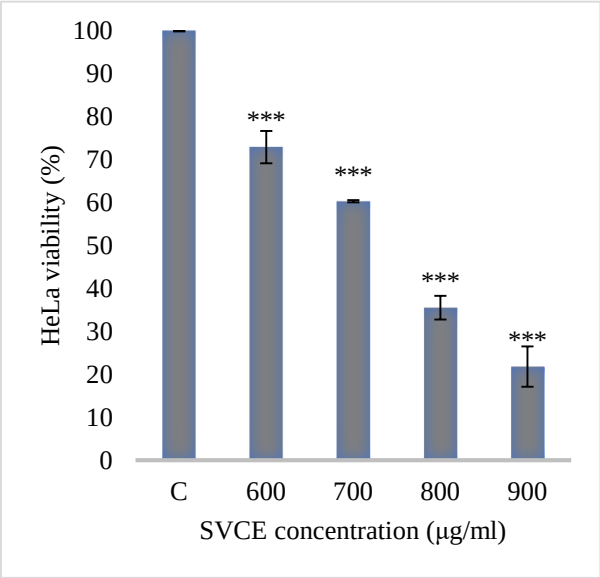

Figure 1. The control group (C = 100% viability) received no SVCE treatment, while the experimental groups received SVCE at various concentrations (600, 700, 800, and 900 µg/ml) for 24 hours. HeLa cells viability (%) test results; IC_{50} of SVCE for HeLa was 723.1 ± 9.73 µg/ml. The data are shown as Mean ± SE. Mean significant difference (*** $p < 0.005$) compared to control.

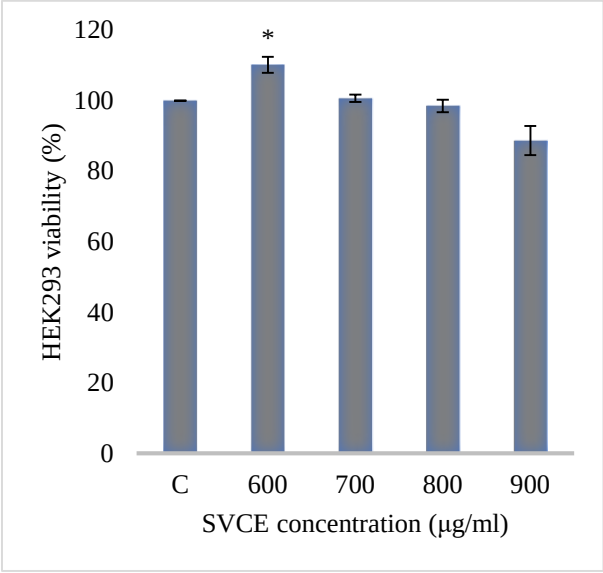

Figure 2. HEK293 cells, control group (C = 100% viability) was not treated at all; experimental groups were treated with different concentrations (600, 700, 800, and 900 µg/mL) of SVCE for 24 h. The result did not show significant difference. The data are shown as Mean ± SE. Mean significant difference (* $p < 0.05$) compared to control.

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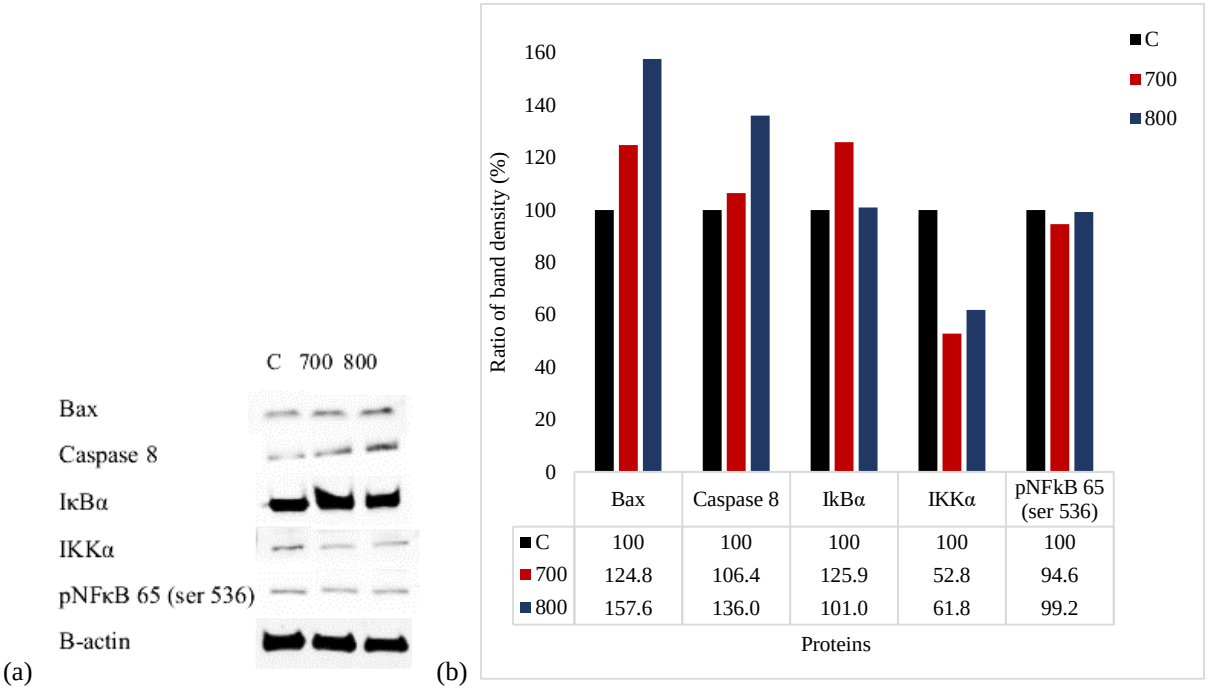
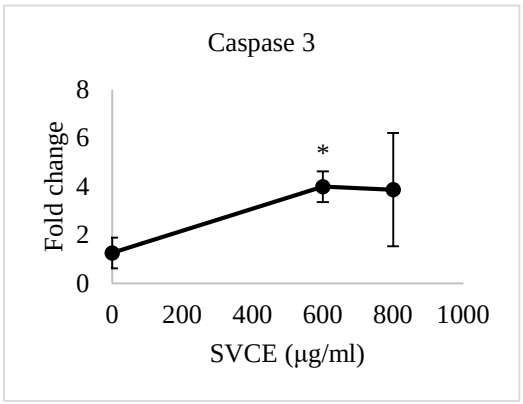
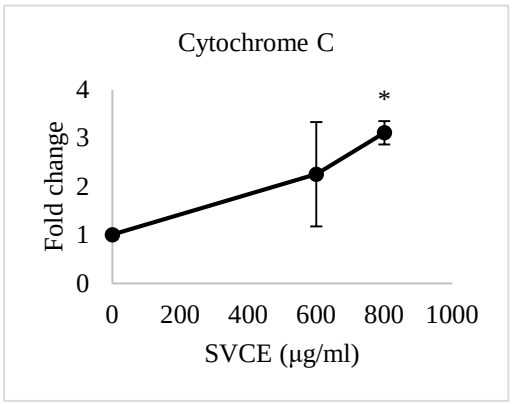
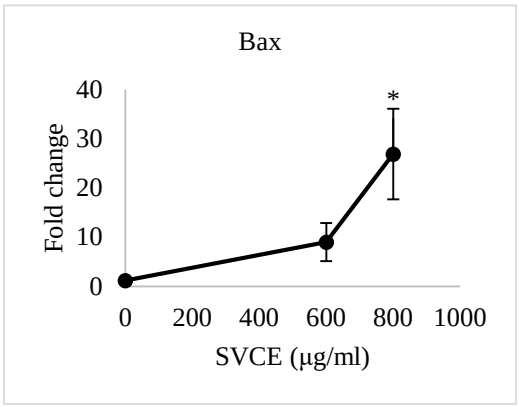
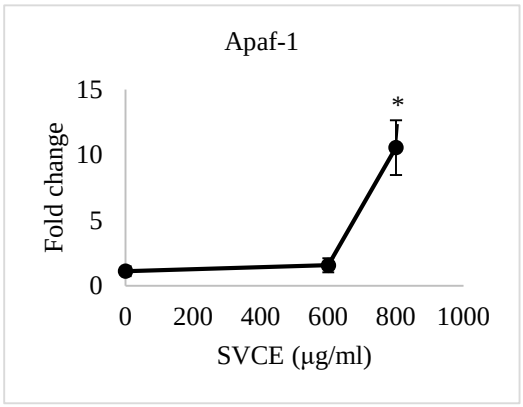


Figure 3. (a) Westernblot analysis of expressed protein signals by HeLa cells and (b) ratio of band density in the control group (no treatment) and experimental groups treated with SVCE at various concentrations (700 and 800 μg/ml) for 12 hours.









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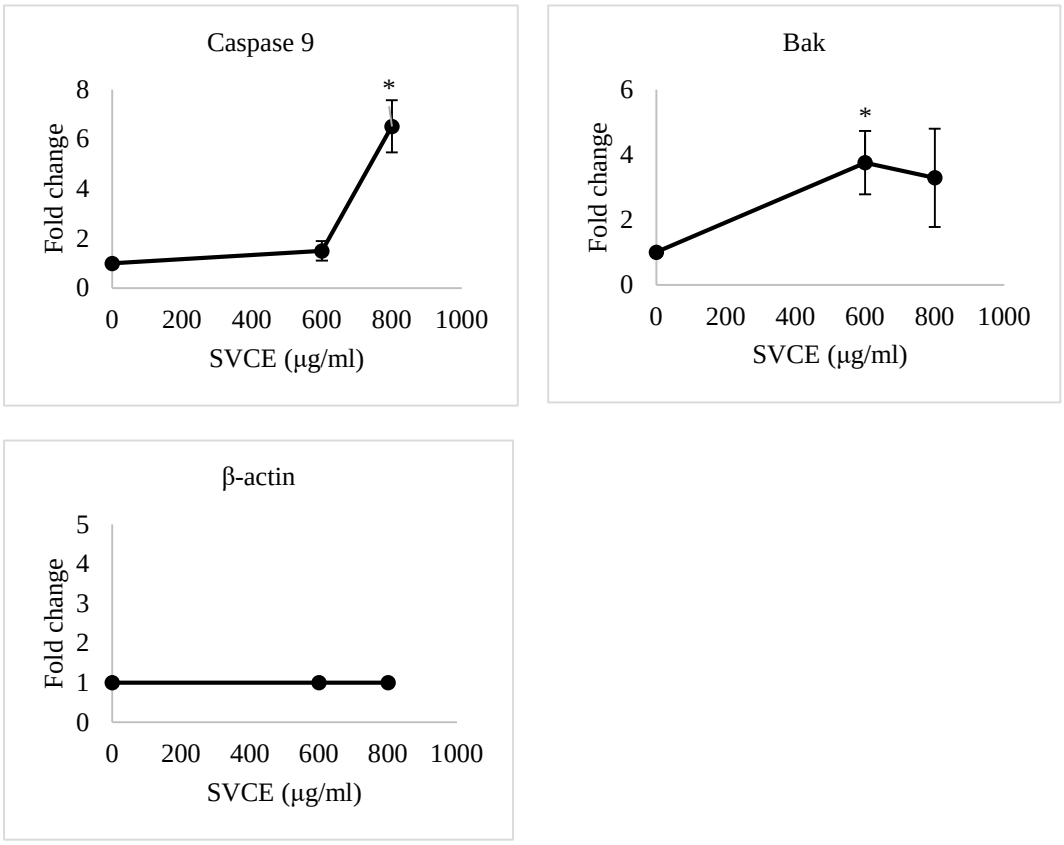

Figure 4. RT-qPCR results of relative gene expressions by the control group (0 µg/ml) with no treatment, and SVCE (600 and 800 µg/ml) treated HeLa cell lines for 12 hours. The data is shown as Mean ± SE. Mean significant difference (* $p < 0.05$) compared to control.

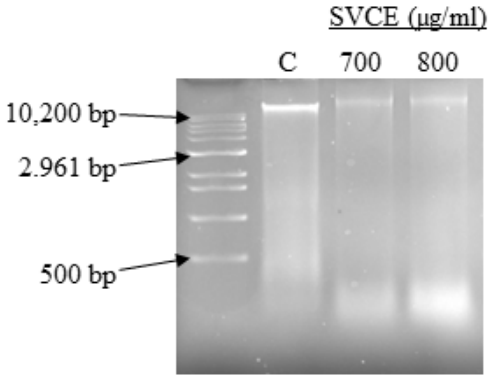

Figure 5. DNA fragmentation of the Control and SVCE-treated HeLa cells with (700 and 800 µg/ml) for 24 h, HeLa genomic DNA was extracted and separated by 1.5% agarose gel electrophoresis with 100V run for 30 min. .

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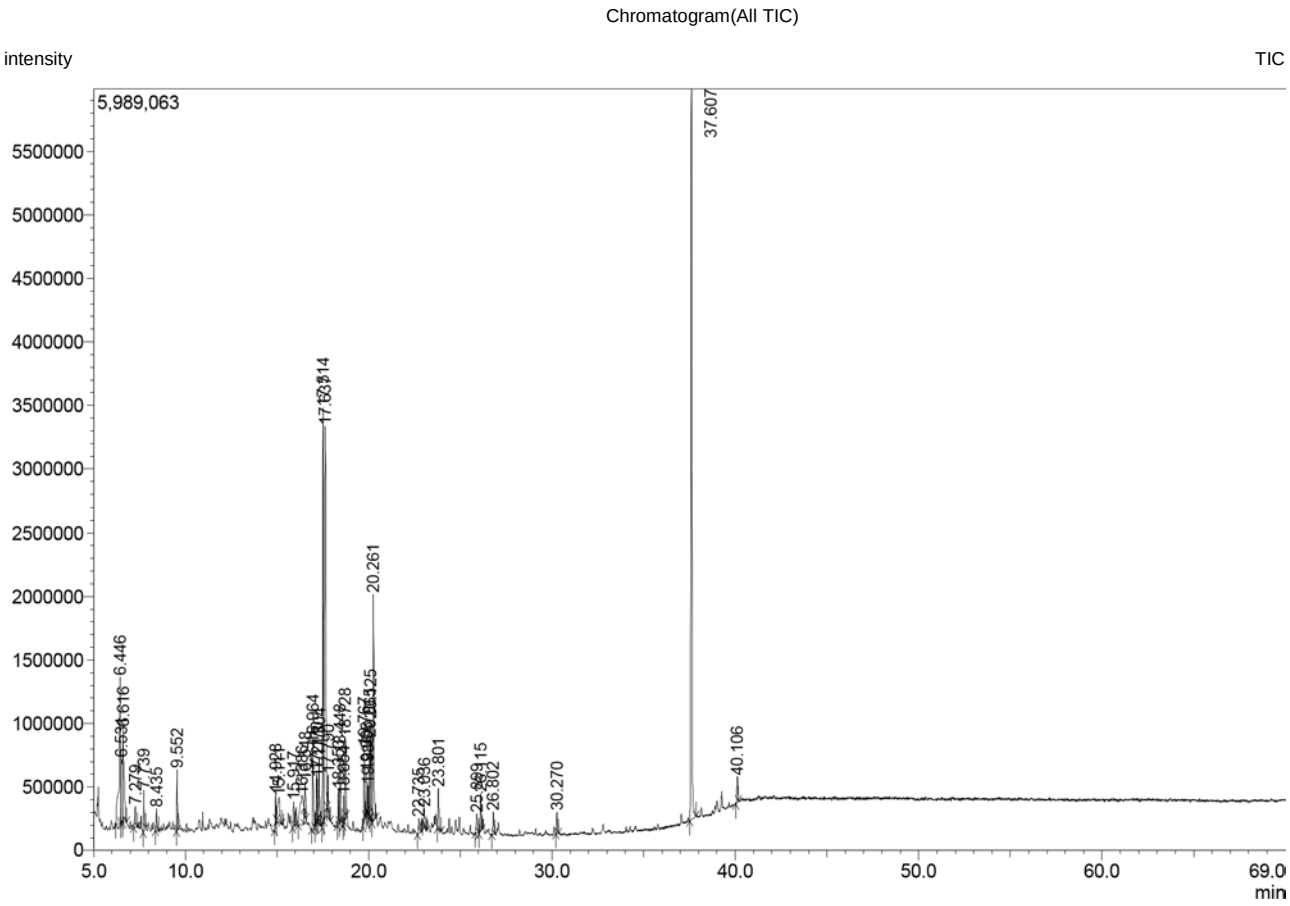

Figure 6. GC-MS chromatogram of crude extract of spike from *S. variolaris.*

Table 2. List of compounds identified in SVCE by using GC-MS.

Peak	Retention time	Area%	Name
1	6.446	5.86	2-Hydroxy-gamma-butyrolactone
2	6.531	2.33	Glycerol
3	6.616	3.90	-
4	7.279	0.64	-
5	7.739	0.85	2-Pyrrolidinone (CAS)
6	8.435	0.58	-
7	9.552	1.19	2-Piperidinone
8	14.928	0.45	Dodecanoic acid (CAS)
9	15.111	1.64	Cycloglycylalanine
10	15.917	0.75	-
11	16.386	3.02	-
12	16.548	0.89	-
13	16.964	2.18	-
14	17.170	1.37	-
15	17.211	0.70	(3S,36)-3-Butyl-6-methylpiperazine-2,5-dione
16	17.304	1.64	-
17	17.514	8.23	Tetradecanoic acid (CAS)
18	17.637	17.15	Pyrrolo(1,2-a)pyrazine-1,4-dione, hexahydro
19	17.790	0.81	-
20	18.353	0.65	-
21	18.448	1.46	-
22	18.654	0.51	-
23	18.728	2.30	-
24	19.767	1.87	-

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25	19.908	0.80	Pyrrolo(1,2-a)pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)
26	19.986	0.51	Cis-9-Hexadecenoic acid
27	20.065	1.91	Pyrrolo(1,2-a)pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)
28	20.125	2.41	-
29	20.261	5.40	Hexadecanoic acid (CAS)
30	22.735	0.59	-
31	23.036	0.51	-
32	23.801	1.26	-
33	25.899	0.76	-
34	26.115	1.06	Pyrrolo(1,2-a)pyrazine-1,4-dione, hexahydro-3-(2-phenylmethyl)
35	26.802	0.69	Pyrrolo(1,2-a)pyrazine-1,4-dione, hexahydro-3-(2-phenylmethyl)
36	30.270	0.57	-
37	37.607	21.76	Cholest-5-en-3-ol (3.beta)- (CAS)
38	40.106	0.80	-

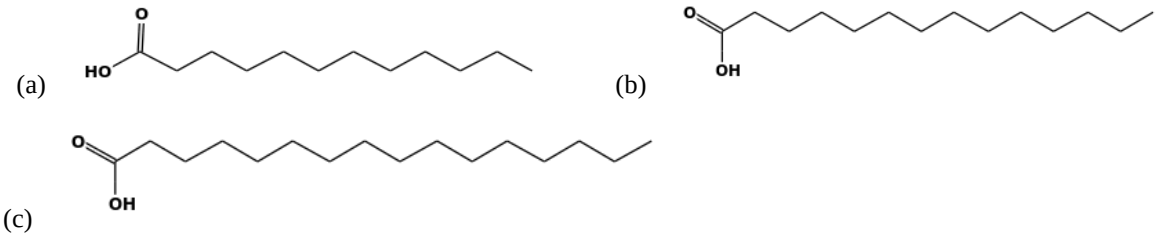

Figure 7. Chemical structure of (a) dodecanoic acid, (b) tetradecanoic acid, (c) hexadecanoic acid, and.

4. Discussion

The goal of this study is to contribute humanity in the discovery of appropriate and effective cancer treatment sources. This study employs a crude extract from the spike of the marine species *S. variolaris* as a natural component to treat and cure cervical cancer. Crude extracts from natural sources were discovered in anticipation of biological functions, such as anticancer activity, and research publications on the subject are on the rise. The crude extract produced from the spike of *S. variolaris* has anticancer function. *In vitro*, the crude extract was found to have anticancer action in HeLa cells, killing 50 percent of the cells at the concentration of 723.1 ± 9.73 μg/ml. This finding demonstrated the crude extract's cytotoxic and antiproliferative effectiveness in HeLa cells.

Apoptosis is a process of regulated cell death which can be triggered by death receptor activation (extrinsic apoptosis) or internally (intrinsic apoptosis) (16). NFκB is a heterodimer protein complex that is required for cell viability, regulates DNA transcription, innate immunity regulators (17), and inflammatory cytokines (18). Because of its essential impact on cell differentiation and proliferation in malignancies, a growing body of data shows that NFκB activation is linked to apoptosis resistance, the production of angiogenic proteins, and carcinogenesis (18). In a latent condition, NFκB dimers are present in the cytoplasm of most cells, attached to a vast variety of IκB blockers, including IκBα (19). The proteasome quickly phosphorylates, ubiquitinates, and degrades IκB, releasing the NFκB dimer, which is subsequently transported to the nucleus (20). Phosphorylation and degradation of IκB have gotten a lot of attention as important stages in the regulation of NFκB complexes (21). Numerous substances retain large amounts of IκB protein in the cytosol, inhibiting NFκB and therefore preventing NFκB translocation to the nucleus after therapy (22). Some of these compounds stimulate IκBα production, while others inhibit IκBα ubiquitination and yet others prevent IκBα breakdown (19). As a result, inhibitors of any stage of the ubiquitin-proteasome limit NFκB activation by sustaining IκB (23). IKK protein is phosphorylated at two serine residues (Ser176/180 of IKKα or Ser 177/181 of IKKβ) and

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eventually promotes the phosphorylation of IκBα at Ser 32/36 (24). Significant suppression of p-IKKα (Ser176/180) phosphorylation results in a reduction in nuclear levels of NFκB p65 (24). Because IKKα is required for both IKK activity and NFκB activation (25). When comparing the treatment and control groups, the westernblot findings revealed that IKKα and p-NFκB 65 (Ser 536) were downregulated, whereas IκBα protein was increased. As stated by the above-mentioned cell physiology, the conclusion reached in this study is that blocking the displacement of NFκB into the nucleus was occurred, that is required for cell proliferation and survival, causes apoptosis.

In the death receptor-mediated apoptotic cascade, activated caspase-8 is a key initiator caspase. The mitochondrial route is activated when active caspase-8 localizes toward the mitochondria and triggers cytochrome-c release (26). Caspases-3 and caspase-7, which are effector caspases, are dimeric zymogens that are triggered by cleavage of their large and small subunits. These zymogens are activated by fully developed caspase-8 cleaving them. In some circumstances, however, XIAP inhibits the activity of the effector caspases, although caspase-8 can overcome this inhibition in the following way. Caspase-8 can also cleave the protein BID and cBID, resulting in the activation of the MOMP effector proteins Bax and Bak. MOMP releases anti-XIAP proteins (such as SMAC) from the mitochondrial intermembranous gap, allowing the executioner to induce apoptosis (27). According to this scientific evidence, when comparing the treatment and control groups, westernblot results revealed that caspase-8 and Bax were activated and upregulated in the treated groups. Hence, this might be one of the causes of the apoptosis of HeLa cells.

Anti-apoptotic and pro-apoptotic members interacting and govern mitochondrial stability and play a key role in cytochrome-c release (28). Moreover, the leakage of cytochrome-c from mitochondria towards the cytoplasm causes apoptotic cell death under a variety of intracellular stress situations. The Bcl-2 family of proteins plays a crucial role in apoptosis. When Bak and Bax form holes on the outer mitochondrial membrane, cytochrome-c is leaked into the cytoplasm, they are thought to be cis (29)(30). Throughout the intrinsic apoptosis, cytochrome-c released from mitochondria binds to Apaf-1 and oligomerizes, triggering caspase-9 and eventually caspase-3 activation in huge complex apoptosomes (30). The mitochondrial pathway's initiator caspase, procaspase-9, is activated and recruited by the apoptosome, which then initiates caspase-3 downstream (16). The activated caspase-8 or caspase-9 then triggers caspase-3, causing apoptosis to occur (30). If membrane potential is reduced, the expression of caspase-3 in drug-treated cells is likely to vary. Changes in the Bcl-2 family, involving elevated Bax and suppressed Bcl-2 level, are linked to increased caspase-3 activation (28). After treating HeLa cells with SVCE, qPCR revealed caspase-3, caspase-9, Bax, Bak, Apaf-1, and cytochrome-c, and these apoptotic genes were higher. Additionally, in the experimental group, westernblot results showed that the intrinsic apoptotic protein Bax level was larger in comparison to the control. These findings suggest that apoptosis followed within the cells as a result of the therapy. Because these apoptotic genes and proteins are naturally potent and capable of inducing apoptotic cell death impulses, apoptosis signal induction is triggered by SVCE therapy.

Depending on the apoptogenic inducer, apoptotic characteristics can be accomplished in a variety of ways, resulting in slightly varied final nuclear morphologies. Despite the fact that it occurs late and may be a dispensable stage in apoptosis, nuclear fragmentation may be a significant event and one of the hallmarks of evidence of apoptosis (31),(32). Chromatin condensation and protein and nucleic acid breakdown are common changes in the nucleus during programmed cell death. This is sometimes accompanied by chromatin fragmentation into a number of smaller masses, which could indicate nuclear fragmentation. The nucleus of apoptotic cells is frequently fragmented, with each part remaining enclosed by a membrane, and the fragments are transferred to apoptotic bodies, observed by transmission electron microscopy (33). The nuclei of the control group and the treated cells with SVCE were seen under a laser scanning confocal microscope after staining with DAPI following a 24-hour incubation period in this investigation. In

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comparison to the control group, the treated group had a significant number of HeLa cells with fragmented nuclei.

Apoptosis is biochemically defined by the disintegration of chromosomal DNA into oligonucleosomal fragments. Apoptotic stimuli could indeed induce cells to die despite producing genomic DNA instability, although DNA fragmentation can speed this process up. Furthermore, the caspase family is primarily responsible for mediating apoptotic signal pathways that ultimately culminate in DNA shattering. Apoptotic DNA fragmentation occurs in both caspase-dependent and caspase-independent ways. Gel electrophoresis technique was used in this study which revealed that SVCE treated groups had significantly more DNA fragmentation than the control group.

Fatty acids operate as chemotherapeutic drugs, inhibiting cancer cell proliferation or inducing apoptosis through a variety of transduction routes. Gene expression, which leads to changes in metabolism, growth, and cell differentiation, is one of these routes, and it plays a role in a variety of pathological reactions. Fatty acids have an impact on a variety of disorders, including cancer. Dodecanoic acid (DA) therapy considerably upregulates the expression of several tumor suppressor miRNAs while suppressing the expression of oncogenic miRNAs. By changing the expression of miRNAs, DA acts as an anticancer drug. Cell proliferation, mitochondrial volume, lactate generation, and oxidative stress were all reduced in DA-expressing mice, CT26 colon cancer cells. DA has been used in several studies as an anticancer fatty acid (34). DA has been shown to have anticancer properties in cancers of the reproductive system (35). The EGFR inhibitor cetuximab was sensitized better with DA. In breast and endometrial cancer cells, DA has antiproliferative and pro-apoptotic effects through boosting the production of ROS, and alterations in gene expression (35). Apoptosis was linked to a decrease in GSH and a rise in ROS production (36). A branched-chain saturated FA, 13-methyltetradecanoic acid (13-MTA), in which tetradecanoic acid is substituted at position 13 by a methyl group and is utilized as a cancer therapy supplement, is a low-toxicity natural substance. 13-MTA has been shown to trigger apoptosis in a variety of human cancer cells. Down-regulation of AKT phosphorylation and up-regulation of MAPK, JNK, and p38 phosphorylation co-stimulates higher amount of Bax and lower amount of Bcl-2, activating the mitochondrial apoptotic cascade through cytochrome-c leakage and caspase involvement (37) and inhibiting NFκB phosphorylation (38). The cleavage of caspase-3, as well as cell cycle arrest in the G1 phase, were observed during 13-MTA-induced apoptosis in Jurkat cells (38). 13-MTA kills tumor cells by fragmenting their DNA during apoptosis (39). N-hexadecanoic acid's cytotoxic activity is thought to be owing to its interaction with DNA topoisomerase-I, which stops proliferation (40). Hexadecanoic acid (HA) appears to cause cellular apoptosis in Chinese hamster ovary cells, according to growing evidence. The principal method by which HA causes apoptosis is by the generation of ROS. The accumulation of ROS in cells treated with HA is recognized to play a role in HA-induced lipotoxicity. Apoptosis occurs in a variety of cell types in response to HA therapy, including breast cancer cell lines (41). These scientific findings imply that the anticancer and apoptotic activity in HeLa cells could be attributed to the DA, TA, and HA contents of the crude extract of *S. variolaris*. Further investigation regarding the particular effects of each of the above-mentioned fatty acid extracts from *S. variolaris* on HeLa is recommended to strengthen the proposition.

5. Conclusion

The cells were treated with a crude extract of a spike of *S. variolaris,* which resulted in observable and objective changes in cell survival, gene expression, protein expression, and DNA fragmentation assays. These compelling findings support the conclusion that the crude extract of *S. variolaris* spike does have inherent clinical significance and potential to do with the therapy of cervical cancer in regard to inducing suicidal apoptotic signals, inhibiting transcription factors that control DNA survival and proliferation, and fragmenting the nucleus and the DNA, thereby halting their growth and metastatic potential.

Rather than relying on this early findings, more research is needed to examine the applicability and other qualities of the crude extract and particular separated components, as well as to commercialize anticancer therapeutic products as medications or supplements.

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