

Evaluation of Anticancer Activity of Ethanolic Extract of *Strobilanthes ciliatus* Leaves on HT-29 Cell Lines

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ABSTRACT

Aim: The present research is aimed to highlight the cytotoxic potential of *Strobilanthes ciliatus* on H T-29 cell lines. **Materials and Methods:** Extraction done with ethanol (SCLE) and Acetone (SCLA) by soxhlet method and anticancer by MTT assay. GCMS was used to analyze the phytochemicals of a particular plant, and MTT flow cytometry was used to assess cytotoxicity. **Results:** A total of 38 distinct compounds were found in this study's analysis of the plant's entire chemical profile, and three of those compounds were found in two different solvent extractions. LD was assessed using the MTT and flow cytometry methods, and the transmittance of the extraction-treated HT29 cell was determined using a plate reader at a wavelength of 570 nm. SCLE had the largest anti-proliferative effect of the 2 investigated extractions, with an IC₅₀ value of 33.62 g/mL, while the acetone extract had less impact. **Conclusion:** The results showed significant cytotoxic activity only on ethanol extraction, not by acetone extract, due to the concentration and diversity of compounds. The studies reveal that natural plant extract showed effective anticancer activities *in vitro* that can be further subjected to the development of a potential therapeutic anticancer agent.

Keywords: MTT assay, Cytotoxicity, Anticancer activity, HT-29 cell lines, *Strobilanthes ciliatus* IC₅₀, Anticancer, Cytotoxic.

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INTRODUCTION

The incidence of cancer is steadily increasing, making it one of the leading causes of mortality around the globe. Currently, there are a number of treatment options for cancer. But there are some pitfalls too. The most significant one is the rise of medication resistance.

Molecular methods based on cancer cell pathology are currently employed to treat cancer cases and lower mortality rates. The use of substances to suppress, postpone, or reverse carcinogenesis prior to invasion is known as cancer chemoprevention. A diet rich in vegetables and fruits confers more protection against cancer than individual micronutrients.^[1] Natural plant products have a significant impact on the chemoprevention of cancer.^[2] Over about 50% of the anticancer medications now on the market are either natural compounds or have a natural connection.^[3] In diverse *in vivo* and *in vitro* models, a variety of secondary plant metabolites, including alkaloids, flavanoids, phenolics, carotenoids,

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gingerols, and organosulfur molecules, have the ability to inhibit the early and late stages of tumorigenesis.^[4] Therefore, it is crucial for anticancer research to discover antitumor medicines derived from natural materials. The traditional medicinal *Strobilanthes ciliatus* is extensively employed in the Ayurvedic, Unani, and Siddha systems of medicine.^[5] Many Ayurvedic remedies, including Sahacharadithailam, Sahacharadikashayam, and Varanadikashayam, contain the herb as a key component.^[6] This medication is highly suggested by “Sahasrayogam” for treating pain, particularly sciatica, lumbar spondylitis, and low back pain. Additionally, the herb has been suggested for the treatment of neurological disorders.^[7] The leaf and bark can treat fever, diphtheria, dropsy, leucoderma, leprosy, pruritis, inflammatory diseases, and a variety of other conditions by acting as a diaphoretic, expectorant, depurative, and febrifuge. The leaves are administered topically for the treatment of gout, lumbago, and joint discomfort. They are also used to cure jaundice, dropsy, rheumatism, and diseases of the urogenital tract. Plant flowers and bark preparations are recommended for treating itching, leprosy, diabetes, toothaches, and urological diseases. Folk medicine has always recommended drinking leaf tea and covering the afflicted area with leaf paste to relieve rheumatic discomfort. Another medication given to new mothers for their health is kurinjhi kuzhambu. Seeds are utilized to cure gonorrhoea, spermatorrhea, dropsy, rheumatism, jaundice, and diseases of the genitourinary tract.^[8,9] Although the plant’s essential oil is commonly used to treat neurological diseases, no research has been done to separate and assess the substance. The pharmacokinetic properties of *S. ciliatus* include antioxidant, antibacterial, anti-inflammatory, analgesic, antidiabetic, and hepatoprotective properties. Numerous phytoconstituents, including terpenoids, phytosterols, flavanoids, sugars, and tannins, are abundant in the plant.^[10,11]

MATERIALS AND METHODS

Collection of the plant

The plant *Strobilanthes ciliatus* was collected from Kaduthuruthy, Kottayam district of Kerala. The botanical survey of India verified the plant’s authenticity, and it was then deposited in the herbarium with voucher number BSI/SRC/5/23/2021/Tech/258.

Chemicals and Reagents

The analytical reagent grade chemicals and reagents were all purchased from reputable vendors.

Preparation of the extracts

The Soxhlet extraction method was used to create the plant extracts. The plant leaves were subsequently dried under the shade and powdered using an electric blender. Acetone and ethanol were used as the solvents for the continuous hot percolation method of extraction. After being stored at 4°C until use, the extracts were then concentrated using a rotary evaporator.

Phytochemical screening^[12,13]

According to standard protocol, a phytochemical examination of ethanolic extract of leaves of *Strobilanthes ciliatus* was completed. GC MS evaluation All of the *Strobilanthes ciliatus* leaf extracts underwent. Analysis was conducted using a Shimadzu GC - MS Model Number: QP2010S instrument and GC - MS solutions software. A pace of 5°C/min is used to maintain the oven’s temperature at 280°C. A 30m-long, 0.25mm-ID, 0.25-micrometer-thick Elite - 5MS column was employed. These extracts were chosen for further research since the ethanol extract of *Strobilanthes ciliatus* had more phytoconstituents.

In vitro cytotoxicity determination by MTT assay

Cell lines and maintenance

HT 29 Cell lines were procured from the National Centre for Cell Sciences (NCCS), Pune, India.

Cell culture media and maintenance^[14]

The cells were cultured in Dulbecco’s Modified Eagles Medium (DMEM-Himedia), supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) and 1% antibiotic cocktail containing Penicillin (100U/mL), Streptomycin (100µg/mL), and Amphotericin B (2.5µg/mL). The cell containing TC flasks (25cm²) were incubated at 37°C at a 5% CO₂ environment with humidity in a cell culture incubator (Galaxy170 Eppendorf, Germany).

MTT assay

Human colon adenocarcinoma cell line (HT 29 cells) were seeded on 96 well plates (2500 cells/well) and allowed to acclimatize to the culture conditions such as 37°C and 5% CO₂ environment in the incubator for 24 hr. The assay preparations were prepared in DMEM medium (100 mg/mL), and a 0.2 m Millipore syringe filter was used to filter sterilize them. At final concentrations of 6.25, 12.5, 25, 50, and 100 g/mL, respectively, all samples were further diluted in DMEM medium and introduced to the wells containing grown cells. Wells that hadn’t been treated were managed. To reduce errors, each experiment was performed in

three copies, and average results were used. The plates underwent an additional 24 hr incubation period after being treated with the test samples. After the incubation time, the wells' media were aspirated and discarded. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay (MTT) solution in PBS was injected into the wells at a concentration of 0.5 mg/mL. The plates were then left to grow formazan crystals for an additional two hours. Dimethyl Sulfoxide (DMSO) was added in 100 L per well after the supernatant was removed. A microplate reader was used to measure the absorbance at 570 nm. Per plate, a blank consisted of two wells with no cells. Triplicates of each experiment were carried out.^[15]

The cell viability was expressed using the following formula:

$$\text{Percentage cell viability} = \frac{\text{Average absorbance of treated}}{\text{Average absorbance of control}} \times 100$$

IC₅₀ value

The IC₅₀ value is the half-maximal inhibitory concentration of the sample. The IC₅₀ values were calculated using the equation for slope ($y = mx + C$) obtained by plotting the average absorbance of the different concentrations of the test sample (6.25-100 µg/mL) in Microsoft Excel

Cell Cycle Kit by Flow Cytometry

After attaining 80% confluency, cells were treated with LD₅₀ concentration and incubated for 24 hr. Untreated control wells were also maintained. After incubation, the cell sample was transferred to a 12×75-mm polystyrene tube or 50 mL conical flask. 1×10⁶ cells are the bare minimum advised for fixation in a tube. The samples were then centrifuged for 5 min at 3000 rpm. The particle was preserved while the supernatant was taken off. A sufficient amount of PBS (i.e., 1 mL of PBS with 1 X10⁶ cells) was added to each tube, and the contents were mixed by pipetting several times or gently vortexing. After centrifuging, the cell pellet usually forms a visible pellet or a white film on the bottom of the tube. For five minutes, the cells were centrifuged at 3000 rpm. The supernatant was eliminated without disturbing the cell pellet, leaving roughly 50 µL of PBS per 1X10⁶ cells. Resuspend the pellet in the remaining PBS by gentle vortexing or pipetting it several times. The revived cells were incorporated.

Staining of Cell Cycle

The samples were centrifuged at 3000 rpm for 5 min at room temperature following the overnight incubation. After removing the supernatant, 250 µl of

PBS were added to the pellet. The centrifugation was then repeated at the same speed and duration. After removing the pellet and removing the supernatant, 250 µl of cell cycle reagent were applied. This was incubated for 30 min in the dark (which is light-sensitive). It was then examined using a Flow Cytometer. Analysis of the samples followed by gating was done in comparison to untreated control cells.

RESULTS

Strobilanthes ciliatus identified with voucher No. BSI/SRC/5/23/2021/Tech/258. The ethanolic extract of *Strobilanthes ciliates* is designated as SCLE, and the acetone extract is designated as SCLA. The examination of phytochemicals on *Strobilanthes* species were qualitatively evaluated using GCMS. The correlation of mass spectra of data obtained from the National Institute of Standards and Technology (NIST II) and Wiley 8 library served as the basis for the identification of phytoconstituents found in the petroleum ether extract of *Strobilanthes ciliatus*. The flower extracted by ethanol spectrum of GC-MS analysis is given in Figure 1. Table 1 displays the GC-MS analysis's findings with 16 distinctive fragmentation patterns of mass spectra were recorded. phytol, Hexa and octadecanoic acid, eicosanoic acid, squalene, propylure, stigmastan, hexadecen-1-olwere detected in the ethanolic extract of *Strobilanthes ciliatus* leaves by GC- MS. Phytol is the major compound (26% RT 16.81 min) followed by octadecadienoic acid (14% RT 19.360 min), n-hexadecanoic acid (12% RT 18.465 min), 2-octylcyclopropene (10%RT 20.281 min) and squalene (2.54% RT 24.202 min). GCMS of acetone extract (Figure 2) reveals presence of 25 different peaks, and the compounds are listed in Table 2. Stigmasterol (20% RT 47.86min) phytol (16% RT 28.150 min), squalene (13% RT39.424 min), beta-Sitosterol, Pentacosane (12.21% RT 49.485 min) (7% RT 48.34 min) and Campesterolare (5.37% RT 46.987 min) are detected as major constituent followed by other minor compounds.

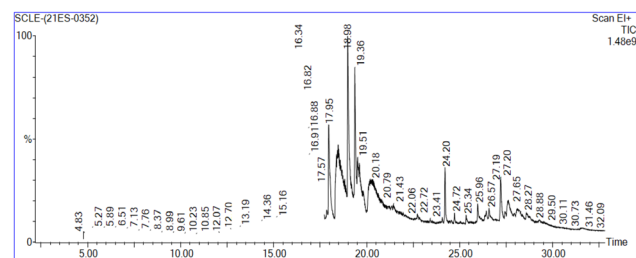


Figure 1: Gas chromatogram of SCLE.

Peak	RT	Area %	Name
1	16.339	17.311	Phytol
2	16.619	6.164	3,7,11,15-tetramethyl-2-hexadecen-1-ol
3	16.819	9.749	Phytol
4	17.575	1.268	2,6,10-trimethylundeca-1,3-diene
5	17.955	7.131	octadecanoic acid, ethyl ester
6	18.370	3.454	n-hexadecanoic acid
7	18.465	8.638	n-hexadecanoic acid
8	18.580	4.730	eicosanoic acid
10	19.360	7.340	9,12-octadecadienoic acid (z,z)
11	19.510	2.428	8,11,14-eicosatrienoic acid, (z,z,z)
12	19.601	1.840	Propylure
13	20.281	10.645	2-octylcyclopropene-1-heptanol
14	24.202	2.554	Squalene
15	27.199	3.101	tetratetracontane
16	27.614	2.509	stigmastan-6,22-dien, 3,5-dedihydro

Peak	R.Time	Area%	Name
1	12.866	0.92	Pentadecane
2	16.325	0.75	2(4h)-benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-
3	22.794	1.62	phytol, acetate
4	22.952	7.14	2-pentadecanone, 6,10,14-trimethyl-
5	24.984	0.67	isophytol
6	25.868	0.48	hexadecanoic acid, ethyl ester
7	28.150	15.84	phytol
8	28.671	1.38	palmitaldehyde, diallylactal
9	29.975	1.59	Isophytol, acetate
10	32.153	1.10	3-methyl-5-(2,6-dimethylheptyl)-1,5-Pent-2-enolide
11	32.330	1.60	4,8,12,16-Tetramethylheptadecan-4-olide
12	33.498	0.45	Tridecanal
13	34.014	0.46	3,4,8-trimethyl-2-nonenal
14	34.508	0.65	pentacosane
15	35.408	0.98	1,2-benzenedicarboxylic acid
16	39.424	13.04	Squalene
17	40.283	0.70	Pentacosane
18	41.302	1.16	Squalene
19	41.790	0.87	nonadecane
20	41.983	1.19	neryl linalool isomer
21	43.571	1.70	pentacosane
22	46.987	5.37	Campesterol
23	47.868	20.43	Stigmasterol
24	48.341	7.70	Pentacosane
25	49.485	12.21	beta.-Sitosterol

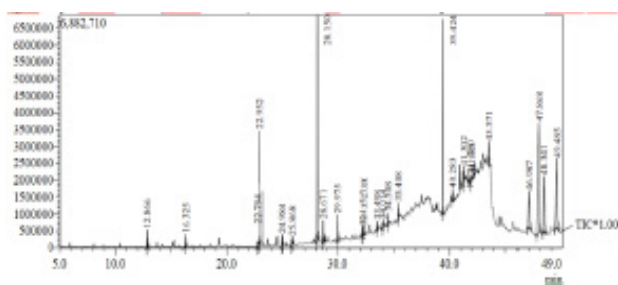


Figure 2: Gas chromatogram of SCLA.

In vitro MTT cytotoxicity of extracts were tested on HT 29 cells and compared with Doxorubicin. The optical densities of proliferated cells are given in Table 3. In control, the 100% viability ensured with 0.8227 ± 0.03 OD. Decreases in OD among SCLE was observed with a maximum of 0.5923 ± 0.009 OD at $6.25 \mu\text{g}$ and a minimum of 0.3118 ± 0.010 OD at $100 \mu\text{g}$. cells treated with SCLA have maximum cell growth 0.7605 ± 0.022 at $6.25 \mu\text{g}$ and 0.4329 ± 0.011 OD at $100 \mu\text{g}$. Cell growth intensity among Doxorubicin was 0.2933 ± 0.006 OD at $6.25 \mu\text{g}$ and 0.0643 ± 0.011 OD at $100 \mu\text{g}$. Table 4 describe the data on the viability percentage of treated cells at different concentration. The data among SCLA, SCLE and standard is compared and represented in Figure 2 shows that the concentration-dependent cytotoxicity is more significant on SCLE than SCLA. At $100 \mu\text{g}$, the death rate was maximum with IC_{50} value of $33.6 \mu\text{g}$. Cells treated with SCLA exhibited IC_{50} $105.18 \mu\text{g}$. Doxorubicin showed IC_{50} of $7.34 \mu\text{g}$. Furthermore, we found that HT-29 cell cycle arrest was induced by *Strobilanthes ciliates*. As illustrated in Figure

Concentration μg	Average OD		
	SCLE	SCLA	Doxorubicin
Control	0.8227 ± 0.03	0.8227 ± 0.03	0.8227 ± 0.03
6.25	0.5923 ± 0.009	0.7605 ± 0.022	0.2933 ± 0.006
12.5	0.544 ± 0.021	0.7125 ± 0.010	0.1831 ± 0.009
25	0.4331 ± 0.010	0.6761 ± 0.029	0.1329 ± 0.016
50	0.3733 ± 0.014	0.5917 ± 0.017	0.1006 ± 0.006
100	0.3118 ± 0.010	0.4329 ± 0.011	0.0643 ± 0.011

5 the population index and DNA content among untreated HT 29 there is 58% gated on G0/G1 phase, 25% in S phase and 12.1% G2/M stage. The HT-29 cells underwent dose-dependent apoptosis after 24 hr treatment with SCLA and SCLE. Moreover, SCLE induced significant apoptosis cancer cells and shows 53% were G0/G1, 17% S phase and 7.9% cells were

Table 4: Growth rate of cells recorded among treatment.			
Concentration	Viability %		
	SCLE	SCLA	Doxorubicin
Control	100	100	100
6.25	72	92.44	53.53
12.5	66.13	86.61	33.41
25	52.65	82.18	24.25
50	45.37	71.92	18.36
100	37.9	52.62	11.73

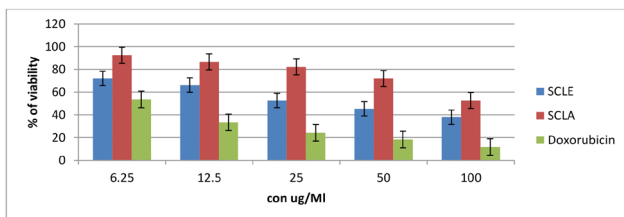


Figure 3: Percentage of viability of HT 29 cells.

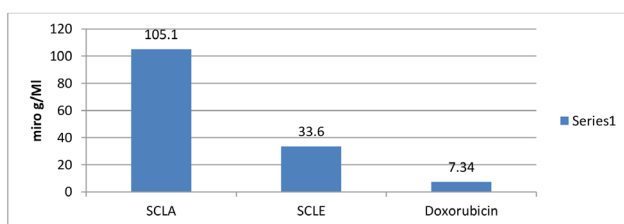


Figure 4: Concentration of half-maximal lethal concentration.

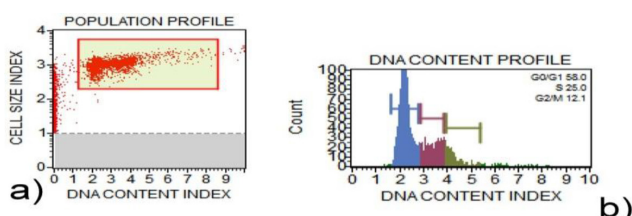


Figure 5: Population index (a) and DNA content (b) untreated.

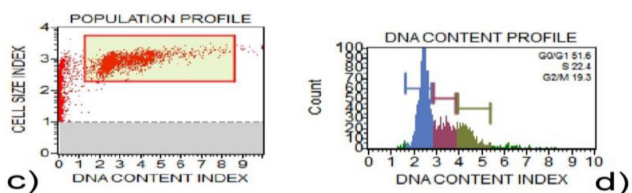


Figure 6: Population index (a) and DNA content (b) SCLE treated.

G2/M phase were recorded from DNA content and population index (Figure 6). Figure 7 data suggested that SCLA-induced suppression of cell growth were

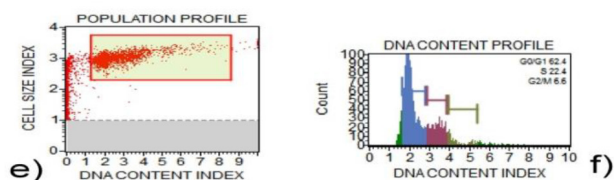


Figure 7: Population index (a) and DNA content (b) SCLA treated.

62% were G0/G1, 22.4% S phase and 6.6% cells were G2/M phase. The percentage of CV on G0/G1 were 10.8, 17% and 14% S phase cells were 9.3, 9.2 and 9.0% where as G2/M phase cells are recorded as 8.9, 9.2 and 10.8%, respectively untreated, SCLE, and SCLA treated.

DISCUSSION

The phytoconstituents were characterized using variables such peak and retention time comparisons, and computer matching. The data of SCLA is completely differing from SCLE metabolites. Both extract showed most common components are phytol, squalene and hexadecanoic acid. Many sterols were found only in SCLA extraction which are absent in SCLE, Our present photochemical finding correlate with the findings of Nurraiha and Norfarizan-Hanoon.^[16] Previous presence of more amount of fatty acids, flavonoids Phyto components were detected in the extract of *Strobilanthes ciliatus* were well documented.^[17,18] The concentration dependednt proliferation inhibition was noted among both tested samples and standard. About 62.1% of cytotoxicity was recorded in ethanol extract, 47.38% in acetone extract 98.27% inhibition by standard drug were recorded at 100µg/mL. To distinguish between cells at various stages of the cell cycle, DNA content histograms were employed. The amount of DNA at IC₅₀ concentrations was reduced in HT-29 cells after exposure to ethanol extract were found to be better than acetone extraction. Additionally, DNA damage was strongly induced in HT-29 by standard and significantly among both extracts. By taking account the results for DNA damage, apoptosis, and cell cycle, both extracts may be able to produce *in vitro* antitumor effects on HT-29 cells. Studies on anti proliferative effect on this flower extract is very scarce. The results obtained in the present study indicate that the leaves of *Strobilanthes ciliatus* are potential source of natural anti-proliferative capacities of the alcoholic leaf extract was correlate with the report of Susi *et al.*^[19] Several biological effects such as anti-inflammatory, anti-microbial, anti-arthritis, anti-tumor activities have been reported among this plant.

[20-24] Sample SCLE treated cells shows 28% death rate at a minimum concentration of 6.25 µg and the viability percentage was 72. Cells treated with SCLA exhibited moderate cytotoxicity and have had death rate 17.66% at 6.25 µg. Doxorubicin showed 46.47% death rate at 6.25µg. On comparing the LC50, SCLE was found to be better than SCLA but less effective than the standard. This denotes that the process required more purification process. The data shows that the ability of anticancer drugs to cause the innate predisposition of the target tumour cells to respond by apoptosis was confirmed by low DNA content. Blocking cell proliferation and inducing apoptosis are thus considered as important properties of chemopreventive and chemotherapeutic agents.^[25] Bakar *et al.*^[26] stated that *Strobilanthes crispus* have promising anti-proliferative and antioxidant activity. DNA, contain key genes act as a carrier, and is crucial for cell development, maturation, and proliferation.^[27] The tumour genes undergo point mutation, fusion, and amplification when a tumour develops, and as the tumour grows, the amount of DNA considerably rises. Our research sought to ascertain whether the DNA content and population index of HT29 demonstrated that the untreated sample had higher DNA content than the sample treated indicates the anti-proliferative action of plant extract was quite different from other plant extract studies.^[28]

CONCLUSION

The results of the phytochemical analysis revealed the detection of esters of fattyacid, phytol, squalene, Sterols, Pentacosane, Pentacosane and nonadecane. Ethanol Extract from plant significantly controlled the Growth rate of cells recorded among the treatment of HT 29 cells was concluded as promising in terms of anticancer potential.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

Plant compounds have historically reported to have a number of unique anticancer medicines, and more are

constantly being created. *Strobilanthes ciliatus* leaf extract showed presence of various phytochemical exhibited potent cytotoxic effects on HT 29 cell lines. Further the extracts and isolated compound need to be evaluated by *in vivo* methods to find out the drug efficiency and toxicity. The present data demonstrated the powerful anticancer properties of *S. ciliatus in vitro*, suggesting that it may serve as a source for a pharmacologically active product that can be developed as a chemotherapeutic drug.

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