Antiproliferative activity of saponin fractions of *Chlorophytum borivilianum*

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ABSTRACT

The purpose of this study was to investigate the anticancer activity of saponin fractions of *Chlorophytum borivilianum* belonging to family liliaceae. The MTT (methylthiazolyldiphenyl-tetrazolium bromide) and SRB method was used to evaluate the antiproliferative activity of the mehanolic extract and crude saponin extract. DNA fragmentation assay was carried out to confirm antiproliferative activity of selected extract. Findings of the study suggested that the crude saponin extract of *C. borivilianum* has potential anticancer activity as very good percentage inhibitions are obtained.

Key words: *Chlorophytum borivilianum*, saponin, antiproliferative, MCF-7

INTRODUCTION

The World Health Organization (WHO) has estimated that approximately 80% of the world's population depends on traditional medicines for meeting their primary health care needs. As an important component of complementary and alternative medicine, traditional Ayurvedic medicine may be a useful model for scientific research because of its standardized system of therapies and long-time practices. Due to the high costs and time-consuming nature of animal cancer model studies, the initial screening of anticancer compounds from natural products is best accomplished using in vitro cancer cell methods. Cultured human cancer cell lines to screen chemicals and natural product extracts in vitro to speed the discovery of new anticancer drugs.[1] *Chlorophytum borivilianum* belonging to family liliaceae is a very well known plant for its aphrodisiac as well as immunomodulatory activity in India.[2] Roots holds very important position in Ayurveda and Unani system where it is mostly used to treat oligospermia, pre and post natal symptoms, arthritis, diabetes and dysuria.[3] *C. borivilianum* root contains carbohydrates, phenolic compounds, saponins and alkaloids.[4]

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As the saponins reported to have anticancer activity [5] hence present study has undertaken to carry out antiproliferative effects on selected human breast cancer cell lines (MCF-7 cell lines). MCF-7 cells are a well-characterized estrogen receptor (ER) positive control cell line (cells are positive for cytoplasmic estrogen receptors) and therefore are a useful in vitro model of breast cancer to study the role of estrogen in breast cancer.

MATERIALS AND METHODS

Plant materials, extraction and isolation

Chlorophytum borivilianum roots roots were purchased from cultivator i.e. Mr. Dharme (Tal. Akot Dist. Akola). Herbarium was prepared as per standard procedure and aunthetified by Dr. Prabha Y. Bhogaonkar (Director, Government Vidarbha Institute of Science and Humanities, Amravati).

The *C. borivilianum* tubers were powdered and defatted by petroleum ether. Marc then extracted with methanol for 3 hour with mild heating. Methanol extract was concentrated to obtain methanol extract (ME). In order to get the crude saponins extract, ME was dissolved in methanol and acetone was added (1:5v/v) to precipitate the saponins as described by yan et al., 1996.[6] The precipitate was dried under vacuum, turning to a whitish amorphous powder named as a crude saponin extract (CSE). CSE was eluted with MeOH: Water by column chromatography. The fractions which have given positive TLC results for saponin only are selected and combine to give PSF (Purified saponin fraction). This PSF hydrolysed with HCL and then unknown

steroidal sapogenin (CS) was isolated with n-hexane-MeOH gradient mobile phase. Tricholimus was used as a standard anticancer drug for MTT assay and ADR for SRB assay.

Antiproliferative Assay[7-9]

SRB assay

The sulforhodamine B (SRB), bright pink Aminoxanthine dye with two sulfonic groups, assay is used for cell density determination, based on the measurement of cellular protein content. Under acidic conditions, SRB binds to basic amino acid residues in TCA (Trichloro acetic acid) fixed cells to provide a sensitive index of cellular protein content.

The monolayer cell culture was trypsinized and the cell count was adjusted to 0.5-1.0 × 105 cells/ml using medium containing 10% new born sheep serum. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed once and 100 µl of different test compound concentrations were added to the cells in microtitre plates. The plates were then incubated at 37°C for 72 hours in 5% CO_2 incubator, microscopic examination was carried out, and observations recorded every 24 hours. After 72 hours, 25 µl of 50% trichloroacetic acid was added to the wells gently such that it forms a thin layer over the test compounds to form overall concentration 10%. The plates were incubated at 4°C for one hour. The plates were flicked and washed five times with tap water to remove traces of medium, sample and serum, and were then air-dried. The air-dried plates were stained with 100 µl SRB and kept for 30 minutes at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then airdried. 100 µl of 10 mM Tris base was then added to the wells to solubilise the dye. The plates were shaken vigorously for 5 minutes. The absorbance was measured using microplate reader at a wavelength of 540 nm 22. The percentage growth inhibition was calculated using following formula,

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$$\% \text{ cell inhibition} = 100 - \left\{\frac{(At - Ab)}{(Ac - Ab)}\right\} \times 100$$

Where, At = Absorbance value of test compound, Ab = Absorbance value of blank, Ac = Absorbance value of control

Three dose-response parameters were calculated for the test extracts: GI50, the drug concentration resulting in a 50% reduction in the net protein increase compared with control cells during the drug incubation; TGI, the drug concentration resulting in total growth inhibition, and LC 50, the concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment compared with that at the beginning, thus indicating a net loss of cells following treatment. These three parameters were calculated if the level of cytotoxicity was reached, whereas if the effect was not reached or was exceeded, the value was listed as greater or less than the maximum or minimum concentration tested.

MTT assay[10]

The different concentration was selected in MTT assay method to confirm the activity of *C. borivilianum* extracts at higher concentrations. The antiproliferative studies were evaluated by the 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay method. Cells were plated in 96-well flat bottom plates at 5000–1000 cells/well and were allowed to adhere to the wells overnight. Then the cells were treated with different concentrations of the fractions (0-1 mg/mL) with a maximal final concentration of 1%. (Concentrations are selected higher than SRB assay to confirm the activity.) After 24 h, MTT was used to monitor cell growth. Control cells were incubated with a media containing an equivalent solvent amount without the test materials. The absorbance of converted dye was measured at a wavelength of 570 nm with a background subtraction at 630 nm by Elisa plate reader (ROBONIK). The extract was screened for its cytotoxicity against selected cell lines at different concentrations to determine the IC_{50} (50% growth inhibition) by MTT assay.

DNA fragmentation assay[11]

Triton X-100 Lysis Buffer

In 96 flat-wells plate, desired concentration of extracts was added to 4 × 10 6 target cells (40 wells of 105 per well) and incubated it for 24 hours at 37°C, while one is kept as control. After incubation, collect the cell sample in 1.5 ml eppendorf tube, spin down, resuspend with 0.5 ml PBS in 1.5 ml eppendorf tubes, and add 55ul of lysis buffer (0.5% Triton X-100 in 5 mM Tris, 20 mM EDTA, pH 8.0) for 20 min on ice (4°C). Centrifuge the eppendorf tubes in cold at 12,000 g for 30 minutes. Transfer the samples to new 1.5 ml eppendorf tubes and then extract the supernatant with 1:1 mixture of phenol:chloroform (gentle agitation for 5 min followed by centrifugation) and precipitate in two equivalence of cold ethanol and one-tenth equivalence of sodium acetate. Spin down, decant, and resuspend the precipitates in 30 ul of deionized water-RNase solution (0.4 ml water + 5 ul of RNase) and 5 ul of loading buffer (20 mM sodium acetate buffer, pH 4.8) for 30 minutes at 37°C. Also insert 2ul of Hindi III marker (12 ul of Stock IV) on the outer lanes. Run the 1.2% gel at 5 V for 5 min before increasing to 100 V. Figure 1 is showing DNA fragmentation pattern by control, extract and standard Hind III marker.

Data Analysis

Data were expressed as the mean + SD. (each point was the result of three independent experiments, performed in triplicate).

RESULTS AND DISCUSSION

Currently chemotherapy is regarded as one of the most efficient cancer treatment approach. Although chemotherapy significantly improves symptoms and the quality of life of patients with cancer, only modest increase in survival rate can be achieved. Faced with palliative care, many cancer patients use alternative medicines, including herbal therapies[12]. Numerous cancer research studies have been conducted using traditional medicinal plants in the form of specific herbal extracts, and combinations to treat specific diseases including cancer, in an effort to discover new therapeutic agents that lack the toxic side effects associated with current chemotherapeutic agents.[13]

Worldwide, breast cancer is the second most common type of cancer after lung cancer and the fifth most common cause of cancer death.[14] Hence it was decided to study anticancer effect of *Chlorophytum borivilianum* extract on breast cancer cell line. The SRB assay was performed for two cell lines i.e. Human Breast Cancer Cell Line MCF-7 and Human Leukemia Cell Line HL60. MTT assay is performed only in MCF-7.

Table 1: Percentage Inhibition of extracts of *Chlorophytum borivilianum* in Human Breast Cancer Cell Line MCF-7 (All values are given in micromolar concentration (µM))

Human Breast Cancer Cell Line MCF-7				
	% Control Growth		Average Values	
Extracts	10	20	40	80
CSE	97.3	105.6	107.3	98.1
PSF	96.4	103.8	97.1	95.7
CS	83.6	77.2	57.5	55.9
ME	91.7	97.4	95.8	91.5
ADR	4.2	3.4	−10.6	−22.4

Table 2: The parameters GI50, TGI, and LC50 obtained for *Chlorophytum borivilianum* for Human Breast Cancer Cell Line MCF-7 in the SRB assay. Parameters below or above the highest or lowest drug concentration used are listed as <0.01 or >100, respectively. All values are given in micromolar concentration (µM)

MCF-7	LC50	TGI	GI50
CSE	>80	>80	>80
PSF	>80	>80	57.9
CS	>80	>80	71.9
ME	>80	>80	>80
ADR	>80	43.7	<10

In SRB method for both cell lines the GI50, TGI, and LC50 values are not comparable with that of standard ADR. In Human Leukemia Cell Line HL60 only PSF has found to be active with only 79.1 GI50 value. But the PSF and CS in MCF-7 cell lines showed accep GI50 values (57.9 and 71.9 respectively) so it is concluded to perform the MTT assay of higher concentrations of these two extracts **(Table 1, 2, 3 and 4).**

Hence in MTT assay, the crude methanol extracts exhibited less growth inhibitory activity against only one a selected cancer cell lines (Human Breast Cancer Cell Line MCF-7) than crude saponin extract and standard drug tricholimus. Dose dependent activity has been observed for both extracts. 1000 µg/ml of concentration of CS and PSF has shown 36.13 and 72.24% inhibition respectively. The results suggested that the main anticancer constituents are saponins which are exist in purified extract.

To confirm antiproliferative activity of selected extract DNA fragmentation assay was preferred as cancer cell DNA is one target of anticancer treatment. Internucleosomal DNA fragmentation is often observed during apoptosis and has become widely accepted as one of the most reliable biochemical markers for the process. The DNA of the rapidly multiplying cells is more exposed than the DNA of normal cells.

Table 3: Percentage Inhibition of *Chlorophytum borivilianum* extracts in Human Leukemia Cell Line HL60 (All values are given in micromolar concentration (µM))

Human Leukemia Cell Line HL60				
	% Control Growth		Average Values	
Extracts	10	20	40	80
CM	118.0	106.5	85.0	48.3
CSE	135.6	125.7	107.1	80.6
PSF	124.9	116.6	76.0	42.6
SS	110.2	105.2	95.3	45.6
ME	130.6	130.4	104.2	81.3
ADR	41.9	26.3	17.3	−22.0

Table 4: The parameters GI50, TGI, and LC50 obtained for *Chlorophytum borivilianum* for Human Leukemia Cell Line HL60 in the SRB assay. Parameters below or above the highest or lowest drug concentration used are listed as <0.01 or >100, respectively. All values are given in micro molar concentration (µM)

HL60	LC50	TGI	GI50
CSE	>80	>80	>80
PSF	>80	>80	79.5
SS	>80	>80	>80
ME	>80	>80	>80
ADR	>80	57.03	<10

Table 5: Absorbance and Percent inhibitions of extracts of *Chlorophytum borivilianum* in Human Breast Cancer Cell Line MCF-7

Name	Concentration of Extracts (µg/ml)				
	200 µg/ml	400µg/ml	600 µg/ml	800 µg/ml	1000 µg/ml
SS	0.324 ± 0.2 (24.75)	0.306 ± 0.4 (28.67)	0.297 ± 0.12 (30.76)	0.286 ± 0.22 (33.33)	0.274 ± 0.7 (36.13)
PSF	0.142 ± 0.1 (26.8)	0.095 ± 0.1 (51.03)	0.080 ± 0.14 (58.76)	0.060 ± 0.18 (69.72)	0.050 ± 0.1 (72.24)
Tricholimus	0.021 ± 0.4 (63.15)	0.016 ± 0.7 (71.92)	0.011 ± 0.03 (80.7)	0.008 ± 0.01 (85.96)	0.007 ± 0.5 (87 .71)

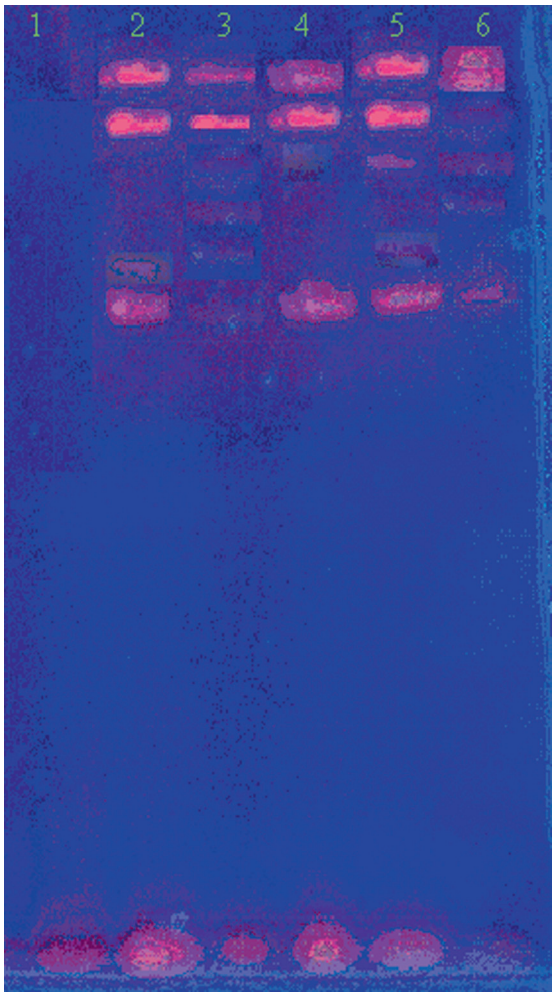

Figure 1: DNA fragmentation in cells treated with different drugs.Human breast cancer cells were Untreated i.e. control: Lane 1, Treated with Hind marker III: Lane 2, Standard Tachrolimus: Lane 3, Saponin CS: Lane 4, CSE: Lane 5 and AL: Lane 6. Lane 1 lacks DNA fragmentation. Lane 4, 5 and 6 shows the comparable DNA fragmentation with that of Lanes 2 and 3.

DNA fragmentation assay confirms that mode of action of extract is due to their fragmentation effect on DNA. Thus extract of *C. borivillianum* possesses significant antiproliferative effect on breast cancer cells. Further experiments are needed to isolate and identify the anticancer compounds (saponins) from methanol extract of *Chlorophytum borivilianum* and also to investigate their mechanism. Electrophoresis of the DNA fraction of the cells treated with CS and PSF revealed a ladder formation of a fragment and its multiples **(Figure 1)** which also confirmed that incubation with extract induced apoptotic cell death.

CONCLUSION

In conclusion, steroid saponins of *Chlorophytum borivilianum* could be regarded as promising drugs for cancer therapy, but the mechanisms of their anti-cancer activity and their toxicity should be further addressed.

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