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IN VITRO ANTICANCER ACTIVITY OF *CAESALPINIA CORIARIA* (JACQ) WILLD (PODS) ON SiHa CELL LINES

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ABSTRACT – In Indian traditional medicine *Caesalpinia coriaria* (Jacq) pods have been widely used for the treatment of bleeding piles, burning skin. It also has hepatoprotective activity, antibacterial activity, antioxidant activity, Xanthine oxidase inhibitory activity. Pharmacological evaluation of partially purified fraction of ethanol extract of *C.coriaria* was studied for anticancer activity on SiHa cell lines. The five fractions significantly reduced cell proliferation with visible morphological changes. Cell cycle analysis suggests that effect of the extract could be due to inhibition of DNA synthesis in SiHa cells. The fraction V of *Caesalpinia coriaria* caused cell death via apoptosis as evident from DNA fragmentation assay. Thus the extract has the potential to be evaluated in detail to access the molecular mechanism-mediated anticancer activities of this plant.

Key words : *Caesalpinia coriaria,* MCF-7, SiHa, A-549, Hoechst 33342, Rhodamine 123, MTT.

INTRODUCTION

The plant kingdom represents an enormous reservoir of biologically active molecules and so far only a fraction of the plants with medicinal activity have been assayed (Qin, 1998). Nearly 50% of the drugs used in medicine are of plant origin. Therefore, much current research devoted to the phytochemical investigation of higher plants which have ethanobotanical information associated with them (Ahmad, 2007). Human use of plants as medicinal agents predates recorded history. Ethno medical plants-use data in many forms has been heavily utilized in the development of formularies and pharmacopoeias, providing a major focus in global health care, as well as contributing substantially to the drug development process (Singh, 2006).

In December 1967, Hartwell published the first of a series of articles in Lloydia on the use of plants by humans against cancer (Graham, 2000). This monumental work, which contains information from published and unpublished sources on over 3000 species of plants which had been reported to have alleged anti-cancer properties.

Cervical cancer is the most common cancer among women in several regions of India (Sharma, 2001). Of the 500.000 new cases of cervical cancer reported worldwide annually, India accounts for one-fifth in terms of overall incidence (Sridhar, 2001). Chemotherapy is one of the methods of treating cancer. However the chemotherapeutic drugs are highly toxic and have devastating side effects. Various new strategies are being developed to control and treat several human cancers (Modha, 2007). The increased popularity of herbal remedies for cancer therapy perhaps can be attributed to the belief that herbal drugs provide benefit over that of allopathy medicines while being less toxic (Gupta, 2004). Natural products are also the lead molecules for many of the drugs that are in use (Cragg, 1997). The continuing search for new anticancer compounds in plant medicines and traditional foods is a realistic and promising strategy for its prevention (Yan-We, 2009). Numerous groups with antitumor properties are plant derived natural products including alkaloids, phenyl propanoids, and terpenoids (Kintzios, 2006, Park, 2008). In order to isolate novel lead active principal from *C.coriaria*, we wanted to explore its antiproliferative action on cervical cancer. So far there is no such report for this plant showing its effect on cervical cancer. This prompted us to investigate the growth inhibitory effect of this plant on cervical cancer cell line.

MATERIALS AND METHODS

Sample Preparation of *C. Coriaria* Extract

The sample were collected from Captain Srinivasa Murti Drug Research Institute Chennai, and allowed to dry under the shade completely. The dried leaves were ground into powder, which was used for further extraction.

Extraction and Drying

About 25gm of dry sample powder was weighed and macerated with 75 ml of each solvent (Acetone, Ethyl acetate, Ethanol and Water) separately and kept overnight in shaker. The extract was collected after filtration using

Whatmann No.1 filter paper and was stored. Another 75 ml of solvent was added to the residual mixture and incubated in shaker for 24 hours and the extract was collected again using a Whatmann No.1 filter paper. This procedure was repeated once again and the extract was evaporated below 40 p C, which was used for further phytochemical analysis.

Bioactivity - Guided Purification

Fractionation of Ethanol Crude Extract

All extracts obtained from three different extraction solvents (acetone, ethanol, ethyl acetate and water) were subjected to antioxidant assay DPPH. From the assay results ethanolic extract, which showed significant scavenging effect was further subjected for purification using column chromatography. 10 grams of ethanol crude extract was chromatographed over silica gel column (100 – 200 mesh, 100 grams). The admixture (60 -120 mesh) was packed on a silica gel column (Merck, India) and eluted with 100% hexane, hexane: ethyl acetate in the ratio of 75:25, 50:50, 25:75, 100% ethylacetate, ethylacetate and methanol in the ratio of 75:25, 50:50, 25:75 and 100% methanol. The eluent was collected in fractions of 100mL each. The chemical composition of each fraction was evaluated by using thin-layer chromatography (TLC) and visualized with UV (254 and 365 nm) and iodine vapours. Based on the TLC profiles, fractions with similar compositions were pooled together and concentrated under reduced pressure. A total of five major combined fractions were obtained from the ethanol extract. The fractions obtained through column chromatography were subjected to the antiproliferation assay on SiHa cell line

Cell Lines and Culture Medium

Caesalpinia coriaria ethanolic pod extract and different fractions I, II, III, IV, V 20 mg/ 10 ml PBS was dissolved. The extracts were vortexed for 30 minutes. 200 ìl of DMSO (Sigma, St. Louis, USA) was added to all the fractions. For all the experiments these prepared extracts were used. SiHa (Cervical Cancer), MCF-7(Breast Cancer), A 549(Lung Cancer) and Vero (Normal Cell Line) were purchased from NCCS, Pune and cultured in Dulbecco's modified Eagle medium(Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Sigma, St. Louis, USA). The cells were incubated in a humidified atmosphere at 5% CO_2 at 37 °C. All the cell lines used in the study were of passage number between 3 to 7.

***In Vitro* Cytotoxicity Assay - MTT** (Mosmann, 1983)

Cell survival was measured using the MTT micoculture tetrazolium assay, according to the method described by (Mosmann, 1983) with slight modifications. Briefly, cells at the exponential growth phase were trypsinized and resuspended in the complete medium to a population of 1 X 10^5 cell mL^{-}. A total of 1X10^5 cells per well were seeded in a 96-well plate. After 48 hr incubation in a 5% humidified CO_2 incubator at 37 °C, varying concentration of *C.coriaria* extract were added to a final volume 100 ìl of standard growth medium per well. After 24 hr incubation at 37 °C, 100 ìl of MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] 5 mg mL^{-} in phosphate buffer saline (PBS) was added to each well and incubated for 12 hr at 37 °C. The medium was removed and formazan crystals thus formed were dissolved in DMSO. The plates were read immediately in a microplate reader (Multiskan Ex Primary EIA) operating at 570 nm. The percentage of viability was calculated using the formulae

$$\% \text{ of viability} = \frac{\text{OD value of Treated}}{\text{OD value of cell control}} \times 100$$

Analysis of DNA Fragmentation (Paola Boss, 1992).

Cell Preparation

1 X 10^5 cells/well was seeded in 6 well microtitre plates and the plates were incubated for 48 hr at 37 °C in 5% humidified CO_2 atmosphere.

DNA fragmentation was analyzed by Paola Boss (1992 a method for DNA separation of fragmented and intact DNA fractions for their analysis by agarose gel electrophoresis. In apoptotic cells, the specific DNA cleavage becomes evident in electrophoretic analysis as a typical ladder pattern due to multiple DNA fragments.

Analysis of Cell Death - Apoptosis & Mitochondrial Depolarization Study (Singh S, 2004)

Chromatin condensation was determined by Hoechst 33342 method. Hoechst 33342(2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'bi-1 H benzimidazole trihydrochloride trihydrate) was a cell permeable DNA stain that was excited by ultraviolet light and emits blue trihydrochloride at 460 to 490 nm. Hoechst 33342 binds preferentially to adenine-thymine(A-T) regions of DNA. A total of 1X10^5 cells were seeded in the six well plates. The plates were incubated at 48 hrs at humidified atmosphere at 5% CO_2 at 37°C. 5 ml of Carnoy's fixative (Methanol:Glacial acetic acid 3:1) was added. Remove the Carnoy's fixative. Wash with 5ml of methanol. Allow the plates to air dry. The cells were now fixed. Add the

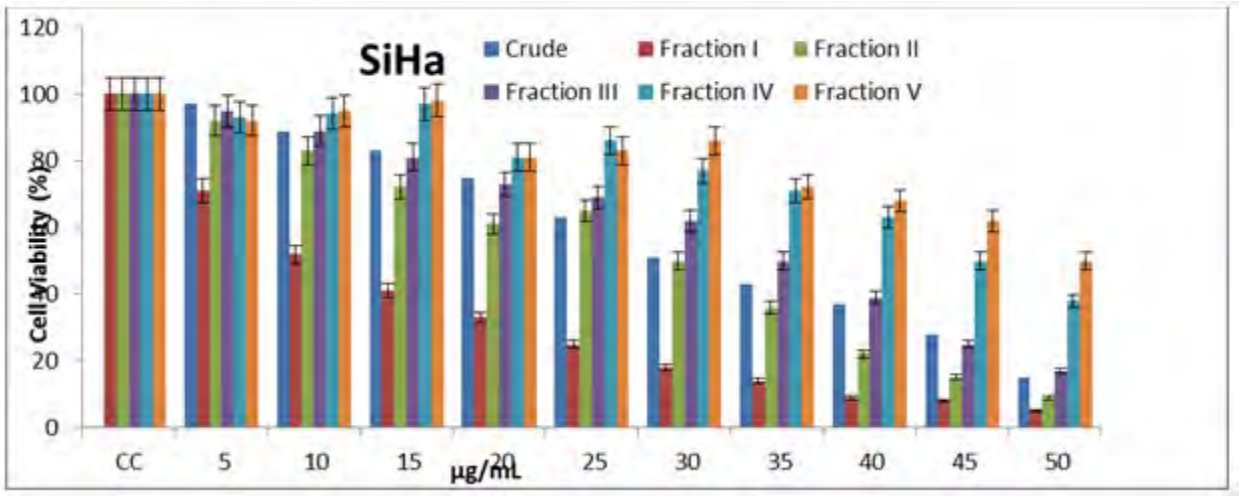


Fig. 1 : Effect of different fraction of *C.coriaria* on SiHa cells (MTT)

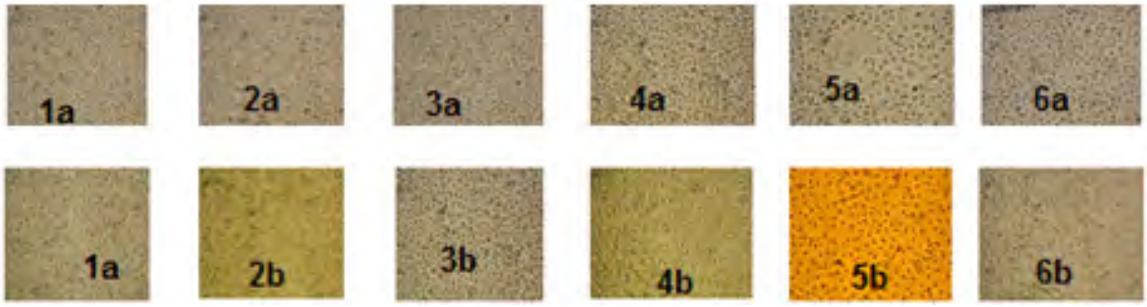
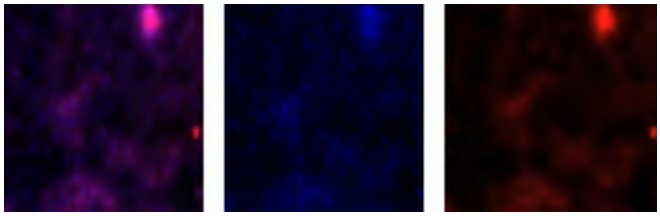
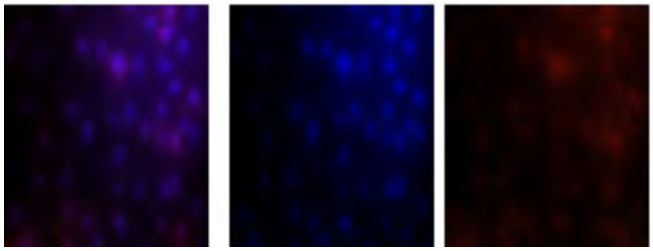


Fig. 2 : Effect of *C.coriaria* fractions on morphology of SiHa cells were examined under phase contrast microscope at 100 x magnification.
1a, 2a, 3a, 4a, 5a, 6a - Control, Fraction I, II, III, IV, V control
2b, 3b, 4b, 5b, 6b - Fraction I, II, III, IV, V Treated



a. Mixed , Hoechst, Rhodamine control SiHa cells.



b. Mixed, Hoechst, Rhodamine Treated Fraction V. SiHa cells

Fig. 3 : Nucleus, mitochondrial membrane potential in SiHa Cells

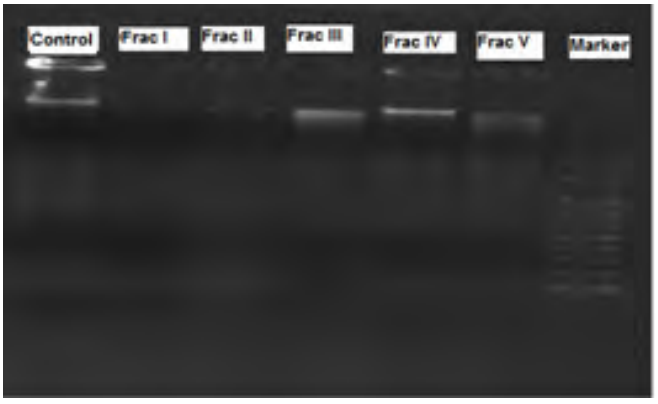


Fig. 4 : DNA fragmentation in *C.coriaria* Fraction III and Fraction V treated SiHa cells. Genomic DNA was extracted with control and fractions.

Hoechst stain(10 ìg/ml) and allow to stand for 15 min. Remove the stain and washed with methanol and allowed to dry. Then add Rhodamine 123 (10 µg/ml) and allow to stand for 15 minutes. Remove the stain and wash with methanol and let it dry. The cells were visualized in a Nikon Eclipse E600 fluorescence microscope.

Cell Cycle Analysis (Dwarakanath , 1999).

The distribution of cells at different stages in the cell cycle was estimated by flow cytometric DNA analysis. Flow cytometric measurements of cellular DNA contents were performed with ethanol (70%)- fixed cells using the intercalating DNA fluorochrome, Propidium iodide as described by (Dwarakanath BS, 1999). $1X10^5$ cells were seeded in culture flask. The plates were incubated at 24 hrs at humidified atmosphere at 5% CO_2 at 37 °C. Harvest the cells from the medium as well as the adherent cell. Make sure the trypsinization is enough to bring the cells in single cell suspension avoid clumping. Pellet out the cells at 2500 rpm/10 min. Discard the supernatant, resuspended the cells with 1.5 ml of ethanol 70% drop by drop to the pellet, vortex the pellet. Store the suspended cell at -20^0C overnight. Centrifuged the cells to remove ethanol at 2500 rpm/ 10 min. To the pellet added PBS-FCS and again centrifuged at 2500 rpm/10 min. Remove the PBS-FCS and add 300 µl of fresh PBS/EDTA to the pellet. Add 20µl of RNase A and incubate at 37 °C for I hr. At the time of analysis add 250 µl of PI (10 µg/ml) and incubate the cells for 20 minutes in the dark at room temperature, and finally analyzed on a FACS cytometer (Calibur, Becton Dickinson, USA). A minimum of 2.5 x 10^3 cells per sample was evaluated, and the percentage of cells in each cell cycle phase was calculated using the CELL QUEST and Modify software (Becton Dickinson).

RESULTS AND DISCUSSION

In Vitro Cytotoxcity Assay - MTT

Cell lines of different origin show different results towards the same compound. Therefore it was necessary to carry out more than one cell line in the initial screening experiment. Three cell lines of human origin, namely MCF-7(Breast cancer), SiHa (Cervical cancer), A549 (Lung Cancer) and Vero (Monkey cell line) were used in the present study. According to the American National Cancer Institute (NCI), the criteria of cytotoxic activity for the crude extracts are IC50 = 30 ìg/ml. In vitro screening of the extract *C.coriaria*, showed that ethanol extract is cytotoxic against all the cell lines at the concentration of 30 ìgmL^{-1}, the crude extract caused 97% cell death in SiHa cell line. The crude extract was then purified by column chromatography using silica gel as stationary phase and increasing the solvent polarity. The fractions obtained through column chromatography were subjected to cell cytotoxicity studies using the five fractions obtained from column chromatography. Characterization for these activity is currently under investigation.

Morphological Changes of Cell Lines

Morphological changes in the cells in detail using a phase contrast microscope. The cells were marked with morphologic changes such as shrinkage, rounding, detachment and membrane blebbing in the SiHa cell lines exposed to the five fractions. These morphological changes may suggest that these five fractions may cause apoptotic cell death in SiHa cell lines.

Analysis of Cell Death - Apoptosis & Mitochondrial Depolarization Study

Hoechst 33342 is used for specifically staining the nuclei of living or fixed cells and tissues. Rhodamine 123 is used for studying the mitochondrial membrane potential of control and treated cells. The nucleus and the mitochondrial membrane potential of the control and treated Fraction V clearly showed the morphological changes induced due to apoptosis are shown in Figure 3.

Effect of *C. Coriaria* Fractions of DNA Fragmentation

DNA fragmentation is a characteristic feature of apoptosis. Therefore *C.coriaria* fractions induced apoptosis was confirmed by the DNA fragmentation assay. Increased DNA fragmentation was apparent in SiHa cells at the fraction III and V. A typical experiment result of agarose gel electrophoresis is shown in Figure 4. Whereas treatment with DMSO (control) did not produce any DNA fragment ladders.

Cell Cycle Analysis

The effect of partially purified fractions on cell cycle analysis of SiHa cells was determined by flow cytometry. SiHa cells treated with *C.coriaria* fraction V at final concentration of

500 µmL^{-1} showed decrease in G1 phase cells from 47.79% to 45.14% and this decrease in G1 phase was accompanied by increase in the population of G2 + M phase from 10.24 to 12.49% in the control and treated cells. *C.coriaria* fraction V showed moderated inhibition in the progression through S phase with a slight decrease in the population of S phase from 9.65 to 7.64% at 48 hr. This was accompanied by decrease in the population of G0-G1 phase of cell cycle (Table 1). A representative histogram for SiHa cells is shown in Figure 5.

The antiproliferative activity of SiHa cell line might result, at least in part from inhibition of DNA synthesis and proliferation and from induction of apoptosis.

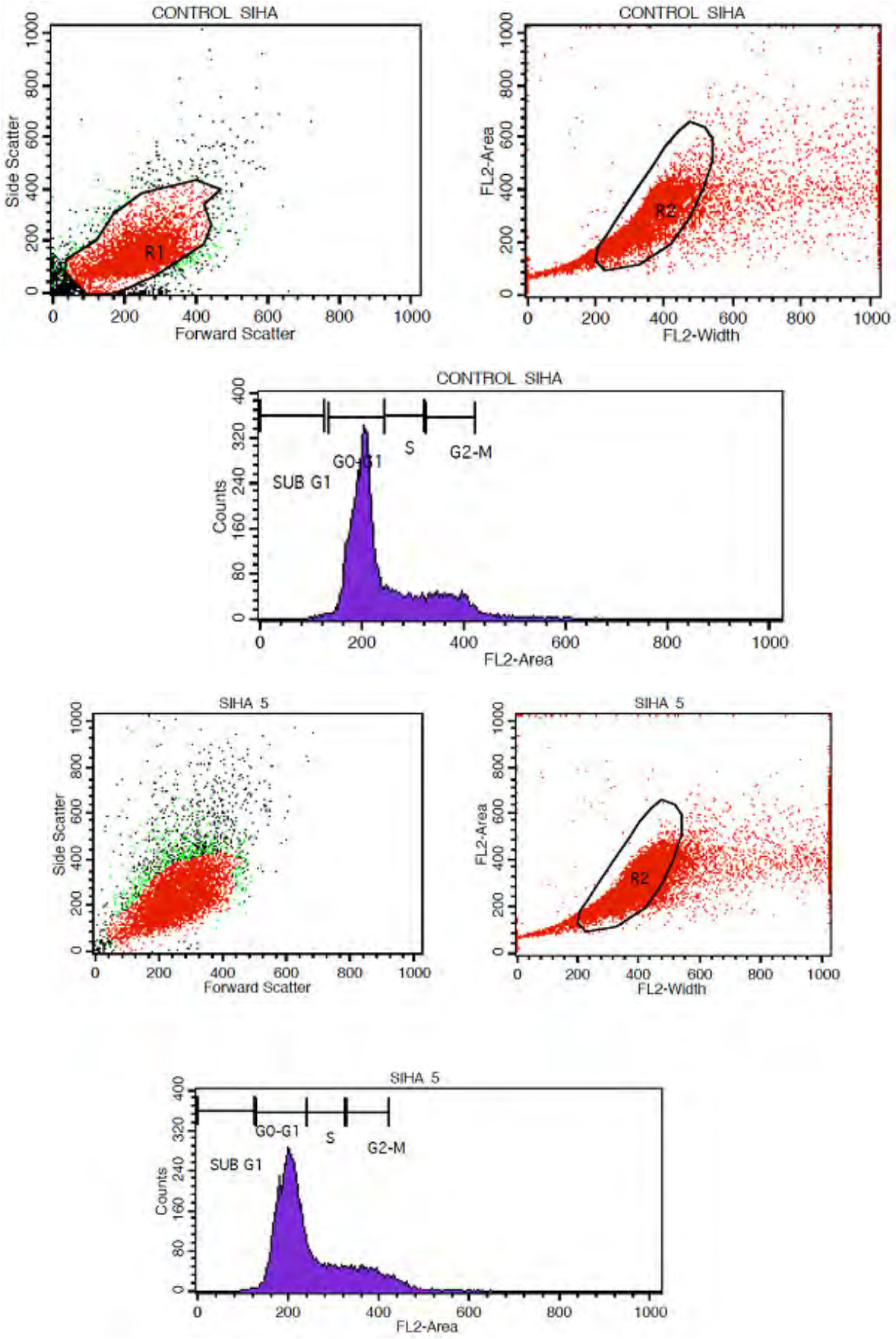


Fig. 5 : Cell cycle analysis of SiHa cells after treatment with Fraction V and control.

Table 1 : Cell cycle distribution of SiHa cell line after treatment with Fraction V and control.

Distribution (% cells)	Control cells	*C.coriaria* Fraction V Cells
G1	47.79	45.14
G2-M	10.24	12.49
S	9.65	7.64
Sub-G1	0.18	0.14

Observations from flow cytometric DNA analysis suggests that mode of cell death induced by *C.coriaria* fraction V is mainly through apoptosis. This has been further strengthened by the DNA fragmentation assay (chromatin fragmentation by internucleosomal DNA cleavage), which is considered as hallmark of apoptosis. The appearance of a DNA ladder was investigated by agarose gel electrophoretic of genomic DNA extracted from control and treated cells. Cell proliferation kinetics clearly showed that *C.coriaria* fraction V retarded the rate of progression through the S-phase and induced an accumulation of cells in G2-M (Table 1). The active components that could induce the growth inhibition which induce apoptosis are under progress. Therefore, it could be concluded that anticancer activity of *C.coriaria* fraction might result in part, from inhibition of DNA synthesis, retardation of cell proliferation and induction of apoptotic death of cancer cells.

CONCLUSION

In conclusion, *C.coriaria* fraction could inhibit the proliferation of human cervical cancer cell line SiHa. Our results demonstrated that cell cycle via S-phase inhibitions play some roles that fraction induced anticancer activity in SiHa cell line. Although scientific studies have been done on large number of Indian botanicals, a considerable smaller number of marketable drugs of phytochemical entities have entered the evidence-based therapeutics (Patwardhan, 2005). The present study ascertains that *C. coriaria* has the anticancer potential and may be a promising drug which would enter the market by evidence-based research for therapeutics. However, further biochemical work and investigation at the molecular level are currently under progress to identify the active components that could induce growth inhibition and to establish the possible explanation of mechanism of DNA synthesis inhibition by *C. coriaria* extract.

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