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Cytotoxicity of alkaloid fraction from *Sphaeranthus amaranthoides* in A549 cell line

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Sphaeranthus amaranthoides **(sivakaranthai) is a weed in paddy fields found in South Asia. The plant is widely exploited for its pharmacological activity. Previous research showed that the crude extract of this plant possess the antioxidant, anti-inflammatory and antibacterial activity. The current study concentrates on the cytotoxic properties of the alkaloid fraction isolated from the *S. amaranthoides.* The cytotoxicity was checked using MTT assay method. The assessment of the morphology of the apoptotic cell was done using the fluorescent microscopy. The percentage of apoptosis was investigated dual staining (EB/AO). Nuclear morphology was assessed using propidium iodide. Finally the cell cycle progression was analyzed using flow cytometer. Alkaloids was found to have the highest toxicity towards A549 lung cancer cell lines (IC_{50}= 29.57 µg). The morphology of the lung cancer cells after treatment showed evidence of apoptosis that included blebbing and chromatin condensation. Dual staining showed the evidence of the early apoptosis induced after treatment. The cell cycle analysis indicated that alkaloids were able to induce G2/M phase arrest in lung cancer cells.**

Key words: *Sphaeranthus amaranthoides*, alkaloids, lung cancer cells.

INTRODUCTION

There are about 20,000 species of tropical plants, of which about 1,300 are said to be medicinal and potential sources for screening of anticancer agents (Said, 1999). Some of the plant extracts from these medicinal plants are reported to have potential to be developed as drugs (Manosroi et al., 2006). *Sphaeranthus amaranthoides* Burm. F known as sivakaranthai (asteracea) is distributed in Asia and Africa. The areal parts of this plant are used in the treatment of tumor by the tribal people. This plant possesses anticancer properties; the alkaloid fraction can help to cure the cancer.

Cancer is the disorder caused by the uncontrolled growth of the abnormal cells in the human body. Lung cancer is one of the most commonly diagnosed cancers worldwide making up 12.7% of all cancer cases. It is also the most common cause of cancer death accounting for 18.2% of all cancer related deaths (Ferlay et al., 2008). The literature suggested that natural compounds can be effective in cancer therapy (Reddy et al., 2003; Gullett et al., 2010). One of the main characteristics of the cancer

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is the resistance of cancer cells towards apoptosis which contributes to the ineffectiveness of anticancer therapies (Tang et al., 2010). Apoptosis is characterized by several biochemical and morphological events, such as nuclear fragmentation, internucleosomal DNA fragmentation (Wyllie, 1980), cell shrinkage (Saraste and Pulkki, 2000), chromatin condensation (Lu et al., 2012), formation of apoptotic bodies, loss of plasma membrane asymmetry (van Engeland, 1998), and disruption of mitochondrial membrane (Biasutto et al., 2010). An attempt is made to understand the mechanism(s) followed by alkaloids from *S. amaranthoides*, we have investigated the effect(s) of alkaloids on the A549 cell lines.

METHODOLOGY

Collection of plant material and preparation of crude extract

S. amaranthoides Burm. F (Asteraceae) plant leaves were collected from Tirunelveli district. The Plant was examined and botanically identified by a botanist V. Chelladurai Research Officer-Botany. The collected leaf was shade dried for three weeks to get consistent weight and made in to coarse powder and was used for further studies.

Preparation of ethanolic extract

The leaf powder was soaked in the petroleum ether for two days to dissolve the chlorophyll and then the leaf material was transferred in to the ethanol for five days. On fifth day, leaf material was filtered. The filtered extract was subjected to rotary evaporator to remove the ethanol and to get the concentrated extract in powder form.

Purification of alkaloids from crude extract

The order of solubility for alkaloids: chloroform>acetone>ethanol>methanol>ethyl acetate>ether>n hexane silica gel G60 emulsion was used as a stationary phase in glass column. (1.5 × 50 cm) and chloroform and acetone (10:1, 9:2, 8:3, 7:4) were used as mobile phases. A larger section of the alkaloids are easily soluble in chloroform and relatively less soluble in other organic solvents. The UV-vis spectrophotometer was used to measure the absorbance of the alkaloids on a Cary E-100 Varion type spectrophotometer. The absorbance was measured between 200-800 nm.

HPLC

HPLC system used consisted of a HPLC binary pump, diode array detector (DAD), and an auto-sampler injector compartment (1200 series, Agilent Technologies, Germany). For separation, C-18, 150 mm, 64.6 mm i.d and 5 mm particle size Thermo Hypersil GOLD column was chosen as the reverse phase while the mobile phase was 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) with the gradient setting of solvent B: 5% (5 min), 5-90% (60 min), 5% (4 min) at a flow rate of 1 ml/min. Detection wavelengths were both set at 280 and 360 nm with constant injection volume of 20 ml. A 3200 QTrap LC/MS/MS system was used for the mass spectrometry analysis, with the iron source and voltage maintained at 500uC and 24.5 kV for negative ionization, respectively. The nitrogen generator was set at 60 psi curtain gas flow, 60 psi exhaust gas flow, and 90 psi source gas flow. The scanning modes selected were enhance mass spectrometer (EMS) and enhance ion product (EIP) for full scan mass spectra that ranged from mass to charge ratio (m/z) of 100-1200.

Cell culture conditions

The A549 lung adenocarcinoma cell line was procured from National Centre for Cell Science (NCCS), Pune, India with the passage number of 11. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, along with 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C. Cells were grown in 75 cm^2 cultures and after a few passages, cells were seeded in a 96-well plate. The experiments were done at 70 to 80% confluence. Upon reaching confluence, cells were detached using trypsin-EDTA solution.

Cell proliferation assay

Proliferation of A549 cells was assessed by MTT assay (Safadi et al., 2003). Cells were seeded into 96-well plates at 5 × 10^4 cells per well 24 h before treatment. After treatment with drugs, cell proliferation was determined using MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, 15 µl (5 mg/ml) MTT solutions was added to each well, and incubated at 37°C for 4 h, after which the MTT solution was discarded and 200 µl of dimethylsulfoxide (DMSO) was added to dissolve the crystals of formazan dye by pipetting up and down. Spectroscopic absorbance of each well was measured at 570 nm using an ELISA reader (BIORD) at 570 and 630 nm as background using a microplate reader (Synergy H1 Hybrid). The IC_{50} value was determined from the dose response curve.

Ethidium bromide/acridine orange (dual staining)

Ethidium bromide/acridine orange staining was carried out by the method of Gohel et al. (1999). A549 cells were plated at a density of 1×10^4 in 48-well plate. They were allowed to grow at 37°C in a humidified CO_2 incubator until they were 70-80% confluent. Then cells were treated with 15.625 and 31.25 µg/ml (selected based on the IC_{50} concentration) of alkaloid fraction for 24 h. The culture medium was aspirated from each well and cells were gently rinsed twice with PBS at room temperature. Then equal volumes of cells from control and drug treated were mixed with 100 µl of dye mixture (1:1) of ethidium bromide and acridine orange and viewed immediately under Nikon inverted fluorescence microscope (Ti series) at 10x magnification. A minimum of 300 cells were counted in each sample at two different fields. The percentage of apoptotic cells was determined by [% of apoptotic cells = (total number of apoptotic cells/total number of cells counted) ×100].

Assessment of nuclear morphology after propidium iodide staining

Propidium iodide staining was carried out by the method of Chandramohan et al. (2007). A549 cells were plated at a density of 1 × 10^4 in 48-well plates. They were allowed to grow at 37°C in a humidified CO_2 incubator until they are 70-80% confluent. Then cells were treated with 15.625 and 31.25 µg/ml of alkaloid for 24 h.

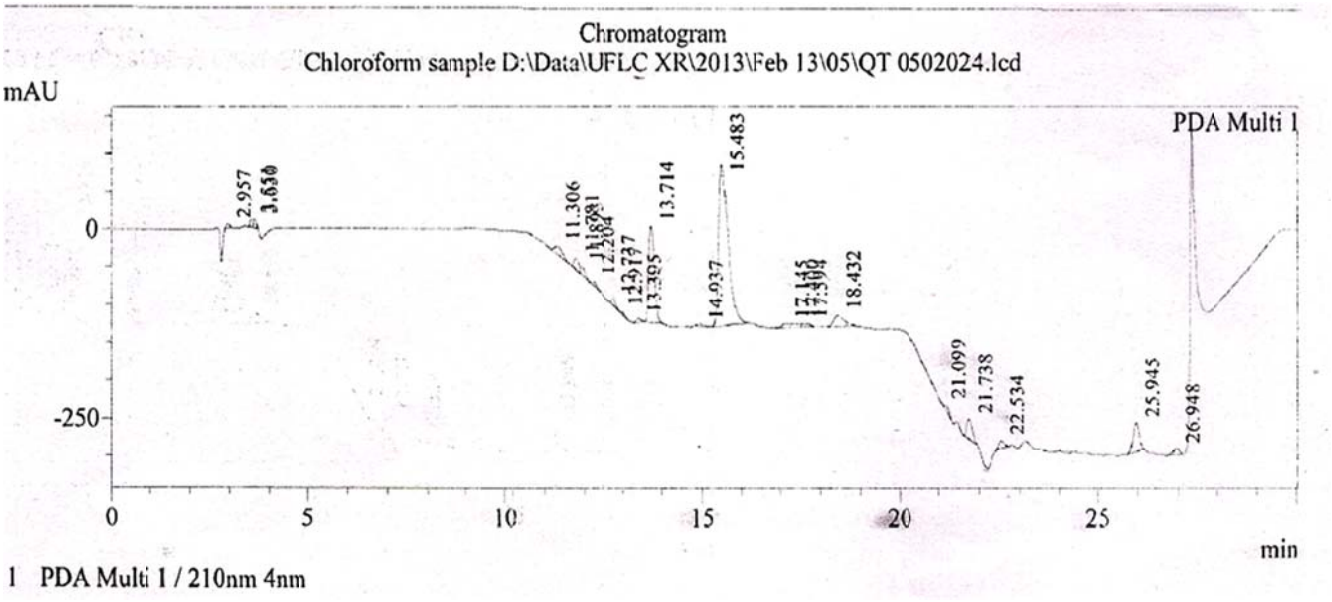

Figure 1. Graph showing the alkaloids retention time (HPLC chromatogram).

Culture medium was aspirated from each well and cells were gently rinsed twice with PBS at room temperature, before fixing in methanol : acetic acid (3:1 v/v) for 10 min, and stained with 50 µg/ml propidium iodide for 20 min. Nuclear morphology of apoptotic cells with condensed/fragmented nuclei was examined by fluorescence microscopy and at least 1 ×10^3 cells were counted for assessing apoptotic cell death.

Cell cycle analysis

To investigate the effect of alkaloid fraction on the cell cycle distribution, A549 cells (1×10^5 cells/ml) were treated with 15.625 and 31.25 µg/ml cultured for 24 h. The treated cells were harvested, washed with phosphate-buffer saline (PBS) and fixed in 75% ethanol at 4°C overnight. After washing twice with cold PBS, cells were suspended in PBS containing 40 µg/ml propidium iodide (PI) and 0.1 mg/ml RNase A followed by shaking at 37°C for 30 min. The stained cells were analyzed with flow cytometer at Ramachandra medical collegeat Chennaiand the data were consequently calculated using WinMDI 2.9 software (Tu et al., 2004).

Statistical analysis

Data was expressed as mean ± S.E.M and analyzed by Tukey's test to determine the significance of differences between groups. A p value lower than 0.05, 0.01 or/and 0.001 was considered to be significant.

RESULTS

The HPLC analysis of the sample revealed 20 different peaks with different retention times in that two major peaks were observed (Figure 1, Table 1).

Cell cytotoxicity assay using MTT method

The A549 lung adenocarcinoma cells were treated with various concentrations (5 - 640 µg) of alkaloid fraction and subjected to MTT assay. As shown in Figure 2, treatment of A549 cells with the drug resulted in significant dose - dependent reduction in cell growth ranging from 12.29±0.01 to 87.98±0.003 after 24 h. The IC_{50} value was found to be 29.57 µg (Table 2), the results obtained reveal that the alkaloids from the plant have strong anticancer activity towards A549 cells. Figure 3 shows the morphological changes due to the alkaloids treatment with various concentrations. The cell morphology in control was observed normal and number was more but in the alkaloid treated cell lines in 20 µg/ml showed spherical shaped. At 80 µg/ml, the number was reduced and the morphology was spherical shaped. Finally with 320 µg/ml, the cells were dead.

Quantification of apoptosis using acridine orange and ethidium bromide assay (apoptosis)

Acridine orange/ethidium bromide (AO/EB) was done to evaluate the type of cell death induced by alkaloid fraction in A549 cells; the morphological variations after double staining were investigated. Live cells stained with AO emitted green fluorescence. Early apoptotic cells had fragmented DNA which exhibited intense green colored nuclei. Late apoptotic and necrotic cell's DNA were fragmented and stained orange and red. From the data it was clear that with increase in the concentration of drug,

Table 1. HPLC DATA (peaktable).

Chl 210 nm 4 nm			
Peak#	**Ret. time**	**Area**	**Area %**
1	2.96	28141	0.41
2	3.55	55842	0.81
3	3.63	46166	0.67
4	11.31	144820	2.11
5	11.78	128760	1.87
6	11.89	79199	1.15
7	12.20	39944	0.58
8	12.74	51948	0.76
9	12.92	14380	0.21
10	13.39	43506	0.63
11	1371	1245617	18.11
12	14.94	54107	0.79
13	15.48	3632779	52.82
14	17.15	62919	0.91
15	17.40	75742	1.10
16	17.59	59726	0.87
17	18.43	259250	3.77
18	21.10	50726	0.74
19	21.74	218746	3.18
20	22.53	96601	1.40
21	25.95	402276	5.85
22	26.95	86320	1.26
Total		6877515	100.00

Shaded values indicate 2 two major peaks which were highest

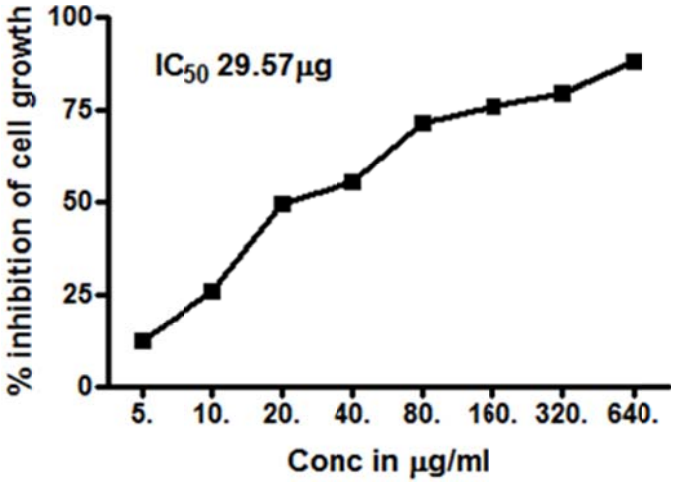

Figure 2. Inhibitory effect of the alkaloid fraction treatment on A549 lung adenocarcinoma cells (MTT ASSAY).

Table 2. Cytotoxicity of alkaloid fraction.

Concentration (µg/ml)	% inhibition of cell growth
5	12.29 ± 0.011
10	26.02 ± 0.002
20	49.28 ± 0.005
40	55.39 ± 0.004
80	71.55 ± 0.005
160	75.76 ± 0.003
320	79.30 ± 0.002
640	87.98 ± 0.003

the number of viable cells decreased tremendously. The percentage of apoptotic cells after treatment with 15.625 and 31.25 µg/ml of drug was drastically increased ($p < 0.001$) to 44 and 70%, respectively. The study reveals that the alkaloids triggered morphological changes in treated A549 cells that indicated possible induction of apoptosis upon treatment in a time dependent manner (Figure 4). The presence of intercalated AO within fragmented DNA indicates early apoptosis. 24 h after the treatment with alkaloids, blebbing and nuclear chromatin condensation were noticeable. Late apoptosis was indicated

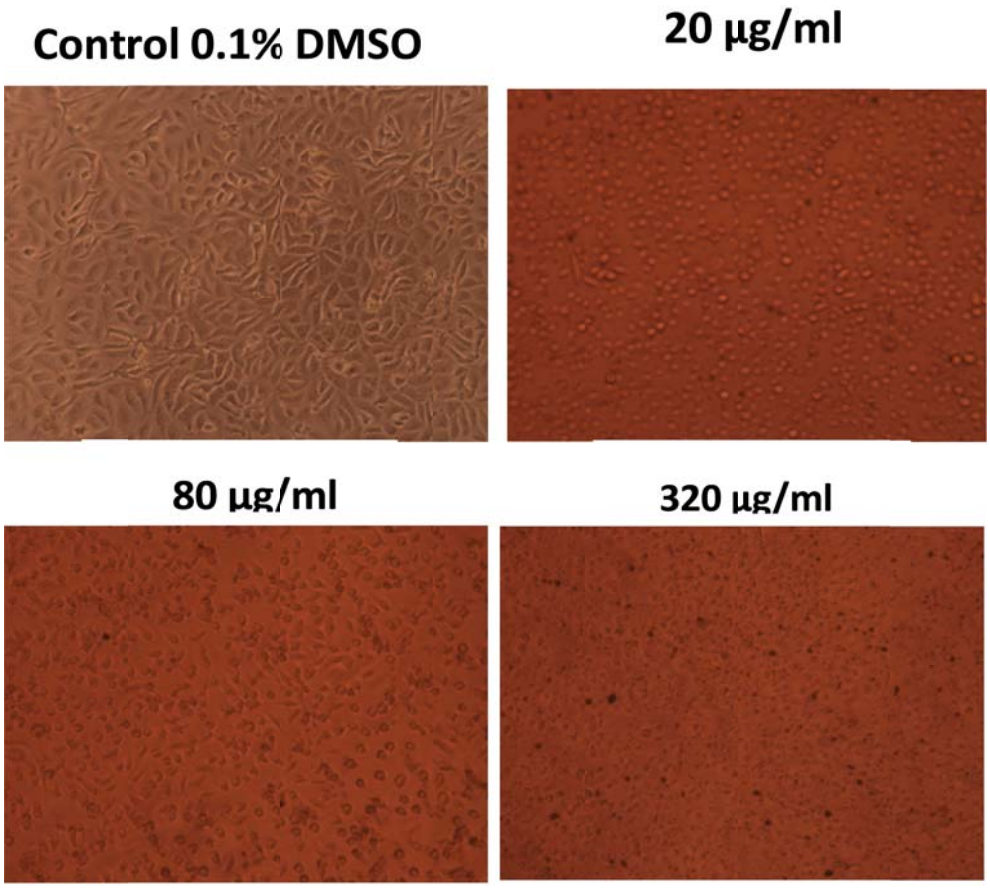

Figure 3. Morphological changes induced by different concentrations of alkaloid fraction.

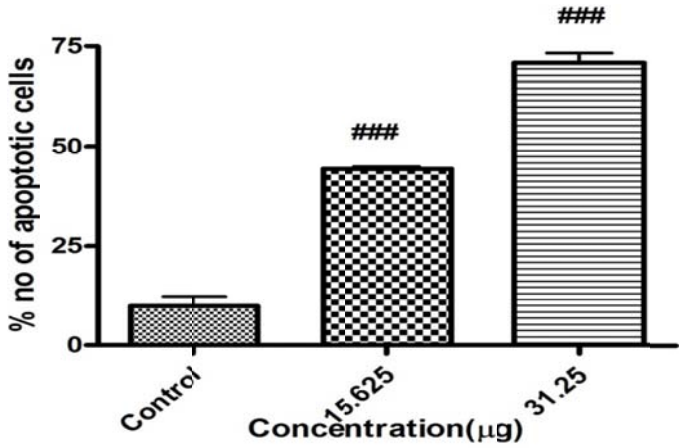

Figure 4. Bar graph showing the percentage number of apoptotic cells.

indicated by the presence of orangish red colour due to the binging of the AO to denatured DNA as observed after 48 h of treatment with alkaloids (Figure 5).

Propidium iodide - nuclear fragmentation assay

Apoptosis was further confirmed by analyzing the nuclear morphology of drug-treated A549 cells. Nuclear morphology was evaluated with membrane-permeable PI stain. The treated cells contained more apoptotic cells while comparing to untreated cells. There was charac-teristic nuclear fragmentation of nuclei in treated A549 cells whereas the untreated control cells did not show any nuclear fragmentation (Figure 6). The apoptotic cells displayed characteristic features of reduced size, intense fluorescence of condensed nuclear chromatin and formation of membrane blebs. The percentage of apoptotic nuclei after treatment with 15.625 and 31.25 µg/ml of drug increased enormously ($p<0.001$) to 53 and 69%, respectively (Figure 7).

FACS analysis

Flow cytometric analysis of the cell cycle was performed to determine the ability of alkaloids to induce cell cycle arrest and apoptosis in A549 cells. After 24 h of incubation,

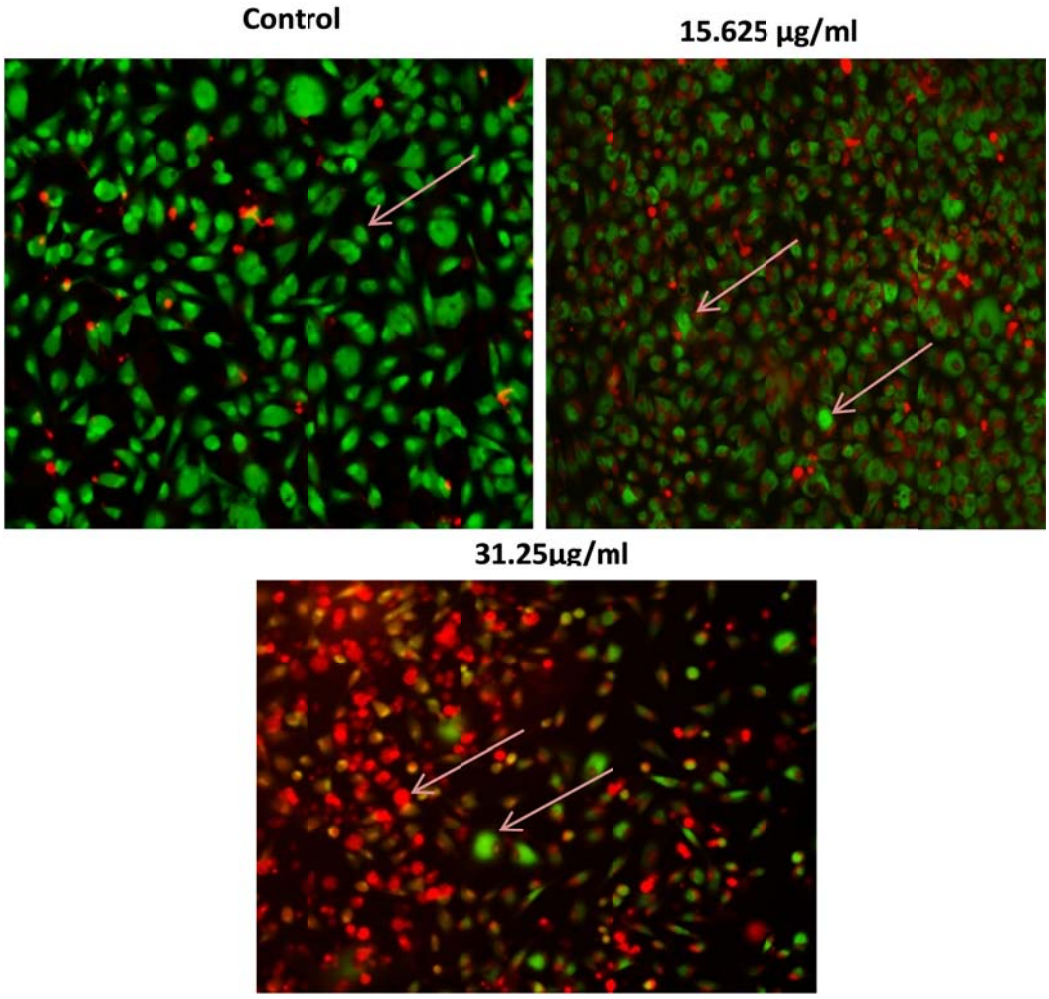

Figure 5. Induction of apoptosis by the drug in A549 cells.

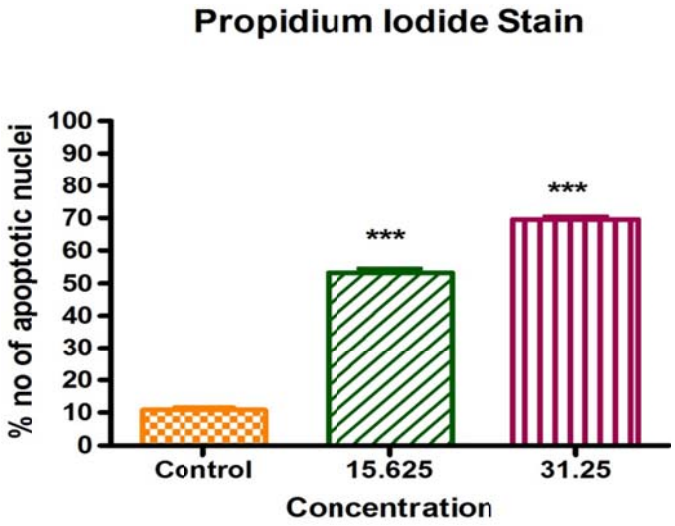

Figure 6. Percentage of apoptotic nuclei.

stability in all the cell cycle populations is generally noticed and compared with the control cell line without treatment. Alkaloid fraction treatment increased the cells in the G2/M phase from 10.45 to 28.32% in 15.625 µg treated cells and 48.79% in 31.25 µg treated cells, respectively. Similarly accumulation of cells in the sub G1 phase from 4.56 to 9.14 and 21.32% in the two treated concentrations was also observed (Figure 8). An increased cell population in the G2/M and sub G1 phase with a concomitant decrease in the G0/G1 and S phase compared to the untreated cells suggest that the alkaloid fraction inhibited the cell cycle progression in G2/M phase and subjected the cells to apoptosis which is evident from cell accumulation in sub G1 phase.

DISCUSSION

In the present study, we report the type of apoptosis induced

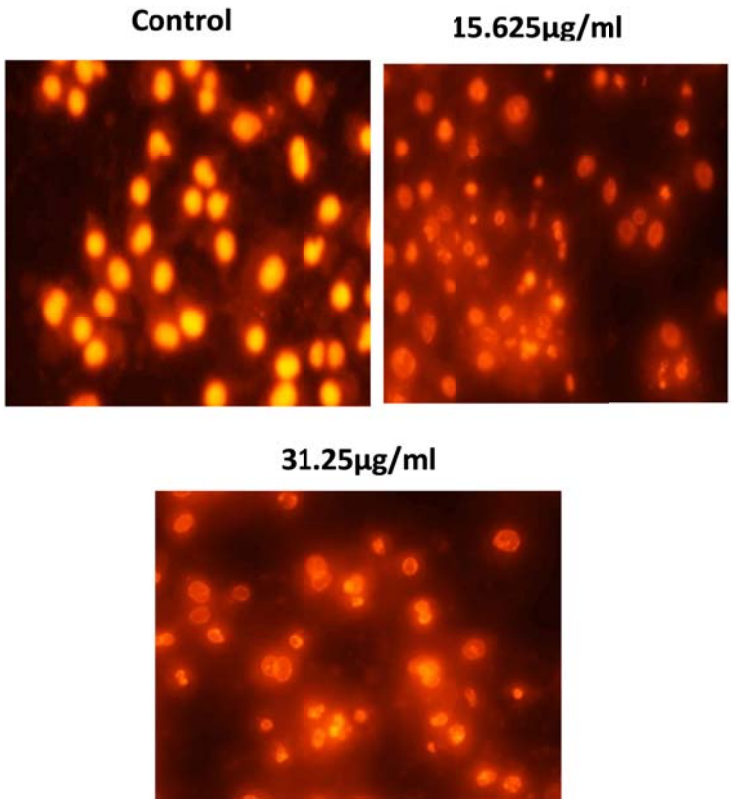

Figure 7. Nuclear localization of A549 cells by propidium iodide staining.

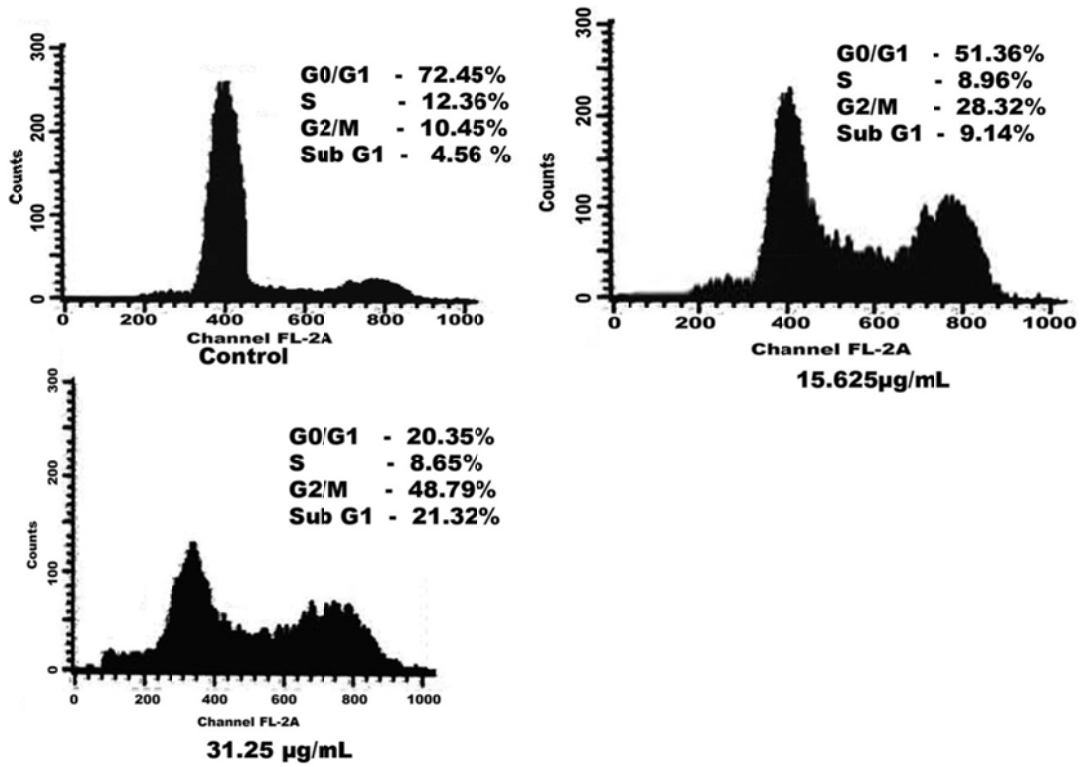

Figure 8. FACS analysis.

by alkaloids from *S. amaranthoides* in A549 cell lines. *S. amaranthoides* is well known plant for the different pharmacological activities which includes hepatoprotective, wound healing, antibacterial activity, anti-inflammatory and antioxidant activity. Cytotoxic activity of *S. amaranthoides* was not reported earlier. In the current report, the type of apoptosis and molecular changes occurred were reported. Depending on the current study the alkaloid fraction showed a good cytotoxic effect on A549 cell line with ic_{50}- 29.78 µg/ml. The alakloids are considered to be active against cancer cell *in vitro* when the IC_{50} is within the concentration range of 1-50 µM (Boik, 2001). Dose and time dependent (Figure 2) cytotoxicity of alkaloids on cancer cell lines were observed. This suggested that treatment with alkaloids inhibited the growth and reduced the viability of these cells. The induction of apoptosis has been described as a standard and best strategy in anticancer therapy (Russo et al., 2006; Hannun, 1997).

The phase-contrast microscopical studies help to identify the early stage of apoptosis which are characterized by the reduced size of the cells, blistering and blebbing of the nuclear membrane (Xu et al., 2004; Willingham, 1999). As seen in the dose and time-dependent treatment (Figure 8), cells get started to separate from the surface of the culture plates. Condensation of the nuclear chromatin is one of the apoptotic character that leads to breakup of the chromatin leading to nuclear fragmentation (Willingham, 1999).

In the present study the detection of early and late apoptosis was performed with dual staining. Based on the results, we have found that alkaloids induced apoptosis with a significant increase the concentration 29.57 µg/ml in A549 cell lines. This was proven with the signs of nuclear fragmentation and chromatin condensation. This can be further supported by the results of cell cycle distribution which showed the accumulation of cells in the S phase and the decrease of cell percentage in the G0/G1phase.

Accumulation of cells in S phase may have contributed to the high level of apoptosis in A549 cells (Pozo-Guisado et al., 2002). The cells blocked in the S phase are now a check point that inhibits the replication due the DNA damage which caused a decrease in cell survival (Bunch and Eastman, 1997; Shi et al., 2001). Hence the above data suggests that the alkaloids altered the cell cycle in a dose-dependent manner, and this could explain the observed correlation cell growth inhibition, cell cycle blockade and cell death (Arora et al., 2011).

Conclusion

The current study states that alkaloids possess strong inhibitory effects on cell growth and is capable of inducing apoptosis in A549 cells. Alkaloids also appear to affect the cell cycle which can induce apoptosis. The present findings provide valuable information in the development of natural compounds for use in cancer therapy.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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