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ANTIPROLIFERATIC EFFECT OF AQUEOUS EXTRACTS OF *ALOE VERA* LEAVES ON VERO CELL LINE

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ABSTRACT

Aloe vera is one of the most popular and widely studied plants for its medicinal properties. In the present study, the cytotoxic effect of aqueous extract of *Aloe vera* was investigated by incubation of different doses of aqueous extract for 24, 48 and 72 hours in vitro using *vero* cells. The present study resulted in cytotoxicity in time- and dose-dependent manner as indicated by MTT assay, fluorescent microscopy, flow cytometric analysis and DNA fragmentation assay. Fluorescent microscopy revealed typical morphological changes i.e. cell pyknosis, chromatin condensation. Nuclear fragmentation was not observed between treated and untreated cells. Flow cytometric profiles of PI-stained cells indicating amount of DNA degradation after treating cells with aqueous extracts at doses 5mg/ml and 10mg/ml for 48hr. DNA fragmentation revealed no detectable typical ladder pattern in vero cells treated with aqueous extracts. From the current study it can be concluded that the aloe extracts, even though showed significant cytotoxicity particularly at higher doses needs to be further investigated regarding its effect on normal cells in conjunction with tumor cells.

KEY WORDS: Aloe Vera, Aqueous Extract, Antiproliferatic, Vero Cell Line

INTRODUCTION

Plants have played a major role as a source of effective anticancer agents and it is significant that over 60% of currently used anticancer agents are derived in one way or another from natural source including plants, marine organisms and microorganisms (Cragg *et al.,* 2005). Several drugs currently used in chemotherapy are derived from the plants for example the vincaalkaloids like vinblastine and vincristine isolated from *catharanthus roseus,* etoposide and teniposide the semi synthetic derivatives of epipodophyllotoxin, isolated from species of the genus *podophyllum*, the naturally derived taxanes from the species of genus *taxus*, the semisynthetic derivatives of camptothecin, irinotecan and topotecan, isolated from *comptotheca acuminate*, and several others (Cragg *et al*., 1993; 1994; Wang, 1998). *Aloe vera* is also one among the various medicinal plants used for the treatment of various disease conditions ranging from dermatitis to cancer. Aloe gel's greatest use is as a skin ointment and for minor burns, abrasions, canker sores and other epithelial injuries. There is also evidence for its use as an antiviral, an ulcer remedy and an adjuvant cancer treatment due to its immune modulating effects. The plants which are screened for their antineoplastic activities should possess cytotoxicity and apoptotic effects on tumorigenic cell lines, while not causing cytotoxicity effects on normal cells and non tumorigenic cell lines. Therefore, the present study was conducted to study the cytotoxic effect of *Aloe vera* aqueous extracts on vero cell line.

MATERIALS AND METHODS

Cell Culture

Vero cell line was maintained in Glasgow minimum essential medium (Sigma, USA) with 10% new born calf serum (Sigma, USA), penicillin 100 IU/ml, streptomycin 100µ/ml. When cells were maintained by routine subculture

MTT Dye Reduction Test

Cell viability was assessed by the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay (Sigma, USA). Cells grown in 96-well plate for 24hours 200µl media with the extracts added per well, with triplicates for each concentration. Range of the concentration of extracts used from 100µg to 40mg/ml (i.e.100µg/ml, 200µg/ml, 500µg/ml, 1mg/ml, 2mg/ml, 5mg/ml, 10mg/ml, 20mg/ml, 30mg/ml, and 40mg/ml) incubated for 24, 48 and 72 hours at 37ºc inside the desiccators for carbon dioxide tension. At the end of each incubation 20µl solution of MTT in phosphate-buffered saline (PBS) (4 mg/ml) was added to each well and re-incubated for 4 hour. After incubation dark blue Formosan crystals was solubilized with the 200µl of acid isopropanol (0.04N HCL) absorbance measured at 570 nm wavelength using computerized Micro Scan ELISA Reader.

Fluorescent Microscopy

Cells were cultured in 6-well plates on cover slips, after 24 hr the cells treated with the extracts for 48 hr. After treatment time media was removed and cover slips washed with Phosphate buffer saline thrice. Cells stained with AO/EB for 5 min (Acridine orange -100µg in 1ml PBS, Ethidium bromide-100µg in 1ml PBS). After staining the cover slips washed with PBS thrice, cells fixed with 4% Para-formaldehyde for 20 min at room temperature. Then the cover slips washed with PBS thrice and mount cover slips using 50% phosphate buffered glycerol and finally observed under fluorescent microscope. AO determines nuclear morphology, while EB distinguishes cell viability.

Gel Electrophoresis of DNA

Extraction of DNA was followed from the method as described by Hermann *et al.,* (1994) with some minor modifications from the treated and untreated cells. DNA samples were subjected to agorose gel electrophoresis of 1.5 % agarose.

Flow Cytometry Analysis of Cells with Presidium Iodide Staining

After treating the cells with extracts, cells were harvested by centrifugation at 8000 rpm 5 min, cells washed with ice cold PBS (pH 7.4) and fixed in ice cold 70% ethanol (added drop wise) -20ºc over night. Pellet was washed twice with ice cold PBS and resuspended in 0.5 ml hypotonic buffer and incubated in the dark room for 15 min and then fixed cells analyzed by flow cytometer and data recorded in FL2 filter. Cells are analyzed by flow cytometer (Becton Dickinson, USA) and data recorded in FL1 filter.

RESULTS

MTT Dye Reduction Test

Aqueous extract caused growth inhibition through the doses of 5,10,20 and 40mg/ml. at time intervals of 24, 28 and 72 hr steadily showing inhibition time and dose dependent. (Table 1).

Fluorescent Microscopy

Effect of the extracts for the detection of typical morphological changes characteristic to the apoptotic cells i.e. cell pyknosis, chromatin condensation and nuclear fragmentation was studied by staining the extract treated cells with the fluorescent dyes acridine orange and ethidium bromide depicted in Figure 1(A &B) where there was no much changes were observed between treated and untreated cells. In untreated cells green fluorescent nucleus was prominent.

Flow Cytometry –PI Staining

Flow cytometric profiles of PI-stained cells indicating amount of DNA degradation after treating cells with water and ethanol extracts at doses 5mg/ml and 10mg/ml for 48hr, is depicted in (Figure 2 C & D) in untreated control 4.66% cells showed PI- fluorescence where as water extract treated cells showed 6.71% at 5mg/ml, and 11.15% at 10mg/ml and in case of ethanol treated cells 7.3% at 5mg/ml, and 13.60% at 10mg/ml cells exhibited fluorescence.

DNA Fragmentation Assay

DNA fragmentation analysis was done according to the method described by Hermann *et al* (1994). There was no detectable typical ladder pattern was observed in vero cells treated with extracts at doses,1.25mg/ml, 2.5mg/ml, 5mg/ml and 10mg/ml. DNA extracted after 48 hours treatment with the extracts (Figure 3).

DISCUSSIONS

Anticancer activity for the Aloe plant has been claimed for various compounds isolated from the plant. Lectin (Glycoproteins) like substances from leaves of *A.vera* and *A. saponaria* and a commercial aloe gel were shown to have haemagglutinating properties and fresh preparations also promoted growth of normal cells in culture but inhibited tumour cell growth (Winters *et al*., 1981). The commercial aloe gel showed an unspecific cytotoxicity (Brasher *et al.,* 1969). MTT assay indicates that the exposure of vero cells to aqueous extracts of aloe plant significantly inhibited the proliferation in dose as well as time dependent manner, higher the concentration of the extracts and longer the time of treatment on cells, the more significant cytotoxicity was achieved. It was observed that at doses from 50µg/ml to 2mg/ml maximum cytotoxicity at 72hr of incubation observed was 20% for both water extract and ethanol extracts. As the dose gradually increased the growth inhibition was also increased with the increased time of exposure to the extracts, Similarly Aloe-emodin- and emodin caused the cell death in a dose- and time-dependent manner of lung carcinoma cells (Hong-Zin Lee., 2001).

Fluorescent microscopic observation of by using acridine orange/ethidium bromide staining showed condensed chromatin, fragmented nuclei and apoptotic bodies. In the present study very few cells treated with the extracts at 5mg/ml showed apoptotic changes.

There was no detectable DNA fragmentation was observed upon treating the cells with extracts at doses 1.25mg/ml, 2.5mg/ml, 5mg/ml and 10mg/ml till 48 hr post exposure. This could attribute to that only little number of cells would have undergone apoptosis which is not detected by fragmentation assay. Flow cytometry is a sensitive and quantitative assay for analysis of apoptotic population and has been widely used (Nicoletti *et al.,* 1991; Zamai *et al*., 1993; Lund *et al.,* 2002). Flow cytometric analysis revealed increase in percentage of cells showing PI fluorescence when compared to control. The present results indicated that the increase in the dose of extracts also increases the percent of cells undergoing cell death .By using the flow cytometry, Sampedro *et al*., (2004) studied the effect of mannan a polysaccharide

obtained from A*loe saponaria, w*here it inhibited the proliferative ability of tumor cells but not affected the normal cells. Aloe emodin a hydroxy anthraquinone, isolated from aloe plant, induced apoptosis in promyelocytic leukemia HL-60 cells which is confirmed by both DNA fragmentation study and flow cytometry (Chen *et al.,* 2004). As with the correlation of results of flow cytometric analysis with the MTT assay, the high rate of cell growth inhibition observed in MTT assay might be due to cytotoxicity and cell death caused is may be due to non apoptotic cell death i.e. necrotic cell death. A study comparing the *in vitro* responses of carcinoma cells, immortalized and non tumorigenic epithelial S-G cells, and normal fibroblasts, to green and black tea extracts, noted that carcinoma and immortalized cells are more sensitive, in terms of growth inhibition, and apoptosis induction, than normal cells (Weisburg *et al*., 2004).

From the current study it can be concluded that the aloe extracts, even though showed significant cytotoxicity particularly at higher doses needs to be further investigated regarding its effect on normal cells in conjunction with tumor cells.

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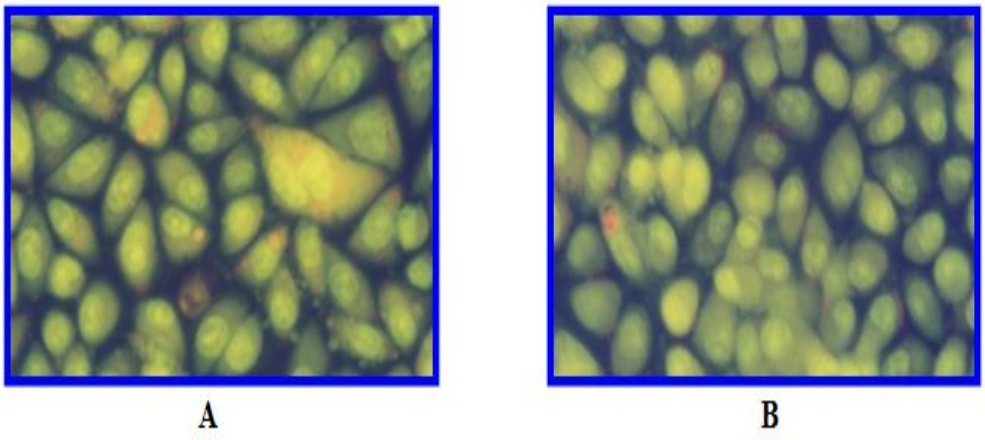
APPENDICES

Table 1: The Rate of Growth Inhibition of Vero Cells Incubated with Aqueous Extract of Aloe Vera at Different Doses and Time Intervals (mean ± SD)

Dose	Rate of Growth Inhibition		
	24h	48h	72h
5 mg/ml	0.815 ± 0.029*[a]	0.752 ± 0.02*	0.694 ± 0.013 *[b]
10 mg/ml	0.749 ± 0.031[a]	0.648 ± 0.023 [a]	0.478 ± 0.039*[b]
20 mg/ml	0.642 ± 0.027*[a]	0.398 ± 0.032 [a]	0.194 ± 0.012*[b]
40 mg/ml	0.442 ±0.033*[a]	0.198 ± 0.011*	0.116 ± 0.013*[b]

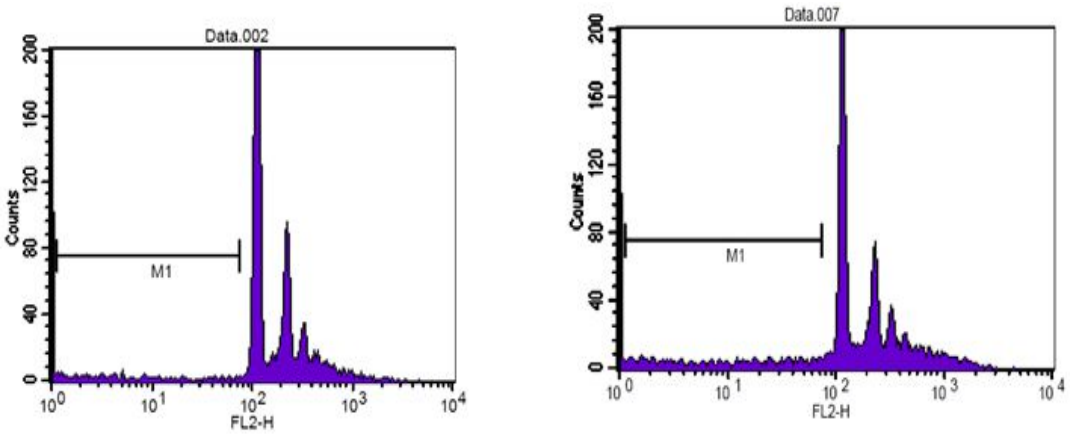
Values with superscript * vary significantly ($p<0.05$) between rows.

Values bearing dissimilar superscript (a/b) vary significantly ($p<0.05$) between column.



A: Control **B: Treated**

Figure 1: Vero Cells Stained with AO/EB, Observed Under Fluorescent Microscope



C: Untreated Cells, D: Treated (10mg/ml).

Figure 2: Flow Cytometric Analysis of Vero Cells Exposed for 48 Hours

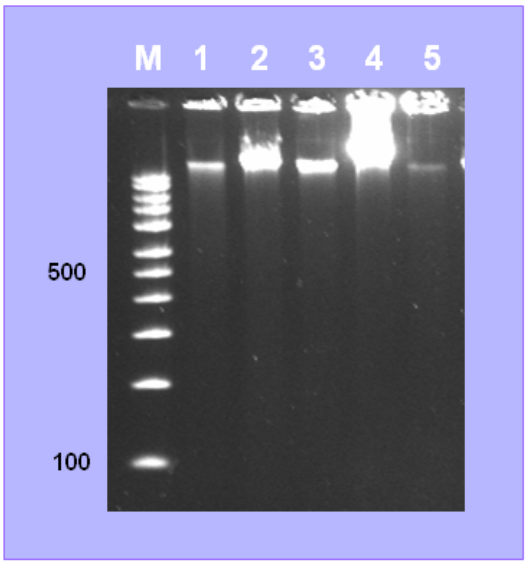


Figure 3: Agarose (1.5%) Gel Electrophoresis of Extracts

1: Negative control, 2: 1.5mg/ml, 3: 2.5mg/ml, 4: 5mg/ml, 5: 10 mg/ml,

M: Molecular weight marker