Induction of Apoptosis and Cytotoxic Activities of
*Apium graveolens* Linn. Using *in vitro* Models

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**Abstract:** Chemoprevention, which includes the use of synthetic or natural agents (alone or in combination) to block the development of cancer in human beings, is an extremely promising strategy for cancer prevention. *Apium graveolens* seeds have been assessed for chemopreventive activity. The antiproliferative effect of the methanolic extract of *Apium graveolens* was evaluated *in vitro* on two human cell lines (DLA, Dalton’s lymphoma ascites; L929, Mouse lung fibroblast). The morphological effects of *Apium graveolens* treated L929 cancer cells were observed under a fluorescence microscope. Typical morphological changes including cell shrinkage, chromatin condensation and characteristic DNA ladder formation in agarose gel electrophoresis were observed. Antitumor screening by the short-term cytotoxicity study with DLA cells showed that the extracts exhibited a dose dependent inhibition of the growth. The extract was found to be cytotoxic towards L-929 cells in 72 hrs MTT assay and concentration required for 50% cell death was 3.85µg/ml. This study confirms that methanolic seed extract of *Apium graveolens* possess cytotoxicity and provoke DNA fragmentation, a sign of induction of apoptosis. Thus *Apium graveolens* may be a potential candidate in the field of anticancer drug discovery.

**Key words:** Anticancer ♦ Apoptosis ♦ Cytotoxic ♦ MTT assay ♦ *Apium graveolens*

**INTRODUCTION**

The search for anti-cancer drugs from plant sources started in earliest in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine and the isolation of the cytotoxic podophyllotoxines. These discoveries prompted the United States National Cancer Institute (NCI) to initiate an extensive plant collection program in 1960, focused mainly in temperate regions. Plant derived natural products such as flavonoids [1], terpenes [2], alkaloids [3] etc. have received considerable attention in recent years due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive effects [4]. India is a rich source of medicinal plants and a number of plant extracts are used against diseases in various systems of medicine such as Ayurveda, Unani and Sidha. Only few of them were scientifically explored.

*Apium graveolens* is a biennial with stems 0.3-2.4 m, erect and branching, belonging to the family Apiaceae, native to foot of north western Himalayas and outlying hills in the Punjab, Afghanistan, which is traditionally used for in ophthalmia, bronchitis, vomiting, hiccough, rectal troubles, ascites, eructations, abdominal pain and toothache. The seed contain 1.5-3% volatile oil (containing the limonene, terpenes, b-selinene and phthalides); coumarins (seselin, ostheno, apigravin, celerin, umbelliferone); furanocoumarins (including bergapten), flavonoids (apigenin, apiin), phenolic compounds [5]. This plant has reported antimicrobial [6], adulticidal, larvicidal, repellent [7] and hepatoprotective activity [8]. The present paper deals with the evaluation of *Apium graveolens* as a cancer chemopreventor.

**MATERIALS AND METHODS**

**Chemicals:** Streptomycin, Penicillin, Ambistyrin, NTE buffer, Trypsin, PBS, Sodium Decyl Sulphate were purchased from Himedia Laboratories Pvt. Ltd. (India).
Dulbecco’s Modified Eagle Medium (DMEM) and 3-(4, 5- Dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma chemical company (St. Louis, MO, USA). DLA (Dalton’s lymphoma ascites) and L929 (Lung fibroblast) cell lines were obtained from Amala Cancer Research Institute (Kerala, India).

Collection of Plant material: Seeds of *Apium graveolens* Linn. Were collected from Institute of Horticulture, Ooty, Tamilnadu district, India during the month of June 2010. The plant material was identified and authenticated by Mr. G. V. S Murthy, Joint Director, Scientist, C-I/C, Botanical survey of India, Tamil Nadu Agricultural University Campus, Coimbatore bearing the reference number BSI/SRC/5/23/10-11/Tech – 730.

Extraction of Plant Material: The fresh seeds of *Apium graveolens* Linn. Were collected, dried in shade under room temperature, powdered mechanically and sieved through no. 40 mesh sieve. Powdered dried seeds (250g) were extracted in round-bottomed flask with 1000 ml petroleum ether (60–80°C) and then repeatedly with methanol using a Soxhlet apparatus. Then, methanol extract was recovered and evaporated to dryness by distillation under reduced pressure in rotatory evaporator. The seed extract was utilized as test material for *in vitro* anticancer activity testing.

Cell Lines: Dalton’s lymphoma ascites (DLA) cells were used for short term *in vitro* cytotoxicity experiments. This cell lines were maintained as ascites tumors in Swiss albino mice. The cells were aspirated, washed thrice in normal saline counted using a haemocytometer and cell suspension of 1 million cells/ml was prepared. One ml of this suspension was injected into peritoneal cavity of Swiss albino mice. Mouse L929 cell lines (Lung fibroblast) were used for long term *in vitro* cytotoxicity experiments and DNA fragmentation assay.

Trypan Blue Dye Exclusion Assay: The viability of cells was checked using trypan blue assay (cell viability should be above 98%) [9]. The cell suspension was added to tubes containing various concentrations of the test compounds and the volume was made up to 1ml using phosphate buffered saline (PBS). Control tubes containing only cell suspension. These assay mixtures was incubated for 3hrs at 37°C and then 1ml of trypan blue was added after incubation and the number of dead cells was counted using a haemocytometer. Only nonviable (dead) cell accepted the blue stain but not the viable cells. Number of viable cells (like water droplets) and non viable cells were counted. The percentage cytotoxicity was calculated using the formula.

\[
\text{% cytotoxicity} = \frac{\text{No. of dead cells}}{\text{No. of viable cell + No. of dead cell}} \times 100
\]

Microculture tetrazolium (MTT) assay: The effect of *Apium graveolens* on cells was determined with MTT (3-(4, 5- dimethylothiazol-2-yl)-2-5- diphenyltetrazolium bromide) assay [10]. Anticancer drug/ cytotoxic compounds damage the cancerous cells and change the mitochondrial activity. When tetrazolium salts comes in contact with the cancerous cells, salts are taken up into the mitochondria due to its net positive charge and plasma membrane potential. In mitochondria, these coloured tetrazolium salts get reduced to formazan dye by NADH+ dependent reaction catalyzed by the Mitochondrial Succinate Dehydrogenase Enzymes (MSDE). As this conversion takes place only in the living cell mitochondria, the amount of formazan formed is proportional to the number of living cells. Hence quantification of viable cells is made possible by quantification of formazan at 570 nm by a spectrophotometer [11-14]. Cells were seeded in a 96-well flat-bottom plate (5000 cells/well) and permitted to adhere for 24hrs at 37° with 5% CO₂ atmosphere. Different drug concentration was added and incubated further for 48hrs. Before 4hrs of the completion of incubation, 20µl of MTT (5mg/ml) was added. Dead cell percentage was determined using an ELISA plate reader set to record absorbance at 570nm. The percentage growth inhibition was calculated using the formula given below.

\[
\text{% Growth inhibition} = 100 - \frac{\text{OD of individual test group}}{\text{OD of Control Group}} \times 100
\]

DNA Fragmentation Assay: Prepare DMEM medium (912mg/100ml) containing benzyl penicillin 1mg/litre, Ambistyrine (1mg/litre) adjust the pH to 7.0 with sodium bicarbonate (colour deep yellowish red: when pink colour comes do not use the medium). To the medium add 2 X 10⁶ L929 cells (suspended in minimal volume), 2µl trypsin and different drug concentration and then make the final volume to 2ml. Incubate at 37°C for various hours (from 1-24 hrs). After incubation centrifuge the cells at 10,000 rpm for 10mins, discard the medium and wash the cells for 2 times in NTE buffer. Suspend the cells in 2ml NTE buffer and 2% trypsin (100µg/ml) and add 20% SDS
(25µl/ml) and proteinase K (100µg/ml). Incubate the cells at 37°C for overnight. Add 1ml NTE buffer saturated phenol and 1ml chloroform and shake the vial 12 times (turning up and down slowly) centrifuge at 10,000 rpm for 10 min (2 phases are seen) and transfer the upper portion to another vial and add 1ml chloroform, repeat this for 4 times. To this RNase was added and incubated at 35°C for 2hrs. Centrifuge the vial at 10,000rpm for 10mins and decant the solvent and take the pellet (DNA) and dissolve in TAE buffer. Then the dissolved DNA is subjected to horizontal electrophoresis [15, 16].

**Data and Statistical Analysis:** All values obtained were expressed as means ± standard error of the mean. Results were subjected to one-way analysis of variance (ANOVA) to assess treatment differences. Significant differences between means were determined at P<0.01.

### RESULTS AND DISCUSSION

Table 1. indicates the results of trypan blue dye exclusion technique and it showed that a dose dependent inhibition of the growth of DLA cells. *Apium graveolens* exhibited 81.95% cytotoxicity at 200µg/ml towards DLA cells. The concentration needed for 50% inhibition of growth of DLA cells was found to be 29.79µg/ml. Curcumin was used as the reference drug and it produced 100% cytotoxicity at 100µg/ml and 200µg/ml and CTC50 value obtained was 14.75µg/ml.

Cytotoxic effect on L929 cells was investigated by MTT assay. Cells were treated with *Apium graveolens* at concentrations ranging from 2-20µg/ml for 48hrs and then the percentage of cell viability was analysed. The plant extract is significantly (P<0.01) inhibited the proliferation of L929 cells in a dose dependent manner (Table 2). The CTC50 of the *Apium graveolens* and curcumin was found to be 10.86µg/ml and 1.70µg/ml, respectively on L929 cell lines.

Microscopical examination of *Apium graveolens* treated L929 cells (Fig.1) revealed that the cell death is due to apoptotic characteristics. It shows characteristic morphology when a cell undergoing apoptosis. Apoptotic cells where distinguished as highly shrunken cell, nuclear condensation, fragmentation, margination, cell blebbing and presence of apoptotic bodies.

Fig. 2 showed the result of electrophoretic run of *Apium graveolens* treated L929 cells exhibited extensive double strand breaks; thereby yielding a ladder appearance was shown in Fig. (2) Of lane 3 and 4 for *Apium graveolens*, while the DNA isolated from fresh L929 cells did not show any double strand breaks in Fig (2) of lane 2 for *Apium graveolens*. The DNA isolated from L929 cells treated with 90µg/ml and 100µg/ml *Apium graveolens* showed fragmentation.

The majority of the cancer treatments are accompanied by a degree of herbal supplements. There are advantageous effects of medicinal plants on cancer [17]. Natural products discovered from medicinal plants have played a vital role in the management of cancer. Natural products or natural product derivatives consist of 14 of top 35 drugs in 2000 based on worldwide sales [18]. Plant based medication has definitely found a role in cancer healing (chemotherapy) and the mechanism of interaction between many phytochemicals and cancer cells has been studied extensively.

The present study was undertaken to assess the cytotoxic activity of AGSE. Chemoprevention, which consists of the use of synthetic or natural agents (alone or in combination) to block the development of cancer in human beings, is an extremely promising strategy for cancer prevention [19]. In vitro confirmation of the extract’s toxicity was done on DLA cell lines.
Fig. 1: Morphological changes and the number of apoptotic nuclei in L929 cells after treatment with *Apium graveolens* for 48 hrs. Fig (a) showing cells begin to shrink and because of break down of proteinacious cytoskeleton rounding are shown. Fig (b) and (c) of *Apium graveolens* showing vacuolation, elongation and margination to one side and DNA fragmented into several chromatin bodies.

Fig. 2: DNA fragmentation in methanolic bark extract of *H. excelsum* with L929 cells for 48 hr of incubation.

In our study, the methanolic bark extracts of *Apium graveolens* in plant constituents may cause cell growth inhibition and induce apoptosis differentially in cancer cells. Apoptosis is a well identified biological response exhibited by cells after suffering DNA damage and is a useful marker for screening compounds for subsequent development as possible anticancer agents [20]. In this study DLA and L929 cancer cells were treated with methanolic seed extract of *Apium graveolens*. The cell growth inhibitory effects of this plant crude extract support to exert their anti-cancer effects in vitro.

We observed *Apium graveolens* inhibits growth of DLA cells in a dose dependent manner and maximum growth inhibitory effects (72.59%) on L929 cancer cells at 20µg/ml. Further studies confirmed that, the cytotoxic potential of *Apium graveolens* is closely associated with chromatin condensation, one of the well markers for apoptosis. The loss of chromatin integrity is often induced by activated caspases. In addition, apoptotic characteristic DNA strand-breaks were observed by means of gel electrophoresis. This induction of apoptosis in cancer cells that make them more render for host phagocytic clearance without initiating inflammation, could be attributed for the extracts tumoricidal activity.

Our present investigations have demonstrated that there has been a growing interest in the alternative medicine and the therapeutic properties of the natural products derived from plants in the recent years. Based on the evaluation done using the various in vitro assay models it may be concluded that *Apium graveolens* Linn. possess anticancer activity. Methanolic extracts of seeds of *Apium graveolens* Linn. is moderately active for the treatment of cancer. Further pharmacological study using other cancer cells is necessary in order to establish whether these plants can be used as a potential source for new anticancer medicine.

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