Anti-proliferative and apoptotic properties of a peptide from the seeds of *Polyalthia longifolia* against human cancer cell lines

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The peptides produced enzymatically from various plants have shown various biological activities including cytotoxicity. Different types of cytotoxic peptides have been reported from the seeds and leaves of Violaceae, Rubiaceae and Annonaceae families. In this study, we report purification and characterization of peptide(s) showing cytotoxic activity against A549 and HeLa cancer cell lines from the seeds of *Polyalthia longifolia* (Annonaceae). Seed proteins of *P. longifolia* were extracted and hydrolyzed using trypsin. The enzyme hydrolysate was applied on to a Sephadex G10 column and eluted using Tris-HCl buffer (pH 7.5). Two fractions F1 and F2 were obtained, of which F2 showed significant cytotoxic activity against lung (A549) cancer cells at 10 µg/mL and cervical (HeLa) cancer cell lines at 30 µg/mL, as revealed by the MTT assay. DNA fragmentation was observed in the tested cancer cell lines treated with F2 peptide at a concentration of 10 µg/mL and 30 µg/mL, respectively. Further, increased number of apoptotic cells was observed in sub-G0 phase of cell cycle of A549 and HeLa cell lines, when treated with 10 µg/mL and 30 µg/mL of F2, as revealed by the flow cytometric analyses. FTIR spectrum of F2 peptide detected the presence of stretching vibrations of carboxylic acid OH residue with peak at 3420 cm\(^{-1}\) and carbonyl (C=O) groups at 1636 cm\(^{-1}\), respectively. RP-HPLC analysis of F2 peptide showed a single peak at a retention time of 12.8 min detected at 280 nm, depicting the purity of F2 to be more than 90%. LC-ESI-MS/MS analysis showed the average theoretical mass of F2 to be 679.8 using m/z ratios. In conclusion, the findings suggest that F2 peptide is an effective inducer of apoptosis of cancer cells, thus offers an important strategy in the development of cancer therapeutics.

Keywords: Cytotoxicity, Flow cytometer, LC-ESI-MS/MS analysis, MTT assay.

Plant-derived compounds and their semi-synthetic as well as synthetic analogs serve as major source of pharmaceuticals for human diseases. The development of more selective agents focused on targeted delivery of imaging probes and drugs to different tumour sites is the current trend in cancer diagnosis and therapies. In the cancer drug discovery program, a paradigm based on ethno botanical and ethno pharmacological data is beneficial for identifying potential anticancer molecules than mass screening of plant species.

Plants containing pharmacologically active polypeptides have been used in natural product-based drug discovery programs. Peptides are small amino acid sequences that can be isolated to bind to a predetermined target and are potentially capable of inhibiting individual signalling components, which are essential in cancer development and progression. Peptides allow structural changes to incorporate protective substitutions, chiral derivatives, non-natural amino acids and other modifications aiming at increased stability, efficiency and resistance to proteolysis. Studies have shown that proteins and peptides from plants exhibit marked inhibitory effects on the proliferation of various tumour cell lines, including murine leukaemia, lung carcinoma, cervical carcinoma, rat osteoblast like sarcoma, human nasopharyngeal carcinoma and ovarian neoplasm. In recent years, plant peptides with cytotoxic activity have gained the potential for research towards the development of anticancer drugs.

Lunasin is a novel and promising chemopreventive peptide derived from soybean seeds. The cancer preventive properties *in vitro* make lunasin a perfect candidate to study an *in vivo* cancer-preventive activity. In search of natural sources of lunasin besides soybean, the isolation and bioassay of lunasin from barley and wheat have also been reported. Recently, a partially purified peptide from *Euphorbia hirta* Linn. with molecular mass less than 3 kDa, has been found to possess significant cytotoxicity against KATO-III, a gastric carcinoma cell line. Also, the peptides derived from the high oleic acid...
soybean meals have shown cell growth inhibition of human colon (HCT-116, Caco-2), liver (HepG-2) and lung (NCL-H1299) cancer cell lines. 

Annonaceae plants are a rich source of bioactive substances, such as flavonoids, alkaloids and acetogenins, extracted from the seeds and other parts of these plants with antitumor properties. Many cyclic peptides have been isolated from the species of Annona genus, including A. glabra and A. squamosa.

Polyalthia longifolia chinensis Benth. & Hook. F, commonly known as Devdaru is a tall tree native to India. The bark of P. longifolia (Annonaceae) is traditionally known to lower blood pressure, stimulate respiration and treat skin diseases, diabetes and hypertension. In addition to antimicrobial compounds, cytotoxic constituents, such as a halimane diterpene-3β, 5β, 16α-trihydroxyhalima-13(14)-en-15, 16-olide (1) and an oxoprotoberberine alkaloid-8-oxopolyalthiaine (2) have been isolated from the methanolic extract of P. longifolia. In the present study, we report purification and characterization of peptide(s) from the seeds of P. longifolia, which show cytotoxic activity against A549 and HeLa cancer cell lines.

Materials and Methods

Materials

Seeds of Polyalthia longifolia were collected in the month of May and authenticated by Dr. P. Jayaraman, from Plant Anatomy Research Centre, Chennai. Buffers used for FPLC and HPLC analysis were of analytical grade and were procured from Sigma-Aldrich, St.Louis, MO, USA. Tumour cell lines A549 (human lung adenocarcinoma epithelial cell line) and HeLa (human cervical adenocarcinoma epithelial cell line) used for cytotoxicity assay were procured from National Centre for Cell Science, Pune.

Protein extraction and purification

Seeds were washed with distilled water and shade-dried. The dried seeds were ground to fine powder and the proteins were extracted with extraction buffer (10 mM Na2HPO4, 15 mM NaH2PO4, 10 mM KCl, 2 mM EDTA, pH 7.0) by stirring overnight at 4°C. The crude protein extract was filtered through a 0.45 µm filter and used for further study. Ammonium sulphate was added to a concentration of 80% saturation in the filtrate and incubated overnight at 4°C. The precipitate was centrifuged at 12,000 rpm for 20 min and the pellet was dissolved in Tris-HCl buffer (pH 7.5). The protein concentration was determined according to Lowry et al using bovine serum albumin as the standard. The pellet containing the trypsin hydrolysate was applied on to fast protein liquid chromatography (FPLC, Akta purifier GE Healthcare) using Sephadex G-10 gel filtration column (1.5 x 50 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The peptide fractions F1 and F2 were eluted using the same buffer at a flow rate of 0.1 mL/min.

Evaluation of cytotoxic activity

The isolated peptides were further used for cytotoxicity activity against lung (A549) and cervical (HeLa) cancer cell lines. Vero (green monkey kidney) cells which are the normal growing cells were used as non-cancerous control cells to examine the inhibitory action of the peptide fraction (F2) in the in vivo system. All the cells (Vero, A549 and HeLa) used for this study were cultured in Dulbecco’s modified eagle medium (GIBCO), supplemented with 10% fetal bovine serum (GIBCO) and 1% antibiotic antimycotic solution at 37°C in a humidified atmosphere of 5% CO2. The A549 cells were seeded at a density of 3500 cells/well, while HeLa and Vero cells were seeded at a density of 3000 cells/well in a 96 well microtitre plates in a total volume of 200 µL/well.

The isolated peptide fractions F1 and F2 were added at a concentration ranging from 10-1000 µg/mL and the cells were incubated for 24 h. The morphology of treated cells was observed using a phase contrast microscope (Leica, Wetzlar, Germany). MTT assay was also performed to evaluate the cytotoxic activity of peptide fractions F1 and F2. To each of the wells, 50 µL (5 mg/mL) of 0.5% MTT was added and incubated for 4 h. The formazan crystals formed were dissolved in dimethyl sulfoxide (DMSO) and the absorbance was read at 570 nm using a microplate reader (Bio-Rad, CA). Seeded wells without added peptides served as blanks. Doxorubicin (Sigma) was used as positive control. Untreated A549 and HeLa cells were used as control. The percentage inhibition of cell viability was determined by using the formula:

\[ \frac{A_{\text{control}} - A_{\text{treatment}}}{A_{\text{control}}} \times 100 \]

where ‘A’ denotes the absorbance read at 570 nm.

The trypsin hydrolysate was filtered through 0.45 µm filter and used for further study. Ammonium sulphate was added to a concentration of 80% saturation in the filtrate and incubated overnight at 4°C. The precipitate was centrifuged at 12,000 rpm for 20 min and the pellet was dissolved in Tris-HCl buffer (pH 7.5). The protein concentration was determined according to Lowry et al using bovine serum albumin as the standard. The pellet containing the trypsin hydrolysate was applied on to fast protein liquid chromatography (FPLC, Akta purifier GE Healthcare) using Sephadex G-10 gel filtration column (1.5 x 50 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The peptide fractions F1 and F2 were eluted using the same buffer at a flow rate of 0.1 mL/min.

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DNA Fragmentation analysis

Based on the results obtained in the MTT assay, DNA fragmentation analysis was carried out to evaluate the mechanism of cell death in cancer cell lines treated with peptide fraction F2. The A549 cells were seeded at a density of 3500 cells/well and HeLa cells of 3000 cells/well in a 6-well titre plate containing 2 mL of minimal essential medium and incubated at 37°C for 48 h in a CO2 incubator with 5% CO2. A549 and HeLa cells were treated with F2 fraction at a concentration of 10 µg/mL and 30 µg/mL and incubated for 24 h in 5% CO2 incubator. The media was discarded, followed by the addition of 1 mL of TPVG (trypsin, PBS, versene, and glucose) and 1mL of phosphate buffered saline (PBS) to each well. The contents of wells were collected and DNA isolation was performed using DNA isolation kit (Genei, India). The pattern of bands for DNA fragmentation was visualized on 1% agarose gel under UV light and photographed.

Cell cycle analysis

A549 and HeLa cells were seeded in 6-well culture plates at a density of 0.3 × 10⁶ cells/well and incubated at 37°C, 5% CO2 in an incubator for 24 h. The cells were then treated with 10 µg/mL and 30 µg/mL of F2, respectively and incubated at 37°C, 5% CO2 for 24 h and 48 h. The treated cells were trypsinized after 24 h and 48 h, washed with fresh medium and centrifuged at 1000 rpm for 5 min at 4°C. The supernatant was discarded and the cells were suspended in 300 µL of PBS and 700 µL of ethanol. The cells were then washed and centrifuged again at 1500 rpm for 10 min at 4°C. The supernatant was discarded and cells were resuspended in 600 µL of PBS containing 0.5% Triton X-100, 20 µg of RNase and incubated at room temperature for 1 h. After incubation, 40 µg/mL of propidium iodide (1 mg/mL stock) was added to the wells and incubated at room temperature for 45 min in dark. The cells were observed in a flow cytometer (FACSCalibur, Beckton Dickinson), equipped with an air cooled argon laser providing 15 mW at 488 nm (blue laser) with standard filter setup. About 10,000 events were acquired and the percentage of each phase of the cell cycle was analyzed using Cell Quest Pro software (Becton Dickinson, USA).

FTIR analysis

Identification of functional groups of F2 fraction was performed using Shimadzu Fourier transform infrared spectrophotometer. The peptide fraction was homogenized with KBr in a mortar and pestle. The sample mixtures were then pressed by vacuum hydraulic at 1.2 psi to obtain a transparent pellet. The pellet was scanned in the infrared absorption region between 400 cm⁻¹ and 4000 cm⁻¹ with a resolution of 4 cm⁻¹.

Reverse-phase HPLC analysis

The purity of peptide fraction F2 was tested using HPLC analyses performed on an Agilent series 1100 HPLC system fitted with a reversed-phase high performance liquid chromatography (RP-HPLC) cartridge, C₁₈ column (250 x 4.6 mm, 5 µm, 300 Å), with solution A - 0.1% trifluoro acetic acid (TFA) and solution B - Acetonitrile (90%) as the mobile phase at a flow rate of 1 mL/min and detected at 280 nm.

Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS/MS) analysis

The purified F2 fraction was dissolved in 50% methanol and 50% water of HPLC grade, infused into a Finnigan TSQ Quantum ultra AM mass spectrometer (Thermo, USA) at a flow rate of 30 µL per min, operated in the positive electrospray ionization (ESI + ve) mode via the electrospray interface.

Statistical analysis

The results were expressed as mean ± S.E.M. Comparisons between treated groups and control were performed with the Dunnett’s multiple comparison test using Graph Pad Prism software.

Results and Discussion

Purification and characterization of peptide

The protein content of crude supernatant from the seeds of P. longifolia was found to be 4.27 mg/mL, as determined according to Lowry et al. The peptide fractions obtained from the trypsin hydrolysate of crude supernatant were precipitated up to 80% saturation limit with ammonium sulphate and further purified using Sephadex G-10 gel filtration chromatography. Two peptide fractions, namely peaks 1 and 2 designated as F1 and F2, respectively were eluted from Sephadex G-10 chromatography (FPLC, Aktaand GE) and detected at 280 nm (Fig. 1). Two mL of each fraction was collected and tested for cytotoxic activity against A549 and HeLa cells.

Cytotoxic activity of peptides

Cytotoxicity is one of the chemotherapeutic hallmarks of anticancer activity. MTT assay is a well-established in vitro model for cytotoxicity
against cancer cell lines and is used as one of the conventional methods for the screening of compounds with potential anticancer properties. We used MTT assay to screen the in vitro anticancer activity of the purified peptide fractions using A549 and HeLa cell lines.

The F1 fraction eluted at retention time of 25 min didn’t show cytotoxic effect on selected cell lines. However, fraction F2 eluted at retention time of 50 min showed significant inhibition on the proliferation of A549 (Fig. 2A) and HeLa (Fig. 2B) cells treated with a concentration ranging from 10-1000 µg/mL (Table 1), warranting further characterization of F2 fraction.

Morphological changes showing the apoptotic phenotype was observed in A549 (Fig. 2A) and HeLa cells (Fig. 2B), where the nucleus was degenerated into discretely spherical fragments of

Table 1—Growth inhibitory effects of peptide (F2 fraction) from seeds of *P. longifolia* against human cancer cell lines along with positive control

<table>
<thead>
<tr>
<th>Sample conc. (µg/mL)</th>
<th>F2 peptide A549</th>
<th>HeLa</th>
<th>Vero</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>58.33 ± 6.46****</td>
<td>29.89 ± 1.98****</td>
<td>10.01 ± 1.06****</td>
</tr>
<tr>
<td>30</td>
<td>69.36 ± 3.77****</td>
<td>51.12 ± 0.39****</td>
<td>11.24 ± 1.77****</td>
</tr>
<tr>
<td>100</td>
<td>78.34 ± 1.33****</td>
<td>74.85 ± 0.89****</td>
<td>14.35 ± 1.13****</td>
</tr>
<tr>
<td>300</td>
<td>77.12 ± 3.36****</td>
<td>78.09 ± 1.69****</td>
<td>16.12 ± 0.36****</td>
</tr>
<tr>
<td>1000</td>
<td>80.91 ± 3.86****</td>
<td>82.32 ± 1.35****</td>
<td>19.21 ± 0.86****</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>52.82 ± 1.15****</td>
<td>46.38 ± 0.43****</td>
<td>40.32 ±1.15****</td>
</tr>
<tr>
<td>0.3</td>
<td>70.92 ± 2.81****</td>
<td>55.85 ± 1.56****</td>
<td>51.83 ± 1.81****</td>
</tr>
<tr>
<td>1</td>
<td>85.17 ± 1.37****</td>
<td>62.10 ± 1.65****</td>
<td>75.17 ±1.35****</td>
</tr>
<tr>
<td>3</td>
<td>87.35 ± 4.33****</td>
<td>84.89 ± 2.97****</td>
<td>89.72 ± 0.24****</td>
</tr>
<tr>
<td>10</td>
<td>99.11 ± 0.53****</td>
<td>99.54 ± 0.46****</td>
<td>92.37 ± 0.82****</td>
</tr>
</tbody>
</table>

***p < 0.0001, **p < 0.001 and *p < 0.01 indicate significant difference compared to positive control.

Fig. 1—Size-exclusion chromatogram (Sephadex G-10) of the peptides obtained by trypsin digestion from the seeds of *P. longifolia*

Fig. 2—Photomicrographs (40x) of A549 cells (A) and HeLa cells (B) treated with different concentrations of peptide (F2 fraction) ranging from 10-1000 µg
highly condensed chromatin, suggesting that fraction F2 induced A549 and HeLa cell apoptosis. Further, A549 and HeLa cells were also treated with doxorubicin, which served as a positive control with concentration ranging from 0.1-10 µg/mL (Table 1). Addition of peptide (F2) showed 58% growth inhibition of A549 cells at 10 µg/mL and 51% inhibition of proliferation of HeLa cells at 30 µg/mL, depicting the cytotoxic effect of the peptide F2, when compared with the cell control (untreated A549 and HeLa cells), showing 100% cell growth. Further, Vero cells exhibited negligible inhibition when treated with F2 at 10-1000 µg/mL, indicating the absence of cytotoxic effect of F2 in Vero cells (Table 1).

The results obtained in this study were in agreement with the apoptotic activity of a small peptide (with a molecular mass of 1,050 Da) purified from Cycas revoluta seeds against human epidermoid cancer (Hep2) and colon carcinoma cells (HCT15)27. Similarly, microcolin A, a linear peptide isolated from Lyngbyama juscula is found to inhibit cell proliferation via the induction of cell apoptosis, suggesting it as potentially active against human cancer cells28. Previous studies have also reported the cytotoxic effect of vitri A, varv A, varv E peptides isolated from Viola tricolor29 and vphi A peptide from Viola philippica30 of Violaceae family against MM966, HeLa and BGC-823 cells.

A novel anticancer peptide derived from rice bran enzymatic hydrolysate is found to possess growth inhibitory properties on colon, breast, lung and liver cancer cells31. Dianthin peptides, isolated from Dianthus superbus have shown potent cytotoxic effect against the Hep G2 cancer cell line32. Kahalalide O, a cyclopeptide from the Sacoglossan Elysiaornata is reported to possess significant inhibitory effects on P-388 cells and human lung cancer A549 cells33. A tridecapeptide from Papaver somniferum inhibits the proliferation of human liver (Bel-7402) and mammary gland cancer cell lines (MCF7)34.

DNA fragmentation analysis

Apoptosis, a kind of cellular death is characterized by the early activation of endogenous proteases, cell shrinkage and DNA fragmentation35. The nuclear DNA of apoptotic cells shows a characteristic laddering pattern of oligonucleosomal fragments36. In this study, the effect of the purified F2 fraction on the induction of apoptosis in A549 and HeLa cells was analysed. Agarsose gel electrophoresis of DNA isolated from A549 cells treated with 10 µg/mL concentration of F2 peptide (Fig. 3A, lane 1) showed a ladder pattern, indicating cell death due to DNA fragmentation. Similar fragmentation pattern of DNA was observed with HeLa cells treated with 30 µg/mL of F2 peptide (Fig. 3B, lane 1). These results were similar with the previous study which reported DNA fragmentation, indicating the cell death of the nuclear DNA of HeLa cells treated with isolated proteins from the leaves of Mirabilis jalapa at IC50 value of 0.65 mg/mL37.

Effect of cytotoxic peptide on A549 and HeLa cell cycle

Cell cycle modulation by various anticancer agents from plants is gaining widespread attention in recent years. A number of plant peptides have shown the ability to induce apoptosis and play an important role in cancer prevention and therapy. The percentage of cells in each phase of cell cycle in A549 and HeLa cells treated with the peptide F2 was determined by flow cytometry38. The sub-G0 group of A549 cells treated with F2 at 10 µg/mL depicted increased apoptotic cell count as 2.03% and 2.89% at 24 h and 48 h, respectively (Fig. 4A). Similarly, an increased number of apoptotic cells were observed in Sub-G0 group of HeLa cells treated with F2 at 30 µg/mL as 2.41% and 3.43% for 24 h and 48 h (Fig. 4B). These results suggested that F2 caused apoptotic-associated chromatin degradation in both the tested cancer cell lines, as revealed by the increase in the number of apoptotic cells in Sub-G0 phase from 24 to 48 h, when compared with Sub-G0 phase of cell cycle in control cells of both the cancer cell lines, indicating
the non-apoptotic population. Our findings in this study were in accordance with the anti-proliferative activity against human lymphoma cell (U937) of peptide fraction from anchovy sauce at 500 µg/mL.

Identification of functional groups by FTIR analysis

The FTIR spectrum of F2 showed the bands of O-H stretching vibrations at 3420 cm$^{-1}$, confirming the presence of carboxylic acid OH residue in the peptide (Fig. 5). Appearance of similar peak bands at 3379 cm$^{-1}$ assigned to O-H stretching vibrations indicated the presence of hydroxyl groups associated with proteins in Ananus comosus peel. The peak at 1636 cm$^{-1}$ in the F2 indicated the presence of C=C and carbonyl (C=O) stretching vibration. Some studies have reported that amino acids, peptides and proteins display IR absorption bands in the range of 800-2500 cm$^{-1}$ and such bands include N-H and C=O.

RP-HPLC analysis

RP-HPLC analysis of cytotoxic F2 fraction showed a single peak (Fig. 6), indicating the presence of...
single peptide with a retention time of 12.8 min detected at 280 nm.

**Identification of peptide by LC-ESI-MS/MS**

The cytotoxic peptide (F2) was subjected to LC-ESI-MS/MS analysis to identify the peptide ions obtained from the tryptic digest. The MS/MS spectrum of a single charged ion with m/z at 679.8 is illustrated in Fig. 7, depicting the fragmentation spectrum containing major ions at m/z 100.4, 122.4, 182.0, 209.3 and 326.4, respectively. Similar studies have demonstrated that the molecular weight, amino acid type and sequence of peptides play a key role on the biological activity of the peptides.

In conclusion, a cytotoxic peptide F2 was isolated from the seeds of *Polyalthia longifolia* with average mass of 679.8 which showed significant anti-proliferative activity against lung (A549) cancer cells at concentration of 10 µg/mL and cervical (HeLa) cancer cell lines, at 30 µg/mL, respectively. However, F1 fraction did not exhibit any cytotoxic activity against the tested cell lines. Besides, F2 did not show any inhibitory effects on the proliferation of Vero cells. Further, treatment of A549 and HeLa cells at 10 µg/mL and 30 µg/mL with F2 peptide resulted in DNA fragmentation, a significant feature of apoptotic cell death. Morphologic changes in cellular nuclei revealed that F2 induced A549 and HeLa cell apoptosis. Thus, the results suggest that F2 peptide is an effective inducer of apoptosis of cancer cells, which offers an important strategy to create new anticancer therapeutics with promising potential in clinical practice.

**References**

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