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Original Article

ANTI-CANCER ACTIVITY OF PHENYL-1,3,5-HEPTATRIYNE IN HUMAN LIVER CANCER

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ABSTRACT

Bidens pilosa, commonly known as Ottrancedi, is a medicinal plant from the ancient period. It contains polyacetylenes, flavonoids, terpenoids, phenylpropanoids, which are well known molecules present in high concentration in medicinal plants with many antibacterial, antifungal and anticancer properties. To study the detailed mode of action of phenyl-1,3,5-heptatriyne which showed promising results on human liver cancer cell line HepG-2, we performed many assays like DNA fragmentation assay, Caspase-3 assay, Cell cycle analysis and fluorescent microscopic studies. During over studies we found that the molecule causes apoptosis in cancer cells lines by expressing high amount of Caspase-3 enzyme and it arrests the cells in G2/M phase. As it is already reported that the infusion of *B. pilosa* L. is non-toxic for single and repeated dose also for dermal structures so the molecule phenyl-1,3,5-heptatriyne can be a good candidate for a anticancer drug in future.

KEYWORDS: liver cancer; phenyl-1,3,5-heptatriyne; apoptosis; anticancer

1. Introduction

The natural compounds isolated from the plants have been used as drugs for many decades. *Bidens pilosa L.* is one of them; it is used in folk medicine for various applications due to the presence of polyacetylenes, flavonoids, terpenoids, phenylpropanoids [1]. In India, it is commonly known as "Ottrancedi" grown in southern parts of India and is frequently used in traditional medicine as a remedy to treat glandular sclerosis, wounds, colds and flu, acute or chronic hepatitis and urinary tract infection [2,3]. The bioactivity-guided fractionation of different extracts of *Bidens pilosa L.* leaf showed potential *in-vitro* anticancer and antimalarial activity and led to the identification of a potential marker compound, phenyl-1, 3, 5-heptatriyne [4]. The present study is to evaluate the mechanism of action of the marker compound phenyl-1,3,5-heptatriyne on human liver cancer cell line HepG-2, as this molecule was observed to be most effective on this cell line in *in-vitro* anticancer assay. It has already been proved that the infusion of *B. pilosa L.* is non-toxic for single and repeated dose also for dermal structures [5, 6].

2. Materials and Methods

2.1. Cells and Chemicals

Pure phenyl-1,3,5-heptatriyne (R4363978) was purchased from RR Scientific, China. Human cancer cell line HepG2 was procured from National Centre for Cell Sciences, Pune, India and grown in DMEM media supplemented with 10% FBS and 100 units/mL penicillin, 100 µ streptomycin in a humidified atmosphere in 5% CO_2 at 37°C. MTT, DAPI stains were purchased from Sigma Aldrich, DNA fragmentation assays kit, Cell cycle assay kit and caspase assay kits were procured from BD Biosciences.

2.2. Cytotoxicity assay:

Cytotoxicity of phenyl-1,3,5-heptatriyne was measured by MTT assay [7]. In brief; approximately 2.0–2.5x10^4 cells, suspended in DMEM containing 10% fetal bovive serum (FBS), 100 units/mL penicillin and 100 mg/mL streptomycin was plated onto each well of a 96-well plate. The plate was then incubated in 5% CO_2 at 37 °C for 24 h. Different concentrations of phenyl-1,3,5-heptatriyne were applied in different wells for 4 h. After 4 h incubation the medium was replaced with normal DMEM. The plate was again incubated for 48 h in same condition. After 48 hours, 10 µl of MTT (5 mg/mL) was added to each well and the plate was kept for 4 h in 5% CO_2 at 37 °C, then the medium was replaced with 100 µL of DMSO. The absorbance of the reaction solution at 570 nm was recorded.

2.3. DNA Fragmentation assay

Apoptosis was determined by using DNA fragmentation [8]. In brief, phenyl-1,3,5-heptatriyne treated cells were scraped off from the wells and centrifuged at 1,200 rpm for 10 min, then washed with phosphate buffer saline. The pellet was collected and treated with lysis buffer for 10 seconds followed by centrifugation at 1600xg for 5 minutes, then DNA was collected from the supernatant.

500 µl of 1% SDS and 8 µl RNase (5 µg/ µL) were added, and the lysate was incubated at 56°C for 2 hours. After first incubation 8 µl proteinase K (2.5 µg/ µl) was added and the lysate was incubated for 2 hours at 37°C. After incubation half volume ammonium acetate was added and DNA was precipitated with 2.5 volumes of ethanol by centrifugation at 12000 RPM for 5 minutes. DNA was dissolved in gel loading buffer and separated by electrophoresis on 1.5 % agarose gel. Pictures of the gels were taken by UV Tran illumination.

2.4. DAPI Staining

Caspase activity assay was carried out using Caspase-3 activation assay kit [10]. Briefly, phenyl-1,3,5-heptatriyne treated cells were trypsinized and pelleted by centrifugation at 2000 RPM for 5 minutes. Cells were washed twice with cold PBS, then resuspended in Cytofix/ Cytoperm solution at a concentration of $1x10^6$ cells/0.5 ml and incubated for 20 min on ice. Cells were pelleted, aspirated, and Cytofix/Cytoperm solution was discarded; cells were washed twice with Perm/Wash Buffer at a volume of 0.5 ml buffer / $1x10^6$ cells at room temperature. Perm /Wash buffer and antibody solution was prepared and the cells were resuspended in the Perm/Wash Buffer plus antibody and incubated for 30 min at room temperature. Cell pellet was washed within 1.0 ml Perm/Wash Buffer, then resuspended in 0.5 ml Perm/Wash Buffer and analyzed by flow-cytometry.

2.5. Caspase activity assay

Caspase activity assay was carried out using Caspase-3 activation assay kit [10]. Briefly, phenyl-1,3,5-heptatriyne treated cells were trypsinized and pelleted by centrifugation at 2000 RPM for 5 minutes. Cells were washed twice with cold PBS, then resuspended in Cytofix/Cytoperm solution at a concentration of $1x10^6$ cells/0.5 ml and incubated for 20 min on ice. Cells were pelleted, aspirated, and Cytofix/ Cytoperm solution was discarded; cells were washed twice with Perm/Wash Buffer at a volume of 0.5 ml buffer / $1x10^6$ cells at room temperature. Perm/Wash buffer and antibody solution was prepared and the cells were resuspended in the Perm/Wash Buffer plus antibody and incubated for 30 min at room temperature. Cell pellet was washed within 1.0 ml Perm/Wash Buffer, then resuspended in 0.5 ml Perm/Wash Buffer and analyzed by flow-cytometry.

2.6. Cell cycle analysis

$5x10^5$ cells were treated with phenyl-1,3,5-heptatriyne for 4 h. After the treatment, the cells were washed with wash buffer (500 mL) twice. The pellet was then treated with trypsin buffer (250 mL). After treatment, 200 µl of trypsin inhibitor & RNase buffer were added to each tube and gently mixed by tapping the tube (Trypsin buffer was not removed) and incubated for 10 minutes at room temperature. 200 µl of cold (2°C to 8°C) propidium iodide solution was added to each tube, gently mixed by tapping, incubated for 10 minutes in dark on ice and analyzed by FAC Scan flow cytometry (Beckman, USA) [11].

2.7. Statistical Analysis

The ANOVA was performed by the IBM SPSS statistical software. Means were calculated by the least significant difference (LSD) method at p less than 0.05.

3. Results

3.1. Cell proliferation assay

The cytotoxicity assay was carried out for the extract, fractions and pure molecule from the plant *Bidens pilosa L.* using four human cancer cell lines: HepG-2 (liver), KB (oral), MCF-7 (breast) and Caco2 (colon). The cytotoxicity results indicated the significant activity of phenyl-1,3,5-heptatriyne towards liver cancer cell lines. The inhibitory concentrations (IC_{50} and IC_{90}) were reported as 0.49 µg/mL and 4.0 µg/mL, respectively [12] (Figure 1).

3.2. DNA Fragmentation

DNA fragmentation assay was carried out to detect the activation of apoptotic pathway in the cell-lines. The cells treated with phenyl-1,3,5-heptatriyne showed ladder formation in the DNA, which is the marker of apoptosis [13] (Figure 2).The nuclear fragmentation was also evaluated by fluorescence microscopy using the DNA-binding fluorescent dye DAPI. The result indicated that the cell-lines treated with phenyl-1,3,5-heptatriyne were showing nuclear fragmentation while in the non-treated cells, the nucleus was intact (Figure 3).

3.3. Expression of Caspase 3

The expression of caspases is the clear cut indication of cells that are undergoing apoptosis. The results of Caspase 3 expression assay showed that phenyl-1,3,5-heptatriyne causes the activation of caspase 3 in cells (Figure 4).

3.4. Cell cycle distribution

The Cell cycle distribution assay is used to detect the cells undergoing apoptosis or necrosis. The results were showing the apoptosis in the cell lines treated with phenyl-1,3,5-heptatriyne. The flow-cytometric analysis confirmed that phenyl-1,3,5-heptatriyne is arresting the cells at G2/M phase [14] (Figure 5).

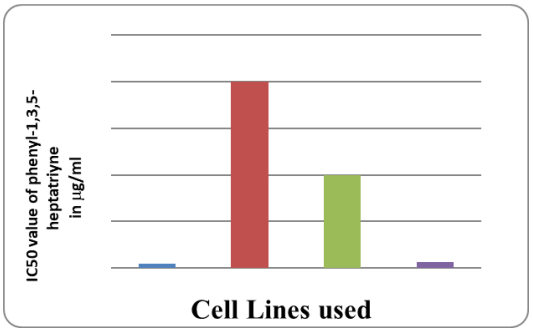

Fig. 1. The IC 50 value of phenyl-1,3,5-heptatriyne against liver cancer (Hepg-2), oral cancer (KB), breast cancer (MCF) and colon cancer (CaCO2) cell-lines.

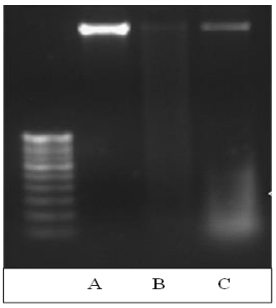

Fig. 2. DNA fragmentation in HepG-2 cell-lines; Negative control cell-lines (A), phenyl-1,3,5-heptatriyne treated cells (B) as compared to positive control camptothecin (C)

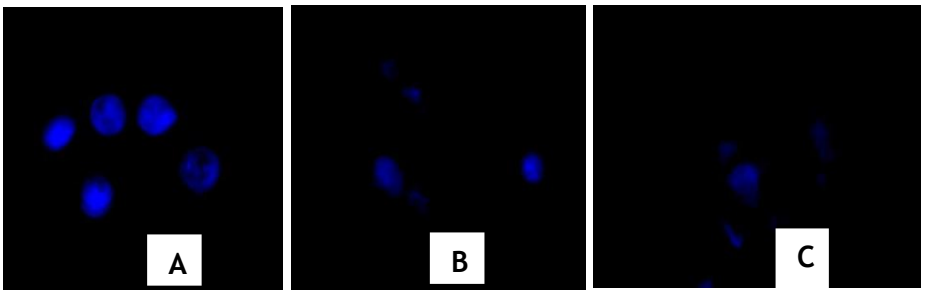

Fig. 3. DAPI staining of HepG-2 cell-lines: negative control cell-lines(A); phenyl-1,3,5-heptatriyne treated cells (B) as compared to positive control camptothecin (C)

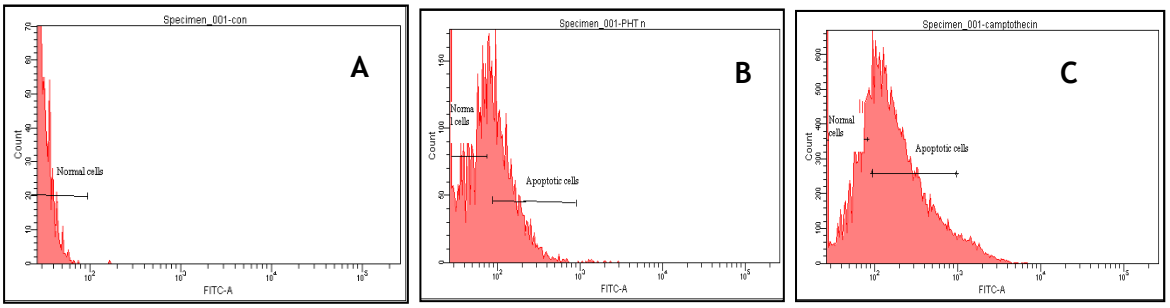

Fig. 4. Caspase 3 expression in HepG2 cell lines treated with phenyl-1,3,5-heptatriyne. Negative control cells (A); phenyl-1,3,5-heptatriyne treated (B); Positive control camptothecin (C).

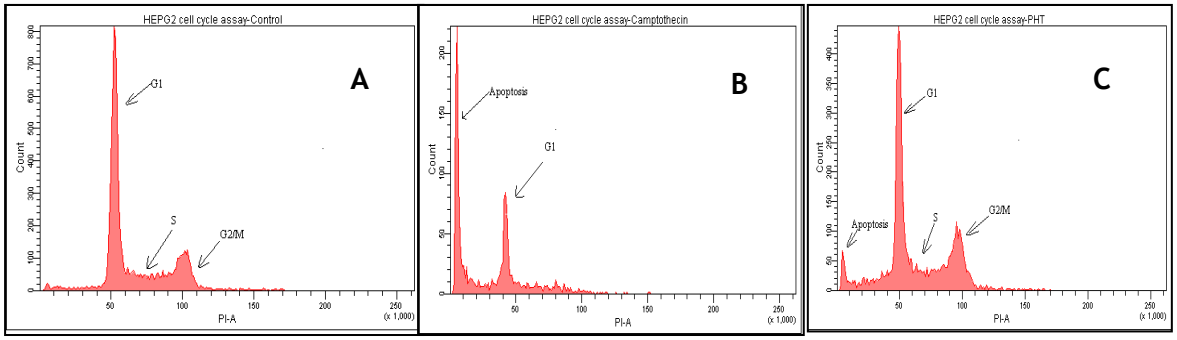

Fig. 5.7 Cell cycle distribution analysis in HepG2 cell lines treated with phenyl-1,3,5-heptatriyne. Negative control cells (A); phenyl-1,3,5-heptatriyne treated (B); positive control camptothecin (C).

4. Discussion

Cancer chemotherapy leads to the development of effective and relatively safe drugs, but unfortunately, low selectivity and long-term treatment of clinical use decrease the effectiveness of these drugs [15]. There are many significant targets for anticancer drugs. Cell cycle inhibitors have been suggested as potential therapeutic approaches to arrest cell growth and induce apoptosis of various cancer cells [16]. In present study, we have demonstrated phenyl-1,3,5-heptatriyne as significantly effective inhibitor of human HepG-2 liver cancer cells. Phenyl-1,3,5-heptatriyne significantly inhibits HepG-2 cell proliferation at a concentration of 0.52 µg/mL. The DNA fragmentation in response to phenyl-1,3,5-heptatriyne indicates the expression of CAD protein, which is responsible for the fragmentation of chromatin into the fragments [17]. The caspase-3 activity results clearly indicated that the cells treated with phenyl-1,3,5-heptatriyne are expressing caspase-3 which is the marker of apoptosis [18]. Moreover, phenyl-1,3,5-heptatriyne significantly induced apoptosis of G2/M phase cells and increased apoptotic cell death through the induction of casapase-3. Apoptosis of G2/M phase cells induced by phenyl-1,3,5-heptatriyne might be associated with the regulation of various gene expression related to cell cycle regulators especially of G2/M phase [19].

5. Conclusions

Phenyl-1,3,5-heptatriyne is a phytomolecule isolated from the sustainable sources. Our studies confirmed its *in-vitro* anticancer activity on liver cancer cell-line and, the mechanism of action studies indicated that phenyl-1,3,5-heptatriyne causes the apoptosis in cancer cells. Many studies confirm that the infusion of *B. pilosa L.* is non-toxic for single and repeated dose also for dermal structures [5]. Overall, this study suggested that phenyl-1,3,5-heptatriyne possesses potential activity as an anticancer agent against liver cancer cell-lines with less toxicity against normal cells.

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Conflict of Interest: The author declares no conflict of interest.

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