Cytotoxic effect of *Cuscuta reflexa* Roxb. and induction of apoptosis in human promyelocytic leukemia HL-60 cells

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The anticancer potential of *Cuscuta reflexa* (whole plant extract), a holoparasitic plant collected from its host plant *Euphorbia royleana* was evaluated by testing its in vitro cytotoxicity and induction of cell death by apoptosis. Among three extracts (95% alcoholic, 50% hydro-alcoholic and aqueous extracts) and four fractions (*n*-hexane, chloroform, *n*-butanol and water) of alcoholic extract, the 95% alcoholic extract (A001) and its chloroform fraction (F002) showed significant cytotoxic activity human cancer cell lines. Both A001 and F002 inhibited cell proliferation and showed dose-dependent cytotoxicity against cervix (HeLa), prostate (DU-145), promyelocytic leukemia (HL-60), colon (50273) and ovary (OVCAR 5) human cancer cell lines. Both A001 and F002 induced apoptosis in HL-60 (human promyelocytic leukemia) cell line, as revealed by several biological end points viz., DNA ladder formation, annexin-V-FITC binding, cell cycle analysis and caspases induction. The F002 showed better activity than A001 by inducing apoptotic bodies formation, apoptotic DNA fragmentation, enhanced annexin-V-FITC binding of the cells, increase of G2/M phase cell cycle and induction of caspase-9,-8 and-3 activities, indicating the involvement of both intrinsic and extrinsic pathways of apoptosis. The results suggested that both A001 and F002 induced apoptosis through both mitochondrial-dependent and independent pathway in HL-60 cells. Thus, apoptotic effect of F002 against HL-60 cells suggests its potential in development as an anticancer drug.

Keywords: Apoptosis, *Cuscuta reflexa*, Cytotoxicity, HL-60, Human cancer cell lines, Tumor growth inhibition

Cancer is a leading cause of mortality and morbidity all over the world. It is the second leading cause of death among non-communicable diseases. Plant-derived anticancer agents are widely used for the treatment of cancer, considering being safe and possessing lesser side effects. The traditionally well-known medicinal plant *Cuscuta reflexa* Roxb., (Fam.: Convolvulaceae) commonly called Dodder plant (*Amar bel* in Hindi) is a parasitic plant with slender yellow stems and is widely distributed in temperate and tropical regions and commonly found throughout India. It also grows on different host plants. The plant has been indigenously used in Indian system of medicine for the remedy for various ailments. The various parts of the plant are used by tribes for treating ailments like fits, protracted fever, diaphoretic, demulcent, purgative, melancholy and insanity and to control fertility. It is also used externally against itch and internally in fevers, ‘retention of wind’ and indurations of the liver in the indigenous system of medicine. The plant is reported to possess α-glucosidase inhibitory, free radical scavenging, antibacterial, psychopharmacological, anti-steroidogenic, hair-growth promoting, antiandrogenic, antimicrobial, anti-diabetic, anti-inflammatory activities. The chemical compounds isolated from the plant are mainly flavonoids.

In this study, we have evaluated the anticancer potential of *Cuscuta reflexa* (whole plant) collected from its host plant *Euphorbia royleana* by testing for its in vitro cytotoxicity against various human cancer cell lines and have also examined the induction of apoptosis (the favored cell death mechanism) by studying apoptotic bodies formation, DNA ladder, enhanced annexin-V-FITC binding of the cells, increased sub-G₀ DNA fraction and caspase-9,-8 and-3 activities.

Materials and Methods

RPMI-1640, Dulbecco’s minimum essential medium (DMEM), fetal calf serum (FCS), trypsin, gentamycin, penicillin, EDTA, 5-flourouracil, camptothecin, propidium iodide, DNase-free RNase, dimethyl...
sulphoxide and sulforhodamine-B (SRB) were purchased from Sigma Chemical Co., USA. All other chemicals were of high purity and obtained locally.

**Plant collection, extraction and fractionation**

Whole plant of *Cuscuta reflexa* was collected from the host plant *Euphorbia royleana* locally in the month of December and authenticated at source by the taxonomist of the institute. A voucher specimen was deposited at the herbarium of the Institute (vide IIIM collection no. 17148, Acc. no. 17719). The freshly collected whole plant was chopped and dried under shade. Three extracts of the plant material were prepared with 95% alcohol, alcohol-water (1:1) and water using repeated solvent extraction procedure. Dried powdered plant material (1 Kg) was percolated in 95% ethanol (5 L) at ambient temperature for 16 h. The solvent was decanted and the process was repeated four-times. The pooled solvent was evaporated under reduced pressure to obtain alcoholic extract (160 g). Similarly, hydro-alcoholic extract was prepared; the dried plant material (200 g) was soaked in alcohol-water (1:1, 1 L) and the extract obtained was 72 g. For preparation of aqueous extract, dried powdered plant material (200 g) was heated with distilled water (1.5L) on steam bath for 2 h, the supernatant was decanted and filtered through celite powder and the process was repeated four-times; pooled extract was concentrated on rotavapour and dried in a lyophilizer. The yield obtained was 40 g.

The alcoholic extract was fractionated sequentially with *n*-hexane, chloroform, butanol and water. The dried alcoholic extract (20 g) was macerated with *n*-hexane (4 × 500 mL). The combined solvent portion was evaporated under reduced pressure to yield *n*-hexane fraction (1.5 g). The residue was further macerated with chloroform (4 × 500 mL). The combined organic layer was evaporated under reduced pressure to yield chloroform fraction (2.25 g). The residue obtained was dissolved in distilled water (1 L) and partitioned between *n*-butanol and water. The process was repeated four-times (4 × 500 mL) the organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure to yield *n*-butanol fraction (8.55 g). The aqueous part was concentrated under reduced pressure to give aqueous fraction (6.4 g).

**Cell lines and culture**

The human cancer cell lines viz., 502713 (colon), OVCAR-5 (ovary), HeLa (cervix), DU-145 (prostate) and HL-60 (leukemia) were obtained either from National Center for Cell Science, (NCCS), Pune, India or National Cancer Institute (NCI), Fredrick, USA.

**In vitro cytotoxicity against human tumor lines**

The in vitro cytotoxicity of extracts and fractions was determined using sulforhodamine-B (SRB) as described previously. In brief, the stock solution (20 mg/mL) of three extracts was prepared in dimethylsulfoxide (DMSO), DMSO-water (1:1) and hot water, respectively and were further diluted with growth medium (RPMI-1640/DMEM with 2 mM glutamine, pH 7.4, 10% FCS, 100 µg/mL streptomycin and 100 U/mL penicillin) to obtain desired concentrations. The stock solutions of *n*-hexane, chloroform and *n*-butanol fractions were prepared in DMSO, while aqueous fraction was dissolved in distilled water.

Cells were grown in tissue culture flasks in growth medium at 37°C in an atmosphere of 5% CO₂ and 95% relative humidity in a CO₂ incubator. Cells at sub-confluent stage were harvested from the flask by treatment with trypsin (0.05% trypsin in PBS containing 0.02% EDTA) and suspended in growth medium (RPMI-1640/DMEM with 2 mM glutamine, pH 7.4, 10% FCS, 100 µg/mL streptomycin and 100 U/mL penicillin) to obtain desired concentrations. The stock solutions of *n*-hexane, chloroform and *n*-butanol fractions was prepared in DMSO, while aqueous fraction was dissolved in distilled water.

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Flow cytometric analysis of apoptosis and necrosis

Extent of apoptosis was measured using annexin-V-FITC apoptosis detection kit as described by the manufacturer’s instruction. Briefly, HL-60 cells (1 × 10^6/1.5 mL/well) were seeded in 12-well plates and treated with 95% alcoholic extract (A001) and its chloroform fraction (F002) at 30 and 100 µg/mL. After 24 h, cells were collected, washed with (7.4) PBS twice, gently resuspended in annexin-V binding buffer and incubated with annexin-V-FITC/PI in dark for 15 min and analyzed by flow cytometry using cell quest software (BD Biosciences, USA). The fraction of cell population in different quadrants was analyzed using quadrant statistics. Cells in the lower right quadrant represented apoptosis and in the upper right quadrant represented necrosis or post-apoptotic necrosis.22

DNA gel electrophoresis

DNA fragmentation was determined by electrophoresis of extracted genomic DNA from HL-60 cells. Cells (2 × 10^6 cells/3 mL) in 6-well plate were treated with A001 and F002 at 10 and 30 µg/mL and staurosporine at 5 µM for 24 h. Cells were lysed in lysis buffer (10 mM EDTA, 50mM Tris-buffer, pH 8.0, 0.5% (w/v) SDS and proteinase K 0.5 mg/mL) and incubated at 50°C for 1 h. Lysate was further incubated with RNase A (0.5 mg/mL) at 50°C for 1 h. Finally, the DNA obtained was heated rapidly at 70°C, supplemented with DNA loading dye and immediately resolved on 1.5% agarose gel at 50 V for 2-3 h.

Cell cycle phase distribution of nuclear DNA

Effect of alcoholic extract (A001) and its chloroform fraction (F002) on DNA content was assessed by cell cycle phase distribution in HL-60 cells. HL-60 cells (1×10^6/1.5 mL/well) in 12-well plate were incubated with A001 and F002 at 30 and 100 µg/mL and staurosporine at 5 µM for 24 h, washed twice with ice-cold PBS (pH-8), harvested, fixed with ice-cold 70% ethanol and stored at −20°C for 30 min. After fixation, the cells were incubated with RNase A (0.1 mg/mL) at 37°C for 30 min, stained with propidium iodide (50 µg/mL) for 30 min in dark.23 Cells were analyzed immediately on a LSR flow-cytometer (Becton Dickinson, USA). The fluorescence intensity of sub-G0 cell fraction represented the apoptotic cell population.

Caspase assays

Cells (2 × 10^6/3 mL/well, 6-well plate) were incubated with 95% alcoholic extract (A001) and its chloroform fraction (F002) for 24 h. At the end of treatment, cells were washed in (7.4) PBS and pellets lysed in cell lysis buffer. Activities of caspase-3, -9/6 and -8 in the cell lysates were determined fluorometrically using BD ApoAlert caspase fluorescent assay kits. Caspase-3 and -8 used fluorochrome conjugated peptides DEVD-AFC (amino acid sequence Asp-Glu-Val-Asp conjugated to 7-amino-4-trifluoromethylcoumarin) and IETD-AFC (Ac-Ile-Glu-Thr-Asp conjugated 7-amino-4-trifluoromethylcoumarin) as substrates, respectively, while caspase-9 employed LEHD-AMC (leucine-glutamic acid-histidine-aspartic acid-7-amino-4-methylcoumarin). Release of AFC (7-amino-4-trifluoromethyl coumarin) and AMC (7-aminoethylcoumarin) was assayed according to the instructions provided in the Manual by the supplier (BD Pharmingen™).

Specific inhibitors (Z-FA-FMK) were used as negative control to determine whether fluorescence intensity changes were specific for the activity of caspases. The peptide based inhibitors used were: DEVD-CHO (N-Ac-Asp-Glu-Val-Asp-CHO) for caspase-3, IETD-FMK (Z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone) for caspase-8 and LEHD-CHO (Ac-Leu-Glu-His-Asp-CHO) for caspase-9/6.

Statistical analysis

Data were expressed as mean ± SD, unless otherwise indicated. Comparisons were made between control and treated groups unpaired Student’s t-test and P values <0.01 were considered significant.

Results

In vitro cytotoxic effect against human cancer cell lines

The activity guided fractionation of all the three extracts (95% alcoholic, 50% hydro-alcoholic and aqueous extracts) of Cuscuta reflexa was monitored by in vitro cytotoxic assay against human cancer cell lines viz., 502713 (colon), OVCAR-5 (ovary), HeLa (cervix), DU-145 (prostate) and HL-60 (leukemia). After 48h, SRB cell viability assay was performed to determine the growth inhibition and cytotoxic activity of extracts and fractions. The growth inhibition in a dose-dependent manner was observed against all cell lines by all the extracts. Of the three extracts, 95% alcoholic extract (A001) and its chloroform fraction (F002) exhibited significant and dose-dependent inhibitory effects against all the human cancer cell lines in 48 h of treatment (Table 1).
IC_{50} values of all the extracts and chloroform fraction of 95% alcoholic extract showed that 95% alcoholic extract had highest growth inhibition against cervix cancer cell line (HeLa) with IC\textsubscript{50} 22 µg/mL, followed by HL-60, OVCAR-5, 502713 and DU-145, respectively, whereas chloroform fraction of 95% alcoholic extract exhibited IC\textsubscript{50} 14 µg/mL for Hela and DU-145, followed by HL-60, 502713 and OVCAR-5 cancer cell lines, respectively. However, 50% hydro-alcoholic and aqueous extracts were comparatively less effective (Table 1).

**Flow cytometric estimation of apoptosis and necrosis**

The exposure of phosphotidyl serine on the surface of cells is an early event in the onset of apoptosis, which has strong binding affinity for annexin-V in the presence of calcium. HL-60 cells were incubated with different concentrations (10 and 30 µg/mL) of A001 and F002 extracts. The cells were stained with annexin-V-FITC and propidium iodide to assess the apoptotic and necrotic cell population (Fig. 1). Both A001 and F002 produced dose-dependent increase in the apoptotic cell population. The basal apoptotic population in the untreated culture was 1%, which increased to 7% and 37% µg/mL at 10 and 30 µg/mL, respectively with A001. Apoptosis also increased from 34% to 80% at dose of 10 and 30 µg/mL, respectively with F002. Thus, apoptosis appeared to be the primary mode of cell death induced by A001 and F002.

**F002 induces DNA fragmentation, a hallmark of apoptosis**

We found that F002 induced early phosphotidyl serine exposure, a sign of apoptosis. To confirm further, genomic DNA was isolated from the treated and untreated HL-60 cells. Cells treated with F002 exhibited typical DNA ladder formation, which is a hallmark of apoptosis. DNA ladder was clearly visible at the concentration of 30 µg/mL (Fig. 2). When treated with 10 µg/mL concentration of F002, the

<table>
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<th>Tissue</th>
<th>Cell line</th>
<th>95% Alcoholic extract</th>
<th>50% Aqueous-alcoholic extract</th>
<th>Aqueous extract</th>
<th>Chloroform fraction (95% alcoholic extract)</th>
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<tbody>
<tr>
<td>Colon</td>
<td>502713</td>
<td>61</td>
<td>74</td>
<td>76</td>
<td>19</td>
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<tr>
<td>Ovary</td>
<td>OVCAR-5</td>
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<td>Cervix</td>
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<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>Prostate</td>
<td>DU-145</td>
<td>68</td>
<td>73</td>
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<td>HL-60</td>
<td>38</td>
<td>65</td>
<td>79</td>
<td>22</td>
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Fig. 1—Flow cytometric analysis of alcoholic extract (A001) and its chloroform fraction (F002) showing induction of apoptosis in HL-60 cells using annexin-V-FITC/PI [Data are representative of one of three similar experiments]

DNA laddering was diffused interspersed with smear, indicative of some post-apoptotic necrosis cells.

**Increases sub-Go DNA fraction of cell cycle phase distribution**

The effect of A001 and F002 on DNA content by cell cycle phase distribution was assessed against human leukemia HL-60 cells. Both A001 and F002 exhibited concentration-dependent increase in hypodiploid sub-Go DNA fraction (<2n DNA) (Fig. 3). The sub-Go fraction, which was 4% in control cells, increased to ~41% and 44% at 10 and 30 µg/mL of A001, respectively. When treated with F002, it increased up to 78% at 30 µg/mL of treatment. Further, the cell cycle G2/M phase was not affected, indicating that F002 did not produce any mitotic block or cause delay in cell cycle.
Upregulation of caspase-3, -8 and -9 activities

The caspase family plays key roles in the execution of apoptosis. We measured caspase-3, -8 and -9 activities induced by A001 and F002 at 30 µg/mL after 24 h. Both A001 and F002 caused an increase of 2.16- and 5.17-fold in the level of caspase-3 in HL-60 cell line. Similarly, 1.61-fold and 4.11-fold increase in caspase-8 activity and 1.66-fold and 4.35-fold increase in caspase-9 activity was observed in HL-60 cells (Fig. 4). Thus, the activation of caspases revealed that A001 and F002 induced apoptosis through intrinsic or mitochondria-dependent pathway.

Discussion

A considerable proportion of people in developing countries depend on traditional medicines for their primary health needs. The plants besides possessing various medicinal benefits have also shown anticancer activity, and thus may have potential for identification of anti-tumor compounds. In this study, we observed the induction of apoptosis in human leukemia (HL-60) cell line by the 95% alcoholic extract (A001) and its chloroform fraction (F002) of holoparasitic plant C. reflexa (whole plant), collected from the host plant Euphorbia royleana. This is for the first time observed the anticancer potential and pro-apoptotic effect of alcoholic extract (A001) and its chloroform fraction (F002) was observed against human leukemia cell line (HL-60). Both A001 and F002 inhibited the cell proliferation against human cancer cell lines viz., 502713 (colon), OVCAR-5 (ovary), HeLa (cervix), DU-145 (prostate) and HL-60 (leukemia) of four different tissues. The cancer growth inhibition by these extracts was cell line-specific and concentration-dependent. Both A001 and F002 were further explored to understand its mechanism of anticancer action.
Inducing apoptosis in tumor cells is considered a preferred way for treatment of cancer. The deregulation of apoptosis has been identified in many human cancers and recognized as an important event during carcinogenesis, involving death of cancer cells. The anticancer drugs preferred for the treatment of cancer are required to activate apoptotic pathway. Apoptosis, or programmed cell death is characterized by a number of well-defined features which include condensation and fragmentation of the chromatin, internucleosomal DNA cleavage, membrane blebbing, caspase activation, and translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane. Both A001 and F002 induced apoptosis, as revealed by flow cytometric analysis. They significantly induced annexin-V binding and showed increase in hypo-diploid sub-G₀ DNA fraction (<2n DNA) of HL-60 cells. F002 also induced DNA fragmentation, a hallmark of the apoptosis.

Another important biochemical modification exhibited by the apoptotic cells are protein cleavage by the caspases. The caspases are ubiquitous family of cysteine proteases that include both upstream (initiator) and downstream (effector) caspases. These are activated during apoptosis and cleave specific proteins, resulting in the irreversible commitment to cell death. Both A001 and F002 efficiently activated the caspase-8 and caspase-9. The activation of caspase-8 has been associated with upstream increased expression of apical death receptors TNFR1 and DR4 (TRAIL-R1). The activation of these receptors might engage Fas-associated death domain (FADD) and death inducing signaling complex (DISC), allowing the release of active form of caspase-8.

Activation of caspase-3 is an important step in the execution phase of apoptosis and its inhibition blocks cell apoptosis. PARP is a well-established substrate for caspase-3 and is a nuclear DNA-binding protein that detects DNA strand breaks and functions in base excision repair. PARP is activated upon binding to DNA strand breaks. During apoptosis, PARP is cleaved into 85 and 29 kDa fragments that no longer support the enzymatic DNA repair function. Upregulation of caspase-8 and -9 leads to the activation of downstream executioner caspase-3 and its cleavage of PARP is consequential events culminating into apoptosis; both A001 and F002 were significantly effective in these actions.

In conclusion, alcoholic extract (A001) and its chloroform fraction (F002) of Cuscuta reflexa (whole plant) exhibited in vitro cytotoxicity against human cancer cell lines and also induced apoptosis in human promyelocytic leukemia (HL-60) cells by intrinsic, as well as extrinsic pathways. However, both A001 and F002 did not exert any cytotoxic effect on the normal cells.

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